SUPPLEMENTARY DATA

Antibodies

Antibodies against Acetylated-lysine, SIRT3, Hexokinase II, phospho-Akt (Ser473), Akt, phospho-IRS1 (Ser612), IRS1, phospho-AS160 (Thr588), GSK3β, phospho-GSK3β (Ser9) were purchased from Cell Signaling.
The antibodies against p85 subunit of phosphoinositide 3-kinase and AS160 were from Millipore.
Anti-GLUT4 and total Rodent OXPHOS antibody were from Abcam.
Anti-IRS1 and anti-VDAC antibodies for immunoprecipitation were purchased from Santa Cruz.
Equal loading was assessed using β-actin (Cell Signaling), GAPDH or VDAC (abcam).
The anti-GLUT4 antibody used for immunofluorescence was a gift from Dr. Jeffrey E. Pessin (Albert Einstein College of Medecine). Anti-Cav3 was from Santa Cruz.

Buffer compositions

BIOPS: CaK_2EGTA (2.77 mM), K_2EGTA (7.23 mM), Na_2ATP (5.77 mM), $MgCl_2*6H_2O$ (6.56 mM), Na_2Phosphocreatine (15 mM), Imidazole (20 mM), Taurine (20mM), K-MES (50 mM), pH 7.1
Buffer Z: KCl (30 mM), K-MES (105 mM), K2HPO4 (10 mM), $MgCl_2*6H_2O$ (5 mM), EGTA (1 mM), K_2EGTA (7.23 mM), , Imidazole (20 mM), Taurine (20mM), Dithiothreitol (0.5 mM), fatty acid free BSA (0.5g/L), pH 7.1
Lysis Buffer: 25mM Tris-HCl pH7.4, 10mM EDTA, 10% Glycerol, 1% Triton-X100, HALT protease and phosphatase inhibitor cocktail (Pierce) and deacetylase inhibitors Nicotinamide (10mM) and Trichostatin A (1µM).
Buffer M: 100mM KCl, 40mM Tris HCl, 10mM Tris base, 5mM MgCl2, 1mM EDTA, 1mM ATP, pH = 7.5 with HALT protease and phosphatase inhibitor cocktail (Pierce) and deacetylase inhibitors Nicotinamide (10mM) and Trichostatin A (1µM).
Loading Buffer: 6% SDS, 350mM Tris pH 6.8, 45% Glycerol, 5% β-mercaptoethanol, bromophenol blue.

Hyperinsulinemic-euglycemic clamp

One week before hyperinsulinemic-euglycemic clamps, catheters were surgically placed in a carotid artery and jugular vein for sampling and infusions respectively. Erythrocytes were replaced to prevent a decline in hematocrit with repeated blood sampling. [3-^{3}H]glucose was primed and continuously infused between *t*=-90-0min (0.04µCi/min). The clamp was initiated at *t*=0min with a continuous insulin infusion (4mU/kg/min) maintained for 155min. Arterial glucose was monitored every 10min, to provide feedback to adjust the glucose infusion rate (GIR). [3-3H]glucose (0.06µCi/µL) was added to the glucose infusate to clamp both arterial glucose and glucose specific activity. [3-3H]glucose kinetics were determined at −10min and at 10min intervals between 80 and 120min as insulin action is in a steady state. A 13µCi bolus of 2[^{14}C]deoxyglucose ([^{14}C]2-DG) was administered at 120min and used to determine the glucose metabolic index (Rg), a measure of tissue-specific glucose uptake. Blood was collected at 2, 5, 15, 25 and 35min after injection to measure the disappearance of [14C]2-DG from the plasma. At t=155min, mice were anesthetized with Pentobarbital and tissues were freeze-clamped for subsequent analyses. Whole-body glucose appearance (Ra) and endogenous glucose production (EndoRa), a measure of hepatic glucose production, were calculated as described (1; 2). Radioactivity of [3-^{3}H]glucose, [^{14}C]2DG and [^{14}C]2DG-6-phosphate were determined by liquid scintillation counting (3). Glucose appearance (Ra) and disappearance (Rd) rates were determined using non-steady state equations (4). Endogenous glucose production (EndoRa) was determined by subtracting the GIR from total Ra. The glucose metabolic index (Rg) was calculated as previously described (5). The ratio of 2[^{14}C]deoxyglucose-6-phosphate to 2[^{14}C]deoxyglucose within tissues was calculated as an index of hexokinase activity. Glycogen was determined using the method of Chan and Exton (6). A full description of the surgery, clamp method and isotope calculations are publicly accessible on the Vanderbilt Mouse Metabolic Phenotyping Center website (https://labnodes.vanderbilt.edu/resource/view/id/10764/community_id/1418).

Immunoprecipitations

For IRS1 immunoprecipitation (IP), 500µg of gastrocnemius protein lysate was incubated 1h at 4°C with 3µg IRS1 antibody (Santa Cruz Biotechnology). Then, 20µL protein A/G PLUS-Agarose (Santa Cruz Biotechnology) was added and incubated overnight at 4°C. The mixture was centrifuged at 1,000*g* and the supernatant removed. The beads were washed four times with cold PBS. Beads were resuspended in 30µL SDS PAGE loading buffer and heated at 80°C for 5min before immunoblot was performed on 15µL. For VDAC IP,

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400µg of the mitochondrial protein fraction from vastus lateralis was incubated 1h at 4°C with anti-VDAC antibody (Santa Cruz Biotechnology). 30µL protein A/G PLUS-Agarose (Santa Cruz Biotechnology) was added and incubated overnight at 4°C. The mixture was centrifuged at 1,000*g* and the supernatant removed. The beads were washed three times with cold PBS + 0.05% lauryl maltoside. For elution of proteins, beads were resuspended in 40µL 1% SDS for 10min, centrifuged at 1,000*g* and supernatant was mixed with 10µL SDS PAGE loading buffer. Immunoblot was performed on 25µL.

Immunostaining

Immunohistochemistry

CD31 staining was assessed by immunohistochemistry in paraffin-embedded tissue sections. Sections (5µm) were incubated with anti-CD31 primary antibody (BD Biosciences) for 60min. Slides were lightly counterstained with Mayer hematoxylin. The EnVision+HRP/DAB System (DakoCytomation) was used to produce localized, visible staining. Images were captured at 20x magnification using a Q-Imaging Micropublisher camera mounted on an Olympus upright microscope. Quantification of capillary density was performed using stereological techniques. Specifically, a test point grid was applied to each micrograph using ImageJ software. To quantify capillary density, the numbers of points falling within muscle tissue and on CD31+ structures were compared (7).

Immunofluorescent staining

Following hyperinsulinemic-euglycemic clamps, gastrocnemii were excised, mounted in optimal cutting temperature medium (Sakura), and flash frozen in liquid-nitrogen cooled isopentane. 5µm sections were cut using a cryostat, mounted on charged slides, and stored at -80°C. On the day of immunostaining, slides were thawed for 30min at room temperature followed by a 5 minute fixation in 4% paraformaldehyde in Tris-buffered saline (TBS). After a series of TBS washes, non-specific secondary antibody binding was blocked by incubating slides in 5% normal goat serum (NGS; Jackson ImmunoResearch) for 1 hour at room temperature. To prevent mouse antibodies from binding endogenous immunoglobulins, sections were incubated in TBS containing 'mouse on mouse' blocking reagent (Vector Laboratories). Then, sections were incubated overnight at 4°C in rabbit anti-Glut4 (1:100; gift from Dr. Jeffrey E. Pessin, Albert Einstein College of Medecine) and mouse anti-Cav3 (1:100; Santa Cruz) in 5%NGS/TBS. The next day sections were washed in TBS followed by incubation in an AlexaFluor 555 conjugated goat anti-rabbit (1:100 dilution; Life Technologies) and an AlexaFluor 647 conjugated goat anti-mouse secondary antibody (1:100 dilution; Life Technologies) in 5%NGS/TBS. Finally, sections were rinsed, coverslipped with Vectashield mounting medium (Vector Laboratories), and stored at 4°C in the dark. Control sections stained in the same manner as described above but lacking primary antibodies were used to demonstrate specificity of the secondary antibodies. Similarly, sections stained with only one primary antibody were used to ensure that secondary antibodies only recognized the correct primary antibody.

Image Acquisition

Images were acquired using an inverted Zeiss LSM 510 confocal microscope equipped with two helium-neon (HeNe) lasers (543nm and 633nm) and a 40X 1.3NA oil immersion Plan Neofluar objective (Zeiss). Alexa Fluor 555 was excited with the 543nm laser line of the HeNe laser and emitted light was collected using a 560-615nm bandpass emission filter. Alexa Fluor 647 was excited with the 633 nm laser line of the HeNe laser and emitted light was collected using a longpass 650nm filter. Sampling was performed at a resolution fulfilling the Nyquist criteria and with the pinhole set to give an optical section of 2µm. The two fluorophores were excited sequentially to prevent bleed-through and frame averaged 4 times. Detector settings were kept constant throughout imaging to allow for quantitative comparisons. 2 images were collected per section from 4 serial sections per mouse.

Image Analysis

All image analysis was performed using ImageJ (NIH). Initially, images were background corrected using the rolling ball radius background subtraction method. Then, automatically thresholded CAV3 immunostaining was used to create a mask demarcating the myofiber plasma membrane. This mask was applied to the GLUT4 (AlexaFluor 555) channel and the mean GLUT4 fluorescence intensity within this mask was measured. All image acquisition and analysis was performed by an investigator blind to diet and genotype.

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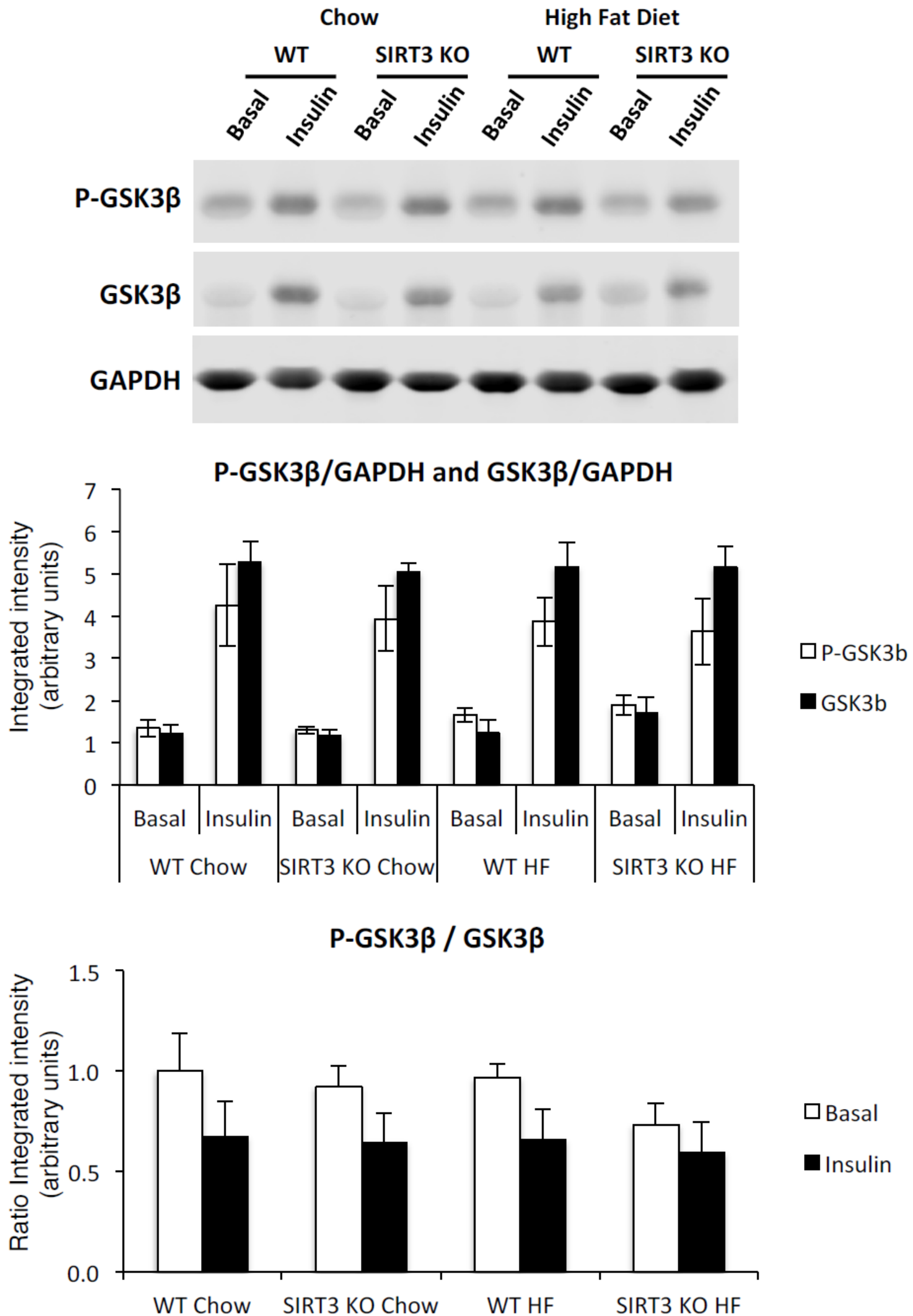
SUPPLEMENTARY DATA

Supplementary Figure S1. Gastrocnemius protein homogenates obtained from 5h-fasted (Basal) or clamped (Insulin) HF-fed mice were applied to a 4-12% SDS-PAGE (n=4). Western blotting was performed for GSK3β, P-GSK3β (Ser9) and GAPDH. Integrated intensities were obtained by the Odyssey software and normalized to GAPDH.



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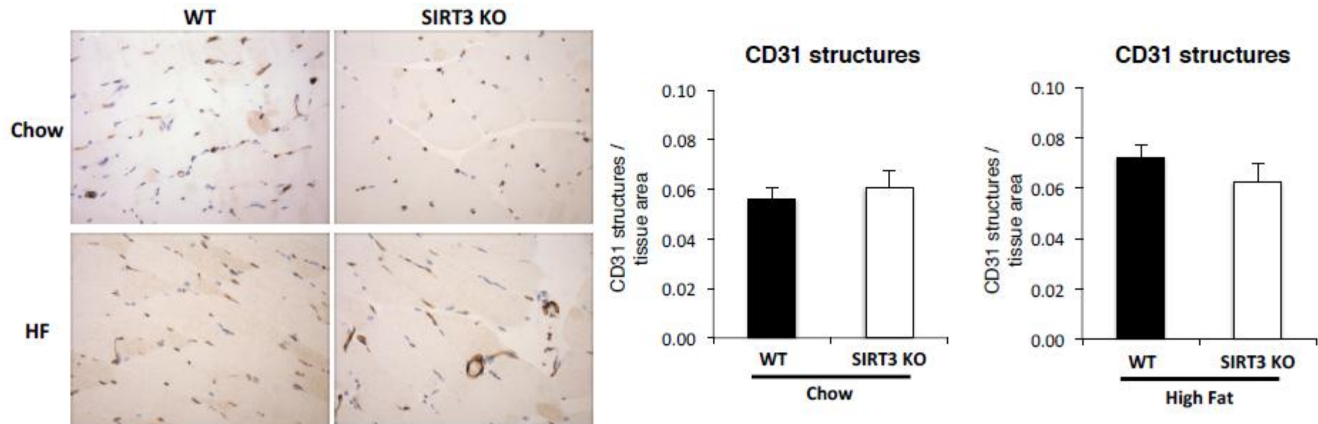
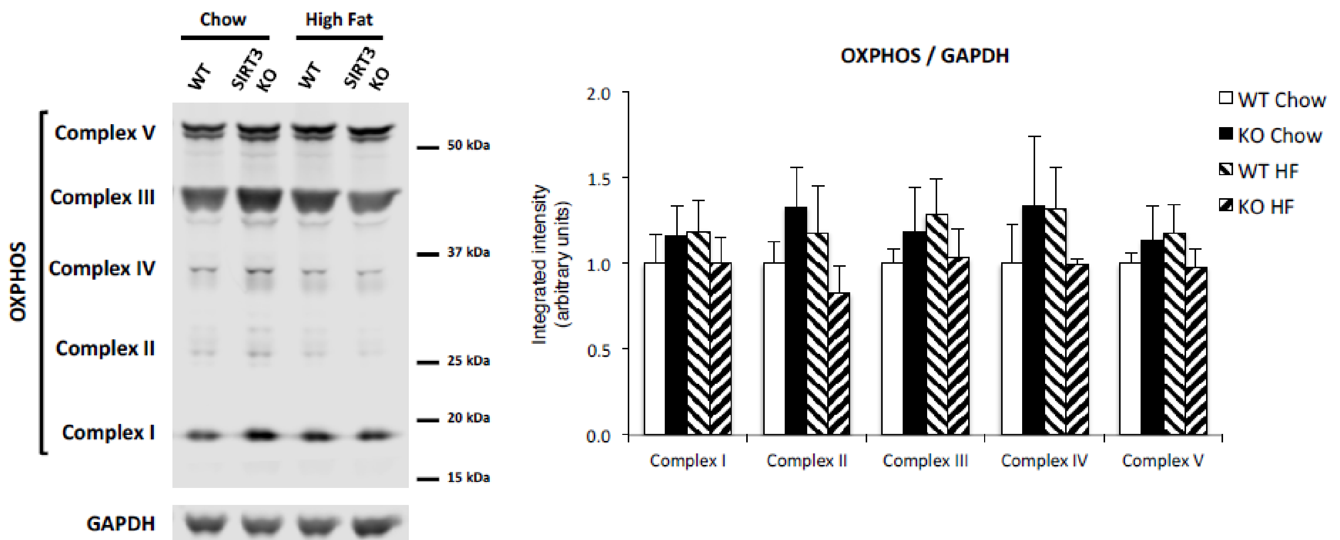
SUPPLEMENTARY DATA

Supplementary Figure S2. Paraffin-embedded gastrocnemius were stained for CD31. Representative images are presented. Number of CD31 positive structures were normalized to tissue area (n=8).



Supplementary Figure S3. Gastrocnemius protein homogenates obtained from 5h-fasted mice were applied to a 4-12% SDS-PAGE (n=4). Western blotting was performed for total OXPHOS. Integrated intensities were obtained by the Odyssey software and normalized to GAPDH.



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