Supplemental Table 1. Body weight, food intake, and plasma concentrations of leptin and lipids in MNUAK1KO and control mice under normal chow and high-fat diet conditions.

Mice at the age of 13-15 weeks were used for the determination of food intake and plasma concentrations of leptin and lipids. Data are mean ± SEM, n = 8, *: P < 0.05 (Student's t-test).

	Normal chow		High-fat diet	
	Control	**MNUAK1KO**	**Control**	**MNUAK1KO**
Body weight (g), 13-15 week-old	26 ± 1.5	27 ± 1.0	38 ± 0.7	37 ± 1.5
Body weight (g), 18-19 week-old	27 ± 0.9	28 ± 0.8	43 ± 1.7	39 ± 2.1
Food intake (g/ animal/ day)	3.8 ± 0.1	3.9 ± 0.2	3.3 ± 0.1	3.2 ± 0.1
Leptin (ng/mL)	0.4 ± 0.1	0.4 ± 0.1	10.5 ± 2.7	8.7 ± 3.6
Free Fatty Acides (µEq/ L)	1039 ± 51	1012 ± 72	978 ± 37	875 ± 31 *
Triglycerides (mg/ dL)	87 ± 10	84 ± 6	148 ± 11	125 ± 7
Cholesterol (mg/ dL)	71 ± 4	72 ± 3	124 ± 8	132 ± 7

Supplemental Table 2. Phosphoproteome analysis in soleus muscles from MNUAK1KO and control mice fed a NC. Phosphorylation status of proteins shown in table 1. [a]Protein isoforms that could not be distinguished by unique peptides. [b]Phosphorylation sites previously identified using site-specific methods in reference to a phosphorylation database (www.phosphosite.org). [c]Ambiguous phosphorylation-sites (i.e., those that could not be determined from MS-MS spectra). Data are mean ± SEM, n = 3.

Symbol	Name	Potential phosphorylation-sites in unique peptides	KO/Control ratio
Phosphoproteins decreased in MNUAK1KO			
Glucose metabolism			
PKCtheta	Protein kinase C theta type	S676[b]	**0.39** ± 0.21
IRS1	Insulin receptor substrate 1	S1097[b]	**0.07** ± 0.05
GYS1	Glycogen [starch] synthase, muscle	S652, S657[b]	**1.03** ± 0.39
		S653[b], S657[b]	**0.85** ± 0.10
Actin-myosin cytoskeleton			
MYH1/ 4/ 6/ 7/ 8[a]	Myosin-1/ 4/ 6/ 7/ 8	S1044/ S1041/ S1039/ S1037/ S1040	**0.46** ± 0.11
TTN	Titin	S322	**0.66** ± 0.10
		S2078, S2080	**0.88** ± 0.08
TCAP	Telethonin	S39	**0.46** ± 0.03
CTNNA1	Catenin alpha-1	S641[b]	**0.41** ± 0.02
SYNPO2L	Synaptopodin 2-like protein	T138, S140	**0.80** ± 0.17
CMYA5	Cardiomyopathy-associated protein 5	S769	**1.13** ± 0.56
PLEC	Plectin	S4629, Y4618 or Y4619 or S4620[c]	**0.45** ± 0.20
		S4392, S4393, S4396	**0.49** ± 0.07
		S4620, S4633	**0.51** ± 0.06
		Y4622, S4629	**0.43** ± 0.10
		Y4622, S4627	**0.35** ± 0.02
		Y4619, S4627	**0.30** ± 0.05
		Y4622, T4630	**0.39** ± 0.11
		S4392, S4393	**0.48** ± 0.02
		Y4622, S4625	**0.61** ± 0.12
Protein biosynthesis			
EIF5B	Eukaryotic translation initiation factor 5B	S108, S114	**0.54** ± 0.06
RPLP2	60S acidic ribosomal protein P2	S105[b]	**0.67** ± 0.20
DNAJC1	DnaJ homolog subfamily C member 1	S477, S478	**1.20** ± 0.31
Miscellaneous			
LNP	Protein lunapark	S411	**0.46** ± 0.06
STEAP3	Metalloreductase STEAP3	S17, S20	**1.44** ± 0.46
GOGA4	Golgin subfamily A member 4	T39, S41	**0.60** ± 0.18
UBP2L	Ubiquitin-associated protein 2-like	S497, S480 or T481 or S482[c]	**0.76** ± 0.18
Phosphoproteins increased in MNUAK1KO			
Actin-myosin cytoskeleton			
LRRC39	Leucine-rich repeat-containing protein 39	S328	**2.78** ± 0.27
ANKRD2	Ankyrin repeat domain-containing protein 2	S347, T351	**1.47** ± 0.13
Protein biosynthesis			
EIF5B	Eukaryotic translation initiation factor 5B	S137	**1.92** ± 0.03
Miscellaneous			
HMGA1	High mobility group protein HMG-I/HMG-Y	S102, S103	**1.02** ± 0.17
PACSIN3	Protein kinase C and casein kinase II substrate protein 3	S354	**1.47** ± 0.15
2310046A06RIK	Uncharacterized protein C6orf142 homolog	S85	**0.74** ± 0.08

Supplemental Experimental Procedures

Phosphoproteome analysis

[Sample preparation for phosphoproteome analysis] Mouse soleus muscles were frozen in liquid nitrogen and then disrupted with Multi-beads shocker (MB400U, Yasui kikai, Osaka, Japan). The disrupted cells were suspended with 1 mL of 0.1 M Tris-HCl (pH 9.0), and then treated with protein phosphatase inhibitor cocktails 1 & 2 (Sigma-Aldrich Japan, Tokyo, Japan) and protease inhibitors (Sigma-Aldrich Japan, Tokyo, Japan) according to the manufacture's protocol. The homogenate was centrifuged at 1,500 *g* for 10 min and the supernatant was added with urea at a final concentration of 8 M. The protein amount in the solution was measured with a BCA protein assay kit (Thermo Fisher Scientific, MA). The solution was reduced with 10 mM dithiothreitol for 30 min at room temperature (rt), alkylated with 50 mM iodoacetamide for 30 min at rt in dark and digested with Lys-C (1/100 w/w) for 3 h at rt, followed by dilution with 4-fold volume of 50 mM ammonium bicarbonate and digestion with trypsin (1/100 w/w) overnight at rt. These digested samples were acidified with addition of trifluoroacetic acid (TFA) and desalted as described below.

[Desalting with SDB-XC stage tip] For desalting after tryptic digestion, a disk cut out from SDB-XC Empore membrane (3M Company, MN) with 10 gauge syringe needle was inserted into a pipette tip D-1000 (GILSON, WI). The tip was conditioned with 100 μL of 0.1% TFA, 80% acetonitrile and then equilibrated with 100 μL of 0.1% TFA, 5% acetonitrile by centrifuge at 1000 *g* for 1 min. The tryptic digest corresponding to 100 μg proteins was loaded onto the tip by centrifuge at 1000 *g* for 5 min. The tip was washed with 100 μL of 0.1% TFA, 5% acetonitrile by centrifuge at 1000 *g* for 1 min. The peptides were eluted with 100 μL of 0.1% TFA, 80% acetonitrile (ACN) by centrifuge at 1000 *g* for 1 min. For desalting after dimethyl labeling and phosphopeptides-enrichment steps, a disk cut out from the membrane with 16 gauge syringe needle was inserted into a pipette tip D-200 (GILSON, WI). Desalting was performed as same manner as described above except volume of solvent and loading amount of sample were changed. In each step, 20 μL of solvents were used and whole sample eluted from one HAMMOC tip was loaded to one desalting tip.

[Enrichment of phosphopeptides with hydroxy acid-modified metal oxide chromatography] Custom-made hydroxy acid-modified metal oxide chromatography (HAMMOC) tips were prepared as follows. A disk cut out from C8 Empore disk membranes (3M Company, MN) with 20 gauge syringe needle was inserted into a 0.1-10μL size pipette tip (Eppendorf Japan, Tokyo, Japan) as a frit. And then, slurry of 0.5 mg of bulk titania (particle size: 10 μm, GL sciences, Tokyo, Japan) in 10 μL of methanol was packed into the tip by centrifuge at 1000 *g* for 1 min. Prior to loading samples, the HAMMOC tips were equilibrated with 20 μL of 0.1% TFA, 80% ACN with 300 mg/mL lactic acid (solution A) by centrifuge at 2000 *g* for 1 min. The desalted tryptic digest from a total 100 μg of isotope-labeled tryptic digest was diluted with 100 μL of solution A and 50 μL of aliquot was loaded to the HAMMOC tip four times by centrifuge at 1000 *g* for 5 min. After successive washing with solution A and 0.1% TFA, 80% acetonitrile by centrifuge at 2000 *g* for 1 min, the peptide was eluted with 50 μL of 0.5% ammonium hydroxide or 1.0% di-sodium hydrogenphosphate by centrifuge at 1000 *g* for 5 min. The eluted fraction was acidified with TFA, and desalted using SDB-XC StageTips as described above. The desalted sample was concentrated in a vacuum evaporator, followed by the dissolution with 10 μL of solution A for subsequent nanoLC-MSMS analysis.

[Nano LC-MS/MS] NanoLC-MS/MS analyses were conducted by using an Orbitrap system (LTQ-Orbitrap XL (Thermo Fisher Scientific, Bremen, Germany), Ultimate3000 pump with FLM-3000 flow manager (Dionex, Germering, Germany) and HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland)). ReproSil-Pur 120 C18-AQ materials (3 μm, Dr. Maisch, Ammerbuch, Germany) were packed into a self-pulled needle (150 mm length x 100 μm I.D., 6 μm opening) with a nitrogen-pressurized column loader cell (Nikkyo Technos, Tokyo, Japan) to prepare an analytical column needle with "stone-arch" frit (1). A PTFE-coated column holder (Nikkyo Technos, Tokyo, Japan) was mounted on an x-y-z nanospray interface (Nikkyo Technos, Tokyo, Japan) and a Valco metal connector

with a magnet was used to hold the column needle and to set the appropriate spray position. The injection volume was 5 μL and the flow rate was 500 nL/min. The mobile phases consisted of (A) 0.5% acetic acid and (B) 0.5% acetic acid and 80% acetonitrile. A three-step linear gradient of 5% to 10% B in 5 min, 10% to 40% B in 60 min, 40% to 100% B in 5 min and 100% B for 10 min was employed throughout this study. A spray voltage of 2,400 V was applied via the metal connector. The MS scan range was m/z 300-1500. Top ten precursor ions were selected in MS scan by Orbitrap with R = 60,000 for MS/MS scans by ion trap in the automated gain control (AGC) mode where AGC values of 5.00×10^5 and 1.00×10^4 were set for full MS and MS/MS, respectively. To minimize repetitive MS/MS scanning, a dynamic exclusion time was set as 20 sec with a repeat count of 1 and an exclusion list size of 500. The normalized CID was set to be 35.0. A lock mass function was used for the LTQ-Orbitrap to obtain constant mass accuracy during gradient analysis.

[Database search] Mass Navigator v1.2 (Mitsui Knowledge Industry, Tokyo, Japan) was used to create peak lists on the basis of the recorded fragmentation spectra. Mass Navigator v1.2 discarded all peaks with an absolute intensity of less than 10, and with an intensity of less than 0.1% of the most intense peak in MS/MS spectra, and an in-house Perl script converted the *m/z* values of the isotope peaks to the corresponding monoisotopic peaks when the isotope peaks were selected as the precursor ions (2). Peptides and proteins were identified by means of automated database searching using Mascot v2.3 (Matrix Science, Tokyo, Japan) against SwissProt release 2011_04 with a precursor mass tolerance of 3 ppm, a fragment ion mass tolerance of 0.8 Da and strict trypsin specificity allowing for up to 2 missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification and oxidation of methionines, phosphorylation of serine, threonine and tyrosine and [$^1H_4$,$^{12}C_2$ / $^2H_4$,$^{13}C_2$]-dimethylation of *N*-terminus and lysine were allowed as variable modifications. Note that neutral loss products from both precursor and fragment ions were considered for MASCOT scoring in this phosphorylation modification setting although no assignment was indicated for precursor-origin neutral loss peaks in the output results according to the supplier.

Peptides were considered identified if the Mascot score was over the 95% confidence limit based on 'identity' score of each peptide. Note that phosphorylation can be discriminated from sulfation by LTQ-Orbitrap with the lock mass function because the error distribution is within 2 ppm, which is less than the mass difference between sulfation and phosphorylation (9.516 mDa) for most of detectable peptides. Phosphorylation sites were unambiguously determined when b- or y-ions were between which the phosphorylated residue exists were observed in the peak list of fragment ions. Based on peptide information (observed *m/z* of monoisotopic ion and retention time) obtained by database searching, the LC-MS peak area of each peptide in all sample was determined by integration of ion intensity in survey MS scan. The peak integration was performed by Gaussian approximation of an extracted ion chromatogram within 5 mDa of the observed m/z using Mass Navigator v1.2.

Measurements of plasma leptin and lipids

Peripheral blood was collected from mice after 2 h-fasting for leptin or after an overnight fasting for lipids. Leptin was measured by using a Mouse Leptin Assay Kit (IBL, Gunma, Japan). Free fatty acid, triglycerides, and cholesterol were measured by using a NEFA C-test, a Triglyceride E-test, and a Cholesterol E-test (Wako Pure Chemical Industries, Osaka, Japan), respectively. All assays were performed according to the manufacturer's instructions.

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