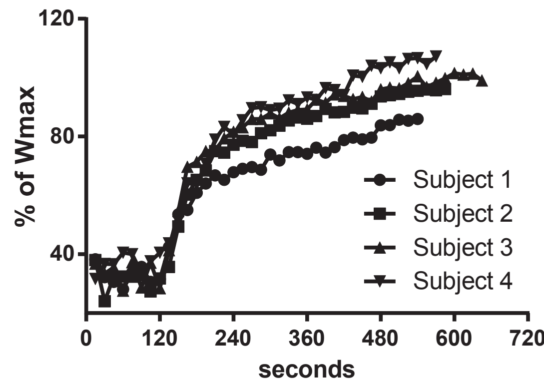
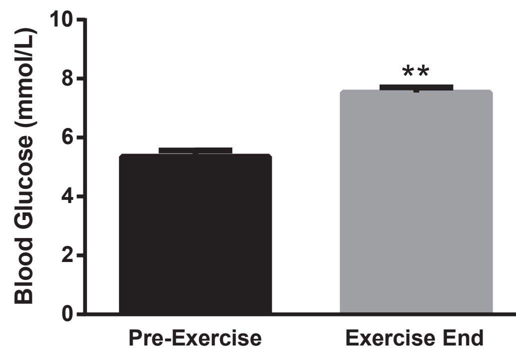
A

Subject	Age	BMI (kg/m2)	Insulin (pre-exercise; uU/ml)	Glucose (pre-exercise; mmol/L)	HOMA-IR
1	27	24.2	4.1	5.8	1.057
2	29	25.3	1.68	4.8	0.358
3	26	25.9	2.22	5.5	0.543
4	24	25.7	3.49	5.3	0.822

B



C



D

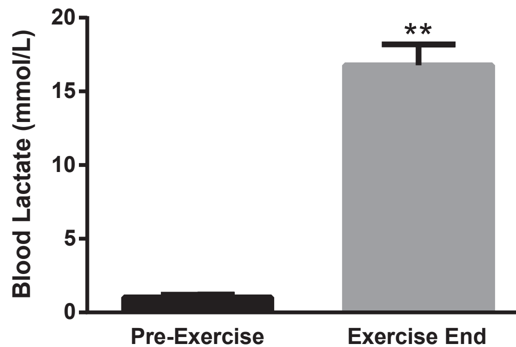


Figure S1, related to Figure 1.
(**A**) Characteristics of the male subjects. (**B**) Subject % Wmax obtained during the exercise trail. (**C**) Subject blood glucose and (**D**) blood lactate pre-exercise and at end of exercise (paired t-test $**P<0.01$; mean ± standard error of mean is shown from n=4 subjects).

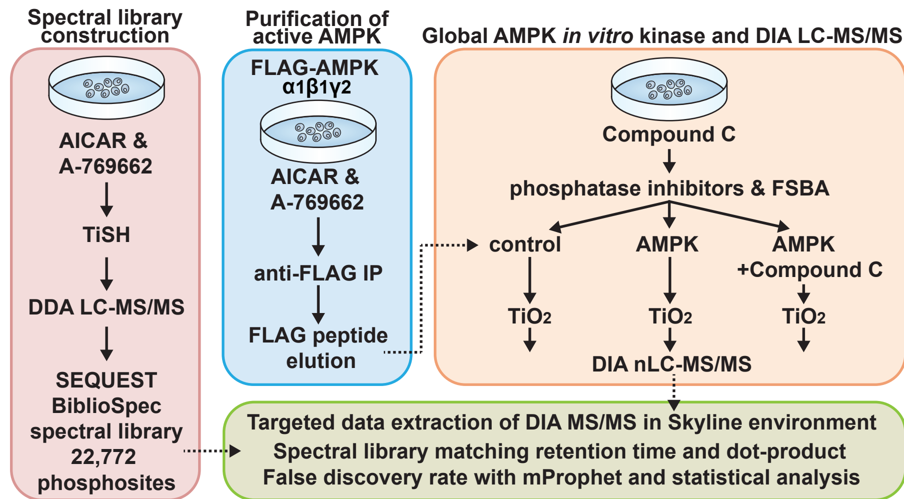


Figure S2, related to Figure 4.
Experimental design of the targeted phosphoproteomic analysis of a global AMPK *in vitro* kinase assay in HEK293 lysates using data indpendent acquisition mass spectrometry (DIA LC-MS/MS)**.** HEK293 cells were treated with a combination of AICAR and A-769662, digested with trypsin and phosphopeptides enriched using titanium dioxide and sequential elution from immobilized metal ion affinity chromatography with fraction by hydrophilic interaction liquid chromatography (TiSH). The enriched phosphopeptide fractions were analyzed by data dependent mass spectrometry (DDA LC-MS/MS), identified by SEQUEST and spectral library generated with BiblioSpec. In a second batch of HEK293 cells, functional FLAG-tagged AMPK was over-expressed and immunoprecipitated. Purified functional FLAG-tagged AMPK was generated with FLAG peptide elution and used in the subsequent *in vitro* kinase analysis. A third batch of HEK293 cells were treated with the AMPK inhibitor, Compound C. Cell lysates were generated in a kinase buffer and treated with a phosphatase inhibitor cocktail and a global pan-kinase inhibitor, fluorosulphonylbenzoladenosine (FSBA). Excess inhibitors were removed by filtration and three aliquots prepared. The lysates were treated with either; i) control (FLAG peptide in kinase buffer), ii) purified FLAG-AMPK (α1β1γ2) and, iii) purified FLAG-AMPK (α1β1γ2) pre-inhibited with Compound C. The reactions were digested with LysC/trypsin, phosphopeptides enriched by titanium dioxide chromatography and analyzed by DIA LC-MS/MS. The spectral library was used to perform targeted data extraction of the DIA MS/MS analysis followed by false discovery rate estimations (Q-values) using mProphet in the Skyline environment. The quantified phosphopeptides were normalized and statistically analyzed (see Supplemental Experimental Procedures).

Supplemental Experimental Procedures

Human subjects and biopsy collection

Four healthy male volunteers (age: 24 - 27; BMI: 24.2 - 25.9 kg/m^2, VO_2 max: 48-59 ml/kg/min, Wmax: 320-375 W) abstained from strenuous exercise for 2 days prior to the experiment. They reported to the laboratory in the overnight fasted state and rested in the supine position for 30 min. A venous catheter was inserted in a forearm vein and a blood sample was obtained. Then local anaesthesia (2-3 ml 2% lidocaine) was administered subcutaneously at 4 incision sites above the vastus lateralis muscle, 2 on each leg spaced 5-6 cm apart. Two biopsies were extracted at rest with a 5mm Bergstrom needle with suction and were immediately flushed out of the needle with ice cold saline, quickly blotted dry and then frozen in liquid nitrogen. Subjects then underwent a single bout of high-intensity cycle exercise. Following warm up subjects exercised for 6 min at 85% of Wmax and then to exhaustion at 92% of Wmax, which occurred after 9-11 min total exercise time following warm up. Venous blood was obtained during the last min of exercise, and 2 muscle biopsies were extracted immediately upon exercise cessation. The study was approved by the regional ethics committee in Denmark (Journal number: H-1-2012-006) and carried out in accordance with the Declaration of Helsinki II. Written informed consent was obtained from each subject.

Human muscle lysis, isobaric labeling and phosphopeptide enrichment

Muscle biopsies were ground under liquid nitrogen and approximately 40 mg of tissue lysed in 6 M urea, 2 M thiourea, 25 mM triethylammonium bicarbonate (TEAB), pH 7.9 containing phosphatase and protease inhibitor cocktails (Roche) by tip-probe sonication (2 x 15 s) on ice.

The lysates were centrifuged at 17,000 x g, 15 min, 4ºC and the supernatant precipitated with 6 volumes of acetone, overnight, -20ºC. Protein pellets were resuspended in 6 M urea, 2 M thiourea, 25 mM TEAB, pH 7.9 and quantified by Qubit fluorescence (Invitrogen). Concentrations were normalized and 1 mg of protein reduced with 10 mM dithiothreitol for 60 min at 25ºC followed by alkylation with 25 mM iodoacetamide for 30 min at 25ºC in the dark. The reaction was quenched to a final concentration of 20 mM dithiothreitol and digested with Lys-C (Wako) at 1:50 enzyme to substrate ratio for 2 h at 25ºC. The mixture was diluted 5-fold with 25 mM TEAB and digested with trypsin at 1:50 enzyme to substrate ratio for 12 h at 30ºC. The peptide mixture was acidified to a final concentration of 2% formic acid, 0.1% trifuoroacetic acid (TFA) and centrifuged at 16,000 x g for 15 min. Peptides were desalted using hydrophilic lipophilic balance – solid phase extraction (HLB-SPE) cartridges (Waters) followed by elution with 50% acetonitrile, 0.1% TFA and dried by vacuum centrifugation. Peptides were resuspended in 30 µl of 100 mM TEAB, quantified by Qubit fluorescence and normalized to 250 µg / 30 µl. This entire procedure was performed in two technical replicates for either iTRAQ or TMT labeling. For iTRAQ labeling, two 4-plex experiments were performed according to the manufacturer’s instructions. The first experiment was labeled as follows; 114 = Subject 1 pre-exercise, 115 = Subject 1 post-exercise, 116 = Subject 2 pre-exercise, 117 = Subject 2 post-exercise. The second experiment was labeled as follows; 114 = Subject 4 post-exercise, 115 = Subject 4 pre-exercise, 116 = Subject 3 post-exercise, 117 = Subject 3 pre-exercise. For TMT labeling, one 10-plex experiment was performed as follows; 126 = Subject 1 pre-exercise, 127N = Subject 1 post-exercise, 127C = Subject 2 pre-exercise, 128N = Subject 2 post-exercise, 128C = Subject 3 pre-exercise, 129N = Subject 3 post-exercise, 129C = Subject 4 pre-exercise, 130N = Subject 4 post-exercise 130C = pool of 62.5 µg of each Subject 1, 2, 3, 4 pre-exercise, 131 =

pool of 62.5 µg of each Subject 1, 2, 3, 4 post-exercise. The labeled peptides were pooled within the respective experiments and dried to approximately 50 µl by vacuum centrifugation. Phosphopeptides were enriched essentially as described previously and consisted of titanium dioxide followed by sequential elution from immobilized metal ion affinity chromatography and fractionation by hydrophilic interaction liquid chromatography (TiSH) (Engholm-Keller et al., 2012). Briefly, peptides were resuspended in 1 ml of titanium dioxide loading buffer (1 M glycolic acid, 80% acetonitrile, 5% TFA), and a 20 µg aliquot was saved for total proteomic analysis. Titanium dioxide beads (15 mg in 150 µl acetonitrile) (GL Science, Japan) were added to the peptide mixture and rotated at room temperature for 20 min. The mixture was centrifuged at 10,000 x g, 1 min and the supernatant was applied to a second aliquot of titanium dioxide (7.5 mg in 75 µl acetonitrile) and rotated at room temperature for 20 min. The mixture was centrifuged at 10,000 x g, 1 min and the supernatant was applied to a third aliquot of titanium dioxide (4 mg in 40 µl acetonitrile) and rotated at room temperature for 20 min. The beads were washed with 100 µL titanium dioxide loading buffer followed by 80% acetonitrile, 2% TFA and finally 16% acetonitrile, 0.4% TFA. The beads were dried briefly by vacuum centrifugation and eluted with 50 µL of 1% ammonium hydroxide by shaking at room temperature for 15 min. The titanium dioxide elution slurry was loaded onto a C8-plugged micro-column and eluted with gentle air pressure to trap beads. The beads were eluted with an additional 50 µL 1% ammonium hydroxide and the elution was pooled. Enriched phosphopeptides were acidified to a final concentration of 10% formic acid and dried by vacuum centrifugation. The enriched phosphopeptides were resuspended in 500 µl of 50% acetonitrile, 0.2% TFA and rotated with 50 µl of Fe(III)-IMAC beads (Sigma-Aldrich) at room temperature for 45 min. The IMAC slurry was loaded onto a crushed GeLoader microcolumn and eluted with gentle air pressure to trap

beads. Mono-phosphorylated peptides were eluted with 50% acetonitrile, 0.1% TFA followed by 20% acetonitrile, 1% TFA; pooled with the flow-through and dried by vacuum centrifugation. Multi-phosphorylated peptides were eluted with 1% ammonium hydroxide, acidified to a final concentration of 10% formic acid, 0.1% TFA and desalted with C18 microcolumns. Enriched mono-phosphorylated peptides were subjected to another round of titanium dioxide as described above. The enriched mono-phosphorylated peptides and non-phosphorylated peptides were fractionated on in-house packed TSK-amide HILIC column as described previously (Palmisano et al., 2010). All isobarically labelled peptides were analyzed by data-dependent acquisition (DDA) as described below.

Animals

For experiments comparing wild type versus AMPK KD mice, female mice were group-housed, kept on a 12:12-h light-dark cycle and had free access to standard rodent chow diet (Altromin no. 1324; Brogaarden, Denmark) and water. All experiments were approved by the Danish Animal Experimental Inspectorate. Soleus muscles from anaesthetized (6 mg pentobarbital sodium/100 g body mass) female C57BL/6 wild type and muscle-specific KD AMPK overexpressing mice 14-16 weeks old (Mu et al., 2001) were quickly dissected out, suspended at resting tension (2-3 mN) in incubation chambers (Multi Myograph system; Danish Myo-Technology, Aarhus, Denmark) containing warm (30°C), and constantly gassed (95% O_2/5% CO_2) Krebs-Ringer-Henseleit (KRH) buffer supplemented with 2 mM pyruvate, and 8 mM mannitol. AICAR (Toronto Research Chemicals) was added as indicated for 1 h to a final concentration of 2 mM. Finally, muscles were quickly removed from the incubation chambers, rinsed in ice-cold saline, blotted dry on gauze and snap-frozen in liquid nitrogen. Frozen muscles were homogenized with

stainless steel pellets using the Tissuelyser II (Qiagen) at 2 x 45 sec at 30 Hz and ice-cold homogenization buffer (10% Glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES (pH 7.5), 1% NP-40, 20 mM ß-glycerophosphate, 10 mM NaF, 2 mM PMSF, 1 mM EDTA (pH 8.0), 1 mM, EGTA (pH 8.0), 10 µg/ml Aprotinin, 10 µg/ml Leupeptin, 2 mM Na_3VO_4, 3 mM Benzamidine, 5 mM Nicotinamide). Homogenates were rotated end-over-end for 1 h at 4°C, and supernatant lysates were obtained by centrifugation at 13000 x g for 20 min at 4°C. Lysate protein content was measured by bicinchoninic acid (BCA) method and lysates were diluted to the same protein concentration (1 µg/µl). Total protein and phosphorylation levels of indicated proteins were determined by immunoblotting as described below.

For mouse treadmill experiments, male C57Bl6 wild type mice were group-housed, kept on a 12:12-h light-dark cycle and had free access to standard rodent chow diet and water. All experiments were approved by The University of Sydney Animal Ethics Committee. Mouse treadmill exercise experiments were performed following a 2-day period of treadmill running acclimatization on an Exer3/6 mouse treadmill (Columbus Instruments) at a 5% incline as previously described (Stockli et al., 2015). Mice 23 weeks old were either rested (n=5) or exercised (n=5) at a starting speed of 8 m/min with increasing speed by 2 m/min every 2 min until exhaustion, defined as falling off the treadmill three times within 15 sec. Exhaustion occurred between speeds of 30 – 34 m/min and times of 23-27 min. Following either rest or exhaustion, each mouse was euthanized and quadricep muscles were dissected. Red quadricep was separated from white quadricep muscle, snap-frozen, and lysed in 2% SDS lysis buffer containing fresh phosphatase inhibitor (Sigma-Aldrich) and protease inhibitor cocktails (Roche) using pulse sonication. Proteins were then precipitated with ice-cold acetone overnight, and total

protein and phosphorylation levels of indicated proteins were determined by immunoblotting as described below.

Cell culture and treatments

Rat L6 myoblasts were maintained in α-Minimum essential medium (α-MEM) containing 5.5 mM glucose (Gibco) and 10% fetal bovine serum (FBS; Hyclone Laboratories) in a 10% CO_2 incubator. L6 myoblasts were differentiated into myotubes with 2% FBS when myoblasts reached ~90-95% confluency. All L6 studies used either 90-95% confluent myoblasts (where indicated) or myotubes between 6 and 8 days post-initiation of differentiation. HEK293 cells were cultured in Dulbecco's modified essential medium (DMEM) containing 25 mM glucose (Gibco), 1X GlutaMax (Gibco) and 10% FBS (Hyclone Laboratories) in a 10% CO_2 incubator. All HEK293 studies were performed 48 hr post-transfection when cells reached between 80-90% confluency. Cells were serum-starved for a total of 2 h prior to all experiments. L6 myoblasts and myotubes were washed twice with PBS and twice with αMEM + 0.2 % BSA prior to serum starvation in αMEM + 0.2% BSA. HEK293 cells were washed twice with PBS and twice with DMEM prior to serum starvation in DMEM. All cells remained in serum starvation medium for 1.5 h and stimulated for the final 30 min with 2 mM AICAR, 100 µM A-769662 or 200 µM DNP, where indicated.

Cell lysis, SDS-PAGE, immunoblot analysis and antibodies

Total cell extracts were prepared from L6 myoblasts, differentiated L6 myotubes and HEK293 cells following two washes with ice-cold PBS. Washed cells were scraped in 2% SDS lysis buffer containing fresh phosphatase inhibitor and protease inhibitor cocktails, and then

homogenized by passing the lysate through an 18-gauge needle ten times. Lysates were then centrifuged to separate the insoluble material from the soluble extract by centrifugation at 13,000 g for 10 min at room temperature. Functional mitochondria were isolated from L6 myotubes for mitochondrial signaling analyses as previously described (Frezza et al., 2007). Protein concentrations were determined via the BCA method (Thermo Fisher), and 10 μg protein was separated by 8% SDS-PAGE. The resolved fractions were transferred to PVDF membrane and subjected to immunoblot analysis with phospho-specific and total antibodies. The phospho-specific AKAP1 S103 antibody was generated by 21st Century Biochemicals. All other phospho-specific and total antibodies were purchased from Cell Signaling Technology, with the exception of the following antibodies: FLAG (Sigma-Aldrich), AMPKα1 and AMPKα2 (Abcam), ACC1 and ACC2 (Merck), VDAC1 (Santa Cruz Biotech), and MitoProfile Total OXPHOS antibody cocktail (Mitosciences). Equal protein loading was confirmed by immunoblot analysis with corresponding total antibodies. Immunoblots of precipitated human muscle proteins and cell lysates were labeled with HRP-conjugated secondary antibodies and analyzed via autoradiography. Immunoblots from mouse muscle samples were imaged by the Bio-Rad ChemiDoc Gel Imager system.

Plasmid and siRNA transfection

Wild type AKAP1 mouse cDNA was kindly provided by John D. Scott (University of Washington, Seattle, Washington). This AKAP1 mouse cDNA was N-terminally FLAG-tagged, and site-directed mutagenesis was performed to target S103 and generate phospho-dead FLAG-tagged AKAP1 S103A mutant cDNA according to manufacturer's instructions (Agilent QuikChange II XL). L6 myoblasts and HEK293 cells were transfected at approximately 70-75%

confluency using Lipofectamine 2000 (Life Technologies) according to manufacturer's instructions, and cells were treated and harvested for experimentation 48 hr post-transfection. Three independent siRNA oligonuceotide sequences were designed and purchased from Shanghai GenePharma Co., Ltd to target mouse and rat AKAP1 for siRNA knockdown. The sense (5'-3') sequences for these rodent AKAP1 siRNA oligonucleotides are as follows: GAGGUGAUGACAACUUUGUTT, CGGGAACAGUAUGGAUUCATT, and CGUGGACUAUGGUGGAUAUTT. For siRNA knockdown of AMPKα, one siRNA oligonucleotide sequences was purchased from Shanghai GenePharma Co. to target either rat AMPKα1 or rat AMPKα2. The sense (5'-3') sequences for rat AMPKα siRNA oligonucleotides are as follows: CGAGUUGACUGGACAUAAATT (AMPKα1) and GCAACUAUCAAAGACAUACTT (AMPKα2). Shanghai GenePharma Co., Ltd Negative Control scrambled siRNA oligonucleotide (sense (5'-3') sequence UUCUCCGAACGUGUCACGUTT) was used as a control in siRNA experiments. For all siRNA knockdown experiments L6 myoblasts were transfected at approximately 48 hr post seeding (or ~60% confluency). Calcium phosphate-based siRNA transfection was utilized for each well of L6 myoblasts seeded in a 6-well plate as follows, 1.5 µl of 20 µM negative control scrambled or 0.5 µl of each AKAP1 siRNA oligonucleotide was added to 44.1 µl Buffer A (1 mM Tris/HCl, 0.1 mM EDTA, pH 7.9). After mixing, 2.4 µl 1M $CaCl_2$ was added dropwise, followed by another 12 µl 1M $CaCl_2$. This siRNA mixture was then added dropwise to 60 µl Buffer B (50 mM HEPES pH 7.0, 280 mM NaCL, 0.75 mM Na_2HPO_4, 0.75 mM NaH_2PO_4). After 30 min incubation at room temperature, 120 µl of the combined siRNA mix was added dropwise to myoblasts in 6-well plates containing 1380 µl αMEM +10% FBS and incubated 12–

16 h. Following 12–16 h incubation, media was aspirated and replaced with αMEM +10% FBS. Cells were harvested for experimentation 72 h after transfection.

Immunofluorescence

L6 myoblasts were co-transfected with pMito-LSSmOrange (Shcherbakova et al., 2012) and either FLAG-tagged AKAP1 WT or S103A cDNA as described above. pMito-LSSmOrange was a gift from Vladislav Verkhusha (Addgene plasmid # 37135). 24 hr post-transfection the cells were trypsinized and seeded into the wells of Ibidi 18 well μ-Slides (Ibidi) pre-coated with Geltrex Matrix (Life Technologies), as per the manufacturer's instructions. 24 hr post-reseeding cells were washed twice with PBS and fixed with 3.7% paraformaldehyde in PBS at 24°C for 15 min. Cells were then blocked and permeabilized with blocking buffer (BB) containing 0.1% (wt/vol) saponin and 2% BSA in PBS. Background autofluorescence was minimized by treatment with enhanceFX (Life Technologies) as per the manufacturer's recommendations. To reduce the high background staining, the anti-FLAG antibody (Sigma) was immunoadsorbed at 4°C overnight on fixed and permeabilized cells not expressing the FLAG epitope. Transfected cells were incubated with anti-FLAG antibody (at ~1.6μg/ml) overnight in BB, washed four times with PBS, and incubated for 30 min with Alexa 488 conjugated Goat anti-Rabbit IgG (H+L) Secondary Antibody at 24°C. After washing, cells were imaged in PBS + 2.5% DABCO on a Nikon A1 laser scanning confocal microscope (Nikon), with a CFI Plan Apo VC 60XWI 1.2 NA water immersion objective at the optimal sampling density as defined by the Nyquist theorem. Image analysis and processing was performed using FIJI (Schindelin et al., 2012).

Mitochondrial respiration assays

High-resolution O_2 consumption measurements were conducted at 37°C using the OROBOROS Oxygraph-2K (OROBOROS Instuments) or the XF24 and XFp analyzers (Seahorse Biosciences). All experiments were performed in respiration buffer containing 110 mM NaCl, 4.7 mM KCl, 2 mM MgSO4, 1.2 mM Na2HPO4, 2.5 mM glucose and 5 mM carnitine (pH adjusted to 7.4). Stocks of sodium palmitate (2 mM) conjugated to fatty acid free BSA (0.34 mM) and BSA alone were prepared in 150 mM NaCl. For O2K experiments, cells were resuspended in respiration buffer supplemented with BSA alone or palmitate (200 μM) at a cellular density of 1 x 10^6 cells/ml. Following an initial baseline reading, A-769662 was added to both chambers, followed by etomoxir (100 μM) to confirm CPT1-dependent fatty acid oxidation. The transient increase in JO_2 induced by A-769662 requires real-time assessment of mitochondrial respiration, for example using the O2K, as it is driven by increased fatty acid delivery into the mitochondrial matrix without concomitant changes in demand for ATP regeneration. Data are expressed as pmol/sec/million cells. For experiments involving the XF24 and XFp, equal cell numbers were seeded for each transfection condition 24 hr prior to the assay at a density of 20,000-40,000 cells/well in a 24-well (XF24) or 20,000 cells/well in an 8-well (XFp) polystyrene Seahorse flux plate. On the day of the experiment cells were switched to respiration buffer supplemented with BSA alone. Respiration was assessed under basal conditions (BSA alone) and in response to palmitate (200 μM). Data are expressed as pmol/min. Following assessment of respiration, cell protein content from each well was quantified with the BCA method. Protein analysis showed no change in protein content with siRNA knockdown and overexpression compared to respective controls.

SILAC and L6 phosphopeptide enrichment

SILAC labeling of L6 myoblasts was performed by supplementing SILAC DMEM (deficient in Lysine, Arginine and Leucine; Thermo Fisher) with 10% FBS (Hyclone Laboratories), 'light' Leucine and either 'light' or 'heavy' Lysine ($^{13}C_8$) and Arginine ($^{13}C_{10}$) (Silantes) to generate two different isotopically labeled cell populations. These cell populations' SILAC labels were switched between the control and AICAR-treated groups in two out of four biological replicates to account for any effects of these isotopically labeled amino acids. Confirmation of AMPK activation was confirmed with immunoblotting (data not shown). L6 myoblasts were cultured for at least five passages to allow sufficient SILAC amino acid incorporation (i.e. > 98%). Following cell harvesting, equal protein content of light and heavy SILAC cell populations were mixed (1:1), trypsinized, and peptides were desalted with C18 Sep-Pak columns (Waters). Peptides were then fractionated with strong cation exchange (SCX), and phosphopeptides were enriched using titanium dioxide chromatography as previously performed and described by our group (Humphrey et al., 2013).

Global AMPK *in vitro* kinase analysis

An overview of the experimental design is presented in Fig. S2 and consists of three steps; i) spectral library construction, ii) purification of active AMPK, and iii) global AMPK *in vitro* kinase analysis. *Spectral library construction* – HEK293 cells were cultured in serum-free media for 1.5 h and stimulated for 30 min with a combination of 2 mM AICAR and 100 µM A-769662 (Ducommun et al., 2014). Cells were lysed in 6 M urea, 2 M thiourea, 25 mM TEAB (Sigma-Aldrich) containing protease (Roche) and phosphatase inhibitor cocktails (Sigma-Aldrich). Peptide generation and phosphopeptide enrichment using TiSH was performed as described above. The enriched phosphopeptides were analyzed by data-dependent acquisition as described

below. *Purification of active AMPK* – HEK293 cells were transiently transfected at 50% confluence with a mixture of PRKAA1-GST, PRKAB1-FLAG and PRKAG2-GST expression vectors (generously provided by Dr. Jonathan Oakhill and Prof. Bruce Kemp and previously characterized). Cells were incubated with 2.5:1 ratio of Lipofectamine-2000 to plasmid DNA in opti-MEM (Gibco) for 24 h and assayed 48 h post-transfection. To maximally activate AMPK, cells were cultured in serum-free media for 1.5 h and stimulated for 30 min with a combination of 2 mM AICAR and 100 µM A-769662. Cells were lysed in NP-40 buffer (1% NP-40, 150 mM NaCl, 50 mM Tris.HCl, 10% glycerol, protease and phosphatase inhibitor cocktail, pH 7.4) using a 22-gauge needle 10 times and then a 27-gauge needle 3 times followed by removal of cellular debris by centrifugation at 16,000 x g, 10 min, 4ºC. A 30 µl slurry of protein G Sepharose (GE Life Sciences) was resuspended in 500 µl of NP-40 buffer and incubated with 2 µg of monoclonal anti-FLAG M2 antibody (Sigma-Aldrich) for 2 h at 4ºC with rotation. The beads were washed briefly with NP-40 buffer and incubated with protein lysate to immunoprecipitate FLAG-tagged AMPK for 2 h at 4ºC. The beads were washed 3 times with NP-40 buffer and once with kinase buffer (25 mM Tris.HCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, 400 µM ATP, pH 7.4). The beads were resuspended in 40 µl of kinase buffer containing 15 µg of 3xFLAG peptide (Sigma-Aldrich) to elute FLAG-tagged AMPK. *Global AMPK in vitro kinase analysis* – A global AMPK *in vitro* kinase assay in HEK293 whole cell lysates was performed as described previously (Knight et al., 2012) with several modifications. To reduce basal levels of AMPK substrates, cells were cultured in serum-free media for 1.5 h and treated for 30 min with 10 µM Compound C. Cells were lysed in NP-40 buffer using a 22-gauge needle 10 times and then a 27-gauge needle 3 times followed by removal of cellular debris by centrifugation at 20,000 x g, 10 min, 4ºC. The lysate was treated with 10 mM 5'-4-fluorosulphonylbenzoladenosine (FSBA), a

potent pan-kinase inhibitor that covalently attacks the catalytic lysine (Zoller et al., 1981), and 5 mM β-glycerophosphate for 30 min, 32ºC. Inhibitors were removed by centrifugation at 4,000 x g, 40 min using a 10 kDa filter (Millipore). Proteins were quantified with BCA, adjusted to 4 mg/ml and diluted 1:1 with 2x kinase buffer. Three equal aliquots of 1.2 mg / 600 µl were prepared and incubated with; i) kinase buffer with FLAG peptide (control), ii) purified AMPK in kinase buffer, or iii) purified AMPK in kinase buffer pre-incubated with 40 µM Compound C. Fifty micrograms were saved for immunoblot analysis and the remainder diluted 1:1 with 6 M urea, 2 M thiourea, 25 mM TEAB, pH 7.9. Peptides were prepared as described above and phosphopeptides were enriched using a single round of titanium dioxide (3 mg of beads) as described above. Enriched phosphopeptides were analyzed by data-independent acquisition as described below.

Mass spectrometry

Peptides were resuspended in 2% acetonitrile, 0.5% acetic acid and loaded onto a 50 cm x 75 µm inner diameter column packed in-house with 1.9 µm C18AQ particles (Dr Maisch GmbH HPLC) using an Easy nLC-1000 UHPLC operated in single-column mode with intelligent flow control loading at 950 bar. Peptides were separated using a linear gradient of 5 – 30% Buffer B over 100 min at 250 nl/min (Buffer A = 0.5% acetic acid; Buffer B = 80% acetonitrile, 0.5% acetic acid). The column was maintained at 50ºC using a PRSO-V1 ion-source (Sonation) coupled directly to a Q-Exactive mass spectrometer (MS). For DDA, a full-scan MS1 was measured at 70,000 resolution at 200 m/z (300 – 1750 m/z; 100 ms injection time; $3e^6$ AGC target) followed by isolation of up to 20 most abundant precursor ions for MS/MS (2 m/z isolation; 8.3e5 intensity threshold; 30.0 normalized collision energy; 17,500 resolution at 200 m/z; 60 ms injection time;

$5e^5$ AGC target). Charge state reduction of isobarically labeled peptides was achieved with a 10% ammonium hydroxide vapour underneath the ESI source (Thingholm et al., 2010). For DIA, a full-scan MS1 was measured at 140,000 resolution at 200 m/z (300 – 1600 *m/z*; 120 ms injection time; $3e^6$ AGC target) followed by 16 x 25 m/z isolations and MS/MS from 450 – 850 *m/z* with a loop count of 8 i.e an intermittent MS1 scan after 8 MS/MS (30.0 normalized collision energy; 17,500 resolution at 200 *m/z*; 60 ms injection time; $3e^6$ AGC target). Window placement was optimized in Skyline (MacLean et al., 2010) to result in an inclusion list starting at 462.9603 m/z with increments of 25.0114 m/z.

Bioinformatics and AMPK substrate prediction

DDA data of human muscle and HEK293 cells were processed using Proteome Discoverer v1.4 and searched with Sequest HT against the human UniProt database (May 2013; 88,767 entries). The data were searched with the following variable modifications; methionine oxidation; serine, threonine and tyrosine phosphorylation; and N-terminus and lysine iTRAQ or TMT labeling. Carbamidomethylation of cysteine was set as a fixed modification. The precursor-ion mass tolerance was set to 20 ppm and product-ion mass tolerance set to 0.02 Da. All results were filtered to 1% FDRs using Percolator (Kall et al., 2007) and phosphosite localisation was performed with PhosphoRS (Taus et al., 2011). A phosphosite localization grouping scheme was devised (<10%, 10-30%, 30-45%, 45-90% and >90%) and median values of peptide spectral matches (PSMs) for each phosphosite were calculated using a script developed in Python. Bioinformatic analysis was performed primarily in the R programming environment [http://www.R-project.org, R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-

900051-07-0]. Phosphosite positions within the protein sequence were determined using an in-house developed R-script, which scanned the quantified peptide sequences against the Uniprot (version May 2013) Fasta formatted protein sequence file to identify corresponding parent proteins and map the highly confident (>90%) phosphorylation positions in the peptides within the parent protein sequences (Uniprot accession name). Protein quantification was performed using the median values of all PSMs of the protein group. All data were normalized to the median of each subject. Significantly regulated phosphopeptides and proteins were determined using a moderated t-test from LIMMA package in R [Smyth, G.K, 2004, Statistical applications in genetic and molecular biology, 3, no.1, article 3]. Linear models were fit to the data and empirical Bayes was used for variance shrinkage in order to determine regulated sites with fold change greater than 1.5 and *P*-values were corrected for multiple testing controlling for 1% FDR using Benjamini and Hochberg method (Benjamini et al., 2001). Pathway analysis was performed with Ingenuity Pathway Analysis (www.ingenuity.com), and prediction of kinase-substrate relationships was performed with NetworKIN v2.0 (Linding et al., 2008) followed by processing and thresholding in PhosphoSiteAnalyzer using default settings (Bennetzen et al., 2012). A kinase-substrate set enrichment analysis was performed to determine if the predicted kinases were associated with increased or decreased activity after exercise. For each kinase, predicted substrates were ranked by fold-change. Using Kolmogorov-Smirnov based gene set tests (Mootha et al., 2003), we evaluated whether the predicted kinase was more or less active after exercise as represented by regulated phosphosites.

DDA data of SILAC labeled rat L6 myotubes was processed using MaxQuant v1.4.0.8 (Cox and Mann, 2008) and searched with Andromeda (Cox et al., 2011) against the rat UniProt database

(October 2013; 34,816 entries). The data were searched with the following variable modifications; methionine oxidation; and serine, threonine and tyrosine phosphorylation. The precursor-ion mass tolerance was set to 20 ppm and 7 pm for first and second searches respectively and product-ion mass tolerance set to 0.02 Da. All results were filtered to 1% FDRs. All data were normalized to the median of each replicate. Significantly regulated phosphopeptides and proteins were determined as stated above using moderated t-test as implemented in LIMMA package in R.

To generate the kinase interactome network, kinase-substrate relationships were first annotated in PhosphoSitePlus for exercise-regulated substrates of 32 kinases. Additionally, 45 kinases were included that contained a regulated phosphorylation site. Six kinases were in common between these two groups (CAMK2A, CAMK2G, MTOR, Src family kinase members, EEF2K and MAPKAPK2). Therefore, we considered 71 unique kinases for the kinome analysis. These 71 kinases and their respective substrates comprised a dataset of 132 interactions (52 unique substrates). We further grouped kinases to consolidate isoforms into their respective kinase group. Next, we curated experimentally validated protein-protein interactions from the Human Protein Reference Database (HPRD) release 9 (April 2010) and the STRING database version 9.1. We identified 127 unique protein-protein interactions within the exercise-regulated kinase-substrate dataset. The resulting kinome and annotations (kinase: substrate, self-phosphorylated, or physical interaction) was graphically represented using Cytoscape version 3.2.0 (Shannon et al., 2003).

All DIA data were processed using Skyline v2.5.0.6157 (MacLean et al., 2010) and targeted data extraction was performed essentially as described previously (Gillet et al., 2012). Spectral libraries were built in Skyline using the BiblioSpec algorithm (Frewen and MacCoss, 2007). Precursor and product ion extraction ion chromatograms (XICs) were generated using extraction windows 2-fold the full width at half maximum for both MS1-filtering and MS2-filtering. Ion match tolerance was set to 0.055 m/z and matched to charges 2+, 3+ and 4+ for MS1-filtering of the first three isotopic peaks and, 1+, 2+ and 3+ for MS2-filtering of b- and y-type ions. Peak scoring models were trained based on mProphet (Reiter et al., 2011) and OpenSWATH (Rost et al., 2014) using a combination of scores and filtered to 1% FDR. The model was trained using the following criteria: i) library intensity dot-product (dot product between library spectrum and product-ions in the DIA data); ii) co-elution count (number of co-eluting fragment ions in the DIA data); and iii) weighted shape of product-ions in the DIA data. These were used to rescore peak picking and peak integration boundaries as described in *Advanced Peaking Picking Models* (https://skyline.gs.washington.edu/labkey/wiki/home/software/Skyline/page.view?name=tutorial_peak_picking). These were further analyzed manually and correct identification was assigned based on the following criteria: i) retention time matching to spectral library within 5% of the gradient length; ii) dot-product between observed phosphopeptide precursor-ion isotope distribution intensities and theoretical >0.95; iv) dot-product between library spectrum intensities and DIA fragment ions >0.90 and; iv) matching peak shape for precursor and product ions. All data were normalized using the total area sum (TAS) and then median absolute deviation method (MAD) was applied within the replicates of each treatment group as previously performed with DIA data (Lambert et al., 2013). Significantly regulated phosphopeptides were determined by calculating the moderated t-statistic using LIMMA package in R where eBayes method was used

for global variance shrinkage. Significance was defined by P-values < 0.05 controlling for 1% FDR.

For comparison of human and rat phosphorylation sites, a new algorithm and automated tool was developed in-house. Based on global pairwise sequence alignment of orthologous proteins from human and rat, sites quantified from independent cross-species experiments can be easily mapped to each other using this tool. Global pairwise sequence alignment between each orthologous protein pair was performed using the BLOSUM62 (Styczynski et al., 2008) substitution matrix, gapOpening of 10, gapExtension of 0.5 using Biostrings package in R [Pages H, Aboyoun P, Gentleman R, DebRoy S: Biostrings: String objects representing biological sequences, and matching algorithms. R package version 2.26.3]. We scanned this sequence alignment using the human phosphorylation site numbers from the MS-based phosphoproteomics experiment as the reference to identify the aligned amino acid residues and their positions in the rat MS-phosphoproteomics data. If there was a match in the residue type and if the corresponding aligned position existed in the rat phosphoproteomics data, we considered this as a 'match' and the Uniprot protein accession name, residue and site number match was retained as a modified site that could be mapped between human and rat.

Data visualisation was performed using base packages in R and Adobe Illustrator.

All DIA data are deposited at the Panorama Repository for Targeted Proteomics (Sharma et al., 2014) and are available via web viewing or direct download for analysis in Skyline (https://panoramaweb.org using the email login: metabolicsystemsbiology@outlook.com

password: USydney1). All DDA data are deposited in the ProteomeXchange using the identifier PXD001543.

Support vector machine learning

As previously described (Humphrey et al., 2013), support vector machine (SVM) based ensemble algorithm was trained to model the motifs as well as the AICAR-induced responses of positive AMPK examples. The learning features used for prediction are the position-specific scoring matrix of a window size of 13 amino acids surrounding the phosphorylation site and the log2 fold enrichment of each phosphorylation site upon AICAR stimulation. Each base classifier was trained on a training set of positive AMPK substrates curated from PhosphoSitePlus and a negative set sampled randomly from all identified phosphorylation sites. The ensemble aggregates 1000 base classifiers and the final predictions were made by using combined prediction probabilities from all base classifiers.

Purified AMPK *in vitro* kinase analysis

HEK293 cells were transiently transfected at 50% confluence as described above with either a mixture of the AMPK expression vectors or FLAG-tagged AKAP1 expression vector (generously provided by Prof. John Scott). To maximally activate AMPK, cells were cultured in serum-free media for 1.5 h and stimulated for 30 min with a combination of 2 mM AICAR and 100 μM A-769662. Cells were lysed in NP-40 buffer and anti-FLAG immunoprecitation was performed as described above. Biotinylated ACC was enriched from a separate batch of HEK293 cells using streptavidin-agarose beads (Sigma-Aldrich). FLAG-tagged AMPK was eluted with FLAG peptide in kinase buffer and added directly onto the enriched FLAG-tagged AKAP1 or

biotinylated ACC beads. Kinase reactions were performed for 30 min at 33ºC with gentle agitation and quenched with SDS-PAGE loading buffer.

Statistical Analysis

Statistical tests are detailed in each figure legend. Error bars represent either mean ± standard deviation (Fig. 4) or mean ± standard error of mean (Fig. 6 and 7), as detailed with the corresponding number of experiments in each figure legend.

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