

Supplemental Detailed Methods

Quantification of IMCL by histology

The ankle plantarflexor muscles of the mouse (gastrocnemius, soleus and plantaris) and the portion of the human muscle biopsies allocated to histology were affixed to cork with tragacanth gum and frozen in liquid nitrogen cooled isopentane. Axial sections were cut at 10µm on a cryostat (Leica Biosystems, Wetzlar, Germany) and stained with Oil Red O (ORO) with serial immunostaining for myosin heavy chain isoforms as previously described (4) (Supplementary Detailed Methods) or fluorescent BODIPY (0.05 mg ml⁻¹ BODIPY 493/503; Life Technologies, Carlsbad, CA) with myosin heavy chain type 1 (1:30 BA-F8) and laminin (ab11575; Abcam, Cambridge, UK). Mean ORO intensity was averaged over 25 fibers, or all fibers if less than 25 were present in a 10x image, of each type per muscle. BODIPY positive particles between 0.1 and 5 µm² within a laminin boundary were identified as lipid droplets (LD) and the average area of individual LDs, the density of LDs (number/fiber area) and the LD area fraction (sum of all LDs/fiber area) were averaged over 20 fibers, or all fibers if less than 20 were present in a biopsy, of each type per muscle.

Oil Red O. Sections were mounted on pre-chilled slides and placed in a staining dish containing 37% formaldehyde such that the section remained out of the liquid in the vapor phase. The staining dish was kept closed at -20°C for 48 hours. Slides were then transferred to a pre-chilled solution of Oil Red O (ORO) (60% ORO stock solution (0.5% w/v Oil Red O in isopropanol)) for 5 minutes and then transferred to a pre-chilled dish of distilled water to warm to room temperature prior to imaging. A serial section was mounted on a second slide for fiber typing by immunostaining isoforms of myosin heavy chain. Fresh mouse muscle sections were incubated with primary antibodies against myosin heavy chain type 1, 2a and 2b (1:30 BA-F8, SC-71, and BF-F3; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) and human sections were incubated with primary antibodies against myosin heavy chain type 1, 2a and 2x (1:30 6H1; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) followed by species and isotype specific fluorescent secondary antibodies. ORO and immunostained sections were imaged in the same region and then images were registered to visualize and quantify ORO stained IMCL by fiber type. In mouse plantarflexor muscles, IMCL was quantified using one 10x image each from the soleus, plantaris and deep gastrocnemius. To normalize intensity between images,

background was subtracted using a portion of the image with no section as “background.” Fiber regions of interest (ROI) were drawn on the background subtracted image in Image J. Then average intensity was measured for each ROI on the inverted red channel, such that higher mean intensity values represent more IMCL. Reproducibility of this method was examined in two sections that were included in two different staining batches. Between batch variability in ORO mean intensity was $10.97 \pm 10.73\%$.

BODIPY. 10 μ m axial sections of the mouse plantarflexors and human biopsies were incubated with fluorescent BODIPY (0.05 mg ml⁻¹ BODIPY 493/503; Life Technologies, Carlsbad, CA) and primary antibodies against myosin heavy chain type 1 (1:30 BA-F8) and laminin (ab11575; Abcam, Cambridge, UK) followed by species and isotype specific fluorescent secondary antibodies. 20x and 40x image stacks were acquired using a confocal microscope (DMi8 Leica with TCS SPEII confocal module, Leica Biosystems, Wetzlar, Germany). Stacks were acquired at 0.8 μ m steps through a depth of 12 μ m to capture the full section thickness. Analysis was performed on the background subtracted Z-projection image with IsoData thresholding in Image J. Fiber ROIs were generated by a custom ImageJ script using the laminin signal as fiber boundaries, then lipid droplets, defined as particles with areas between 0.1 and 5 μ m², were quantified in each ROI using the Analyze Particles algorithm in ImageJ. Fiber ROIs were subdivided into type 1 and type 2 based on the myosin heavy chain type 1 signal and lipid droplet metrics were averaged to yield a single value per fiber. Reproducibility of this method was examined in two sections that were included in two different staining batches. Between batch variability in LD area fraction was $14.70 \pm 7.85\%$.

Measurement of isolated myofiber mechanics

Soleus muscles from mice and the portion of the human muscle biopsies allocated to isolated myofiber experiments were permeabilized in a 0.5% w/v Brij 58 (Sigma Aldrich, St. Louis, MO) solution with agitation for 30 minutes at 4°C and stored in a glycerinated storage solution at -20°C until the day of testing. Recipes for testing solutions can be found in (2). For testing, individual myofibers were teased from the muscle bulk in a low [Ca²⁺] (pCa 9) relaxing solution and affixed in a chamber within the Aurora 1400A permeabilized fiber system (Aurora Scientific, Aurora, Ontario, Canada) bathed in relaxing solution with one end tied to a lever arm and the other to a force transducer with 10-0 nylon suture. An inverted light microscope was

used to set sarcomere length to 2.5 μm and to measure fiber diameter at three places along the length of the fiber and the length of the fiber between suture knots. The fiber was then transferred to a weakly buffered, low $[\text{Ca}^{2+}]$, pre-activating solution followed by a high $[\text{Ca}^{2+}]$ (pCa 4.5) activating solution by chamber switching in the 1400A system with simultaneous force recording. This was repeated 3 times. At the end of active testing, fibers were returned to relaxing solution, allowed to equilibrate for 5 minutes and then were subjected to an incremental stress relaxation test. In this test, fibers were stretched in 200 μm increments at 4 mm/sec with 2-minute holds for 8 stretches or until failure, with simultaneous force recording. Following testing, fibers were removed from the chamber, incubated with primary antibodies against myosin heavy chain type 1, 2a and 2x (1:30 BA-F8, SC-71, 6H1) followed by appropriate secondaries and classified as either type 1 or type 2 based on this signal. 10-20 fibers were tested per muscle or biopsy.

Forces were converted to stresses by normalizing to fiber cross-sectional area using the average of the diameter measurements. Passive stretch was converted to sarcomere strain using the initial sarcomere length, measured fiber length and length change. Peak active tension was quantified as the average of the three activation trials. Stress relaxation data was fit to a 3-element Hill type model with a time dependent viscosity (3).

$$\sigma(t) = (k_p + k_s \exp(\frac{-k_s t(\eta_0 t + \eta_\infty \alpha)}{\eta_0 \eta_\infty (t + \alpha)})) \varepsilon(t)$$

K_p and K_s reflect the parallel and series components of elasticity which are generally considered to reflect sarcomeric proteins (primarily titin) and other cytoplasmic proteins, respectively. The initial viscosity (η_0) reflects the instantaneous viscosity during stretch, the rest viscosity (η_∞) reflects the final viscosity at the end of the hold (rest) and α reflects the rate of change in viscosity over the course of stress-relaxation. Peak active and passive forces were normalized to fiber cross sectional area using measured diameter and assuming a cylindrical shape. Following testing, fiber type was identified by immunostaining for myosin heavy chain isoforms. 10-20 fibers were tested per muscle or biopsy. The sample size available for some of these assessments in human muscle is less than that for histology due to technical challenges with isolated fiber mechanics. Reasons for this include insufficient biopsy material (fibers too short or too little sample for both histology and mechanics) or less than 5 fibers with quality data of a given type.

Test-retest variability over the 3 active tests averaged $3.68 \pm 1.72\%$ and was not significantly different between experimental groups (Control vs Pre-Diabetes vs Diabetes) by 1-way ANOVA ($p=0.45$). Between experimenter variability was assessed in two samples and averaged $14.44 \pm 19.83\%$ for mean peak active tension across both fiber types.

Measurement of muscle contractility

Soleus muscles were isolated from experimental mice under a deep plane of anesthesia (2% isoflurane). The distal tendon was attached with 8-0 suture to a lever arm connected to a dual-mode force and length transducer and the proximal end was attached to a fixed post in an ex-vivo muscle stimulation system (1300A Aurora Scientific, Aurora, Ontario, Canada). The muscle was bathed in a Mammalian Ringer's solution (mM: 137 NaCl, 5 KCl, 2 CaCl₂, 1 MgSO₄, 1 NaH₂PO₄, 24 NaHCO₃, 11 glucose containing 10 mg/l curare) maintained at 37°C. Stimulation was elicited through parallel plate electrodes flanking the muscle with 20V controlled voltage. Muscles were tensioned to slack length (set as first detectable passive tension) and twitch forces were recorded with increasing length at increments of 10% slack fiber length (measured through a microscope reticule) until a plateau in force was observed. Then, isometric tetanic contractions (300 ms train of 0.3 ms pulses at 225 Hz) were measured increasing length by 5% slack fiber length to determine peak tetanic tension and peak fiber length. A final twitch contraction was obtained at peak fiber length to determine twitch kinetics. Finally, the muscle was removed from the system, blotted and weighed. Tensions were normalized to physiological cross-sectional area (PCSA) with published values for pennation angle and muscle density (1).

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4. Collins KH, Gui C, Ely EV, Lenz KL, Harris CA, Guilak F, Meyer GA: Leptin mediates the regulation of muscle mass and strength by adipose tissue. *J Physiol* 2022;600:3795-3817