

# Identification of the sucrose non-fermenting related kinase SNRK, as a novel LKB1 substrate

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**Abstract** Recent work has shown that the LKB1 tumour suppressor protein kinase phosphorylates and activates protein kinases belonging to the AMP activated kinase (AMPK) subfamily. In this study, we identify the sucrose non-fermenting protein (SNF1)-related kinase (SNRK), a largely unstudied AMPK subfamily member, as a novel substrate for LKB1. We demonstrate that LKB1 activates SNRK by phosphorylating the T-loop residue (Thr173), and that the LKB1 regulatory subunits STRAD and MO25 are required for LKB1 to activate SNRK. We find that SNRK is not active when expressed in HeLa cells that lack expression of LKB1, and its activity is restored by expression of wild type LKB1, but not catalytically deficient LKB1. We also present evidence that two other AMPK-related kinases more distantly related to AMPK than SNRK, namely NIM1 and testis-specific serine/threonine kinase-1 (TSSK1) are not substrates for LKB1. Tissue distribution analysis indicates that SNRK protein is mainly expressed in testis, similar to TSSK isoforms, whereas NIM1 is more widely expressed. These results provide evidence that SNRK could mediate some of the physiological effects of LKB1.

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## 1. Introduction

LKB1 was originally identified as a gene mutated in the inherited Peutz Jeghers Syndrome (PJS), in which subjects are predisposed to developing benign and malignant tumours [1,2]. Subsequent work demonstrated that overexpression of LKB1 in various cell lines induced a G1 cell cycle arrest [3,4] and that mice lacking one allele of the LKB1 gene developed tumours similar to those found in PJS in humans (reviewed in [5]). Genetic analysis in *Caenorhabditis elegans* [6], *Drosophila* [7], *Xenopus* [8] and mammalian cells [9], also indicated that LKB1 also plays an important role in regulating cell polarity.

In vivo, LKB1 is complexed to STRAD an inactive pseudokinase [10] and MO25 [11], an armadillo repeat domain scaffolding protein [12]. The binding of LKB1 to STRAD and MO25, localises it in the cell cytosol and activates LKB1 by a mechanism not involving T-loop phosphorylation [13]. The

first physiological substrate of LKB1 was identified as the AMP activated protein kinase (AMPK) [14–16], which functions as a regulator of cellular energy charge [17]. LKB1 complexed to STRAD and MO25 phosphorylates the T-loop Thr residue of AMPK (Thr172), leading to activation of this enzyme. Further evidence that LKB1 is a key regulator of AMPK in cells, comes from the finding that AMPK cannot be activated in LKB1 knockout fibroblasts or HeLa cells which do not express LKB1, in response to a variety of treatments that activate AMPK in LKB1 expressing cells.

Inspection of the human genome indicates that in addition to the 2 isoforms of AMPK, 12 other less studied kinases (MARK1, MARK2, MARK3, MARK4, QIK, QSK, SIK, NUA1, NUA2, BRSK1, BRSK2, and MELK), are highly related to AMPK (Fig. 1A). Apart from MELK, which autophosphorylates its own T-loop residue, LKB1 complexed to STRAD and MO25, phosphorylated the T-loop Thr residue of the other members of the AMPK-related kinases, resulting in activation of these enzymes [18]. Moreover, the AMPK-related kinases activated by LKB1 possessed vastly lower activity in LKB1-deficient cell lines compared to LKB1-expressing cells, providing genetic evidence that these enzymes are indeed physiological substrates of LKB1 [18].

Examination of the human kinome [19] shows that there are eight other largely unstudied kinases (NIM1, SNRK, TSSK1, TSSK2, TSSK3, TSSK4, SSTK, and HUNK), on the same branch as AMPK on the human kinome tree (see Fig. 1A). Sequence analysis of these enzymes indicates that they are less related to AMPK than the AMPK-related enzymes that have previously been shown to be substrates for LKB1. Nevertheless, alignment of the T-loop sequences of these enzymes, indicate that they possess a Thr-residue located at an identical position to the T-loop of AMPK subfamily kinases phosphorylated by LKB1, and many of the surrounding residues are also conserved (Fig. 1A). This suggests that LKB1 might also phosphorylate these enzymes and in this study, we provide evidence that one of these enzymes, namely, the sucrose non-fermenting related kinase (SNRK) is a substrate for LKB1.

## 2. Materials and methods

### 2.1. Materials

Protease-inhibitor cocktail tablets and sequencing grade trypsin were obtained from Roche; P81 phosphocellulose paper was from Whatman; [ $\gamma$ - $^{32}$ 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. The

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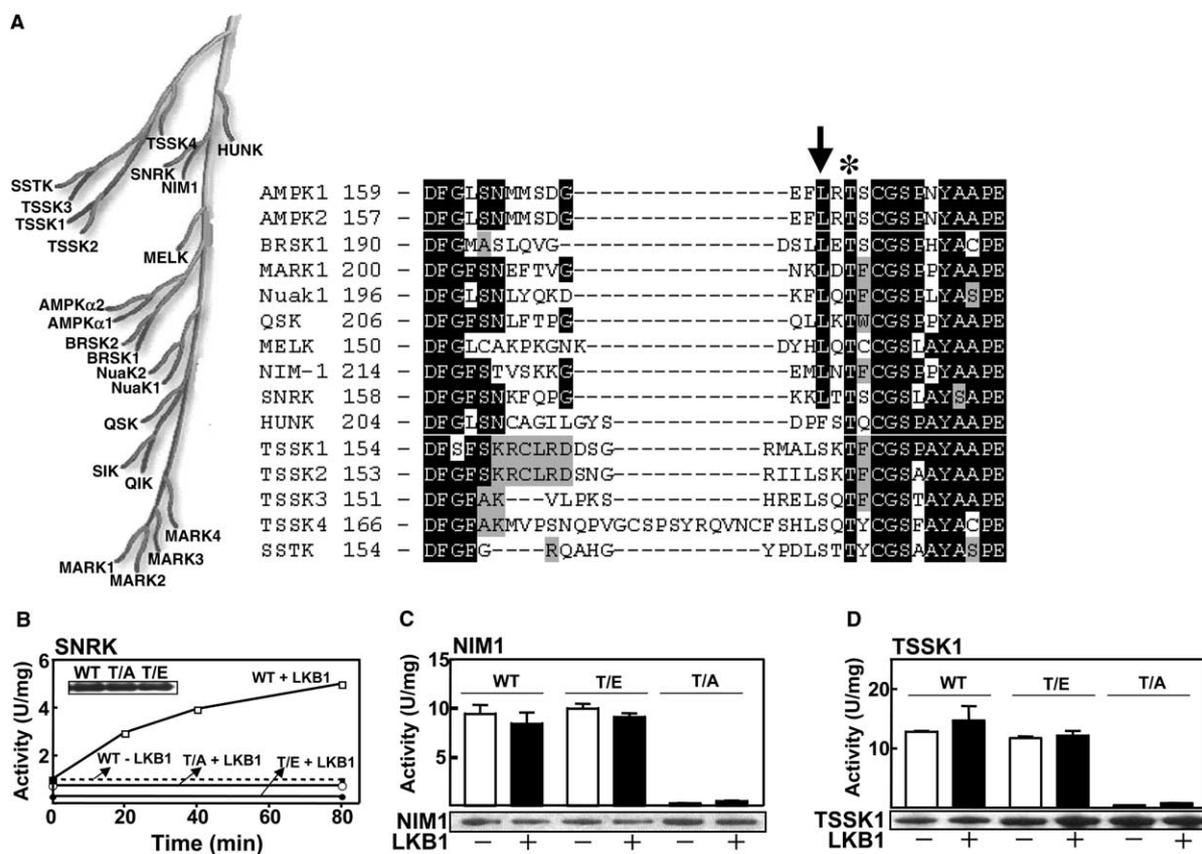


Fig. 1. Activation of SNRK by LKB1. (A) Dendrogram and alignment of the T-loop sequences of AMPK subfamily protein kinases [19]. The T-loop Thr residue is marked with an asterisk and the -2 Leu is marked with an  $\downarrow$ . The identical residues are shaded black and the conserved residues in grey. (B) Wild-type (WT) SNRK, SNRK[T173A] (T/A) or SNRK[T173E] (T/E), were incubated in the presence or absence of the LKB1:STRAD:MO25 complex in the presence of  $Mg^{2+}$  and ATP. At the indicated time points SNRK activity was quantified utilising the *AMARA* peptide and the results are expressed as specific activities. (C) Wild-type NIM1, NIM1[T229A] (T/A) or NIM1[T229E] (T/E) was incubated in the presence or absence of LKB1:STRAD:MO25 complex as in (B). After 30 min, the activity of the NIM1 towards the *AMARA* peptide was quantified. (D) Similarly, Wild-type TSSK1, TSSK1[T174A] (T/A) or TSSK1[T174E] (T/E) was incubated either in the absence or presence of LKB1:STRAD:MO25 complex. After 30 min, the activity of the TSSK1 was quantified employing the *AMARA* peptide as a substrate. Results shown are means  $\pm$  S.D. of assays carried out in duplicate and representative of three independent experiments.

following antibodies were raised in sheep and affinity purified on the appropriate antigen: SNRK (residues 1–20 of human SNRK) and NIM1 (residues 1–28 of human NIM1). Secondary antibodies coupled to horseradish peroxidase were from Pierce. Immortalised LKB1<sup>+/+</sup> and LKB1<sup>-/-</sup> mouse embryonic fibroblast cells from E9.5 kindly provided to us by Tomi P. Mäkelä and co-workers [14], have been previously described.

## 2.2. General methods

Tissue culture, transfection, immunoblotting, restriction enzyme digests, DNA ligations, and other recombinant DNA procedures were performed using standard protocols. All mutagenesis were carried out using the Quick-Change site-directed mutagenesis method (Stratagene). DNA constructs used for transfection were purified from *Escherichia coli* DH5 $\alpha$  using Qiagen plasmid Mega or Maxi 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.

## 2.3. Buffers

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, 10 mM sodium- $\beta$ -glycerophosphate, 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.

## 2.4. Cloning of SNRK, TSSK1, and NIM1

The full length coding region of human SNRK cDNA (NCBI Acc NM\_017719) containing a 5' HA tag sequence were amplified from overlapping IMAGE consortium EST clone 813590 and 6189654 using primers 5'-gcaattctaccatcacgatgtgccagattacccgaggattaaagcagggtatgatgg-3' and 5'-cgaattctcagatgacatggcaacagctggc-3'. The resulting PCR product was ligated into pCR2.1-TOPO vector (Invitrogen), sequenced, and subcloned as an *Eco*R1-*Eco*R1 insert into the pGEX6P-1 expression vector. Full length SNRK with 5' HA tag sequence was amplified from the pCR2.1-TOPO clone using primers that incorporated *Not*I sites at the 5' and 3' ends for subcloning into the pEBG2T expression vector as a *Not*I/*Not*I insert. The coding region of human NIM1 cDNA (NCBI Acc NM\_153361) with an N-terminal HA tag was amplified from IMAGE consortium EST 4797792 using primers 1. 5'-actagtggccaccatgaccatcacgatgtgccagattacgccaactgcatgtatgaatggagggtggc-3' and 2. 5'-actagtgtttataaaatcgagcaaaattggatgtgtg-3'. The resulting PCR product was ligated into pCR2.1-TOPO vector sequenced and subcloned as a *Spe*I-*Spe*I insert into pEBG2T and *Spe*I-modified pGEX6P-1 expression vectors. The coding region of human TSSK1 cDNA (NCBI Acc NM\_032028.2) with an N-terminal HA tag was amplified from IMAGE consortium EST 5298216 using primers 1. 5'-ggatccgccaccatgaccatcacgatgtgccagattacccgatgacgctgctgctcaagcagcagg-3' and 2. 5'-ggcggcctcactggcggcggctctgaggcgtg-3'.

The PCR product was ligated into pCR2.1-TOPO vector, sequenced, and subcloned as a *Bam*H1–*Not*I insert into pGEX6P-1 and pEBG2T expression vectors.

### 2.5. Expression and purification of AMPK-related kinases in *E. coli*

The pGEX constructs of the full-length wild-type or mutant forms of GST-HA tagged AMPK-related kinases were transformed in *E. coli* BL21 cells. One litre cultures were grown at 37 °C in LB broth supplemented with 100 µg/ml ampicillin and induced at an OD<sub>600</sub> of 0.8 with 100 µM IPTG. Cultures were incubated for a further 16 h at 26 °C before harvesting by centrifugation at 4000 × *g* at 4 °C for 30 min. Enzymes were purified by glutathione–sepharose affinity purification as described previously [18].

### 2.6. Activation of SNRK by LKB1

1–2 µg of *E. coli*-expressed GST-SNRK (or GST-NIM1 or GST-TSSK1), was incubated with or without 0.3 µg LKB1 in buffer A containing 5 mM Mg-acetate and 0.1 mM ATP in a final volume of 20 µl. After incubation at 30 °C for the times indicated, 5 µl of the incubation mix was quenched in 25 µl of ice-cold buffer A containing 2 mM EDTA. The activities of the quenched AMPK-related kinase were determined by adding 20 µl of 10 mM Mg-acetate, 0.1 mM [ $\gamma$ -<sup>32</sup>P] ATP, and 200 µM *AMARA* peptide (AMARAASAAALARRR) as substrate [18]. After 15 min incubation at 30 °C, incorporation of <sup>32</sup>P-phosphate into the *AMARA* peptide substrate was determined by using the P81 phosphocellulose paper method [20]. One unit of activity was defined as that which catalysed the incorporation of 1 nmol of <sup>32</sup>P into the *AMARA* peptide.

### 2.7. Immunoprecipitation and assay SNRK, NIM1 or TSSK1

0.1–1 mg of the indicated cell lysate was incubated at 4 °C for 1 h on a shaking platform with 5 µg of antibody conjugated to 5 µl of protein G–sepharose. Immunoprecipitates washed twice with Lysis buffer containing 0.5 M NaCl, and twice with buffer A. Kinase activity of the immunoprecipitates towards the *AMARA* peptide was then measured in a total assay volume of 50 µl as described above.

### 2.8. Mapping phosphorylation sites

SNRK (10 µg) was incubated for 40 min with 2 µg of wild type LKB1:STRAD $\alpha$ :MO25 $\alpha$  in buffer A containing 5 mM Mg-acetate and 100 µM [ $\gamma$ -<sup>32</sup>P]-ATP (5000 cpm/pmol) in a total reaction volume of 50 µl. The reaction was 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. The sites phosphorylated on SNRK were mapped using an Applied Biosystems 4700 Proteomics Analyser (MALDI–TOF–TOF) [18] and on solid-phase Edman degradation on an Applied Biosystems 494A sequenator of the peptide coupled to Sequelon-AA membrane (Milligen) [21]. To identify the sites of phosphorylation on the T-loop of TSSK1, a tryptic peptide digest of GST-TSSK1 that had been expressed and purified from *E. coli* was analysed by electrospray mass spectrometry on a Q-TOF2 (Waters) electrospray mass spectrometer. Tandem ms/ms spectral peak lists were searched using the Mascot search engine (Matrix Science) against a local database. Phosphorylation sites predicted from the Mascot search were validated by manual interpretation of the ms/ms spectra.

## 3. Results

### 3.1. Activation of SNRK by LKB1

We initially cloned and attempted to express in *E. coli* GST fusion proteins encoding full length human NIM1, SNRK, TSSK1 and HUNK as representative members of the AMPK-related kinases that had not been previously tested as LKB1 substrates. We were able to express soluble NIM1, SNRK, and TSSK1, but were unable to express a soluble form of HUNK in *E. coli* or mammalian 293 cells. We next developed an assay for NIM1, SNRK, TSSK1 using the *AMARA* peptide [22], a substrate for all the AMPK-subfamily kinases

tested thus far, which is not directly phosphorylated by the LKB1:STRAD:MO25 complex [18]. Interestingly, incubation of SNRK with LKB1:STRAD:MO25 complex and Mg-ATP resulted in time dependent 5-fold activation of SNRK (Fig. 1B). In contrast, NIM1 (Fig. 1C) and TSSK1 (Fig. 1D) when expressed in *E. coli*, were highly active in the absence of LKB1 and their activity not significantly increased by incubation with amounts of LKB1:STRAD:MO25 complex that activated SNRK.

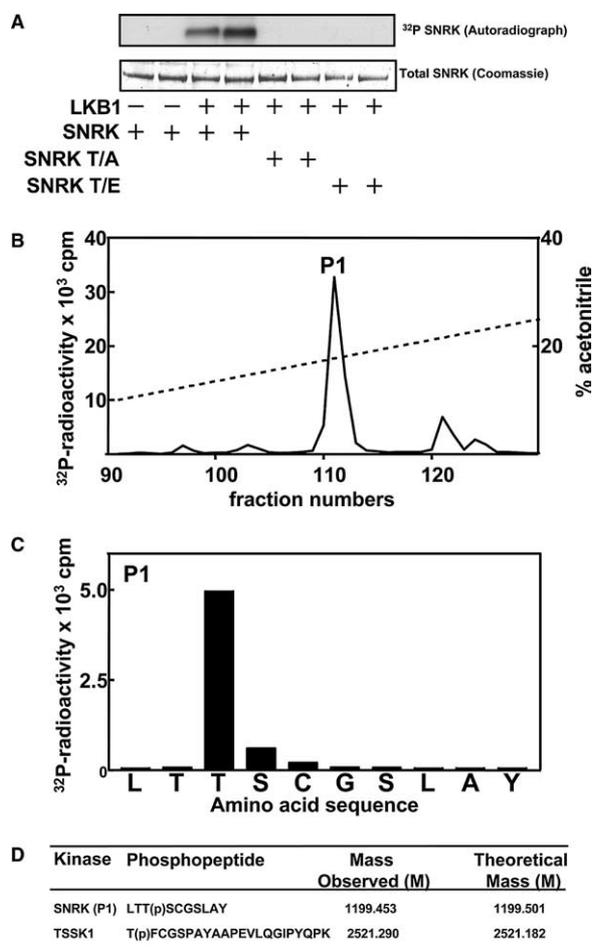
### 3.2. Mechanism of activation of SNRK by LKB1

To determine whether LKB1 activated SNRK by phosphorylating its T-loop Thr residue (Thr173), this was mutated to either Ala to prevent phosphorylation or Glu to mimic phosphorylation. Both the SNRK[T173A] and SNRK[T173E] mutants possessed ~2-fold lower activity than wild-type SNRK in the absence of LKB1 phosphorylation, and their activity was not elevated following incubation with LKB1 complex and Mg-ATP (Fig. 1A). A catalytically inactive point mutant of SNRK that does not autophosphorylate, was phosphorylated by LKB1 to a stoichiometry of 0.7 mol of phosphate per mole of SNRK, and mutation of Thr173 to Ala or Glu prevented phosphorylation of SNRK by the LKB1 complex (Fig. 2A). The LKB1-phosphorylated <sup>32</sup>P-labelled SNRK, was digested with trypsin and the resulting peptides separated by chromatography on a C<sub>18</sub> column and a single major <sup>32</sup>P-eluting peptide termed P1 was observed (Fig. 2B). Solid phase Edman sequencing (Fig. 2C) and MALDI–TOF mass spectrometry (Fig. 2D), confirmed the identity of P1 as the SNRK tryptic T-loop peptide phosphorylated at Thr173.

Mutation of the T-loop Thr of NIM1 (Fig. 1C) or TSSK1 (Fig. 1D) to Ala inactivated these enzymes, and the mutants could not be activated by incubation with LKB1 complex and Mg-ATP. In contrast, mutation of the T-loop Thr on NIM1 and TSSK1 to Glu to mimic phosphorylation, restored catalytic activity to a similar level as that of wild-type enzyme. Electrospray Q-TOF2 mass spectroscopy analysis of a trypsin digested TSSK1 purified from *E. coli*, revealed that it was phosphorylated at its T-loop residue (Fig. 2D), indicating that TSSK1 as was previously shown for MELK [18], can autophosphorylate its T-loop Thr residue.

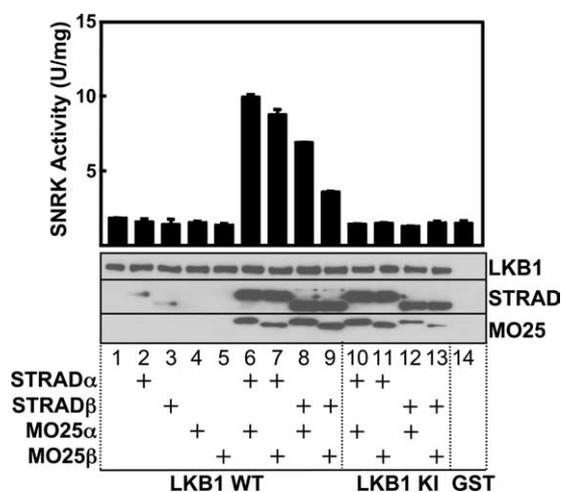
### 3.3. Activation of SNRK requires LKB1 catalytic activity as well as STRAD and MO25

To establish the importance of LKB1 catalytic activity and the STRAD and MO25 subunits in enabling LKB1 to activate the SNRK, we expressed wild-type or catalytically inactive GST-tagged LKB1[D194A], FLAG-tagged STRAD $\alpha$ / $\beta$  and *myc*-tagged MO25 $\alpha$ / $\beta$  in various combinations in 293 cells, affinity purified the complexes on glutathione–sepharose and tested the complexes for their ability to activate SNRK (Fig. 3). Heterotrimeric LKB1 complexes containing a catalytically inactive mutant of LKB1 were unable to activate SNRK, confirming that LKB1 mediated the activation of SNRK in this reaction (Fig. 3, compare lanes 6–9 with lanes 10–13). Non-complexed LKB1 failed to activate SNRK (Fig. 3, compare lanes 1 and 14). The same result was obtained with LKB1 that had been co-expressed with MO25 $\alpha$  or MO25 $\beta$  (Fig. 3, lanes 4 and 5), which was as expected, since these proteins do not interact with LKB1 in the absence of STRAD $\alpha$ / $\beta$  [11]. LKB1:STRAD complexes



**Fig. 2.** *LKB1 phosphorylates SNRK specifically at the T-loop.* (A) *E. coli* expressed kinase inactive SNRK[D158A], SNRK[D158A,T173A] (T/A) or SNRK[D158A,T173E] (T/E), were incubated with LKB1:STRAD:MO25 complex in the presence of  $Mg^{2+}$  and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . Phosphorylation of the SNRK protein was determined following electrophoresis on a polyacrylamide gel and subsequent autoradiography of the Coomassie blue-stained bands corresponding to each substrate. Similar results were obtained in three separate experiments. (B)  $^{32}\text{P}$ -labelled catalytically inactive SNRK [D158A] after phosphorylation with the LKB1:STRAD:MO25 $\alpha$  complex, was digested with trypsin and chromatographed on a  $C_{18}$  column. Fractions containing the major  $^{32}\text{P}$ -labelled tryptic peptide (P1) possessing 64% of the applied  $^{32}\text{P}$ -radioactivity are shown. No other major  $^{32}\text{P}$ -labelled peptides were observed in other fractions of the chromatography. (C) Peptide P1 was subjected to solid-phase sequencing and  $^{32}\text{P}$ -radioactivity was measured after each cycle of Edman degradation. (D) Peptide P1 was analysed by MALDI-TOF-TOF mass spectrometry and the deduced amino acid sequence and the site of phosphorylation was indicated, together with the observed and theoretical mass. The P1 peptide sequence was deduced as LTT(p)SCGSLAY ((p) indicates preceding Thr residue is phosphorylated) in which the Cys residue was pyridethylated from alkylation with 4-vinylpyridine prior to digestion. P1 was generated by a mixed tryptic/chymotryptic cleavage resulting from low level chymotryptic activity in the trypsin employed. The peptide labelled "TSSK1" is derived from a tryptic digest of unlabelled TSSK1 that had been expressed and purified from *E. coli* and analysed by electrospray mass spectrometry on a Q-TOF2 as in Section 2. This peptide encompasses the T-loop of TSSK1 phosphorylated at the Thr residue. The results in (D) are Mass of peptide calculated from  $m/z$  observed in either MALDI-TOF-TOF analysis for SNRK or electrospray for TSSK1. Abbreviations: (p) indicates preceding Thr residue is phosphorylated.

also failed to induce a detectable activation of SNRK (Fig. 3, compare lanes 2 and 3). Heterotrimeric complex containing LKB1, STRAD $\alpha$  or STRAD $\beta$ , and MO25 $\alpha$  or MO25 $\beta$



**Fig. 3.** *Activation of SNRK by LKB1 requires STRAD and MO25.* The indicated combinations of GST-tagged wild-type (WT) LKB1 (lanes 1–9) or kinase inactive (KI) LKB1[D194A] (lanes 10–13) or GST alone (lane 14), FLAG-tagged STRAD $\alpha$  or STRAD $\beta$ , and *myc*-tagged MO25 $\alpha$  or MO25 $\beta$ , were co-expressed and affinity purified on glutathione-sepharose from 293 cells as described previously [14]. The complexes were assayed for their ability to activate SNRK and results are expressed as specific activity employing the *AMARA* peptide as substrate. Results shown are means  $\pm$  S.D. of assays carried out in duplicate and representative of four independent experiments. Samples of the incubations were also analysed by Western blotting and probed using anti-GST to detect LKB1, anti-Flag to detect STRAD isoforms and anti-*myc* to detect MO25 isoforms.

was required to obtain significant activation of SNRK (Fig. 3, lanes 6–9). As previously reported for AMPK $\alpha$ 1 [14] and AMPK-related kinases [18], the LKB1:STRAD $\alpha$ :MO25 $\alpha$  complex activated SNRK most efficiently.

### 3.4. Activation of SNRK in HeLa cells requires LKB1

In order to investigate SNRK activity in cells, we generated peptide antibodies that detect <1 ng of recombinant SNRK in an immunoblot analysis and readily immunoprecipitate SNRK in an active form that had been expressed by transient transfection in 293 cells (data not shown). Using this antibody, we were unable to detect endogenous SNRK protein or immunoprecipitate detectable SNRK activity in all cell lines tested, including 293 cells, LKB1 $^{-/-}$  or LKB1 $^{+/+}$  mouse embryonic fibroblasts cells or HeLa cells that do not express LKB1 (data not shown). In order to obtain evidence that LKB1 regulated SNRK in cells, we overexpressed SNRK by transient transfection in control HeLa cells that lack LKB1 as well as HeLa cells that stably express either wild-type or catalytically inactive LKB1 (Fig. 4). SNRK purified from HeLa cells expressing wild-type LKB1 was substantially more active than SNRK expressed in control HeLa cells lacking LKB1 or HeLa cells expressing catalytically inactive LKB1 (Fig. 4A).

We also generated a NIM1 peptide antibody, and in contrast to SNRK, were readily able to detect, immunoprecipitate and assay endogenously expressed NIM1 in all cell lines tested. Consistent with the notion that NIM1 is not regulated by LKB1, NIM1 possessed the same catalytic activity when immunoprecipitated from control HeLa cells or cells expressing catalytically active LKB1 (Fig. 4B). We also observed that NIM1 immunoprecipitated from

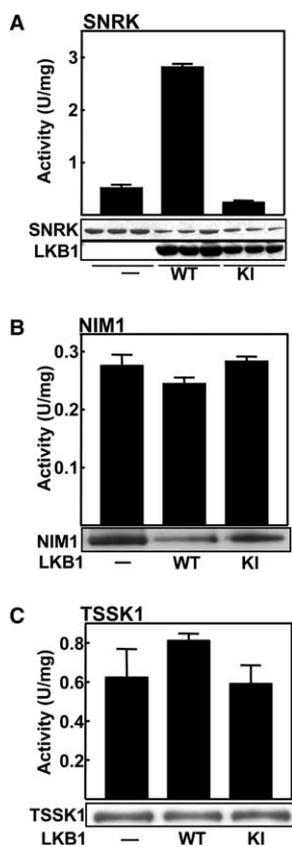


Fig. 4. *SNRK* requires *LKB1* to be active in cells. (A) GST-*SNRK* was overexpressed in Control HeLa cells (–) lacking *LKB1* expression or HeLa cells stably expressing wild-type (WT) *LKB1* or kinase inactive (KI) *LKB1*[D194A]. Forty-four hours post-transfection cells were lysed and GST-*SNRK* affinity purified from the cell lysates and its activity quantified employing the *AMARA* peptide as substrate. Cell lysates were immunoblotted with an anti-HA antibody to detect *SNRK* or anti-*LKB1* was used to detect *LKB1*. (B) The activity of endogenous *NIM1* immunoprecipitated from the indicated HeLa cell lysates was determined using the *AMARA* peptide as substrate. (C) As in (A) except that *TSSK1* was expressed rather than *SNRK*. Results are shown as means  $\pm$  S.D. of 2–4 assays and are representative of two independent experiments.

*LKB1*<sup>−/−</sup> mouse embryonic fibroblast cells, possessed the same activity as that isolated from wild-type *LKB1*<sup>+/+</sup> cells (MJ & AM, data not shown). We were unable to generate an antibody recognising *TSSK1*, but observed that *TSSK1* in contrast to *SNRK*, possessed high basal activity when expressed in control HeLa cells, which was not further stimulated by overexpression of *LKB1* (Fig. 4C).

### 3.5. Tissue distribution of *SNRK* and *NIM1* protein

To investigate the protein distribution of *SNRK* and *NIM1*, we attempted to immunoprecipitate these enzymes from 9 rat tissues, and subjected these to immunoblot and *AMARA* peptide kinase activity analysis. We were only able to detect *SNRK* protein and activity in testis extracts (Fig. 5A). In contrast, *NIM1* protein could be detected at varying levels in all tissues, but *AMARA* peptide kinase activity of *NIM1* immunoprecipitates was most abundant in brain and testis (Fig. 5B).

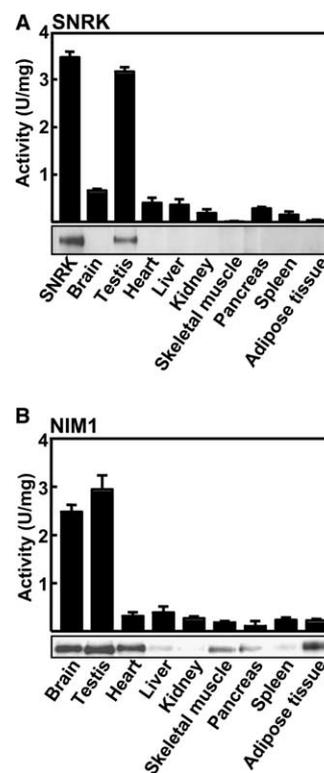


Fig. 5. Tissue distribution of *SNRK* and *NIM1*. *SNRK* (A) or *NIM1* (B) was immunoprecipitated from 1 mg of the indicated rat tissue lysates using specific antibodies as described in Section 2. The immunoprecipitates were either subjected to an in vitro *AMARA* peptide kinase activity or immunoblotted with antibodies recognising the *SNRK* (A) or *NIM1* (B) proteins. Results of kinase activities are means  $\pm$  S.D. of assays carried out in triplicate and representative of two independent experiments.

## 4. Discussion

*SNRK* was originally identified in a PCR-based screen designed to identify novel protein kinases [23]. Subsequent studies indicated that *SNRK* mRNA was induced 3-fold when granule neurons were cultured in low potassium, indicating that it could play a role in regulating survival responses in these cells [24]. Analysis of *SNRK* mRNA suggested that it was expressed in hematopoietic progenitor cells and several leukemic cell lines [25]. Northern blot analysis of *SNRK* mRNA in rat tissues indicated that it was expressed mainly in brain and testis [24]. Analysis of rat *SNRK* mRNA expression in NCBI UniGene ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)), indicates that it is only detected in testis tissue consistent with the results presented in Fig. 5A. In contrast, analysis of human *SNRK* mRNA expression in NCBI UniGene, indicates that it is found in diverse tissues. Although *SNRK* mRNA has been detected in Jurkat cells [25], we have been unable to detect *SNRK* protein in these cells using our antibody (MJ data not shown), suggesting that *SNRK* protein levels may not correlate with mRNA levels. Our finding that *SNRK* is abundant in testis, indicates that it may have similar tissue distribution to the 4 isoforms of *TSSK*, whose mRNA are almost exclusively expressed in the testis [26–29], and have thus been proposed

to play a role in regulating spermatogenesis and/or sperm motility.

Our finding that TSSK1 is active and phosphorylated at its T-loop residue when expressed in *E. coli*, and not further activated by LKB1 suggests that it is not a substrate for LKB and is able to autoactivate itself. As the 3 other isoforms of TSSK are highly homologous in sequence to TSSK1 [26], it is likely that these will also not be activated by LKB1.

Cantley and colleagues have determined the optimal substrate phosphorylation motif for LKB1, employing degenerate peptide library screen and found that LKB1 possesses a strong preference to phosphorylate Thr residues which possess a Leu residue at the  $-2$  position [16]. Interestingly, SNRK together with the 13 other AMPK subfamily kinases that are phosphorylated and activated by LKB1 (AMPK $\alpha$ 1, AMPK $\alpha$ 2, NUA1, NUA2, BRSK1, BRSK2, QIK, SIK, QSK, MARK1, MARK2, MARK3 and MARK4), possesses a Leu residue at the  $-2$  position from the T-loop Thr (Fig. 1A). In contrast, TSSK1, TSSK2, TSSK3, TSSK4, SSK1 as well as HUNK (hormonally up regulated Neu-associated kinase [30]) do not possess such a  $-2$  Leu residue before the T-loop Thr residue, suggesting a reason for why these proteins may not be LKB1 substrates. Unfortunately, we were unable to express significant levels of HUNK, that has been suggested contribute to changes in the mammary gland that occur during pregnancy in response to ovarian hormones [31]. However, as HUNK is the least related AMPK subfamily kinase to AMPK and also lacks the  $-2$  Leu residue (Fig. 1A), it may not comprise an LKB1 substrate.

To our knowledge NIM1 has not been previously been studied and its DNA sequence has only been reported from human mouse and rat genome sequencing projects. NIM1 possess only 436 amino acids and is thus over 200 residues shorter in sequence than the other AMPK-related kinases phosphorylated by LKB1. Interestingly, NIM1 does possess a Leu residue at the  $-2$  position from the T-loop Thr. However, as NIM1 expressed in *E. coli* (Fig. 1C) or HeLa cells lacking LKB1 (Fig. 4B) is active, it is unlikely to be a physiological substrate for LKB1. The finding that mutation of the T-loop Thr residue of NIM1 to Ala, inactivated the enzyme, whereas, its mutation to Glu to mimic phosphorylation restored activity, also suggests that NIM1 activates itself by autophosphorylating its own T-loop residue. In this regard, NIM1 is similar to the MELK AMPK-related kinase, which also possesses a Leu residue at the  $-2$  position, but is capable of autophosphorylating itself at this residue [18]. It should be noted that although we can detect NIM1 protein by immunoblot analysis in all rat tissues analysed, significant levels of NIM1 activity following immunoprecipitation were only observed in brain and testis tissues. This may indicate that NIM1 is maintained in an inactive form in tissues such as muscle and adipose tissue (Fig. 5B). Further work is required to establish the physiological roles of SNRK. In conclusion, we provide evidence that SNRK is activated by LKB1 and could therefore mediate cellular effects regulated by LKB1. Moreover, a significant number of inherited forms of PJS do not possess mutations in the LKB1 gene [32], suggesting that there are other causative loci for PJS. It would be interesting to test whether these PJS families possess mutations in the SNRK gene.

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