Functional analysis of Peutz-Jeghers mutations reveals that the LKB1 carboxy-terminal region exerts a crucial role in regulating both the AMPK pathway and cell polarity

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Published by Oxford University Press 2005
Germline mutations of the *LKB1* gene are responsible for the cancer-prone Peutz-Jeghers syndrome (PJS). *LKB1* encodes a serine/threonine kinase that acts as a regulator of cell-cycle, metabolism and cell polarity. The majority of PJS missense mutations abolish LKB1 enzymatic activity and thereby impair all functions assigned to LKB1. Here, we have investigated the functional consequences of recurrent missense mutations identified in PJS and in sporadic tumors which map in the LKB1 C-terminal non-catalytic region. We report that these C-terminal mutations do not disrupt LKB1 kinase activity nor interfere with LKB1-induced growth arrest. However, these naturally occurring mutations lessened LKB1-mediated activation of the AMP-activated protein kinase (AMPK) and impaired downstream signalling. Furthermore, C-terminal mutations compromise LKB1 ability to establish and maintain polarity of both intestinal epithelial cells and migrating astrocytes. Consistent with these findings, mutational analysis reveals that the LKB1 tail exerts an essential function in the control of cell polarity. Overall, our results ascribe a crucial regulatory role to the LKB1 C-terminal region. Our findings further indicate that LKB1 tumour suppressor activity is likely to depend on the regulation of AMPK signalling and cell polarization.
**Introduction**

Peutz-Jeghers syndrome (PJS) is an autosomal dominant disorder typified by multiple hamartomatous polyps of the gastrointestinal tract and by mucocutaneous pigmented macules (1, 2). PJS patients are predisposed to the development of various neoplasms commonly affecting the breast and the intestine but also testis, cervix and pancreas (3, 4). Germline mutations of the \(LKB1\) gene (also called \(STK11\)), which is located on chromosome 19p13.3, have been identified in the majority of PJS patients (5, 6). Somatic mutations of \(LKB1\) seldom occur in sporadic tumors but appear relatively common in adenocarcinoma of the lung (7). Consistent with a causative role of \(LKB1\) in PJS, heterozygous mice carrying a disrupted \(lkb1\) allele develop gastric polyps resembling PJS hamartomas (8-11). The inactivating nature of PJS mutations together with a frequent loss of the wild type allele in PJS tumors sustain the notion that \(LKB1\) is a tumor suppressor.

The human \(LKB1\) gene encodes a serine/threonine kinase of 433 amino acids that contains a nuclear localisation signal in its N-terminal region (12, 13). The subcellular localization of \(LKB1\) is determined by its association with STRAD (or “Ste20 related adaptator”), a STE-20-related pseudokinase (14), but also with LIP-1, a leucine-rich repeat molecule (15). Both STRAD and LIP-1, direct \(LKB1\) outside the nucleus and anchor the protein kinase in the cytoplasm. STRAD binds MO25, a small scaffolding protein, and the formation of a heterotrimeric complex containing \(LKB1\) markedly stimulates \(LKB1\) catalytic activity (16). Finally, STRAD stabilizes the \(LKB1\) protein, although the chaperones Hsp90/Cdc37 are also involved in the regulation of \(LKB1\) protein turnover (17, 18).
LKB1 has been conserved across evolution, and the *Caenorhabditis elegans* (*par-4*) and *Drosophila melanogaster* orthologues are necessary for establishing cell polarity during embryogenesis (19, 20). An LKB1-directed polarising function was recently demonstrated in human cells, and the formation of an apical brush border along with the sorting of markers to their respective membrane domains was induced in non-polarized intestinal cells upon expression of LKB1 and STRAD (21). LKB1 has also been implicated in the control of a TGFβ and Wnt signalling pathways (15, 22, 23). Moreover, reintroduction of *LKB1* in tumor cells that lack expression of *LKB1* blocks cells in the G1 phase of the cell cycle (24-26).

The recent demonstration that the AMP-activated protein kinase (AMPK) is phosphorylated and activated by LKB1 has provided important clues on LKB1 function (27-29). In response to various metabolic stress situations that lead to an increase in the intracellular AMP/ATP ratio, AMPK phosphorylates and switches off anabolic pathways while inducing catabolic pathways (30). Accordingly, LKB1 senses conditions of low cellular energy and protects cells from apoptosis by stimulating the AMPK signaling cascade (29). In addition to its AMPK kinase function, LKB1 activates the 11 other AMPK-related members that constitute the AMPK subfamily (31). Thus, LKB1 exerts pleiotropic functions through modulation of the cell cycle, metabolism and polarity.

The vast majority of PJS missense mutations are located in the region coding for the kinase domain and result in the abolition of the enzymatic activity, thus disrupting all functions attributed to LKB1 (6, 32-34). However, several recurrent mutations identified in PJS and in sporadic tumors are located in the non-catalytic LKB1 C-terminal part (35-38). The C-terminal region of LKB1 consists of 124 residues which is processed by several posttranslational modifications. Five phosphorylation sites have been mapped: two residues
are autophosphorylation sites (Thr 336, Thr 402) and three others (Ser 325, Thr 363, Ser 428) are phosphorylated by upstream kinases, although only a few of the kinases involved have been identified (13, 14, 39). Thr 363 is phosphorylated by both the ataxia telangiectasia mutated kinase (ATM) and the DNA-PK kinase (40), whereas Ser 428 is phosphorylated by the cAMP-dependent kinase and p90RSK (41). In addition, LKB1 has been shown to undergo farnesylation at a cysteine residue located in the C-terminal region (Cys430 in human LKB1) (13, 41). It is therefore likely that the LKB1 C-terminal region serves as a regulatory domain mediating dynamic interactions with several classes of proteins and promoting subcellular targeting.

Here, we report that recurrent mutations identified in PJS and in sporadic tumours, which are located in the C-terminal region of LKB1, do not impair LKB1 kinase activity and preserve its ability to promote growth arrest. However, these mutations did lead to an impairment of both AMPK signalling and LKB1 polarity function. We further show that the C-terminal region of LKB1 is crucial for the regulation of cell polarity.
Results

Disease-associated mutations affecting the LKB1 C-terminal region

The C-terminal non-catalytic region of the human LKB1 protein is encoded by exons 8 and 9 and encompasses amino acids 309 to 433. To date, one single in frame interstitial deletion and four missense mutations, which map in exon 8, have been identified in PJS and/or in sporadic tumors (35-38). The in-frame deletion, which removes codons 331 to 333 and substitutes the aspartate encoded by codon 330 for a glycine, was found in a family with several affected members on three generations (36). The four missense mutations are located at codons 314, 324, 354 and 367. Mutation at codon 324 which results in the substitution of Pro324 for a Leu (P324L) has been identified in the germline of PJS patients and in sporadic carcinoma (38). In this work, we report the identification of a novel germline mutation at codon 354 which substitutes the phenylalanine for a leucine (see the families, material and methods section), a mutation which was previously characterized in a sporadic colon cancer (35). Mutations at codons 314 (Pro 314 His) and 367 (Thr 367 Met) were both identified in sporadic colon cancers (35, 38). In this study, we have investigated the functional consequences of mutations at codons 324, 354 and 367.

Kinase activity and subcellular localization of LKB1 are not altered by C-terminal mutations

To examine the functional consequences of LKB1 C-terminal mutations, the missense mutations, resulting in the substitution of P324L, F354L, T367M (Figure 1), were singly introduced in the cDNA coding for a chimeric protein containing the green fluorescent protein (GFP) fused to human LKB1 (Figure 1). The GFP-LKB1 proteins were also tagged N-terminally with a Flag epitope. These constructs were co-transfected with a vector expressing
STRAD in Bosc23 embryonic kidney epithelial cells. Two days post-transfection, LKB1 was immunoprecipitated with the anti-Flag antibody and assayed for autokinase activity. Quantification of the immunokinase assay (Figure 2A) revealed that the three LKB1 C-terminal mutations did not alter LKB1 autokinase activity compared to LKB1 wild type. However, as expected, introduction of a mutation which disrupts the ATP-binding site (K78A), abolished the LKB1 kinase activity (Figure 2A).

The binding of STRAD directs LKB1 from the nucleus to the cytoplasm and this re-localization is required for LKB1-mediated growth arrest (14). Accordingly, all the PJS mutations whose functional consequences have been investigated so far, interfere with the binding of STRAD, thus preventing the translocation of LKB1 from the nucleus to the cytoplasm (14). To examine whether C-terminal mutations alter LKB1 subcellular localization, Bosc cells were co-transfected with the GFP-LKB1 constructs and either with a vector expressing STRAD or with a control vector. As previously demonstrated (14), LKB1 was relocalized in the cytoplasm upon expression of STRAD, whereas the LKB1 kinase-dead (K78A) was retained within the nucleus (Figure 2B). The LKB1 C-terminal mutants were translocated to the cytoplasm when coexpressed with STRAD (Figure 2B) as LKB1 wild type. The mutation T367M was found to decrease the efficiency of the nucleus export when compared to LKB1 wild-type (Figure 2B, data not shown). We further confirmed that the three C-terminal mutations did not impair the ability of LKB1 to interact with STRAD (data not shown), as recently reported (42). Taken together, these results provide evidence that the C-terminal mutations do not disrupt STRAD binding to LKB1, and that LKB1 mutant forms can be exported to the cytoplasm, albeit less efficiently for one of the mutants.
C-terminal mutations do not affect LKB1-mediated suppression of cell growth

Expression of LKB1 in G361 melanoma cells and HeLa cells is known to promote cell growth arrest (25, 26). Previous studies have shown that mutations identified in PJS and in sporadic tumors impair LKB1-induced growth suppression (32-34). G361 cells were transfected with the LKB1-GFP constructs and subjected to G418 selection for 2 weeks. LKB1 expression resulted in a dramatic reduction of the number of colonies and this biological effect required a functional kinase domain since the LKB1 K78A mutant did not inhibit proliferation of G361 cells (Figure 3). By contrast, the three C-terminal mutations did not affect LKB1-induced growth arrest (Figure 3). These results, which are consistent with the interaction with STRAD and the kinase activity, demonstrate that the C-terminal mutations do not perturb LKB1-mediated suppression of cell growth.

LKB1 C-terminal mutations interfere with the activation of AMPK and downstream pathways

It has been previously reported that LKB1 phosphorylates threonine 172 within the α catalytic subunit of AMPK and thereby contributes to the activation of this protein kinase (27-29). To determine whether the C-terminal mutations might impair LKB1-mediated activation of AMPK, HeLa cells were co-transfected with vectors expressing STRAD and either LKB1 wild-type or LKB1 mutant forms. Western blot analysis performed on HeLa cell lysates with a phosphospecific anti-Thr172 antibody showed that LKB1 stimulated the level of Thr172 phosphorylation (Figure 4A), whereas cells expressing the LKB1 C-terminal mutants had a marked decrease of Thr172 phosphorylation (Figure 4A). To confirm these results, we assayed the activity of AMPK in HeLa cells transiently co-expressing LKB1 C-terminal mutants with STRAD. AMPK was immunoprecipitated and the kinase activity toward the SAMS peptide substrate was measured in presence of [γ-32P]ATP. As shown in Figure 4A,
AMPK activity was significantly reduced in cells expressing LKB1 C-terminal mutants versus LKB1 wild type.

Recent studies have demonstrated that LKB1 negatively regulates the mTOR kinase which controls protein synthesis and cell growth. This effect is mediated via AMPK which phosphorylates and activates the function of the Tuberous Sclerosis Complex (TSC) (43, 44). To further investigate the functional consequences of LKB1 C-terminal mutations on the AMPK signalling cascade, we examined the regulation of S6 kinase (p70S6K) and 4E-BP1, two known substrates of mTOR. For that purpose, we used an anti-phospho Thr389 antibody which recognizes a residue in p70S6K whose phosphorylation is critical for the kinase function. For 4E-BP1, we assayed the retarded electrophoretic mobility on SDS-PAGE gels of the hyperphosphorylated form of the molecule. Using this approach, we found that co-expression of LKB1 wild type and STRAD resulted in a dramatic decrease of the level of phosphorylation of both S6K and 4E-BP1 compared with cells expressing either LKB1 C-terminal mutations or the catalytically inactive LKB1 K78A (Figure 4B). We next examined whether the regulation of acetyl CoA carboxylase α (ACCA), the rate-limiting enzyme that catalyzes the first step of long chain fatty acid synthesis, was also affected in cells expressing LKB1 C-terminal mutants. ACCA is negatively regulated by the LKB1 and this inhibition is achieved through the AMPK-dependent phosphorylation of ACCA at serine 79 and serine 1200 (29). By using an antibody raised against phospho-Ser79, we confirmed that LKB1 promotes phosphorylation of ACCA. Conversely, expression of LKB1 C-terminal mutants led to a significant diminution of phosphorylation at Ser79 but not to a complete loss, as observed with the LKB1 78A (Figure 4B). Collectively, these data support the hypothesis that the C-terminal mutations impair the LKB1-mediated activation of the AMPK pathway.
C-terminal mutations impair LKB1 polarizing activity

Two of us (AB, HC) have recently generated an experimental system aimed at studying LKB1 polarizing activity. Human colon cancer cell lines LS174T and DLD-1, which display a very low expression of STRAD and LKB1, were transfected with a vector containing the STRAD cDNA placed under the control of the inducible tetracycline (tet)-repressor promoter (21). Upon STRAD induction, exogeneous LKB1 was stabilized and translocated to the cytoplasm and the intestinal cells underwent dramatic morphologic changes, including remodeling of the actin cytoskeleton, formation of an apical brush border and polarized sorting of proteins to the apical and baso-lateral domains (21).

To address whether C-terminal mutations disturb LKB1 polarizing activity in intestinal epithelial cells, we transfected either the vectors expressing GFP-LKB1 or the C-terminal mutant forms in LS174T cells containing inducible STRAD. As shown in Figure 5A, the GFP-LKB1 protein and the various mutants were expressed at similar levels. 24 h post-transfection, the cells were further treated with doxycyclin for 24 h and the actin cytoskeleton was visualized with phalloidin-TRITC. As previously described, upon co-expression of GFP-LKB1 and STRAD, cells rounded up, stress fibers disappeared and actin formed a cap at the cortex of one pole of the cell. Counting of the cells displaying these characteristic features allowed us to quantify the effect elicited by LKB1. Expression of LKB1 resulted in a two fold augmentation of the number of cells with a polarized phenotype upon doxycyclin treatment whereas no significant differences were observed upon expression of the kinase-dead version of LKB1 (K78A) (Figure 5B). Similarly, co-expression of STRAD and the LKB1 C-terminal mutants did not increase the percentage of the cells with a polarized phenotype (Figure 5B), thus indicating that these mutations impair LKB1-induced polarization in intestinal cells.
To further assess the functional consequences of the C-terminal mutations of LKB1, we used an assay recently designed to study the polarity of migrating cells (45). After wounding of an astrocyte monolayer, the establishment of cell polarity can be readily determined by observing the reorientation of the centrosome. To explore the possible role of LKB1 in this biological process, the LKB1 expression plasmid was microinjected into leading edge cells after scratching the monolayer of primary rat astrocytes. 55% of expressing cells had a correctly polarized centrosome, which is not significantly different from cells injected with a GFP control plasmid (61%) (Figure 6A, B). In contrast, expression of the kinase-deficient mutant of LKB1 (K78A) resulted in only 33% of cells having a polarized centrosome (25% represents completely random polarity in this assay). When the vectors expressing the LKB1 C-terminal mutant forms were micro-injected, we also observed that expression of these mutants inhibit centrosome reorientation during wound-induced astrocyte migration (Figure 6A, B). Statistical analysis of the data obtained with the LKB1 C-terminal mutants showed that the effects observed are significant, even though the magnitude of the inhibition was less prominent with the LKB1 C-terminal mutants than with the catalytically inactive LKB1 (on average 45% of inhibition; see Figure 6B).

**Involvement of the LKB1 C-terminal region in the control of cell polarization**

Our precedent results suggest that the carboxy-terminal region of LKB1 mediates a crucial function in the control of cell polarity. To investigate directly this question, we reasoned that expression of the non-catalytic C-terminal region should exert a dominant-negative effect through the titration of effectors which bind to LKB1 and are required for the polarity function of endogenous LKB1. Astrocytes were wounded and a vector expressing the C-terminal 126 amino acids of LKB1 was microinjected in leading edge cells. As shown in Figure 6C, expression of this protein strongly inhibited polarity, whereas microinjection of the
N-terminal 309 amino acids of the kinase-dead LKB1 (ΔCt KD) had no significant effect on polarity (Figure 6C). Furthermore, expression of the C-terminal domain of LKB1 containing either the P324L or the T367M mutation did not alleviate the polarity inhibitory effect in astrocyte (Figure 6C), thus suggesting that these mutations disrupt the binding of some, but not all, effectors involved in LKB1-mediated regulation of cell polarity. Taken together, these data confirm that the carboxy terminal portion of LKB1 plays a critical role during the establishment of astrocyte polarization.
Discussion

In this study, we report the functional analysis of mutations identified in PJS and in sporadic tumours which are localized in the LKB1 C-terminal non-catalytic region. A number of nonsense and frameshift PJS mutations are supposed to result in the truncation of the LKB1 C-terminal (46). However, it is likely that these truncated forms of LKB1 are not, or are weakly, expressed in vivo due to nonsense-mediated decay (NMD), a surveillance mechanism which eliminates mRNA bearing premature stop codons (47). Therefore, only five non-truncating mutations, including one single in-frame deletion and four missense mutations that affect the non-catalytic C-terminal region of the protein have been reported so far (35-38). We found that carboxy-terminal mutations analysed in this study have distinctive effects compared with kinase-deficient PJS mutations since they impair both LKB1 ability to regulate cell polarity and to activate the AMPK pathway without disrupting LKB1-mediated cell cycle block.

Recent reports have shown that the cytoplasmic localization of LKB1 is sufficient to induce arrest in G1 phase of the cell-cycle (16). LKB1 re-localization depends both on its kinase activity and on its ability to interact with STRAD (14). Consistently, kinase-deficient PJS mutants as well as the SL26 PJS mutant, which specifically disrupts the interaction with STRAD, are retained within the nucleus and are growth arrest defective (33, 34). By contrast, we found that the LKB1 C-terminal mutants were translocated to the cytoplasm upon expression of STRAD and exhibited a growth suppressive effect. These results are in agreement with a recent study which reported that LKB1 C-terminal mutants still interact with the STRADα-MO25α complex (42). Concerning the pathways involved in LKB1 cell cycle control, LKB1 was implicated in the p53-dependent induction of the CDK inhibitor...
p21WAF1 (26, 48). LKB1 was also involved in both Brahma-related gene protein (BRG1)-dependent growth arrest (49) and upregulation of the PTEN tumour suppressor gene expression (50). However, the in vivo relevance of these pathways, with regard to LKB1 ability to restrain tumour development, is unknown yet. In this study, we found that C-terminal mutations did not prevent LKB1-mediated suppression of cell growth. Furthermore, truncation of LKB1 just downstream of the kinase domain reduced but did not abolish, LKB1-induced growth-arrest (data not shown). Therefore, these data suggest that the LKB1 C-terminal region is dispensable for the regulation of cell cycle.

LKB1 is an upstream activator of AMPK and regulation of the AMPK pathway is believed to be directly involved in LKB1 tumour suppressor function (27-29). Consistent with this model, LKB1 has recently been identified as a negative regulator of the mTOR signalling through the sequential stimulation of AMPK and of the TSC1/TSC2 tumour suppressor complex (43, 44). Furthermore, ACCA, a substrate of AMPK, controls fatty acid synthetic metabolism which is frequently dysregulated in tumours (51). We found that C-terminal mutations impair LKB1-mediated activation of AMPK and downstream pathways, thus reinforcing the idea that AMPK plays a key role in the control of cell transformation. These results further suggest that the LKB1 C-terminal region might contribute to the binding of AMPK and that mutations analyzed in this study uncouple this interaction, thereby resulting in a reduced enzyme-substrate recognition. Further studies will be needed to address this question.

The results presented here demonstrate that the C-terminal region of LKB1 plays a crucial role in the regulation of cell polarity. Interestingly, mutation of either the farnesylation-acceptor site or the serine corresponding to Ser428 in the C-terminal region of drosophila LKB1 impairs its ability to pattern the anterior-posterior embryonic axis (20). However,
mutation of either Ser428 or Cys430 does not affect human LKB1 polarizing activity in migrating astrocytes (S.Etienne-Manneville, data not shown), implying that alternative mechanisms are involved. Both AGS3, a protein that promotes the dissociation of Goα from GGDP (or “activate heterotrimeric G protein signaling independently of their associated/coupled receptor”), and the protein kinase GSK3β interact with LKB1 and are supposed to act as polarity transducers downstream of LKB1 (52-54). However, in our study, mutation or truncation of the LKB1 C-terminal region did not prevent the recruitment of AGS3 or GSK3β (data not shown). It is thus possible that LKB1 interacts with additional polarity proteins, which interaction is perturbed by the C-terminal mutations. The C-terminal mutations might then only affect one of the multiple pathways leading to cell polarity downstream of LKB1, thus explaining that the inhibition of polarity observed in astrocytes was less marked than the dominant-negative effect obtained with the kinase-defective LKB1 mutant. Alternatively, the AMPK pathway may be involved in the coordinated regulation of metabolism and polarity. In agreement with this notion, ACCA, which is phosphorylated and downregulated by AMPK, was shown to be essential for the establishment of C. elegans embryonic polarity (55). Also, TOR signalling participates to the establishment of polarity in yeast (56). It is thus conceivable that AMPK and AMPK-related kinases, such as MARK/PAR1, participate to the regulation of polarity. Future work should delineate the molecular mechanisms allowing the C-terminal region of LKB1 to interface with distinct partners and to unravel how the interplay of these effectors propagates polarity signals.

In conclusion, our data demonstrate an important role of the C-terminal region of LKB1 for the control of both the AMPK pathway and cell polarity. Loss of cell polarity has been suspected to constitute one of the oncogenic events contributing to the development of
malignancies. Our results reinforce this model and provide evidence that recurrent mutations identified in PJS and in sporadic cancers severely perturb LKB1-mediated cell polarization.
Families, material and methods

Families

The germline and sporadic mutations located in exon 8 of the LKB1 gene (codon 324: CCG CTG [P324L], codon 354: TTC TTG [F354L], codon 367: ACG ATG [T367M]) have been previously described (35-38). In addition, the same mutation at codon 354 which has been hitherto characterized in a sporadic colon cancer, has now been identified in the germline of two members of a french family (S.Olschwang, unpublished data). The index case, a young male aged of 14 years at diagnosis, displayed a large number of pigmented macules without evidence of intestinal polyps; the mother’s proband, who transmitted the germline mutation, was asymptomatic.

Constructs

The pSG-Flag eukaryotic expression vector was derived from the pSG5 plasmid (Stratagene; La Jolla, CA, USA) and contained the sequence coding for the Flag epitope. pSG construct encoding the human wild-type LKB1 was previously described (18). The pSG/Flag-LKB1 was digested, and the sequence coding for the human flag-LKB1 was subcloned into HindIII/PstI sites of the peGFP-C1 eukaryotic expression vector (Clontech; Palo Alto, CA, USA). The peGFP-C1/Flag-LKB1 vector encodes the human flag-LKB1 fused N-terminally to the eGFP coding sequence. The following missense mutations of human LKB1 were created by site-directed mutagenesis via the Stratagene Quickchange Kit, using the following oligonucleotides:

K78A: 5’GTGCAGGAGGGCCGTCGATCCTCAAGAAGAAGAAG3’ and 5’CTTCTTCTTCTTGAGGATCGCGACGGCCCTCCTGCAC3’
P324L: 5’GCACCAGTGCCCATCCCAGCTAGCCACAGCAGAAG3’ and 5’CTTGGTGTCGTCGGCTAGTCGAGGACTGGAGTCG3’
F354L: 5’GAGGACGAGGACCTCTTGGACATCGAGGATGACG3’ and 5’GTCATCTCAGGATGCTAGGATCCTCGTCG3’
T367M: 5’TACACTCAGGACTTCTAGGTGCTCAGGACAGGTCG3’ and 5’GACCTGTCGGGCACCATGAAGTCCTCAGTGA3’.
The pRK5 constructs expressing mouse wild-type LKB1 and C-terminal domain of LKB1 were a generous gift of A. Ashworth (The Breakthrough Breast Cancer Research Centre, Institute of Cancer Research, Fulham Road, London SW3 6JB, UK). In these constructs, kinase-inactive K78A, P324L and T367M mutants were created by site-directed mutagenesis using the following oligonucleotides:
K78A: 5’GCCGCAGGGCGGTCGCGATCCTCAAGAAGAA3’ and 5’TTCTTTCTTGAGGATCGCG ACCGCCCTGCGGC3’
P324L: 5’GCGCTCGTGACCTATCCCACTGAGGACTAAG3’ and 5’CTTAGTGTCTGGGCTCAGTGGGATAGGTACGCG3’
T367M: 5’TACACCCAGGACTTCTCATGGGTGCTGGACTGGGACAGGTCG3’ and 5’GACCTGTCGGGCACCATGAAGTCCTGGGTGTA3’.
The pcDNA3/Flag-STRAD construct expressing Flag-tagged STRAD protein was kindly provided by H. Clevers (Center for Biomedical Genetics, Utrecht, The Netherlands).

Antibodies
The Anti-Flag monoclonal antibody (clone M2) was purchased from Sigma. Anti-Actin was from MP Biomedicals (Ilikirch, France). Anti-Myc was obtained from Roche Diagnostics (Meylan, France). Anti-p70S6K, anti-phospho p70S6K (T389), anti-4EBP1, anti-phospho ACC (S79), anti-AMPK, anti-phospho AMPK (T172) were obtained from Cell Signaling.
(Beverly, MA, USA). To immunoprecipitate LKB1, the anti-LKB1 (N19, clone sc-8185) obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) was used. HRP-secondary antibodies were purchased from Amersham (Orsay, France). All commercially available antibodies were used according to the manufacturers' recommendations.

**Cell lines and transfection assays**

Bosc human cells were cultured in DMEM with 10% of heat-inactivated fetal bovine serum and 4,5 g/l of glucose, supplemented with 50 U/ml of penicillin and 50 U/ml of streptomycin and incubated at 37°C in 5% CO₂. Hela human cells were cultured DMEM containing 10% of heat-inactivated fetal bovine serum, 1g/l of glucose and supplements as described above. G361 human cells were cultured in McCoy’s medium containing standard supplements. LS174T human cells were cultured in RPMI 1640 media containing standard supplements. LS174T clone expressing tetracycline (tet)-inducible flag-STRAD (clone TR1-6) was described previously (21). Bosc, Hela and G361 cells were transfected using ExGen 500 (Euromedex; Mundolsheim, France), LS174T cells were transfected using lipofectamine 2000 (Invitrogen; Cergy Pontoise, France) according to the manufacturer's instructions.

**Cell lysis and immunoblotting**

Cells were lysed 48 h post-transfection. Bosc cells were lysed in a Nonidet P-40 lysis buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate and 1% Nonidet P-40, supplemented with a mixture of proteases inhibitors. After incubation at 4°C, lysates were clarified by centrifugation at 16000 g for 5 min at 4°C. Hela cells were serum-starved in DMEM for 4h, and scraped with a rubber spatula in Triton X100 lysis buffer containing 20 mM Tris-HCL pH7.5, 150 mM Nacl, 1mM EDTA, 1mM EGTA, 1% triton X100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1mM
sodium orthovanadate supplemented with a mixture of proteases inhibitors. Hela cell lysates were immediately centrifugated. Protein concentration was determined in supernatants by a modified Bradford assay (Bio-Rad). Clarified lysates were then boiled in Laemmli buffer (20 mM Tris-HCl (pH 6.8), 2% SDS, 20% glycerol, 20 µg of bromophenol blue per ml) in the presence of 1.4 M of 2-mercaptoethanol, separated on a SDS-PAGE polyacrylamide gel, and transferred to polyvinylidenedifluoride (PVDF) membranes (Immobilon P Millipore; St Quentin en Yvelines, France). Membranes were incubated with the appropriate antibody as previously described.

**LKB1 kinase assay**

Clarified lysates of Bosc cells were generated as described above. 1-2 mg of soluble proteins were incubated at 4°C for 2 h with 1 µg of anti-LKB1 (N19) antibody. Protein A coupled to sepharose beads (Amersham) was added, and incubation continued for 1 h. LKB1 immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer (20 mM HEPES pH 7.4, 10 mM MgCl₂, 10 mM MnCl₂, 10 mM β-glycerophosphate). The immunoprecipitates were then incubated in kinase buffer containing 10 µCi [γ³²P] ATP (4500 Ci/mmol, Amersham). After 30 min at 30°C, the reaction was stopped by addition of Laemmli buffer. Samples were electrophoresed on a SDS/PAGE polyacrylamide gel, transferred to PVDF membrane and analysed by autoradiography.

**AMPK activity assay**

The kinase activity of AMPK was measured using the SAMS peptide as substrate (29). Clarified lysates of HeLa cells were prepared as described above. 1-2 mg of soluble proteins were incubated at 4°C for 2 h with both 10 µg of anti-Flag antibody and with 1 µg of anti-AMPK antibody, and incubated for an additional 1 h with Protein A-sepharose beads.
Immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer (40 mM HEPES pH 7.4, 80 mM NaCl, 50 mM MgCl₂, 1 mM Dithiotreitol). The immunoprecipitates were then incubated for 20 min at 30°C in the kinase buffer containing 100 µM of SAMS peptide and 10 µCi [γ³²P] ATP (4500 Ci/mmol, Amersham). Following brief centrifugation, 40 µl of the reaction mixture were spotted onto p81 phosphocellulose paper (Upstate Biotechnology; Dundee, UK), which were washed three times with 0.75% phosphoric acid and one time with acetone. The radioactivity was measured using a scintillation counter (Packard).

**Growth suppression of G361 cells**

G361 were cultured to 50% confluence on 6 cm diameter dishes and transfected with 2 µg of a cDNA construct encoding the wild-type and mutant LKB1 in the peGFP-C1 vector or an empty peGFP-C1 vector using Exgen transfection reagent (Euromedex). A duplicate set of dishes was used for each condition. After 48 h, G418 was added to the medium to a final concentration of 1 mg/ml for a further 48 h and then was increased to 2 mg/ml. The medium was then changed every 48 h, maintaining G418. After 15 days, the cells were Giemsa-stained and the average percent of colonies per cm² was counted.

**Immunohistochemistry**

LS174T TR1-6 cells were seeded on laminin (BD Biosciences)-coated coverslips (2 µg/cm²) at 7000 cells/cm² and were either untreated or subjected to a doxycyclin (Sigma Aldrich; Lyon, France) treatment (1 µg/ml) for 24 hr. Subsequently, cells were fixed in 4% paraformaldehyde, followed by permeabilisation in 0.1% triton. The actin cytoskeleton was visualised by staining with TRITC-labelled phalloidin (Sigma Aldrich). Polarized cells were then analysed using a Leica microscope.
**Cell culture and scratch-induced migration**

Primary rat astrocytes were prepared as previously described (45). For scratch-induced assays, cells were seeded on (poly) L-ornithine precoated coverslips or 90 mm diameter dishes, grown in the presence of serum to confluence and the medium changed 16 h before scratching. Individual wounds (around 300 µm wide) were made with a microinjection needle. Wound closure occurred around 16-24 h later. Conventional epifluorescence images were recorded on a CCD camera and processed using the Metamorph software.

**Centrosome reorientation**

Centrosome reorientation was determined as previously described (45, 54). Expression vectors (0.1-0.2 mg/ml) were microinjected into the nuclei of the first row cells immediately after scratching. Cells were fixed after 8 h and the centrosome (MTOC) was visualized using an anti-pericentrin antibody (BabCO) and a fluorescently labeled secondary antibody (Jackson ImmunoResearch Labs, Inc). Nuclei were visualized with Hoescht. GFP fluorescence was monitored to confirm protein expression. Cells in which the centrosome was within the quadrant facing the wound were scored positive and for each point at least 300 cells from 3 independent experiments were examined.
Acknowledgements

We are grateful to Dr A. Ashworth for kindly providing the pRK5 constructs, and to Dr M. Ware for the critical reading of the manuscript. This work was supported by a grant from the Ligue Nationale Contre le Cancer (équipe labellisée). CF and HG are recipients of a fellowship from Association pour la Recherche sur le Cancer and the Ligue Nationale Contre le Cancer, respectively.

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Figure Legends

Figure 1: Schematic representation of wild-type and mutant forms of LKB1
The three missense mutations tested in this study (P324L; F354L; T367M) are depicted. Lys78 is located in the ATP binding site. Thr185, Thr336 and Thr402 are autophosphorylation sites; Ser31, Ser325, Thr363 are phosphorylated by upstream kinases. Cys431 is the farnesylation site. NLS: nuclear localization signal.

Figure 2: Influence of C-terminal mutations on LKB1 kinase activity and on LKB1 subcellular localization
(A) Bosc human cells were transfected with pEGFP-C1 vectors expressing either the wild-type or indicated mutant forms of GFP-Flag-LKB1, and with the pcDNA3 vector expressing Flag-STRAD. Cells were lysed 48 h post-transfection and LKB1 proteins were immunoprecipitated using an anti-LKB1 (N19) antibody. Immunoprecipitates were then incubated for 30 min with $[^{32}P]ATP$, separated by SDS-PAGE and visualized by autoradiography. Levels of LKB1 auto-phosphorylation were quantified and are represented graphically in arbitrary unit (U). The results represent the averages of four independent experiments. (B) Bosc cells were transfected with pEGFP-C1 vectors expressing either the wild-type or indicated mutant forms of Flag-LKB1, and with either the pcDNA3 vector or the pcDNA3 vector expressing Flag-STRAD. At 48 h post-transfection, cells were fixed and GFP fluorescence was analysed using confocal microscopy. Bar, 5 µm.

Figure 3: C-terminal mutations do not affect LKB1 anti-proliferative activity
G361 cells were transfected with pEGFP-C1 constructs encoding wild-type or the indicated mutant forms of LKB1. The pEGFP-C1 construct encodes the neo/G418 resistance gene.
After 15 days of G418 selection, Giemsa-stained colonies were photographed and the number of colonies/cm² of the dish was determined. Separate experiments with each condition were carried out in duplicate. The results represent the averages of three independent experiments.

**Figure 4 : C-terminal mutations impair LKB1-mediated activation of the AMPK pathway**

HeLa cells were co-transfected with pEGFP-C1 vectors expressing either the wild-type GFP-Flag-LKB1 or indicated LKB1 mutant forms along with the pcDNA3 vector expressing Flag-STRAD. Cells were serum-starved and lysed 48 h post-transfection. A) Lysates were immunoblotted with anti-Flag to detect LKB1 and STRAD, and with the anti-phospho-Thr172 antibody to detect activated AMPK. In parallel, *in vitro* AMPK kinase assay was performed employing the SAMS peptide as substrate. LKB1, STRAD and AMPK proteins were immunoprecipitated using anti-Flag and anti-AMPK antibodies. Immunoprecipitates were incubated for 20 min with SAMS peptide and \[^{32}\text{P}]\text{ATP}\), then AMPK activity was quantitated. The scale of the chart is displayed in arbitrary unit (U). The results represent the averages of three independent experiments. B) HeLa cell protein extracts were immunoblotted with antibodies recognizing phospho-Thr389 of p70S6K (P-p70S6K), p70S6K, 4E-BP1, phospho-Ser79 of ACC (P-ACC), Flag (STRAD, LKB1) and actin.

**Figure 5 : Polarization of intestinal epithelial cells is impaired upon expression of LKB1 C-terminal mutants**

LS174T TR1-6 cells, which express the STRAD cDNA placed under the control of the inducible tetracycline (tet)-repressor promoter, were seeded on laminin-coated coverslips and transfected with pEGFP-C1 constructs encoding wild-type or the indicated mutants forms of Flag-LKB1. 24 h post-transfection, LS174T TR1-6 cells were subjected to a treatment with
doxycyclin (1 µg/ml) for a 24 h period. (A) At 48 h post-transfection, untreated cells were lysed and LKB1 proteins were visualized by Western Blot using an anti-Flag antibody. (B) The percentage of LS174T TR1-6 cells displaying actin caps without doxycyclin treatment or after doxycyclin treatment for 24 h was measured. Relative cap formation induction was determined as the ratio between the percentage of polarized cells transfected with pEGFP-C1 constructs encoding wild-type or the indicated mutant forms of LKB1 and that measured in cells transfected with pEGFP-C1. The results represent the averages of five independent experiments.

Figure 6: LKB1 C-terminal region is required for astrocyte polarization

After scratching the rat astrocyte monolayer, leading-edge cells were microinjected with pEGFP-C1 or pRK5 constructs expressing either the wild-type or indicated mutant forms of LKB1. (A) Cells were fixed after 8 h, and stained with an anti-pericentrin antibody to detect the centrosome and with Hoescht to visualize nuclei. Centrosome polarization was assessed in astrocytes of the first row, expressing the indicated GFP-tagged constructs as described in material and methods. red: pericentrin; blue: Hoechst; GFP expressing cells are marked with an asterix; the direction of the wound is indicated by a white line; bars: 5 µm. (B) The percentage of cells having their MTOC in the quadrant facing the wound was measured. Random orientation of the MTOC with respect to the wound edge corresponds to a value of 25% at 0 h. Results shown are representative of 3 independent experiments. (C) After scratching an astrocyte monolayer, leading-edge cells were microinjected with the indicated constructs. The percentage of expressing cells having their MTOC in the quadrant facing the wound was determined 8 h later. Abbreviations: Cter, C-terminal; ΔCter, C-terminal deleted.

*P < 0.05, **P < 0.01, ***P < 0.001 vs. control vector (paired t test). Western Blot using an anti-Myc antibody indicating the expression of the wild-type LKB1 protein, a mutant form.
and the C-terminal domain after transfection in Bosc cells. These experiments were repeated three times with similar results.
Figure 1
Figure 2

A

+ STRAD

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LKB1 activity (U)

0 1 2 3 4

LKB1  K78A  P324L  F354L  T367M

B

- STRAD

+ STRAD

LKB1  K78A  P324L  F354L  T367M
Figure 3

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![Colony images](image-url)
Figure 4

A

\[ + \text{STRAD} \]

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AMPK activity (U)

B

\[ + \text{STRAD} \]

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</table>
Figure 5

A

WB: αFLAG

LKB1

B

Relative cap formation induction

- DOX
+ DOX

EGFP LKB1 K78A P324L F354L T367M
Abbreviations

ACC: Acetyl CoA Carboxylase α
AGS3: Activate heterotrimeric G protein Signalling independently of their associated receptor
AMPK: AMP-activated Protein Kinase
ATM: Ataxia Telangectiasa Mutated kinase
BRG1: Brahma-Related Gene protein
GFP: Green Fluorescent Protein
NMD: Nonsense Mediated Decay
PJS: Peutz-Jeghers Syndrome
PTC: Premature Stop Codons
STRAD: STe 20 Related Adaptor