Supplementary Section

LKB1 is a master kinase that activates 13 protein kinases of the AMPK subfamily, including the MARK/PAR-1 kinases

Jose M. Lizcano[1], Olga Göransson[1], Rachel Toth[2], Maria Deak[1], Nick Morrice[1], Jérôme Boudeau[1], Simon Hawley[3], Lina Udd[4], Tomi P. Mäkelä[4], D, Grahame Hardie[3] and Dario R. Alessi[1]

Materials and Methods.

Materials. Protease-inhibitor cocktail tablets and sequencing grade trypsin were obtained from Roche. N-octyl-glucoside was from Calbiochem, and tissue culture reagents were from Life Technologies. Tetracyclin-free foetal bovine sera (FBS) was from Perbio, and other tissue culture reagents were from Biowhittaker. Precast 4–12% and 10% polyacrylamide Bis-Tris gels were obtained from Invitrogen; P81 phosphocellulose paper was from Whatman; Phenformin from Sigma and AICA riboside from TorontoXX. [γ-^{32}P]ATP, glutathione-Sepharose, Protein G-Sepharose were purchased from Amersham Biosciences. All peptides were synthesised by Dr Graham Bloomberg at the University of Bristol.

Antibodies.

The following antibodies were raised in sheep and affinity purified on the appropriate peptide antigen: BRSK1 (MVAGLTLGKGPESPDGDVS, residues 1-20 of human BRSK1), BRSK2 (LSWGAGLKGQKVATSYESSL, residues 655-674 of human BRSK2), NUAK1 (MEGAAAPVAGDRPDLGLGAPG residues 1-21 of human NUAK1), NUAK2 (TDCQEVTATYRQALRVCSKLT, residues 653-673 of human NUAK2), QIK (MVMADGPRHLQRGPVRVGFYD, residues 1-21 of human QIK), SIK

(MVIMSEFSADPAGQGQGQQK, residues 1-20 of human SIK used for immunoprecipitation from human cells), SIK (GDCEMEDLMPCSLGTFVLVQ, residues 765-784 of human SIK used for immunoprecipitation from mouse cells), QSK (TDILLSYKHPEVSFSMEQAGV, residues 1349-1369 of human QSK), MARK1 (SGTSIAFKNIASKIANELKL, residues 776-795 of human MARK1), MARK4 (MSSRTVLAPGNDRNSDTHGT, residues 1-20 of human MARK4), MELK (MKDYDELLKYYELHETIGTG, residues 1-20 of human MELK). The specific AMPKα1 antibody employed in Figures 7, 8B and 9 was raised against the peptide (CTSPPDSFLDDHHLTR, residues 344-358 of rat AMPKα1) whilst the antibody recognising both AMPKα1 and AMPKα2 employed in Fig 8A, was raised against the peptide (CDPMKRATIKDIRE residues 252 to 264 or rat AMPKα1). The anti MARK2 and MARK3 peptide antibodies we raised were not specific as they immunoprecipitated other MARK isoforms as well. The anti MARK3 antibody from Upstate Biotech (anti c-TAK #05-680), raised against the human MARK3 protein was found to immunoprecipitate MARK2 as efficiently as MARK3 but did not immunoprecipitate MARK1 and MARK4 (OG, data not shown). The Phosphospecific antibodies recognising AMPK phosphorylated on the T-loop were generated against as described previously against the peptide (KFLRT(P)SCGSPNYA residues 168 to 180 of rat AMPKα1) (Sugden et al., 1999).The LKB1 antibody used for immunoblotting was raised in sheep against the mouse LKB1 (Sapkota et al., 2001) and that for immunoprecipitation was raised in sheep against the human LKB1 protein (Boudeau et al., 2003). Monoclonal antibody recognizing the HA epitope tag was from Roche, monoclonal antibodies recognising the GST and the FLAG epitopes were from Sigma. Anti-*myc* antibodies were prepared by ammonium sulphate precipitation of medium from Myc1-9E10 hybridoma cells grown in RPMI 1640 medium supplemented with 2 mM glutamine and 15% (v/v) foetal bovine serum. Secondary antibodies coupled to horseradish peroxidase used for immunoblotting were obtained from Pierce.

General methods and buffers. Restriction enzyme digests, DNA ligations, and other recombinant DNA procedures were performed using standard protocols. All mutagenesis was performed using the QuikChange site-directed mutagenesis method (Stratagene). DNA constructs used for transfection were purified from *E. coli* DH5α using Qiagen plasmid Mega kit according to the manufacturer's protocol. All DNA constructs were verified by DNA sequencing, which was performed by The Sequencing Service, School of Life Sciences, University of Dundee, Scotland, UK, using DYEnamic ET terminator chemistry (Amersham Biosciences) on Applied Biosystems automated DNA sequencers. Lysis Buffer contained 50 mM Tris/HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (w/v) Triton-X 100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1% (v/v) 2-mercaptoethanol and 'complete' proteinase inhibitor cocktail (one tablet/50 ml). Buffer A contained 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, and 0.1% (v/v) 2-mercaptoethanol. SDS sample Buffer contained 50 mM Tris/HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.005% (w/v) bromophenol blue, and 1% (v/v) 2-mercaptoethanol.

Cloning of AMPK-related kinases.

NUAK1. In order to obtain the coding region of human NUAK1 cDNA (NCBI Acc. NM_014840) a PCR reaction was carried out using a brain cDNA library (Clontech) as a template and the sense primer 5'- actgcagccctggagcccaggaagc-3' and the antisense primer 5'- ctagttgagcttgctgcagatctccagcgc-3'. The resulting PCR product covered the coding region of NUAK1 from amino acid residues 31-661. The cDNA of the missing N-terminal 31 amino acids was incorporated into the 5' primer of another PCR reaction, then the HA tag was added by PCR using the sense primer 5'-actagtgccaccatgtacccatacgatgtgccagattacgccgaaggggccgccgcgcctgtggcgggg-3' and antisense primer 5-ctagttgagcttgctgcagatctccagcgc-3'. The resulting PCR product was

ligated into pCR2.1-TOPO vector (Invitrogen), and subcloned as a *Spe1-Not1* fragment into pEBG2T (Sanchez et al., 1994) and as an *EcoR1-EcoR1* insert into pGEX6P-1 (Amersham) expression vectors.

NUAK2. The coding region of human NUAK2 cDNA (NCBI Acc. NP_112214) with an N-terminal HA tag, was amplified by PCR from IMAGE consortium EST clone 6718982 (NCBI Acc. CA487493) with primers 5'-actagtgccaccatgtacccatacgatgtgccagattacgccgagtcgctggttttcgcgcggcgctcc-3' and 5'-tcaggtgagctttgagcagaccctcagtgcctg-3'. The resulting PCR product was ligated into pCR2.1-TOPO vector, sequenced, and subcloned as a *Spe1-Spe1* fragment into pEBG2T and as an *EcoR1-EcoR1* insert into pGEX6P-1 expression vectors.

QIK. The coding region of human QIK cDNA (NCBI Acc. XM_041314) with an N-terminal HA tag was amplified from IMAGE consortium EST clone 5495545 (NCBI Acc. BM799630) using sense primers 5'-gcgtcgactacccatacgatgtgccagattacgccgtcatggcggatggcccgag-3' or 5'-gcactagttacccatacgatgtgccagattacgccgtcatggcggatggcccgag-3' for subsequent cloning into vectors pGEX6P-3 and pEBG2T respectively, and antisense primer 5'-gagcggccgctaattcaccaggacatacccgttgtg-3'. Amplified PCR products were ligated into pCR2.1 TOPO vector, sequenced, and subcloned as a *Sal1-Not1* or *Spe1-Not1* insert into pGEX6P-3 or pEBG2T respectively.

SIK. The coding region of human SIK cDNA (NCBI Acc. NM_173354) with an N-terminal HA tag was amplified from IMAGE consortium EST clone 4831049 (NCBI Acc. NM_173354) using primers 5'-gcggatcctacccatacgatgtgccagattacgccgttatcatgtcggagttcagcgcgg-3' and 5'-gagcggccgctcactgcaccaggacaaacgtgcc-3'. The PCR product was ligated into pCR2.1 TOPO vector, sequenced, and subcloned as a *BamH1-Not1* insert into pGEX6P-1 and pEBG2T.

MELK. The coding region of human MELK cDNA (NCBI Acc. NM_014791) with an N-terminal HA tag was amplified from IMAGE consortium EST clone 4547136 (NCBI Acc. BC014039) using primers 5'-gcggatcctacccatacgatgtgccagattacgccaaagattatgatgaacttctcaaatattatgaattacatg-3' and 5'-gtgcggccgcttataccttgcagctagataggatgtcttcc-3'. The PCR product was ligated into pCR2.1 TOPO vector, sequenced, and subcloned as a *BamH1-Not1* insert into pGEX6P-1 and pEBG2T.

BRSK1. Nucleotides 60-1010 of the coding region of human BRSK1 cDNA (NCBI Acc. NM_032430) were amplified from IMAGE consortium clone 6154749 (NCBI Acc. BQ434571) using primers 1. 5'-ccacccccacccacccagcacgcccaatatgtgggcccctatcggctggagaagacgctgggcaaagg-3' and 2. 5'-cgatgcagcctctcgcggtccctgaagcagc-3', nucleotides 60-106 being added by primer 1. Nucleotides 980-2337 of BRSK1 were amplified from the same EST clone using primers 3. 5'-gctgcttcagggaccgcgagaggctgcatcg-3' and 4. 5'-tcagggcagaggggtcccgttggtggcc-3'. Each PCR product was ligated into pCR2.1 TOPO vector and sequenced. A single nucleotide difference compared with BRSK1 NM_032430 sequence that is present in the EST clone was 'corrected' by site-directed mutagenesis in the pCR2.1 TOPO clone containing the 5' half of BRSK1. The remaining 5' 59 nucleotides of BRSK1 and the HA tag were added to the 5' half of BRSK1 by PCR using sense primers 5. 5'-ggtgggggctctcccgcctaccacctcccccacccccacccccacccacccagcacgcccaatatg-3' and 6. 5'-ggatcctacccatacgatgtgccagattacgcctcgtccggggccaaggagggaggtgggggctctcccgcctacc-3'. The final PCR product was ligated into pCR2.1 TOPO vector and sequenced. Overlap PCR was then performed using the 5' half and 3' half of BRSK1 as templates and primer 7. 5'- gcggatcctacccatacgatgtgcc-3' and primer 4. and the PCR product ligated into pCR2.1 TOPO vector and sequenced. Full length BRSK1 cDNA with N-terminal HA tag was then subcloned into pGEX6P-1 and pEBG2T as a *BamH1-BamH1* insert.

BRSK2. The coding region of HA-tagged human BRSK2 cDNA (NCBI Acc. AF533878) was amplified by PCR from IMAGE consortium EST clone 6144640 (NCBI Acc. BU193218) using primers 5'-ggatccgccaccatgtacccatacgatgtgccagattacgccacatcgacggggaaggacggcggcgcg-3' and 5'-gcggccgctcagaggctactctcgtagctggtggccaccttctggcccttaagccca-3'. The resulting PCR product was cloned into pCR2.1-TOPO vector, sequenced, and subcloned as a *BamH1-Not1* insert into pEBG2T and pGEX6P-1 vectors.

QSK. Nucleotides 55-640 of the coding region of human QSK cDNA (sequence obtained from Sugen database www.kinase.com (Manning et al., 2002)) were amplified from IMAGE consortium EST clone 4396995 (NCBI Acc.BF983268) using primers 1. 5'-ggagccgggcccgcgggccgcctgctgcctccgcccgcgccggggtccccagccgccccgctgccgtgtcccctgcggccggccagccg-3' and 2. 5'-tgaagaggttactgaaaccaaaatctgctattttgatattc-3', nucleotides 55-110 being added by primer 1. The remaining 5' 54 nucleotides and HA tag were subsequently added by PCR using primers 3. 5'-gattacgccgcggcggcggcggcgagcggagctggcggggctgccggggccgggactgggggagccgggcccgcgggccgcctgctg-3' and 4. 5'-gcggatcctacccatacgatgtgccagattacgccgcggcggcggcggcgagcgg-3'. The final PCR product was ligated into pCR2.1 TOPO vector and sequenced to produce pCR2.1 QSK clone 1. Nucleotides 610-865 of QSK were amplified from IMAGE consortium EST clone 6247938 (NCBI Acc. BQ685213) using primers 5. 5'-atagcagattttggtttcagtaacctcttcactcctgggcagctgctgaagacctggtgtggcagccctccctatgctgcacctgaactc-3' and 6. 5'- ctgtggacataaaaaatgggatgcggaactttcc-3', nucleotides 610-674 being added by primer 5. The PCR product was ligated into pCR2.1 TOPO vector and sequenced. A single nucleotide deletion at QSK open reading frame position 696, which is present in the EST clone, was corrected by site-directed mutagenesis to produce pCR2.1 QSK clone 2. QSK PCR products from pCR2.1 QSK clones 1 and 2 were input into an overlap PCR using primers 4. and 6. This product (QSK nucleotides 4-865 with N-terminal HA tag) was ligated into pCR2.1 TOPO vector and sequenced to produce pCR2.1 QSK clone 3.

QSK nucleotides 832-4110 were amplified from IMAGE consortium EST clone 360441 (NCBI Acc. AA015726) using primers 7. 5'-ggaaagttccgcatcccatttttttatgtccacag-3' and 8. 5'-gagcggccgcttacacgcctgcctgctccatgc-3'. The PCR product was ligated into pCR2.1 TOPO vector and sequenced to produce pCR2.1 QSK clone 4.

QSK PCR products from pCR2.1 clones 3 and 4 were input into an overlap PCR using primers 4. and 8. to generate full length QSK cDNA with N-terminal HA tag. The PCR product was ligated into pCR2.1 TOPO vector and sequenced to produce pCR2.1 QSK clone 5. Full length QSK with N-terminal HA tag was subcloned from pCR2.1 QSK clone 5 as a *BamH1-BamH1* insert into pGEX6P-1 and pEBG2T vectors. In order to generate the T-loop mutations in QSK, mutagenesis was performed on pCR2.1 QSK clone 3, followed by overlap PCR to generate the full length mutant forms of QSK. The overlap PCR product was ligated into pCR2.1 TOPO, sequenced, and subcloned into pGEX6P-1 and pEBG2T as a *BamH1-BamH1* insert.

MARK1. Nucleotides 4-1078 of the coding region of human MARK1 cDNA (NCBI Acc. NM_018650) with an N-terminal HA tag were amplified from a human brain cDNA library using primers 5'- gcgaattctacccatacgatgtgccagattacgcctcggcccggacgccattgc-3' and 5'- catcatacttctgatttattaaggcatcatttatttc-3'. Nucleotides 1042-2388 of MARK1 were amplified from IMAGE consortium EST clone 48109 (NCBI Acc. H11850) using primers 5'- gaaataaatgatgccttaataaatcagaagtatgatg-3' and 5'-gagtcgacttacagcttaagctcatttgctatttttgatgc-3'. Amplified PCR products were input into an overlap PCR to generate the full length HA-tagged MARK1 cDNA. The PCR product was ligated into pCR2.1 TOPO vector, sequenced, and subcloned as an *EcoR1-Sal1* insert into pGEX6P-1. Full length HA-tagged MARK1 was amplified from the pCR2. 1 TOPO MARK1 clone for subsequent cloning into pEBG2T as a *Kpn1-Not1* insert using primers 5'-gcggtacctacccatacgatgtgccagattacgcctcggcccggacgccattgc-3' and 5'-gagcggccgcttacagcttaagctcatttgctatttttgatgc-3'.

MARK2. The coding region of human MARK2 cDNA (NCBI Acc. NM_004954) was amplified from IMAGE consortium EST clone 4591688 (NCBI Acc. BG419875) using sense primers 5'-gcgtcgactacccatacgatgtgccagattacgccattcggggccgcaactcagcc-3' or 5'-gcactagttacccatacgatgtgccagattacgccattcggggccgcaactcagcc-3' for subsequent cloning into vectors pGEX6P-3 and pEBG2T respectively, and antisense primer 5'-gagcggccgcttaaagcttcagctcgttggctattttgg-3'. Amplified PCR products were ligated into pCR2.1 TOPO vector, sequenced, and subcloned as a *Sal1-Not1* or *Spe1-Not1* insert into pGEX6P-3 or pEBG2T vectors respectively.

MARK3. The coding region of human MARK3 cDNA (NCBI Acc. NM_002376) with an N-terminal HA tag was amplified from IMAGE EST 5104460 (NCBI Acc. BI223323) using primers 5'-gcggccgcagccaccatgtacccatacgatgtgccagattacgcctccactaggaccccattgccaacggtga-3' and 5'-gcggccgcttacagctttagctcattggcaattttggaagc-3'. The resulting PCR product was cloned into pCR2.1-TOPO vector, sequenced, and subcloned as a *Not1-Not1* insert into pEBG2T and pGEX6P-2 vectors.

MARK4. In order to obtain the full length coding region of human MARK4 cDNA (NCBI Acc.AK075272) two EST clones were used as templates for PCR reactions. The first 228 amino acids with an N-terminal HA tag was amplified from IMAGE consortium EST clone 6301902 (NCBI Acc.BQ709130) using primers 1. 5'-agatctgccaccatgtacccatacgatgtgccagattacgcctcttcgcggacggtgctggccccggg-3' and 2. 5'-tgccctgaaacagctccggggcggc-3'. The remaining coding region was amplified from IMAGE consortium EST clone 5503281 (NCBI Acc. BM467107) with primers 3. 5'-gggatcgaagctggacacgttctgc-3' and 4. 5'- gcggccgctcacactccaggggaatcggagcagccgggg-3. The resulting PCR products were used as templates in an overlap PCR reaction with primers 1. and 4. The PCR product was ligated into pCR2.1-TOPO, sequenced, then subcloned further as a *Bgl2-Not1* insert into the *BamH1-Not1* site of pEBG2T and pGEX6P-1 vectors.

Other DNA constructs. The DNA constructs encoding wild type GST-LKB1 or catalytically-inactive GST-LKB1[D194A] in the pEBG-2T vector (Sapkota et al., 2001), FLAG-STRADα, FLAG-STRADβ, *myc*-MO25α, *myc*-MO25β in the pCMV5 vector (Boudeau et al., 2003) or the kinase domain of AMPKα1[residues 1-308] in the pGEX vector (Scott et al., 2002) have been described previously.

Cell culture, stimulation and cell lysis. The generation of HeLa cells stably expressing wild type or kinase dead LKB1 has been described previously (Sapkota et al., 2002). The cells were cultured in Minimum Essential Medium Eagle supplemented with 10% (v/v) tetracycline-free-FBS, 1 X non-essential amino acid solution, 1X penicillin/streptomycin solution (Invitrogen), 100 μg/ml zeocin and 5 μg/ml blasticidin (Invitrogen). The production and culture of immortalised $LKB1^{+/+}$ and $LKB^{-/-}$ MEF cells from E9.5 embryos, was described previously (Hawley et al., 2003). Human kidney embryonic 293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS. Cells cultured on a 10 cm diameter dish in 10% (v/v) serum were left unstimulated, stimulated with 10 mM phenformin or 2mM AICA riboside for 1h and lysed in 1 ml of ice-cold Lysis Buffer after quick rinsing in PBS. MEF cell lysates were snap frozen in liquid nitrogen, thawed prior to use and centrifuged at 4°C for 30 min at 23000 x g to remove cell debris. HeLa cell lysates were centrifuged immediately at 4°C for 15 min at 13 000 rpm. Protein concentrations were determined by the Bradford method with bovine serum albumin as the standard (Bradford, 1976).

Immunoblotting. Total cell lysates (10-50 μg) or immunoprecipitated protein (from 1 mg of cell lysate) were heated in SDS Sample Buffer, and subjected to SDS-PAGE and electrotransfer to nitrocellulose membranes. Membranes were then blocked in 50 mM Tris/HCl pH 7.5, 0.15M NaCl (TBS), 0.5 % (by vol) Tween containing 10 % (by mass)

skimmed milk, and probed for 16 h at 4°C in TBS, 0.5 % (by vol) Tween, 5 % (by mass) skimmed milk and 1 μg/ml of the indicated antibodies. Detection of proteins was performed using horse radish peroxidase conjugated secondary antibodies and the enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Little Chalfont, UK).

Expression and purification of AMPK-related kinases in *E. coli*. Unless otherwise indicated, pGEX constructs encoding full length wild type or mutant forms of GST-HA tagged AMPK-related kinases, or GST-AMPKα1[1-308], were transformed into *E. coli* BL21 cells. One-litre cultures were grown at 37°C in Luria broth containing 100 μg/ml ampicilin until the absorbance at 600 nm was 0.8. 100 μM isopropyl-β-D-galactoside was added, and the cells were cultured for a further 16h at 26°C. For pGEX constructs encoding the wild type or mutant GST-HA-tagged QSK, induction of protein expression was carried out by culturing the cells until the absorbance at 600 nm was 1, and then 1 mM isopropyl-β-D-galactoside was added, and the cells cultured further for 1h at 30°C. Pelleted cells were resuspended in 35 ml of ice-cold lysis buffer and lysed in one round of freeze/thawing, and the lysates were sonicated to fragment DNA. The lysates were centrifuged for 30 min at 20,000 x g, and the recombinant proteins were affinity purified on glutathione-Sepharose and eluted in Buffer A containing 20 mM glutathione and 0.27 M sucrose.

Expression and purification of AMPK-related kinases in 293 cells. Twenty 10 cm diameter dishes of 293 cells were cultured and each dish transfected with 5 μg of the pEBG-2T construct encoding wild type AMPK-related kinase using a modified calcium phosphate method (Alessi et al., 1996). 36 h post-transfection, the cells were lysed in 1 ml of ice-cold Lysis Buffer, the lysates pooled and centrifuged at 4ºC for 10 min at 13,000 x g. The GST-fusion proteins were purified by affinity chromatography on

glutathione-Sepharose and eluted in Buffer A containing 20 mM glutathione and 0.27 M sucrose.

Expression and purification of LKB1:STRAD:MO25 complex in 293 cells. Different combinations of GST-tagged LKB1, FLAG-tagged STRADα or STRADβ, and Myc-tagged MO25α or MO25β were expressed in 293 cells and the complexes purified on glutathione-Sepharose as described previously (Boudeau et al., 2003).

Measurement of activation of AMPK-related kinases. AMPKα1 catalytic subunit and AMPK-related kinases was measured following their phosphorylation with LKB1 as follows. 1-2 μg of AMPKα1 catalytic domain or AMPK-related kinase, were incubated with or without 0.1-1 μg of wild type of the indicated LKB1 complex in Buffer A containing 5 mM MgAcetate and 0.1 mM ATP, in a final volume of 20 μl. After incubation at 30°C for the times indicated in the figure legend, AMPKα1 catalytic domain or AMPK-related kinase activities were determined by adding 30 μl of 5 mM magnesium acetate, 0.1 mM [γ-^{32}P]-ATP (300 cpm/pmol) and 200 μM *AMARA* peptide (AMARAASAAALARRR(Dale et al., 1995)) as substrate . After incubation for 5-20 min at 30°C, incorporation of ^{32}P-phosphate into the peptide substrate was determined by applying the reaction mixture onto P81 phosphocellulose paper and scintillation counting after washing the papers in phosphoric acid as described previously (Alessi et al., 1995). One Unit (U) of activity was defined as that which catalysed the incorporation of 1 nmol of ^{32}P into the substrate. Time course reactions for the second stage of the assay were performed in order to ensure that the rate of phosphorylation was occurring linearly with time. Kinetic data was analysed according to the Michaelis-Menten relationship by non-linear regression using the computer program GraphPad Prism (GraphPad Software Inc, San Diego, USA).

Mapping the sites on BRSK2, NUAK2 and MARK3 phosphorylated by the LKB1 complex. The wild type and mutant AMPK-related kinases (5 μg) were incubated for 30 min with 1-2 μg of wild type LKB1:STRADα:MO25β in Buffer A containing 5 mM magnesium acetate and 100 μM [γ-^{32}P]-ATP (5000 cpm/pmol) in a total reaction volume of 30 μl. After 30 min, the reactions were terminated by adding SDS to a final concentration of 1% (w/v) and dithiothreitol to 10 mM and heated at 100 °C for 1 min. After cooling, 4-vinylpyridine was added to a concentration of 50 mM, and the samples were left on a shaking platform for 30 min at room temperature to alkylate cysteine residues and then subjected to electrophoresis on a BisTris 4-12% polyacrylamide gel. The gels were stained with Coomasie R250, autoradiographed and the bands corresponding to phosphorylated AMPK-related kinases excised and cut into smaller pieces. There were washed sequentially for 15 minutes on a vibrating platform with 1 ml of the following: water, a 1:1 mixture of water and acetonitrile, 0.1 M ammonium bicarbonate, a 1:1 mixture of 0.2 M ammonium bicarbonate and acetonitrile and finally acetonitrile. The gel pieces were dried by rotary evaporation and incubated in 0.1 ml of 50 mM ammonium bicarbonate, 0.1% (by mass) n-octyl-glucoside containing 1μg of alkylated trypsin. After 16 h, 0.1 ml of acetonitrile was added and the mixture incubated on a shaking platform for 10 min. The supernatant was removed and the gel pieces were further washed for 10 min in 0.3 ml of 50 mM ammonium bicarbonate, and 0.1% (v/v) trifluoroacetic acid. The combined supernatants, containing >90% of the ^{32}P-radioactivity, were chromatographed on a Vydac 218TP5215 C_{18} column (Separations Group, Hesperia, CA) equilibrated in 0.1% (by vol) trifluoroacetic acid in water. The column was developed with a linear acetonitrile gradient (diagonal line) at a flow rate of 0.2 ml/min and fractions of 0.1 ml were collected.

Phosphopeptide sequence analysis. Isolated phosphopeptides were analysed on an Applied Biosystems 4700 Proteomics Analyser (MALDI-TOF-TOF) using 5mg/ml alpha

cyannocinnamic acid as the matrix. Spectra were acquired in both reflectron and linear modes and the sequence of potential phosphopeptides were confirmed by performing MALDI-MS/MS on selected masses. The characteristic loss of phosphoric acid (M-98 Da) from the parent phosphopeptide as well as the neutral loss of dehydroalanine(-69) for phosphoserine or dehydroaminobutyric acid (-83) for phosphothreonine were used to assign the position of the phosphorylation site(s). The site of phosphorylation of all the ^{32}P-labelled peptides was determined by solid-phase Edman degradation on an Applied Biosystems 494A sequenator of the peptide coupled to Sequelon-AA membrane (Milligen) as described previously (Campbell and Morrice, 2002).

Identification of T-loop Phosphorylation Site in MELK. The tryptic digest of GST-MELK that had been expressed and purified from *E.coli* was acidified with 0.25M acetic acid in 30% acetonitrile (v/v) and 2 μl (settled volume) of PHOS-select Iron chelate gel (Sigma) was added. The sample was shaken for 30 min and the resin collected in a microC18 ZipTip (Millipore), washed with 2 x 25 μl 0.25 M acetic acid in 30% acetonitrile (v/v) and eluted with 25 μl 0.4 M NH_4OH. The eluate was analysed by MALDI-TOF-TOF mass spectrometry and T-loop phosphopeptide was identified and sequenced by ms/ms.

Immunoprecipitation and assay of endogenous AMPK and AMPK-related kinase
0.1-1 mg of Hela or MEF lysate protein was incubated at 4ºC for 1h on a shaking platform with 5 μg of the corresponding antibody, which had been previously conjugated to 5 μl of protein G-Sepharose. The immunoprecipitates were washed twice with 1 ml of Lysis Buffer containing 0.5 M NaCl, and twice with 1 ml of Buffer A. Phosphotransferase activity towards the *AMARA* peptide was then measured in a total assay volume of 50 μl as described above.

Immunoprecipitation and assay of endogenous LKB1 employing LKBtide substrate. 0.1-1 mg Hela or MEF cell lysate protein was incubated at 4°C for 1h on a shaking platform with 5 μl of protein G-Sepharose conjugated to 5 μg of human LKB1 antibody. The immunoprecipitates were washed twice with 1 ml of Lysis Buffer containing 0.5 M NaCl, and twice with 1 ml of Buffer A. Phosphotransferase activity towards the LKBtide peptide (SNLYHQGKFLQTFCGSPLYRRR residues 241-260 of human NUAK2 with 3 additional Arg residues added to the C-terminal to enable binding to P81 paper), was 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 vol) 2-mercaptoethanol, 10 mM magnesium acetate, 0.1 mM [γ^{32}P]ATP (~200 cpm/pmol) and 200 μM LKB1tide peptide. The assays were carried out at 30°C with continuous shaking, to keep the immunoprecipitates in suspension, and were terminated after 10 min by applying 40 μl of the reaction mixture onto p81 membranes. The p81 membranes were washed in phosphoric acid, and the incorporated radioactivity was measured by scintillation counting as described previously for MAP kinase (Alessi et al., 1995).

Supplementary References

Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P. and Hemmings, B.A. (1996) Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J*, **15**, 6541-6551.

Alessi, D.R., Cohen, P., Ashworth, A., Cowley, S., Leevers, S.J. and Marshall, C.J. (1995) Assay and expression of mitogen-activated protein kinase, MAP kinase kinase, and Raf. *Methods Enzymol*, **255**, 279-290.

Boudeau, J., Baas, A.F., Deak, M., Morrice, N.A., Kieloch, A., Schutkowski, M., Prescott, A.R., Clevers, H.C. and Alessi, D.R. (2003) MO25alpha/beta interact with STRADalpha/beta enhancing their ability to bind, activate and localize LKB1 in the cytoplasm. *EMBO J*, **22**, 5102-5114.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, **72**, 248-254.

Campbell, D.G. and Morrice, N.A. (2002) Identification of Protein Phosphorylation Sites by a Combination of Mass Spectrometry and Solid Phase Edman Sequencing. *J. Biomol. Techn.*, **13**, 121-132.

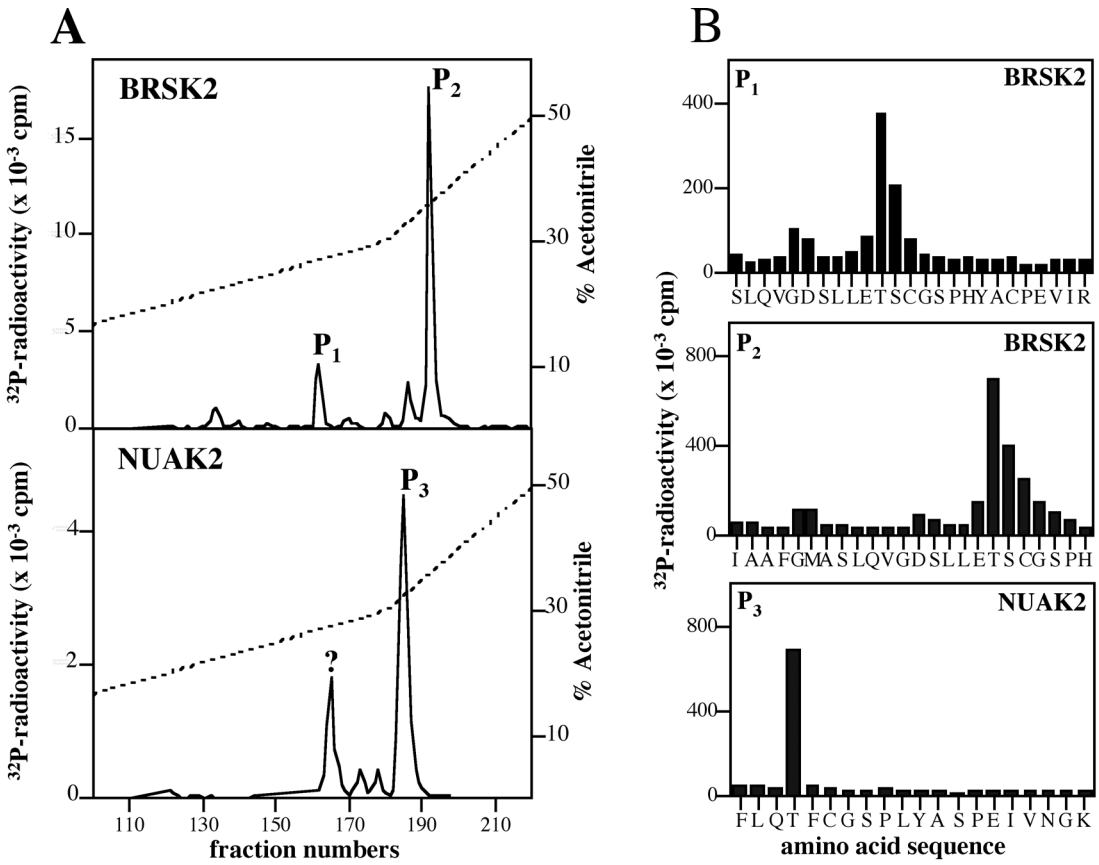
Dale, S., Wilson, W.A., Edelman, A.M. and Hardie, D.G. (1995) Similar substrate recognition motifs for mammalian AMP-activated protein kinase, higher plant

HMG-CoA reductase kinase-A, yeast SNF1, and mammalian calmodulin-dependent protein kinase I. *FEBS Lett*, **361**, 191-195.
Davies, S.P., Carling, D. and Hardie, D.G. (1989) Tissue distribution of the AMP-activated protein kinase, and lack of activation by cyclic-AMP-dependent protein kinase, studied using a specific and sensitive peptide assay. *Eur J Biochem*, **186**, 123-128.
Hawley, S.A., Boudeau, J., Reid, J.L., Mustard, K.J., Udd, L., Makela, T.P., Alessi, D.R. and Hardie, D.G. (2003) Complexes between the LKB1 tumor suppressor, STRADalpha/beta and MO25alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. *J Biol*, **2**, 28.
Ross, H., Armstrong, C.G. and Cohen, P. (2002) A non-radioactive method for the assay of many serine/threonine-specific protein kinases. *Biochem J*, **366**, 977-981.
Sapkota, G.P., Deak, M., Kieloch, A., Morrice, N., Goodarzi, A.A., Smythe, C., Shiloh, Y., Lees-Miller, S.P. and Alessi, D.R. (2002) Ionizing radiation induces ataxia telangiectasia mutated kinase (ATM)- mediated phosphorylation of LKB1/STK11 at Thr-366. *Biochem J*, **368**, 507-516.
Sapkota, G.P., Kieloch, A., Lizcano, J.M., Lain, S., Arthur, J.S., Williams, M.R., Morrice, N., Deak, M. and Alessi, D.R. (2001) Phosphorylation of the protein kinase mutated in Peutz-Jeghers Cancer Syndrome, LKB1/STK11, at Ser431 by p90RSK and cAMP-dependent protein kinase, but not its farnesylation at Cys433, is essential for LKB1 to suppress cell growth. *J Biol Chem*, **276**, 19469-19482.
Scott, J.W., Norman, D.G., Hawley, S.A., Kontogiannis, L. and Hardie, D.G. (2002) Protein kinase substrate recognition studied using the recombinant catalytic domain of AMP-activated protein kinase and a model substrate. *J Mol Biol*, **317**, 309-323.
Sugden, C., Crawford, R.M., Halford, N.G. and Hardie, D.G. (1999) Regulation of spinach SNF1-related (SnRK1) kinases by protein kinases and phosphatases is associated with phosphorylation of the T loop and is regulated by 5'-AMP. *Plant J*, **19**, 433-439.

Supplementary Figure 1. **Analysis of phosphorylation of BRSK2, NUAK2 and MARK4 and MELK**. (A) Catalytically inactive mutants of BRSK2[D159A] and NUAK2[D193A], that are unable to autophosphorylate, were incubated with LKB1 complex for 30 min with Mg^{2+} -[γ^{32}P]ATP and separated by electrophoresis on a polyacrylamide gel which was then autoradiographed. The ^{32}P-labelled proteins were digested with trypsin and the resulting peptides were chromatographed on a C_{18} column. Fractions containing the major ^{32}P-labelled peptides are marked. (B) Peptides P_1, P_2 and P_3 were subjected to solid phase sequencing and ^{32}P-radioactivity was measured after

each cycle of Edman degradation. We were unable to determine the identity of the NUAK2 peptide indicated with "?". (C) The indicated peptides were analysed by MALDI TOF-TOF mass spectrometry as described above. The deduced amino acid sequence and the site of phosphorylation was indicated, together with the observed and theoretical mass. The peptide labelled "MARK4" was derived from MARK4 phosphorylated by LKB1 as described in the legend to Fig 5G. This peptide encompasses the T-loop motif of MARK4 phosphorylated at only the Thr residue. The peptide labelled "MELK" is derived from a tryptic digest of unlabelled MELK that had been expressed and purified from *E. coli* as described above. This peptide encompasses the T-loop of MELK phosphorylated at the Thr residue. Abbreviations: m, methionine sulphone; (p) indicates preceding Thr residue is phosphorylated.

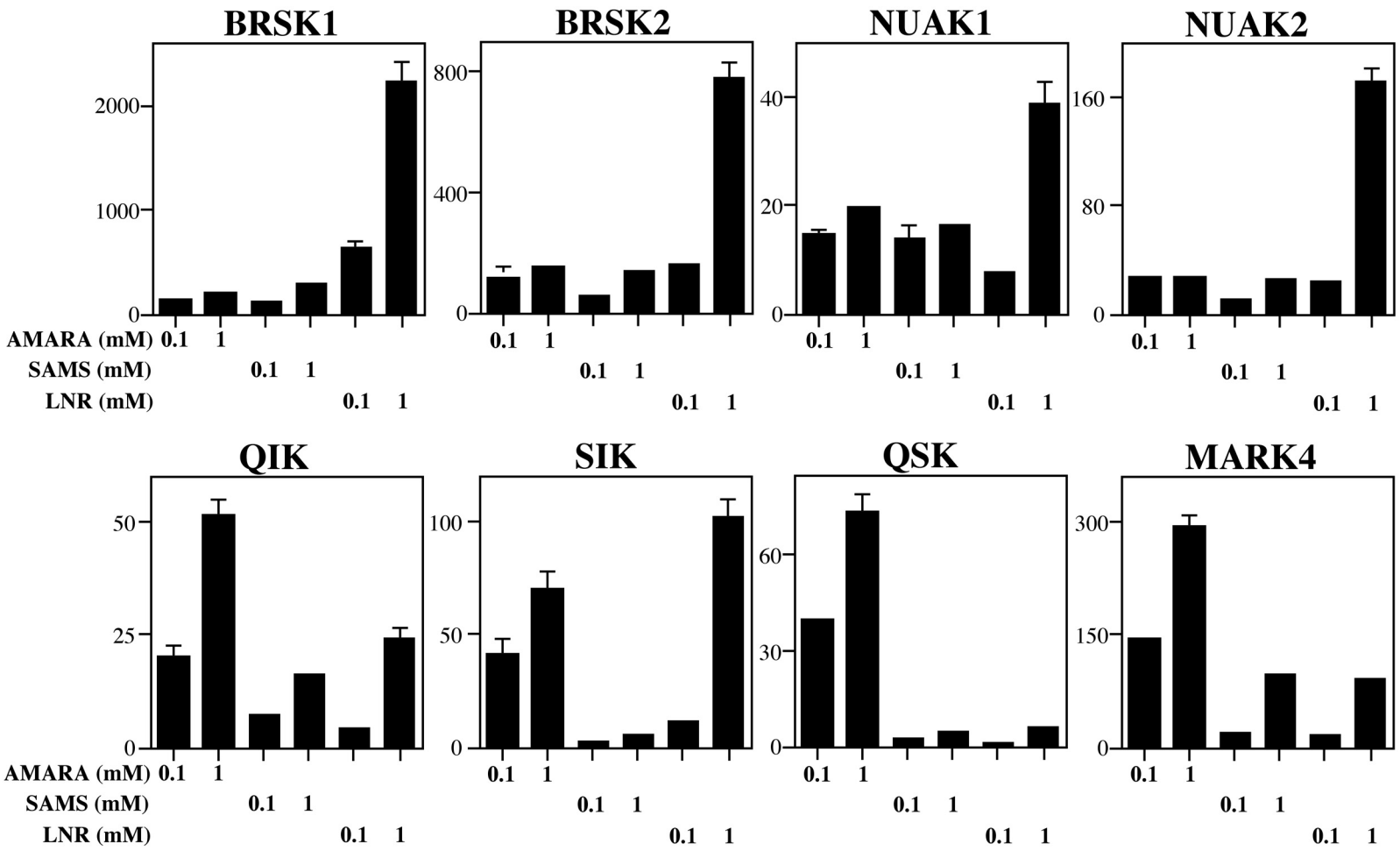
Supplementary Figure 2. **Substrate specificity analysis of the AMPK-related kinases**. The indicated AMPK-related enzymes in which the T-loop was mutated to Glu, in order to activate these enzymes, were assayed using either 0.1 mM or 1 mM of either the AMARA peptide (AMARAASAAALARRR (Dale et al., 1995), the SAMS peptide (HMRSAMSGLHLVKRR (Davies et al., 1989)) or the LNR peptide (KKLNRTLSFAEPG (Ross et al., 2002)). The activities are presented as average ± SD of two separate experiments with each determination performed in triplicate.



C

Kinase	Posphopeptide	Mass observed	Theoretical mass
BRSK2 P_1	IAAFGmASLQVGDSLLET(p)SCGSPHYACPEVIR	3268.7870	3628.6680
BRSK2 P_2	SLQVGDSLLET(p)SCGSPHYACPEVIR	2951.4530	2951.3472
NUAK2 P_3	FLQT(p)FCGSPLYASPEIVNGK	2356.1088	2356.1333
MARK4	LDT(p)FCGSPPYAAPELFQGK	2225.9983	2226.1497
MELK	GNKDYHLQT(p)CCGSLAYAAPELIQCK	2970.4421	2970.3648

Supplementary Figure 1



Supplementary Figure 2

Supplementary Table. Tissue distributions of ESTs encoding the indicated AMPK-related kinases. Abbreviation No., number

BRSK1

Tissue	No
Brain	8
Nervous system	6
Islets of Langerhans	3
Lung tumour	3
Heart	2

Other (epidermis, fetal eye, prostate) 1 per tissue type.

BRSK2

Tissue	No
Eye	14
Nervous system	13
Breast	10
Brain	8
Pooled germ cells	3
Var. tumour cells	7

NUAK1

Tissue	No
Brain	18
Blood/plasma	7
Liver/spleen	5
Eye	4
Uterus	4
Prostate	3
Var. tumour cells	9

Other (lung, head/neck, breast, adipose tissue, placenta, embryonic stem cells) less than 3 per tissue type.

NUAK2

Tissue	No
Blood	18
Placenta	6
Brain	6
Ovary	6
Nervous system	3
Kidney	2
Colon	2
Eye	2
Var. tumour cells	10

Other (including muscle, head/neck, cervix, lung) 1 per tissue type.

MELK

Tissue	No.
Blood	7
Adenocarcinoma	7
Stomach	5
Germinal B cell	5
Germ cell tumour	4
Ascites	4

Other (cervix, eye, nervous system, intestine, heart, brain, uterus, placenta) less than 3 per tissue type.

QIK

Tissue	No.
Eye	17
Liver/spleen	17
Blood	14
Brain	13
Breast	10
Intestine	11
Intestinal tumour	10
Melanocyte	9
Nervous system	9
Insulinoma	7
Lung	7
Placenta	7
Heart	6
Fibroblast	6
Kidney	5
Germ cell tumour	5

Other (prostate, uterus, testis, head, pancreas, bone, stomach) less than 5 per tissue type.

SIK

Tissue	No.
Uterus	12
Adenocarcinoma	12
Intestine tumour	12
Brain tumour	11
Breast	9
Kidney	8
Melanocyte	7
Blood/plasma	7
Stomach	6
Brain	5
Fetal heart	5
Heart	5
Head/neck	5

Other (placenta, cervical tumour, thymus, liver, lung, prostate, marrow, eye, bladder tumour) less than 5 per tissue type,

QSK

Tissue	No
Liver/spleen	25
Lung carcinoma	23
Brain	14
Head/neck	13
Nervous syst. tumour	11
Adenocarcinoma	10
Placenta	10
Melanocyte	9
Pancreas	8
Eye	7
Carcinoid	7
Blood	6
Kidney	6

Other (breast, intestine, testis, lung, uterus, heart) less than 6 per tissue type.

MARK1

Tissue	No
Brain	17
Carcinoid	12
Breast	7
Lung tumour	6
Pancreas	5
Eye	4

Other (heart, head/neck, kidney, nervous system, placenta) less than 3 per tissue type.

MARK2

Tissue	No
Head/neck	18
Brain	17
Intestine	13
Adenocarcinoma	11
Pancreas	9
Placenta	8
Eye	8
Blood	7
Melanoma	6

Other (prostate, kidney, lung, bone, uterus, thymus, nervous system) less than 6 per tissue type.

MARK3

Tissue	No
Brain	29
Nervous system	26
Liver/spleen	23
Eye	21
Placenta	19
Lung	17
Colon	8
Stomach	7
Heart	6
Uterus	6
Kidney	6
Skeletal muscle	5
Cervix	5
Blood	5
Var. tumour cells	102

Other (prostate, testis, pancreas, lymph, morrow, breast) less than 5 per tissue type.

MARK4

Tissue	No
Brain	13
Eye	13
Nervous tissue	11
Liver/spleen	7
Colon	4
Stomach	3
Breast	3
Prostate	3
Testis	2
Lung	2
Var. tumour cells	43