

Supplementary Section

Materials. Protease-inhibitor cocktail tablets were obtained from Roche (#1697498, Lewes, Sussex, UK). Tween-20, pyruvate, cytochalasin B and 5-aminoimidazole-4-carboxamide riboside (AICAR) from Sigma (Poole, UK). 2-deoxy-D-[1-³H]-Glucose, D-[¹⁴C]-Mannitol, [γ -³²P]ATP, and Protein G-Sepharose were purchased from Amersham Biosciences (Little Chalfont, UK). Insulin from Novo-Nordisk and Precast SDS-polyacrylamide Bis-Tris gels were from Invitrogen. Phosphocellulose P81 paper was from Whatman. All peptides were synthesised by Dr Graham Bloomberg at the University of Bristol.

Antibodies. The specific AMPK α 1 antibody was raised against the peptide (CTSPDSDLDDHHLTR, residues 344-358 of rat AMPK α 1), the specific AMPK α 2 antibody was raised against the peptide (CMDDSAMHIPPGLKPH, residues 352 to 366 of rat AMPK α 2), the phosphospecific antibodies recognising AMPK phosphorylated on the T-loop was generated against the peptide (KFLRT(P)SCGSPNYA residues 168 to 180 of rat AMPK α 1). The LKB1 antibody used for immunoblotting was raised in sheep against the mouse LKB1 protein or raised against the N-terminal peptide (TFIHRIDSTEVYQPR, residues 24-39 of human LKB1) and that for immunoprecipitation was raised in sheep against the N-terminal peptide. The Phosphospecific antibody recognising acetyl-CoA carboxylase (ACC) phosphorylated on Ser212 was generated against the peptide (TMRPSMS(P)GLHLVK corresponding to residues 215–227 of human ACC2). ExtrAvidin peroxidase conjugate, used to detect ACC2 that has a naturally conjugated biotin, was from Sigma. Anti-phospho-ERK Thr202/Tyr204 antibodies (#9101) recognising ERK1 and ERK2, anti-total ERK1 and ERK2 antibodies (#9102) were from Cell Signaling Technology. Secondary antibodies coupled to horseradish peroxidase were from Pierce.

Preparation of tissue lysates. Mouse skeletal muscle and other mouse tissues were rapidly excised and frozen in liquid nitrogen and subsequently pulverized to a powder in liquid nitrogen. A 10-fold mass excess of ice-cold lysis buffer containing 50 mM Tris/HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (by mass) Triton-X 100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 mM sucrose, 0.1% (by volume) 2-mercaptoethanol and 'complete' proteinase inhibitor cocktail (1 tablet per 50 ml) was added to the powder tissue and homogenized on ice using KINEMATICA POLYTRON (Brinkmann, CT, USA). Homogenates were centrifuged at 13,000g for 10 min at 4°C to remove insoluble material. The supernatant was collected and the Bradford

Method using serum bovine albumin as the standard measured protein concentration. Lysates snap frozen in aliquots in liquid nitrogen and stored at -80 °C.

Immunoblotting. Muscle extracts (20-50 μg) were heated in SDS sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis and electrotransfer to nitrocellulose. Membranes were then blocked for 1h in 50 mM Tris/HCl pH 7.5, 0.15 M NaCl, 0.1 (by vol) % Tween (TBST), containing 10 % (by mass) skimmed milk for the sheep antibodies and 5 % (by mass) bovine serum albumin for commercial phospho-specific and ExtrAvidin-peroxidase antibodies. The membranes were then incubated for 16 h at 4°C with 1 $\mu\text{g}/\text{ml}$ for the sheep antibodies or 1000-fold dilution for commercial antibodies in TBST, 5 % (by mass) skimmed milk (sheep antibodies) or 5% (by mass) and bovine serum albumin (commercial antibodies). Detection of proteins was performed using horse radish peroxidase conjugated secondary antibodies and the enhanced chemiluminescence reagent.

Quantitative immunoblot by Li-Cor analysis. The immunoblots were incubated with antibodies in 50 mM Tris/HCl pH 7.5, 0.15M NaCl, 0.2% (by vol) Tween containing 5% (by mass) skimmed milk overnight at 4 °C. The blots were washed and incubated for 1 hour with fluorescently labelled anti-mouse secondary antibody at room temperature. The blots were analysed using a Li-Cor Odyssey infrared detection system following the manufacturers' guidelines. The band intensity was quantified using Li-Cor software. Using this approach, a more quantitative analysis of immunoblots can be achieved than using the standard chemiluminescence techniques (see <http://www.licor.com/>).

Immunoprecipitation and assay of LKB1 and AMPK. 500 μg muscle lysate was used to immunoprecipitate LKB1 and 50 μg was used for AMPK α 1 and AMPK α 2 immunoprecipitation. The lysates were incubated at 4°C for 1h on a shaking platform with 5 μl of protein G-Sepharose coupled to 3 μg of LKB1, AMPK α 1 or AMPK α 2 antibodies. The immunoprecipitates were washed twice with 1 ml of Lysis Buffer containing 0.5 M NaCl, and twice with 1 ml of Buffer A (50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, and 0.1% (by volume) 2-mercaptoethanol). Phosphotransferase activity towards the LKBtide peptide (SNLYHQGKFLQTFCGSPLYRRR residues 241-260 of human NUA2 with 3 additional Arg residues added to the C-terminal to enable binding to P81 paper (Lizcano et al., 2004)) for LKB1 or AMARA peptide for AMPK α 1 and AMPK α 2 (Dale et al., 1995), were then measured in a total assay volume of 50 μl consisting of 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1% (by volume) 2-mercaptoethanol, 10 mM magnesium acetate, 0.1 mM [γ ³²P]ATP (~200 cpm/pmol) and 200 μM LKB1tide or 200 μM AMARA peptide. The assays were carried out at 30°C with continuous shaking, to keep the immunoprecipitates in suspension, and were terminated

after 20 min by applying 40 μ l of the reaction mixture onto P81 papers. These were washed in phosphoric acid, and the incorporated radioactivity was measured by scintillation counting. One milliUnit (mU) of activity was defined as that which catalysed the incorporation of 1 pmol of ^{32}P into the substrate per min.

Glucose transport in isolated skeletal muscle. Mice were fasted overnight (16 h) prior to experiment and sacrificed by cervical dislocation, and EDL muscles were rapidly and carefully removed. The method to measure glucose transport was similar to that described previously (Bruning et al., 1998). Tendons from both ends of each muscle were tied with suture and mounted on an incubation apparatus. Muscles were incubated in 8 ml of KRB buffer containing 2 mM pyruvate at 37 °C. Glucose transport was measured in 2 ml KRB buffer containing 1 mM 2-deoxy-D-[1- ^3H]-Glucose (1.5 $\mu\text{Ci/ml}$) and 7 mM D-[^{14}C]-mannitol (0.3 $\mu\text{Ci/ml}$) at 30°C for 10 min. Both incubation and transport buffers were continuously gassed with 95% O_2 -5% CO_2 . To terminate the transport, muscles were immersed in KRB buffer containing 80 μM cytochalasin B on ice. To study the effect of insulin or AICAR on glucose transport, EDL muscles were incubated in KRB buffer for 60 min in the presence or absence of 100 nM insulin or 2 mM AICAR followed by 10 min glucose transport measurement. To study the effect of contraction *in situ*, sciatic nerve stimulation was performed for 5 min (train rate, 1/s; train duration, 500 ms; pulse rate, 100 Hz; duration, 0.1 ms at 2-5 V) and muscles were then removed, washed for 15 min in 8 ml KRB buffer containing 2 mM pyruvate before measuring glucose uptake for 10 min as described above and quantitated as described below. Corresponding EDL muscles from the resting legs were processed in the same way and served as a control. To study the effect of contraction *in vitro*, isolated EDL muscles were incubated in KRB buffer for 50 min and muscles were electrically stimulated during the last 10 min period (train rate, 2/min; train duration, 10 s; pulse rate, 100 Hz; duration, 0.1 ms at 100 V) followed by 10 min of glucose transport measurement as described above and quantitated as described below.

At the end of the glucose transport, muscles were quickly frozen in liquid nitrogen. Muscles were stored at -80 °C. Muscles were processed by incubating in 250 μ l 1M NaOH at 80°C for 10 min, neutralized with 250 μ l 1M HCl, and particulates were precipitated by centrifugation at 13,000 g for 2 min. ^3H and ^{14}C radioactivity present in 350 μ l of the supernatant was measured by scintillation counting. Blanks were subtracted from values obtained from muscle derived from the same mouse that had been through the same treatments in the absence of radioactivity. From the ^{14}C specific radioactivity the extracellular water volume present in the muscle was calculated. This enables the extracellular ^3H volume to be determined and subtracted from the total ^3H radioactivity to

calculate the intracellular amount of 2-deoxy-D-[1-³H]-Glucose transported per gram of muscle per hour.

Contraction force measurement. Tendons from both end of isolated EDL muscle was tied with suture and mounted on an incubation apparatus. Muscle was incubated in KRB buffer containing 2 mM pyruvate for 50 min at 37 °C. Muscle was then transferred to a supporting apparatus with resting tension set at 0.2-0.3 mN. Muscle was electrically stimulated to contract for 10 sets of 10-sec contraction (100 Hz; duration, 0.1 ms at 100 V) with 50-sec interval between set. Force generation during the contraction protocol was monitored with an isometric force transducer (Kent Scientific) which was connected to a data recorder (iWorx) and a computer. The recorded data were analysed using software (iWorx) to calculate total force production for each 10-sec contraction.

Measurement of nucleotides. 50 mg tibialis anterior and EDL muscle powdered in liquid nitrogen was homogenized in 0.2 ml of 5% (by volume) perchloric acid, and then centrifuged at 14,000 rpm for 3 min at 4°C to remove insoluble material. 220 µl of 1:1 tri-n-octylamine and 1, 1, 2,-trichlorotrifluoroethane solution was added to the supernatant and rigorously vortexed. After centrifugation the upper aqueous layer was removed and extracted a second time with a further 220 µl of the same organic solvent mixture. The final aqueous phase (20 µl) was analyzed by capillary electrophoresis with on-column isotachophoretic concentration, using run buffers consisting of 50 mM sodium phosphate, and 50 mM NaCl (pH 5.2; leading buffer); and 100 mM MES/Tris pH 5.2 (tailing buffer). To each buffer was added 0.2% hydroxyethyl-cellulose to decrease the electro-osmotic flow. Before running each sample, a 10% volume of leading buffer was added to the sample. Nucleotide peaks were detected by UV absorbance at 260 nM (ref. 400 nM), and integrated using System Gold software (Beckman). Nucleotide ratios were calculated from peak areas after correction for retention times. Peaks due to added ATP, ADP, IMP and AMP coincided with the particular sample peaks supporting their assignment as such. In addition comparison of the absorbance spectra of individual standard and sample peaks also supported their correct assignment.

Statistical analysis. Data are expressed as means ± SEM. Statistical analysis was undertaken by unpaired Student's *t* test or one-way analysis of variant (ANOVA). When ANOVA revealed significant differences, further analysis was performed using Tukey's post hoc test for multiple comparisons. Differences between groups were considered statistically significant when $p < 0.05$.

Supplementary References.

- Bruning, J.C., Michael, M.D., Winnay, J.N., Hayashi, T., Horsch, D., Accili, D., Goodyear, L.J. and Kahn, C.R. (1998) A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. *Mol Cell*, **2**, 559-569.
- Dale, S., Wilson, W.A., Edelman, A.M. and Hardie, D.G. (1995) Similar substrate recognition motifs for mammalian AMP-activated protein kinase, higher plant HMG-CoA reductase kinase-A, yeast SNF1, and mammalian calmodulin-dependent protein kinase I. *FEBS Lett*, **361**, 191-195.
- Lizcano, J.M., Goransson, O., Toth, R., Deak, M., Morrice, N.A., Boudeau, J., Hawley, S.A., Udd, L., Makela, T.P., Hardie, D.G. and Alessi, D.R. (2004) LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. *EMBO J*, **23**, 833-843.