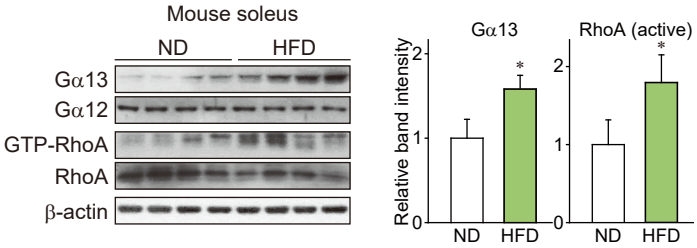
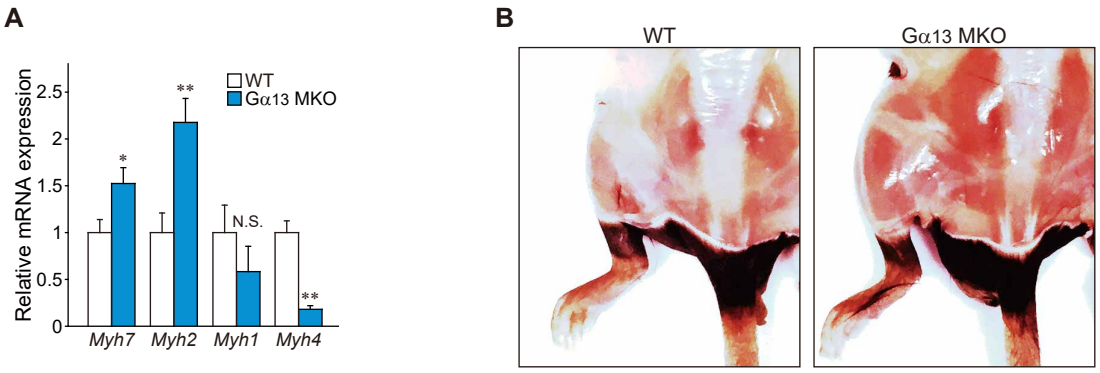


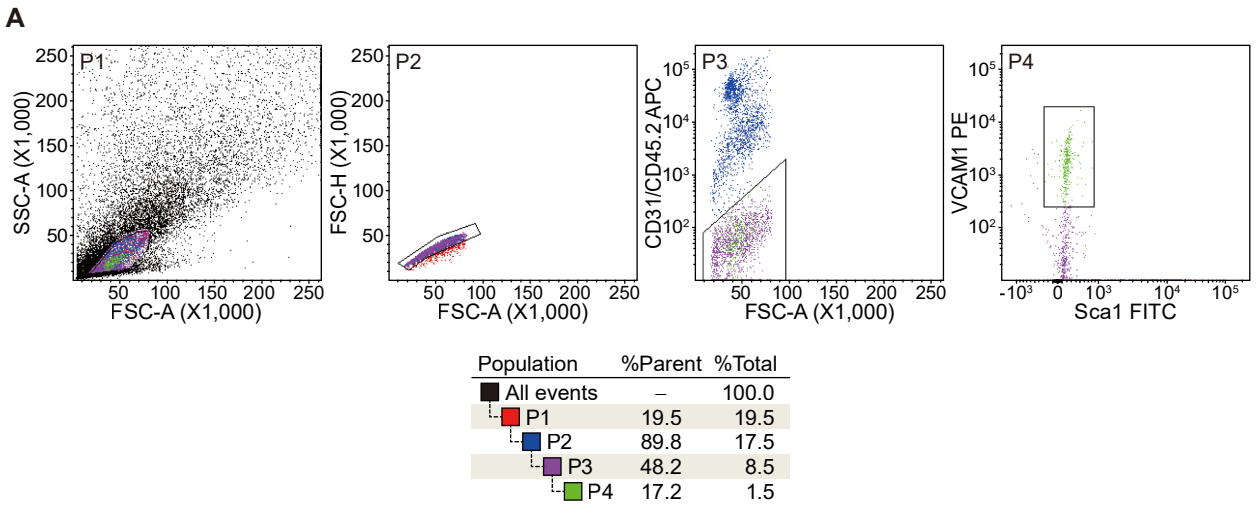
Supplemental Figure 1

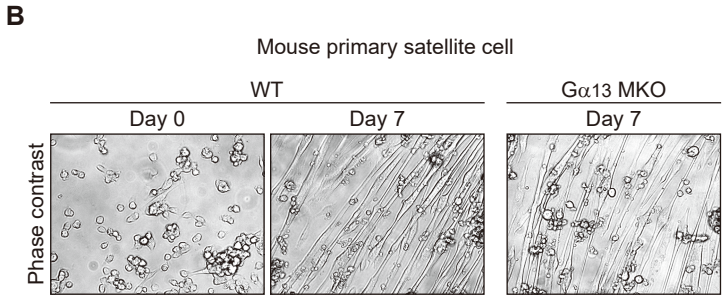
Supplemental Figure 1. Gα13 expression after high-fat diet feeding in soleus muscles. Immunoblottings for Gα13 and Gα12. Mice were fed with either normal diet (ND) or high-fat diet (HFD) for 12 weeks and fasted overnight. The blots were run in parallel using same samples. Quantifications of band intensities are represented as the mean ±SEM (Student's t test).

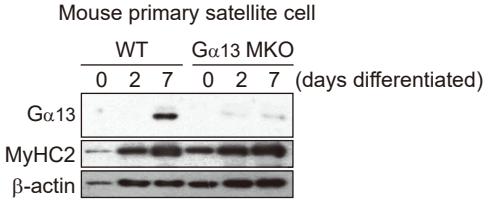


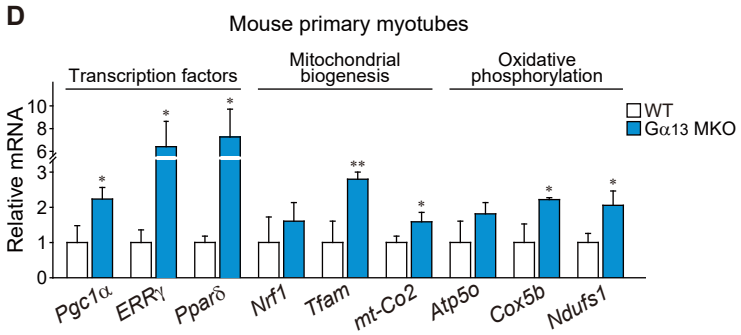
Supplemental Figure 2

Supplemental Figure 2. Muscle-specific Gα13 knockout alters fiber type. (**A**) qPCR assays for myosin heavy chain transcripts. mRNA levels of markers for type 1, 2a, 2x or 2b muscles (*Myh7*, *Myh2*, *Myh1* or *Myh4*, respectively) were examined in tibialis anterior muscles from 16-week-old mice fasted overnight. (**B**) Enhanced photographs for Figure 2**D**. Color contrasts of photographs were adjusted to an equal extent. For **A**, data were shown as the mean ±SEM. *$p<0.05$, **$p<0.01$ (Student's t test).



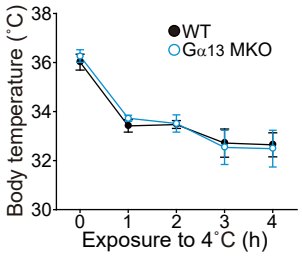






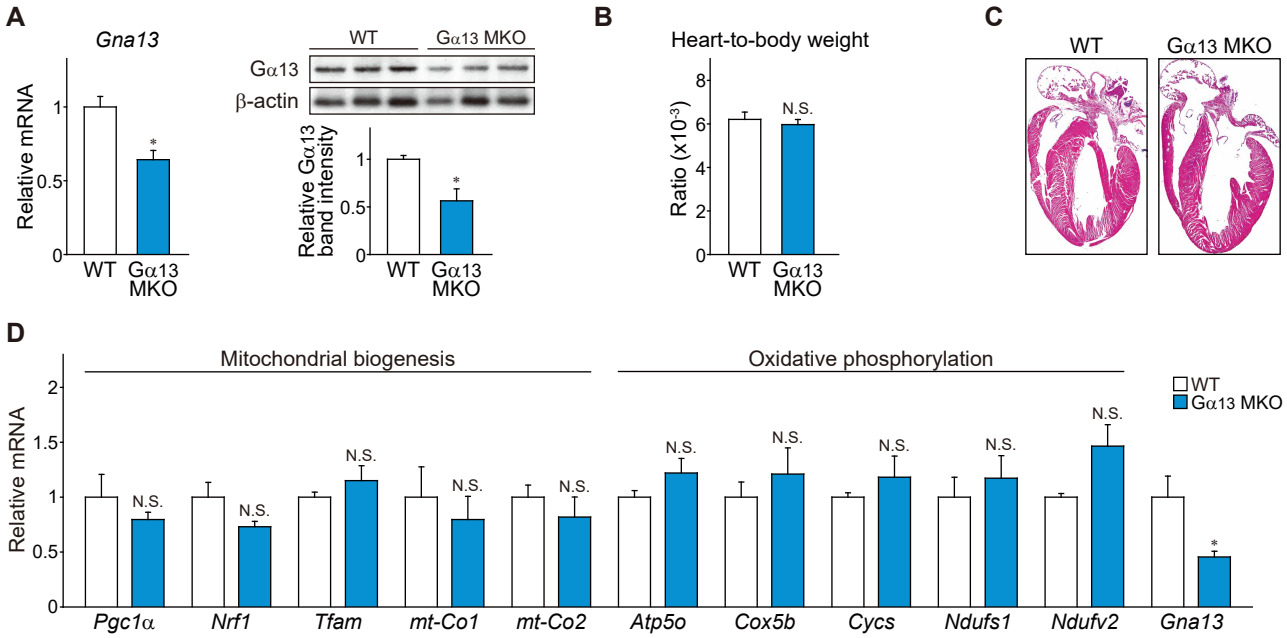
Supplemental Figure 3

Supplemental Figure 3. Isolation and validation of primary satellite cells. (**A**) Representative plots for FACS-based primary satellite cell isolation. After enzymatic digestion of hindlimb muscles pooled from both legs per mouse, cells were labled with APC anti-CD31, APC anti-CD45.2, FITC anti-Sca1 and PE anti-VCAM1 antibodies. Cells in the P4 gate were eventually sorted as satellite cells. The population hierarchy is shown under. (**B**) Phase contrast images of isolated satellite cells. Cells from 12-week-old WT or Gα13 MKO mice were differentiated to myotubes for 7 d. (**C**) Immunoblotting for Gα13. Primary satellite cells from each genotype were harvested before or after differentiation for 2 or 7 d. The blots were run in parallel using same samples. (**D**) qPCR assays. Primary satellite cells from WT or Gα13 KO mice were cultured and collected 7 d after differentiation to myotubes.



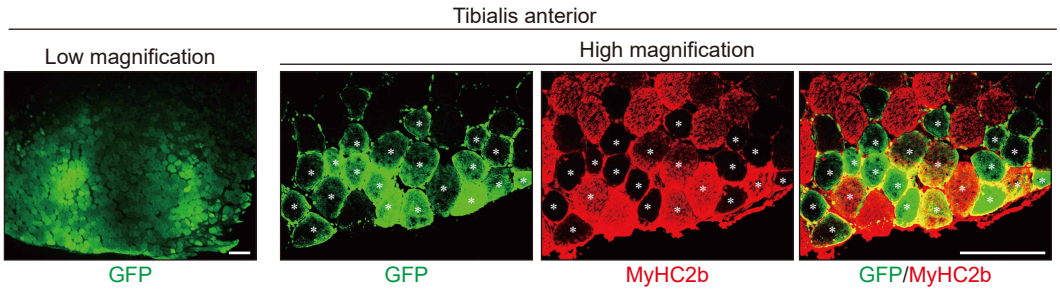
Supplemental Figure 4

Supplemental Figure 4. Core body temperature during acute cold exposure. Following overnight fasting, nine-week-old mice of each genotype were housed individually in a cold room of 4℃. Core temperatures were monitored every 1 h using a rectal thermometer (n=4-5 each). Data were shown as the mean ±SEM (Student's t test).



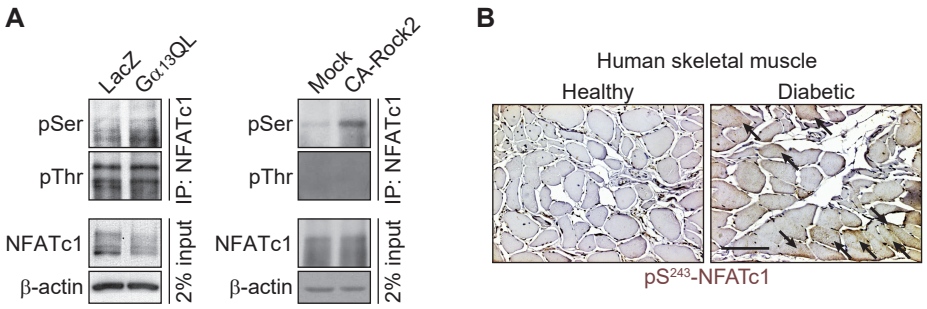
Supplemental Figure 5

Supplemental Figure 5. Gene expression and morphological analysis in heart tissues of WT and Gα13 MKO mice. (**A-D**) Twelve-week-old mice of each genotype were fasted overnight prior to sacrifice. (**A**) qPCR analyses (*left*) and immunoblottings (*right*) for Gα13 in heart tissues of WT and Gα13 MKO mice (n=3 each). The blots were run in parallel using same samples. (**B**) Heart-to-body weight ratio (n=3 each). (**C**) Representative H&E images of whole hearts (n=3 each). (**D**) qRT-PCR assays (n=3 each). For **A**, **B** and **D**, data were shown as the mean ±SEM. *$p<0.05$ (Student's t test).



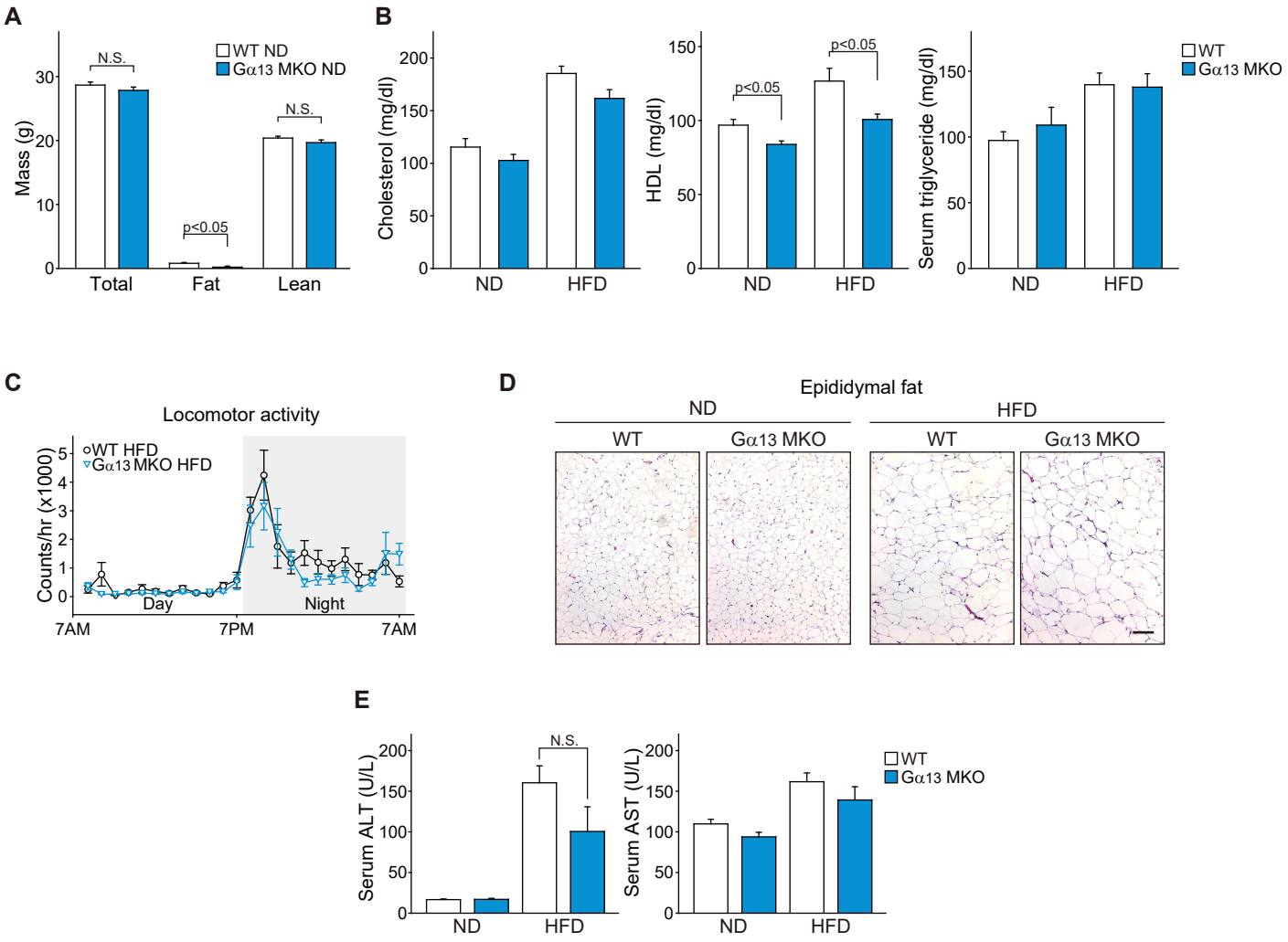
Supplemental Figure 6

Supplemental Figure 6. Validation of in vivo electroporation. Following an intramuscular injection of GFP-expressing plasmid, tibialis anterior muscles received electric shocks for gene delivery. Shown above are the representative images of native GFP fluorescence and MyHC2b immunostaining 5 d after electroporation-mediated gene delivery. Asterisks indicate GFP^+ fibers. Scale bars, 100 μm.



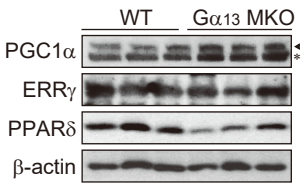
Supplemental Figure 7

Supplemental Figure 7. NFATc1 phosphorylation is promoted by an active mutant of Gα13 (or Rock2), or in a diabetic patient muscle. (**A**) Immunoprecipitation assays. C2C12 myotubes were transfected with the plasmid encoding Gα13QL or CA-Rock2, and were harvested 24 h afterward. NFATc1 immunoprecipitates obtained from the lysates were immunoblotted for phosphoserine or phosphothreonine. (**B**) Immunohistochemistry for $pSer^{243}$-NFATc1 in the human specimens used in Figure 1**G**. Scale bar, 100 μm.



Supplemental Figure 8

Supplemental Figure 8. Changes in the metabolic profile of Gα13 MKO mice. (**A**-**E**) Nine-week-old WT or Gα13 MKO mice were fed on normal (ND) or high-fat diet (HFD). After 9 weeks of HFD feeding, the mice were fasted overnight prior to sacrifice. (**A**) Fat and lean mass of WT and Gα13 MKO mice before high-fat diet feeding (n=8 each). (**B**) Serum lipid levels. Total or HDL cholesterol and triglyceride levels in serum were examined immediately after sacrifice (n=6-8 each). (**C**) Locomoter activities of mice over time. The high-fat fed mice were housed in individual metabolic cages (n=8). (**D**) Representative H&E images of epididymal fat (n=6-8 each). Scale bar, 200 μm. (**E**) Serum transaminase activities. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were examined immediately after sacrifice (n=6-8 each). For **A**-**C** and **E**, data were shown as the mean ±SEM (Student's t test).



Supplemental Figure 9

Supplemental Figure 9. Immunoblottings for PGC1α, ERRγ and PPARδ in tibialis anterior muscles. Twelve-week-old WT or Gα13MKO mice were fasted overnight prior to sacrifice. The blots were from different gels run in parallel using same samples.

Supplemental Table 1 Changes in gene expression related to muscle contractile machinery, glucose/fat metabolism

Lipid metabolism				*Glucose metabolism*				*Muscle contraction*			
Symbol	KO/WT	S.E.*	Adj. p-value[#]	Symbol	KO/WT	S.E.*	Adj. p-value[#]	Symbol	KO/WT	S.E.*	Adj. p-value[#]
Nr1d1	0.63	0.15	2.9E-04	Nr1d1	0.63	0.15	2.9E-04	Myh4	0.19	1.31	7.6E-43
Nr1d1	0.60	0.11	0.061	Nr1d1	0.60	0.11	0.061	Myh4	0.19	1.19	9.1E-41
Nr1d1	0.64	0.11	0.130	Nr1d1	0.64	0.11	0.130	Myh4	0.19	1.24	2.2E-36
Chpt1	0.57	0.38	0.241	Pfkfb2	1.61	0.17	0.785	Myh4	0.19	1.28	2.5E-35
Serinc1	0.63	0.19	0.281	Nr1d1	0.66	0.07	0.899	Myh4	0.19	1.39	3.5E-34
Ctdnep1	1.52	0.17	0.309	Taldo1	0.51	0.25	0.958	Myh4	0.09	1.83	2.6E-26
Sgms1	0.33	0.62	0.314	Inppl1	0.62	0.17	0.968	Myh4	0.09	1.93	2.4E-20
Mtmr2	0.60	0.46	0.439	Ptpn2	2.22	0.33	1	Myh4	0.17	1.50	1.9E-19
Pex7	0.49	0.40	0.535	Smek2	1.83	0.39	1	Myh4	0.40	0.85	5.6E-12
Ptpla	0.41	0.27	0.566	Lcmt1	1.71	0.43	1	Ttn	0.61	0.26	1.9E-03
Lrp1	0.63	0.12	0.652	Pfkfb2	1.63	0.39	1	Myl6b	0.65	0.05	0.17
Dab2	0.62	0.44	0.677	Foxo1	1.57	0.13	1	Myh13	0.65	0.32	0.23
Agps	0.57	0.64	0.731	Sirt1	1.56	0.33	1	Myl6b	0.61	0.07	0.55
Dab2	0.60	0.19	0.733	Pfkfb1	1.56	0.14	1	Nos1	1.71	0.24	0.60
Rbl2	1.51	0.04	0.798	Pfkfb1	1.56	0.12	1	Myl6b	0.64	0.09	0.63
Eci2	0.55	0.23	0.811	Npy1r	1.54	0.40	1	Pde4b	0.36	0.24	0.64
Plcb1	0.55	0.34	0.816	Aldh5a1	1.51	0.23	1	Actn2	0.59	0.30	0.69
Cd74	0.66	0.26	0.843	Prkaa1	0.65	0.32	1	Glra1	0.53	0.25	0.98
Smpd4	0.35	0.28	0.881	Igf1	0.65	0.35	1	Pde4b	0.66	0.24	1.00
Pnpla8	0.61	0.37	0.899	Enpp1	0.65	0.24	1	Nos1	1.68	0.28	1
Nr1d1	0.66	0.07	0.899	Pgd	0.64	0.33	1	Tpm1	1.61	0.39	1
Aldh1a2	0.50	0.18	0.922	Rpe	0.64	0.13	1	Cacna1s	0.66	0.41	1
Crot	1.59	0.11	0.934	Pik3ca	0.63	0.26	1	Camk2d	0.64	0.53	1
Dab2	0.64	0.31	0.935	Ganc	0.63	0.17	1	Mybpc2	0.64		1
Agps	1.98	0.28	0.938	Prkaa1	0.62	0.17	1	Mybpc2	0.63		1
Pigc	2.02	0.32	0.940	Ganc	0.62	0.84	1	Actn3	0.59	0.78	1
Inppl1	0.62	0.17	0.968	Pgd	0.61	0.47	1	Pde4b	0.58	0.24	1
Rbl1	0.49	0.33	0.993	Nr3c1	0.61	0.20	1	Mybpc2	0.58		1
C3	0.63	0.13	0.993	H6pd	0.60	0.37	1	Actn3	0.57	0.78	1
Acsl3	3.18	0.96	1	Fabp5	0.58	0.33	1	Cald1	0.56		1
Mtm1	2.08	0.61	1	Atf3	0.58	0.33	1	Actn3	0.56	0.56	1
Pdss1	1.96	0.49	1	Enpp1	0.51	0.27	1	Ttn	0.54	0.17	1
Phyh	1.87	0.20	1	Smek2	0.49	0.40	1	Actn3	0.51	0.93	1
Crot	1.85	0.23	1	Gfpt1	0.49	0.53	1	Actn3	0.51	0.88	1
Smek2	1.83	0.39	1					Actn3	0.49	0.76	1
Sphk2	1.81	0.16	1					Actn3	0.48	0.92	1
Hpgd	1.80	0.27	1					Actn3	0.48	0.96	1
Aldh3a2	1.80	0.21	1					Actn3	0.46	1.00	1
Mgll	1.76	0.69	1					Actn3	0.46	1.00	1
Ptplad1	1.73	0.31	1					Actn3	0.46	0.93	1
Tiparp	1.72	0.18	1					Actn3	0.44	0.92	1
Thrsp	1.71	0.16	1					Actn3	0.43	0.87	1
Anxa1	1.71	0.41	1								
Atm	1.70	0.28	1								
Ocrl	1.70	0.51	1								
Serac1	1.69	0.28	1								
Ppt1	1.66	0.23	1								
Ptges2	1.66	0.22	1								
Ces1f	1.66	0.15	1								
Serinc5	1.64	0.58	1								
Ptplad1	1.64	0.14	1								
Oxsm	1.63	0.30	1								
Abca17	1.63	0.16	1								
Gdpd1	1.63	0.32	1								
Pafah2	1.62	0.36	1								

H2-Ke6	1.61	0.17	1
Hacl1	1.61	0.36	1
Mtm1	1.61	0.11	1
Smpdl3a	1.60	0.28	1
Galc	1.60	0.42	1
Ptges3	1.59	0.23	1
Them4	1.56	0.22	1
Pparg	1.56	0.35	1
Sirt1	1.56	0.33	1
Sult1e1	1.54	0.52	1
Naaa	1.54	0.34	1
Pik3c3	1.53	0.21	1
Acly	1.53	0.21	1
Bmpr1b	1.52	0.20	1
Nphp3	1.51	0.21	1
Ehhadh	1.51	0.14	1
Ptpmt1	1.51	0.38	1
Aldh5a1	1.51	0.23	1
Alg13	1.51	0.25	1
Acsl4	1.50	0.32	1
Lta4h	0.67	0.29	1
St8sia4	0.67	0.37	1
Slc35c1	0.67	0.11	1
Dgat1	0.66	0.21	1
Tecrl	0.66	0.28	1
Mtmr3	0.66	0.24	1
Ptges2	0.66	0.34	1
Snca	0.66	0.28	1
Soat1	0.66	0.22	1
Pign	0.66	0.27	1
Gm2a	0.65	0.16	1
Prkaa1	0.65	0.32	1
Igf1	0.65	0.35	1
Ip6k2	0.65	0.47	1
Echs1	0.65	0.19	1
Pi4k2a	0.65	0.26	1
Dgkz	0.65	0.30	1
Rb1	0.65	0.25	1
Mapk9	0.65	0.38	1
Pi4kb	0.64	0.49	1
Dpagt1	0.64	0.24	1
Crtc3	0.64	0.43	1
Hexb	0.64	0.44	1
Abhd12	0.64	0.44	1
Lonp2	0.64	0.45	1
Rb1	0.64	0.41	1
App	0.64	0.27	1
Atm	0.64	0.48	1
Pik3ca	0.63	0.26	1
Thra	0.63	0.22	1
Rbp1	0.63	0.38	1
Prkab2	0.63	0.18	1
St3gal6	0.63	0.17	1
4932438A13	0.63	0.27	1
Pdss2	0.63	0.23	1
Anxa1	0.63	0.22	1
Pld3	0.63	0.32	1
Taz	0.63	0.19	1

Ddhd1	0.62	0.44	1
Thrb	0.62	0.62	1
Impa1	0.62	0.22	1
Prkaa1	0.62	0.17	1
Stub1	0.62	0.36	1
Lrp1	0.61	0.19	1
Nr3c1	0.61	0.20	1
Pigl	0.61	0.32	1
Pex5	0.61	0.40	1
St3gal6	0.60	0.28	1
Crem	0.60	0.43	1
Fgf2	0.60	0.42	1
Tek	0.60	0.35	1
Dhrs9	0.59	0.32	1
Sgpl1	0.59	0.39	1
Elovl5	0.59	0.36	1
4932438A13	0.59	0.52	1
Idi1	0.59	0.61	1
Fabp5	0.58	0.33	1
Fads3	0.58	0.47	1
Ugcg	0.58	0.36	1
Tecrl	0.58	0.35	1
Golm1	0.58	0.16	1
Asah1	0.58	0.61	1
Bscl2	0.58	0.21	1
Acsf2	0.58	0.21	1
Oxsm	0.58	0.30	1
Mtm1	0.57	0.32	1
Dgat1	0.57	0.40	1
Smg1	0.57	0.32	1
Pik3c3	0.55	0.33	1
Asah1	0.55	0.76	1
Fig4	0.55	0.53	1
4932438A13	0.54	0.39	1
Cd74	0.54	0.70	1
Agtr1a	0.54	0.26	1
Pex7	0.53	0.61	1
Ocrl	0.53	0.69	1
Alg13	0.52	0.40	1
Inpp5j	0.52	0.28	1
Bbs4	0.52	0.33	1
Npc1	0.50	0.89	1
Adh5	0.50	0.73	1
Smek2	0.49	0.40	1
Aplp2	0.49	0.32	1
Pi4kb	0.49	0.39	1
Adora1	0.48	0.32	1
Nsdhl	0.48	0.54	1
Pnpla8	0.46	0.80	1
Adh4	0.46	0.15	1
Cyp2j6	0.44	0.74	1
Cyp27a1	0.43	0.44	1
Sgpl1	0.42	0.46	1
Far1	0.41	0.60	1
St8sia4	0.39	0.67	1

* Standard error; # Adjusted p value.

Supplemental Table 2 Primers for qPCR

Gene symbol	Forward	Reverse
Acadl	GCATCAACATCGCAGAGAAA	GGCTATGGCACCGATACACT
Acadm	TTGAGTTGACGGAACAGCAG	CCCCAAAGAATTTGCTTCAA
Acads	TTGCCGAGAAGGAGTTGGTC	AGGTAATCCAAGCCTGCACC
Acox1	ACTACCTGGACAGCCAATGC	CCCGACTGAACCTGGTCATA
Actb	CTGAGAGGGAAATCGTGCGT	TGTTGGCATAGAGGTCTTTACGG
ATP5o	TCTCGACAGGTTCGGAGCTT	AGAGTACAGGGCGGTTGCATA
Cd36	GATGACGTGGCAAAGAACAG	TCCTCGGGGTCCTGAGTTAT
Cox5b	TTCAAGGTTACTTCGCGGAGT	CGGGACTAGATTAGGGTCTTCC
Cpt1b	AAGAGACCCCGTAGCCATCAT	GACCCAAAACAGTATCCCAATCA
Cycs	CCAAATCTCCACGGTCTGTTC	ATCAGGGTATCCTCTCCCCAG
Esrrg	GCAAGGCATTCTTCAAGAGG	GGCTGGGCAGCTGTACTCTA
Gna12	TGCTTCGACGGCATCACA	GAAGTGCTTCTTAATGCTCACA
Gna13	GGAGACTCGAGTGTTCCTGC	TGGAATGTAATCCGGTACTCCAA
Lpl	CCCTACAAAGTGTTCCATTA	CTCGCTCTCGGCCACTGT
Mt-co1	ACTATACTACTAACAGACCG	GGTTCTTTTTTTCCGGAGTA
Mt-co2	AACCATAGGGCACCAATGATAC	GGATGGCATCAGTTTTAAGTCC
Myh1	CTCTTCCCGCTTTGGTAAGTT	CAGGAGCATTTCGATTAGATCCG
Myh2	TCACATCCAACAAGAAGCCAGAGC	CCCTGGCTGACAAATGGGTAATCA
Myh4	AGTCCCAGGTCAACAAGCTG	TTTCTCCTGTCACCTCTCAACA
Myh7	ACCAGGCCCTTTGACCTCAAGAAA	TCTTGTCGAACTTGGGTGGGTTCT
Ndufs1	AGGATATGTTCGCACAACTGG	TCATGGTAACAGAATCGAGGGA
Ndufv2	GCAAGGAATTTGCATAAGACAGC	TAGCCATCCATTCTGCCTTTG
Nfatc1	GGTAACTCTGTCTTTCTAACCTTAAGCTC	GTGATGACCCCAGCATGCACCAGTCACAG
Nrf1	GGAGCACTTACTGGAGTCC	CTGTCCGATATCCTGGTGGT
Ppargc1a	ACGAGGCCAGTCCTTCCTCC	AGCTCTGAGCAGGGACGTCT
Ppard	GACCAGAACACACGCTTCCT	CCGACATTCCATGTTGAGG
Rps18	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Tfam	GCAAAGGATGATTCGGCTCAGGGAA	CCGGATCGTTTCACACTTCGACGG

Supplemental Materials and Methods

Animals.

We bred Ckmm-Cre (Jackson Laboratory, backcrossed with C57BL/6 for 10 generations) mice with $Gna13^{fl/fl}$ mice (a gift from Stefan Offermanns, Max Plank Institute, Bad Nauheim, Germany; backcrossed with C57BL/6 at least six generations)(1) to generate Gα13 MKO mice, which were then bred again with $Gna13^{fl/fl}$ mice to produce control littermates with Gα13 MKO mice. The above control mice developed and behaved the same as WT mice. Mice were maintained in a 12 h dark/light cycle and fed with normal rodent chow or HFD (D12492, Research Diets). All mice used were male and had C57BL/6 background. The group sizes were chosen based on our experience with high-fat diet models without using a statistical method to predetermine sample size. For experiment, mice of similar age and weights were randomly subgrouped and subjected to treatments. Due to the experimental design, the genotypes of the mice could not be blinded. All animal use and studies were approved by the IACUC of the Seoul National University and by the IACUC of Center of Animal Care and Use at Lee Gil Ya Cancer and Diabetes Institute, Gachon University. All human patient samples were obtained from Genetex.

qPCR and western blot analyses.

Total RNA was extracted using TRIzol (Invitrogen). Gene expression was assessed by qPCR analysis using the primers shown in Supplemental Table 2. Protein lysates were quantified, separated by SDS-PAGE and transferred to a nitrocellulose membrane (GE Healthcare) followed by immunoblotting with the primary antibodies listed below. Immunoprecipitation assays were performed with previously described method using indicated antibodies, followed by western blot analysis (2). Rabbit polyclonal antibody against phospho-Ser241-NFATc1 was raised against HSPST(pS)PRASITEEC by Genscript. The antibody was determined to be ten times specific to phosphorylated peptide over non-phosphorylated form, by ELISA. Antibodies to Gα13 (sc-410), Gα12 (sc-409), RhoA (sc-418), $pSer^{259}$-NFATc1 (sc-23979), NFATc1 (sc-7294), His-tag (sc-803), PGC1α (sc-13067) and PPARδ (sc-74517) were from Santa Cruz and used in 1:1000 dilution. Antibodies to Calcineurin A (2614), Lamin A/C (2032), HA-tag (3724), FLAG-tag (8146), Rock2 (9029), pMYPT1 (5163), $pSer^{307}$-IRS1 (2381), IRS1 (3407), pJNK (9251), JNK

(9252), pThr^{308}-Akt (9275), pSer^{473}-Akt (9271), Akt (4685), pGSK3β (9323), GSK3β (9315) and pSer235/236-S6 (2211) were from Cell Signaling and used in 1:10000 dilution. Anti-S6 antibody (GTX113542) from Genetex was used in 1:5000 dilution. Anti-ERRγ antibody (ab128930) was used in 1:10000 dilution. Antibody used to immunoprecipitate GTP-bound RhoA (26904) was from Neweast Biosciences. Anti-β-actin antibody (A5441) was from Sigma. Horseradish peroxidase-conjugated goat anti-rabbit (G21234) and goat anti-mouse IgGs (G21040) were from Invitrogen.

Histological analysis.

Immunohistochemistry. Skeletal muscle samples were immediately frozen with liquid nitrogen–cooled isopentane after dissection and sectioned on a cryostat microtome. Liver, adipose tissue, and pancreas were fixed in 4% formadehyde and analyzed after. Immunofluorescence staining was performed by the routine protocol which was previously described (3), with antibodies to MyHC1 (1:100, Abcam ab11083), MyHC2a (1:200, DSHB SC-71), MHC2b (1:100, DSHB BF-F3), Gα13 (1:250, Abcam ab128900) and pSer241-NFATc1 (Genscript, described above).

Succinate dehydrogenase staining. Cross-sections of individual skeletal muscles were cut from the mid-belly region of the snap-frozen muscle. After drying for 5 min at room temperature, the sections were incubated for 30 min in a 0.2 M sodium phosphate buffer solution containing 0.6 mM nitrotetrazolium blue chloride (Sigma N6876) and 50 mM sodium succinate. The sections were then rinsed three times for 30 s each in physiological saline, for 5 min in 15% ethanol and mounted.

Oil Red O staining. Muscle samples were embedded in OCT and sectioned. Oil Red O stock solution (0.5 g of Oil Red O (Sigma) in 100 ml of isopropanol) was diluted with water (60:40 (vol/vol)), followed by filtration. After staining, samples were washed several times in water. The sections were lightly counterstained with haematoxylin.

Transmission electron microscopy. Soleus was fixed in Karnovsky's fixative immediately after isolation. Samples were then post-fixed by 1% osmium tetraoxide in 0.05M sodium cacodylate buffer. After overnight incubation with 0.5% uranyl acetate, samples were dehydrated in ethanol and propylene oxide and polymerized in Spurr's resin.

Observation was performed by the National Instrumentation Center for Environmental Management at Seoul National University. The images were taken using a LIBRA 120 transmission electron microscope (Zeiss).

Treadmill running.

We performed treadmill running studies using a motorized, speed-controlled treadmill system. Running speed was set to 10 m/min for 30 min and increased by 2 m/min increments every 10 min until 16 m/min. The inclination angle was 5%. Male mice were trained at a speed of 10 m/min in three sessions to acquire running skills before running tests. Mice were determined to be exhausted and excluded if they resist to run and rest on the electric shock grid for over 20 s. WT mice were subjected to run until exhaustion and rested for 4 h. In a separate study, WT and Gα13 MKO mice were allowed to run until exhaustion for the measurements of total running time and distance. Mice were anesthetized, and skeletal muscles were dissected for measure protein levels.

Electroporation-mediated gene transfer.

Under anesthesia, both tibialis anterior (TA) muscles were injected with hyaluronidase (40 μg). After 3 hours, 80 μg of plasmid vector encoding for shNFATc1 (5'-GAGGCTATAAGAGGATGTTGT-3') was injected into one TA muscle and for shControl (5'-TTCTCCGAACGTGTCACGT-3') to the contralateral muscle. In another set of experiment, vectors encoding WT NFATc1 or S243D mutant NFATc1 were used. Eight electric pulses (50 ms, 1 Hz, 62.5 V) were delivered immediately to the injected TA muscle using a square-pulse stimulator (Harvard Apparatus ECM830) through a two-needle array placed on the medial and lateral sides of the TA muscle, so that the electrical field was perpendicular to the long axis of the myofibers. Mice were sacrificed to isolate muscles 14 d following injection.

Glucose tolerance test (GTT) and insulin tolerance test (ITT).

For GTT, mice fed with high-fat diet for 7 weeks or normal chow were fasted overnight (16 h) and injected intraperitoneally with a glucose solution (2g/kg body weight). Blood glucose concentrations were measured before

and 15, 30, 60 and 120 min after glucose injection. For ITT, mice fed on high-fat diet for 9 weeks were prefasted overnight and i.p. injected with insulin at 0.75 U/kg body weight. Blood glucose concentrations were measured before and 30, 60, 90 and 120 min after insulin injection.

Mouse metabolic studies.

Fat and lean body masses were measured by ^{1}H minispec system (LF90II, Bruker Optik) in mice. To measure whole-body energy metabolism in HFD-fed mice, a comprehensive laboratory animal monitoring system (CLAMS; Columbus Instruments) was used for 4 d (2 d of acclimation followed by 2 d of measurement).

Hyperinsulinemic–euglycemic clamp.

After an overnight fast, [3-^{3}H]-glucose (HPLC purified; PerkinElmer, USA) was infused at a rate of 0.05 μCi/min for basal 2 h to assess the basal glucose turnover. Following the basal period, hyperinsulinemic-euglycemic clamp was conducted for 150 min with a primed/continuous infusion of human insulin (21.4 mU per kg during priming and 3 mU per kg per min during infusion, Eli Lilly), while plasma glucose was maintained at basal concentrations as previously described with slight modification (4). To estimate insulin-stimulated whole body glucose fluxes, [3-^{3}H]-glucose was infused at a rate of 0.1 μCi/min throughout the clamps. In addition, 2-deoxy-D-[1-^{14}C]glucose (2-[^{14}C]DG; PerkinElmer, USA) was injected as a bolus at the 125th min of the clamp to estimate the rate of insulin-stimulated tissue glucose uptake. At the end of the experiment, tissues were taken for the measurement of 2-DG uptake. Rates of basal and insulin-stimulated whole-body glucose fluxes were calculated at the end of the basal period and during the 90-120 min of the clamp experiment, respectively. Tissue glucose uptake was measured during the final 25 min of the clamp study. Rates of basal and insulin-stimulated whole-body glucose fluxes and tissue glucose uptake were determined as previously described (4).

Muscle cell culture and differentiation.

C2C12 myoblasts. Cells were obtained from ATCC free of mycoplasma contamination and cultured in DMEM containing 10% FBS. For CRISPR-mediated gene knockout, vectors encoding for Cas9 with sgRNAs targeting

Gα13 or NFATc1 were obtained from Sigma. The vectors were co-transfected with another vector encoding for puromycin N-acetyl-transferase. After 3 d of selection with puromycin treatment, single colonies were selected for validation of gene knockout and subcultured. Myotube differentiation was initiated by switching confluent C2C12 to DMEM containing 2% horse serum. All studies were performed in differentiated myotubes.

Primary satellite cells. Primary satellite cells were isolated by flow cytometer-based cell sorting. Briefly, hindlimb muscles were first undergone enzymatic digestion with type 2 collagenase and dispase. Then the cells were washed and labeled with APC anti-CD31, APC anti-CD45.2, FITC anti-Sca1, and PE anti-VCAM1 antibodies. Using FACS Aria (BD Biosciences), about 1.5% of total population was $CD31^{-}CD45.2^{-}Sca1^{-}VCAM1^{+}$ cells and they were sorted as satellite cells. For differentiation, the cells were plated on gelatin-coated cell culture miniplates and differentiated with media containing 5% horse serum for 7 d.

Cellular respiration assays.

Oxygen consumption was measured using the XFp Extracellular Flux Analyzer (Seahorse Bioscience). One hour before the experiment, cells were washed and incubated in non-buffered differentiation medium. During the time-course of the experiment, oxygen concentration was measured over time periods of 3 min at 3-min intervals. OCR over the 3 min measurement period was calculated using Fixed Delta technique for determining the slope. In addition to the basal rates, uncoupled (by addition of oligomycin), maximal (with FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone) and non-mitochondrial respiration rates (with rotenone plus antimycin A) were determined.

Triglyceride measurement.

TA muscles were homogenized in 5% NP-40 solution. 0.1 M Tris–acetate buffer (pH 7.4) containing 0.1 M potassium chloride and 1 mM EDTA. Six volumes of chloroform/methanol (2:1) were then added. After vigorous stirring, the mixtures were incubated on ice for 1 h and then centrifuged at 800g for 3 min. The resulting lower phase was aspirated. Quantification of triglycerides was done using a colorimetric quantification reagent (Sigma) under manufacturer's protocol.

***Ex vivo* uptake assays for fatty acid and glucose.**

Fatty acid uptake assay. Muscles were isolated from 50-week-old mice of each genotype and immediately soaked in DMEM supplemented with 0.5 μCi/ml [9,10-^{3}H]-palmitate in presence of 2% BSA and 0.2 mM cold palmitate. After 30-minute incubation in 30℃, tissues were washed thoroughly and lysed with 50℃ 1 M KOH solution for 1 h. Samples were then used for scintillation counting.

Glucose uptake assay. A kit from Abcam was used. Briefly, soleus muscles were isolated from 18-week-old mice and immediately soaked in DMEM supplemented in presence of insulin and 2-deoxyglucose. After 15-min incubation, tissues were washed thoroughly and lysed. The levels of 2-deoxyglucose in lysates were then determined by an enzymatic recycling amplification reaction.

Microarray.

For the quality control, RNA purity and integrity were evaluated by OD 260/280 ratio, and analyzed by Agilent 2100 Bioanalyzer (Agilent Technologies). The Affymetrix Whole transcript expression array was performed according to the manufacturer's protocol (GeneChip Whole Transcript PLUS reagent Kit). cDNA was synthesized using the GeneChip WT (Whole Transcript) Amplification kit as described by the manufacturer. The sense cDNA was then fragmented and biotin-labeled with TdT (terminal deoxynucleotidyl transferase) using the GeneChip WT Terminal labeling kit. Approximately 5.5 μg of labeled DNA target was hybridized to the Affymetrix GeneChip Mouse 2.0 ST Array at 45℃ for 16 h. Hybridized arrays were washed and stained on a GeneChip Fluidics Station 450 and scanned on a GCS3000 Scanner (Affymetrix). Raw data were extracted automatically in manufacturer's data extraction protocol using Affymetrix GeneChip Command Console Software. After importing CEL files, the data were summarized and normalized with robust multi-average method. We exported the result with gene level RMA analysis and performed the differentially expressed gene (DEG) analysis. The comparative analysis between test sample and control sample was carried out using LPE test and fold change in which the null hypothesis was that no difference exists among 2 groups. False discovery rate was controlled by adjusting *p* value using Benjamini-Hochberg algorithm. For a DEG set, Hierarchical cluster analysis was performed using complete linkage and Euclidean distance as a measure of similarity. All statistical test and visualization of differentially expressed genes

was conducted using R statistical language (www.r-project.org). The raw transcriptomics data have been deposited in the Gene Expression Omnibus (GEO) under the accession code GSE83737.

Preparation of nuclear extracts.

Cells were allowed to swell after the addition of hypotonic buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% NP-40, 1 mM DTT, and 0.5 mM PMSF. The lysates were incubated for 10 min on ice and centrifuged at 7,200g for 5 min. Pellets containing crude nuclei were then resuspended in an extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 10 mM DTT and 1 mM PMSF fluoride, and were incubated for 30 min on ice. The samples were centrifuged at 15,800g for 10 min to obtain supernatants.

Transfection of plasmids and siRNAs.

FLAG-Rock2 expression clone was purchased from Genecopoeia. The construct encoding for WT-NFATc1 was a gift from Anjana Rao (La Jolla Institute for Allergy and Immunology, La Jolla, CA)(5), whereas that for NFAT activity luciferase was from Gerald R Crabtree (Stanford University, Stanford, CA)(6). The empty plasmid, pcDNA3.1, was used for mock transfection. For gene knockdown assays, siRNA pools and non-targeting siRNAs for mouse *Rock1* and *Rock2* were obtained from Dharmacon. The cells were transfected with plasmid and/or siRNA using Amaxa Nucleofector system (Lonza) according to supplier's instructions. Adenovirus encoding mouse Gα13QL was infected to cells in growth media with virus encoding LacZ as control.

Kinase assays and mass spectrometry.

In the presence of [^{32}P]-ATP, 0.2 μg recombinant human Rock2 (Abcam) and 1 μg NFATc1 (Sigma) were mixed in a final volume of 20 μl of a kinase buffer containing 20 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 25 mM β-glycerophosphate, and 1 mM Na_3VO_4, and protease inhibitor cocktail (Calbiochem). Proteins were incubated for 15 min at 30℃. For competitive peptide inhibition assay, 500 μg of an 8-mer peptide (HSPSTSPR) was incubated with 0.1 μg Rock2 and 0.5 μg NFATc1. Phosphorylation was visualized by

autoradiography via FLA7000 instrument (GE healthcare) after SDS-PAGE. For LC/MS/MS, NFATc1 was incubated with cold ATP instead of [^{32}P]-ATP, with or without Rock2. Post-translational modifications were detected using Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific) and compared.

Accession code.

Gene Expression Omnibus: the microarray data using soleus muscles of each genotype has been deposited under the accession number GSE83737.

Statistics.

Two-tailed Student's *t* tests were done to assess the significance of differences among treatment groups. Data were expressed as means ±SEM. The criterion for statistical significance was set at $p<0.05$ or $p<0.01$.

Study approval.

All studies were approved by the institutional board of the Seoul National University and by the IACUC of Center of Animal Care and Use at Lee Gil Ya Cancer and Diabetes Institute, Gachon University.

References for Methods

1. Moers A, Nieswandt B, Massberg S, Wettschureck N, Gruner S, Konrad I, et al. G13 is an essential mediator of platelet activation in hemostasis and thrombosis. *Nat Med.* 2003;9(11):1418-22.
2. Yang YM, Lee CG, Koo JH, Kim TH, Lee JM, An J, et al. Galpha12 overexpressed in hepatocellular carcinoma reduces microRNA-122 expression via HNF4alpha inactivation, which causes c-Met induction. *Oncotarget.* 2015;6(22):19055-69.
3. Koo JH, Lee HJ, Kim W, and Kim SG. Endoplasmic reticulum stress in hepatic stellate cells promotes liver fibrosis via PERK-mediated degradation of HNRNPA1 and up-regulation of SMAD2. *Gastroenterology.* 2016;150(1):181-93 e8.
4. Choi CS, Savage DB, Abu-Elheiga L, Liu ZX, Kim S, Kulkarni A, et al. Continuous fat oxidation in acetyl-CoA carboxylase 2 knockout mice increases total energy expenditure, reduces fat mass, and improves insulin sensitivity. *Proc Natl Acad Sci U S A.* 2007;104(42):16480-5.
5. Monticelli S, and Rao A. NFAT1 and NFAT2 are positive regulators of IL-4 gene transcription. *Eur J Immunol.* 2002;32(10):2971-8.
6. Clipstone NA, and Crabtree GR. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature.* 1992;357(6380):695-7.