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LKB1 interacts with and phosphorylates PTEN: a functional link between two proteins involved in cancer predisposing syndromes

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Germline mutations of the *LKB1* (*STK11*) tumor suppressor gene lead to Peutz-Jeghers syndrome (PJS) and predisposition to cancer. *LKB1* encodes a serine/threonine kinase generally inactivated in PJS patients. We identified the dual phosphatase and tumor suppressor protein PTEN as an LKB1-interacting protein. Several *LKB1* point mutations associated with PJS disrupt the interaction with PTEN suggesting that the loss of this interaction might contribute to PJS. Although PTEN and LKB1 are predominantly cytoplasmic and nuclear, respectively, their interaction leads to a cytoplasmic relocalization of LKB1. In addition, we show that PTEN is a substrate of the kinase LKB1 *in vitro*. As PTEN is a dual phosphatase mutated in autosomal inherited disorders with phenotypes similar to those of PJS (Bannayan–Riley–Ruvalcaba syndrome and Cowden disease), our study suggests a functional link between the proteins involved in different hamartomatous polyposis syndromes and emphasizes the central role played by LKB1 as a tumor suppressor in the small intestine.

INTRODUCTION

Syndromes with Mendelian modes of inheritance that predispose to cancers provide excellent opportunities to discover genes implicated in cancer and thus contribute to an understanding of the regulation of cell division. The Peutz-Jeghers syndrome (PJS) is an autosomal dominant disease characterized by mucocutaneous pigmentation and gastrointestinal hamartomatous polyposis (OMIM no. 175200). PJS patients are at an increased risk to develop malignancies, and the organs that are most targeted are the gastrointestinal tract, breasts and female reproductive organs (1,2). The genetic locus responsible for the majority of PJS cases was mapped to chromosome 19p13.3 and found to encode a serine/threonine protein kinase named LKB1 (alias STK11) (3–5). The mutations lead to loss or impairment of kinase activity (6,7). Transfection of *LKB1* into human tumor cell lines having reduced levels of *LKB1* mRNA or impaired LKB1 kinase activity results in a G1 cell cycle arrest (8). This growth inhibitory effect is mediated through signaling of cytoplasmic LKB1 and induction of p21 expression through a p53-dependent mechanism (9).

Lkb1-deficient mice (*Lkb1*−/−) die as embryos displaying neural tube defects, mesenchymal cell death, vascular abnormalities with severe extra-embryonic development and overexpression of vascular endothelial growth factor (VEGF) (10). Heterozygous *Lkb1* knockout mice develop hamartomatous polyps in the gastrointestinal tract without inactivation of the remaining wild-type *Lkb1* allele, suggesting that polyposis results from *LKB1* haplo-insufficiency (11), rather than LOH

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(3). Genetic studies revealed that LKB1 orthologues are required to establish embryonal polarity in *Drosophila melanogaster, Caenohabditis elegans and Xenopus* (12–14). Interestingly, cell polarity is severely compromised in a variety of human cancers but it remains unclear whether loss of polarity is a consequence of uncontrolled proliferation or a causative factor in cancer initiation. Most knowledge about cell polarity mechanisms has been generated in studies using simple, genetically tractable organisms including *Drosophila, C. elegans* and yeast (15). However, genes responsible for cell polarity also regulate cell proliferation in these organisms. Although, LKB1 has been connected through protein–protein interaction to several pathways that influence cell proliferation (16–19), it is not known whether tumorigenesis in human LKB1 mutants is, indeed, influenced by polarity abnormalities. Recently endogenous complexes of human LKB1 with two proteins, STRAD and MO25α were identified and shown to regulate epithelial polarity. This suggested a model in which loss of *LKB1* in humans may cause cellular transformation through disruption of epithelial polarity (20). However, expression of STRAD and MO25α has not been directly demonstrated in the gastro-intestinal organs except for pancreas and liver (21). Hence, the genuine substrate of LKB1 in the gastrointestinal tract, where the PJS phenotype is the strongest resulting in hamartomatous polyps and/or cancer, remains to be identified.

To further assess the biological function of the kinase LKB1, we performed a two-hybrid selection in yeast. We identified the tumor-suppressor protein PTEN as an LKB1-interactor. *PTEN* is frequently mutated in a large number of cancers but also in other autosomal dominant diseases such as Cowden disease (CD) and Bannayan–Riley–Ruvalcaba syndrome (BRRS) (22), both belonging to the same family of hamartomatous polyposis syndromes as the PJS. We confirmed this interaction in mammalian cells and found that PTEN overexpression in cells results in the cytoplasmic retention of LKB1. Moreover, we identified PTEN as a substrate of the kinase LKB1 *in vitro.*

RESULTS

Isolation of PTEN in a yeast two-hybrid selection of LKB1 interactors

The 313 N-terminal amino acids of LKB1 (LKB1-313) comprising the kinase domain were used to screen a human testis cDNA library by the yeast two-hybrid method (Fig. 1A). The rationale for this choice was that most of the natural PJS mutations map to this domain and that no transactivation was observed in yeast with this construct. One LKB1-interacting clone contained almost the entire coding sequence (from codon 39) of the dual phosphatase and tumor suppressor PTEN (Fig. 1B; PTEN39-403) (22). This partial *PTEN* clone does not contain the minimal domain required for the interaction with phospholipids (PIP2 domain, Fig. 1B) (23). To map the minimal LKB1-interaction domain of PTEN, we generated a series of truncated PTEN fusions (Fig. 1B) and tested them for interaction in two different yeast strains (Y190 and PJ69-4A; see Materials and Methods): (i) two C-terminal deletions of PTEN (PTEN39-314 and PTEN39-224, larger deletions were unstable in yeast), (ii) one N-terminal deletion (PTEN173-403) that preserves the integrity of the C2 domain (24) and (iii) a deletion of the phosphatase active site (PTEN39-403ΔAS, deletion of residues 122 to 131, Fig. 1A). The results of these interaction mating experiments are shown schematically in Figure 1B. Most notably, the PTEN AA 173-403 fusions are sufficient for interaction with LKB1. These data suggest that the minimal LKB1-interaction domain may include the N-terminal portion of the PTEN C2 domain (see subsequently). We also tested two truncated fusions of LKB1 for interaction with PTEN39-403 and found that the kinase domain (LKB49-309) was sufficient for this interaction (Fig. 1A).

Influence of pathogenic *LKB1* and *PTEN* missense mutations on the interaction

We used the yeast two-hybrid system to determine the effects of pathogenic *LKB1* or *PTEN* missense mutations on the LKB1–PTEN interaction. The tested mutations are shown in Figure 1. For *LKB1*, they comprised three germline missense mutations causing PJS (L67P, D176N and W308C), one somatic mutation found in a testicular carcinoma (G163D) and an engineered mutation that affects the ATP-binding site of LKB1 (K78I) (2,4,6,25). The LKB1 amino acid substitution mutants L67P, K78I, D176N and W308C and G163D have previously been reported to be kinase-defective or partially defective, respectively (6,25). Remarkably, K78I and D176N maintained the interaction with PTEN, whereas the other kinase-defective mutants L67P and W308C, as well as G163D, lost the capability of interacting with PTEN. These results demonstrate that the LKB1 kinase activity is not necessary for binding to PTEN and suggest the possibility that the pathogenicity of some mutations is not solely due to impaired LKB1 kinase activity but also due to decreased affinity for PTEN.

For *PTEN*, we tested two germline mutations causing Cowden syndrome (C124R, G129E), a somatic mutation found in the glioma cell line U343MG (C124S) and one sporadic mutation causing Bannayan–Riley–Ruvalcaba syndrome (C105Y) (26–29). The *PTEN* point mutations C124R, C124S and G129E are defective in either both protein and lipid phosphatase (C124R/S), the lipid phosphatase (G129E) activity or in both the lipid and the protein phosphatase (C124R/G129E) activities (30–33). In the context of the PTEN39-403 protein, none of these PTEN point mutants nor the deletion mutant PTEN39-403ΔAS showed interaction with LKB1 (Fig. 1B), demonstrating that the N-terminal portion of the PTEN C2-domain (discussed earlier) is either not sufficient for interaction with LKB1 or that a structurally destabilized phosphatase domain interferes with its interaction. Taken together, these results suggest that the LKB1–PTEN interaction requires the N-terminal portion of the PTEN C2-domain and, in the context of the full-length protein, the integrity of the phosphatase domain. We also tested the PTEN39-403T382A/T383A double mutant, because phosphorylation at these residues regulates PTEN stability and function in mammalian cells (34). This double mutant is still capable of interacting with LKB1 (Fig. 1B).

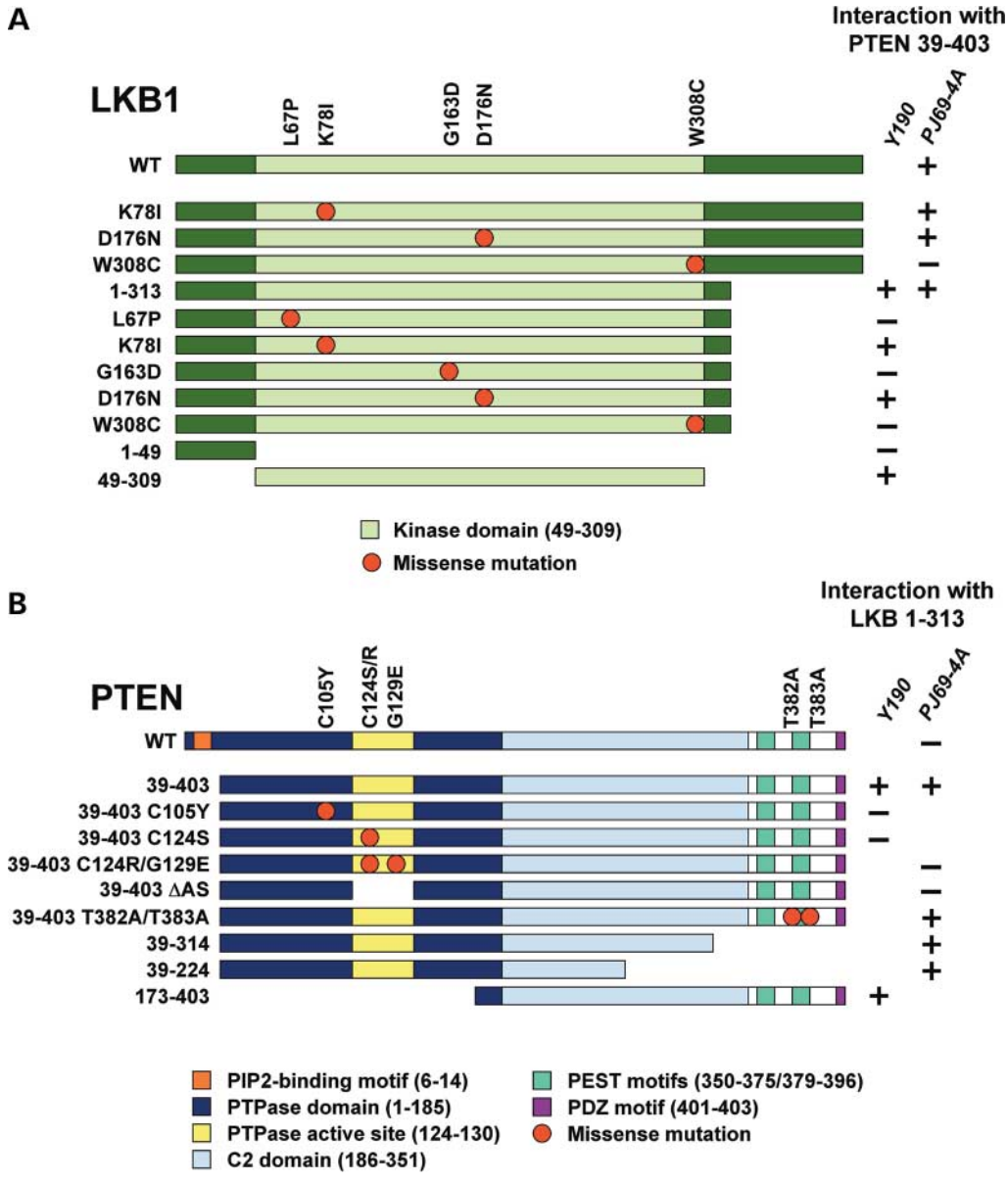

Figure 1. LKB1 and PTEN interactions in yeast two-hybrid assays. Schematic representation of LKB1 and PTEN constructs used in this study. (**A**) LKB1 and (**B**) PTEN proteins and their derivatives were tested for interaction with PTEN 39-403 and LKB1 1-313, respectively, in two different yeast strains (Y190, right column and PJ69-4A, far right column). A ' + ' sign denotes interaction, whereas a ' − ' sign denotes no interaction. ΔAS, deleted active site.

PTEN interacts with LKB1 in mammalian cells and influences its subcellular localization

To confirm the interaction between LKB1 and PTEN in mammalian cells, co-immunoprecipitation experiments were carried out both with exogenously overexpressed and with endogenous proteins. For the former, COS7 cells were transiently transfected with plasmids expressing LKB1 fused to myc and GFP tags and PTEN fused to a V5 epitope (Fig. 2). Lysates were immunoprecipitated with V5-antibodies and probed with anti-LKB1 or anti-PTEN specific antibodies (Figs. 2A and B). LKB1 was exclusively precipitated in the presence of PTEN, demonstrating that LKB1 and PTEN can interact in mammalian cells.

To assess whether endogenous LKB1 and PTEN interact, we used 5 mg of a total protein extract from normal human small intestine and immunoprecipitated endogenous PTEN using anti-PTEN antibodies. Fifty microgram of the total extract was loaded as input (lysate) for comparison and immunoblots were probed with anti-LKB1 antibodies. We found a single band that co-precipitated with PTEN with the same size as LKB1 in total human small intestine extract (Fig. 2B) but not in the control immunoprecipitation with a control antibody of the same isotype. Likewise, anti-PTEN antibodies showed that PTEN was only precipitated with the corresponding antibody (Fig. 2B). Hence, we conclude that endogenous LKB1 physically interacts with PTEN.

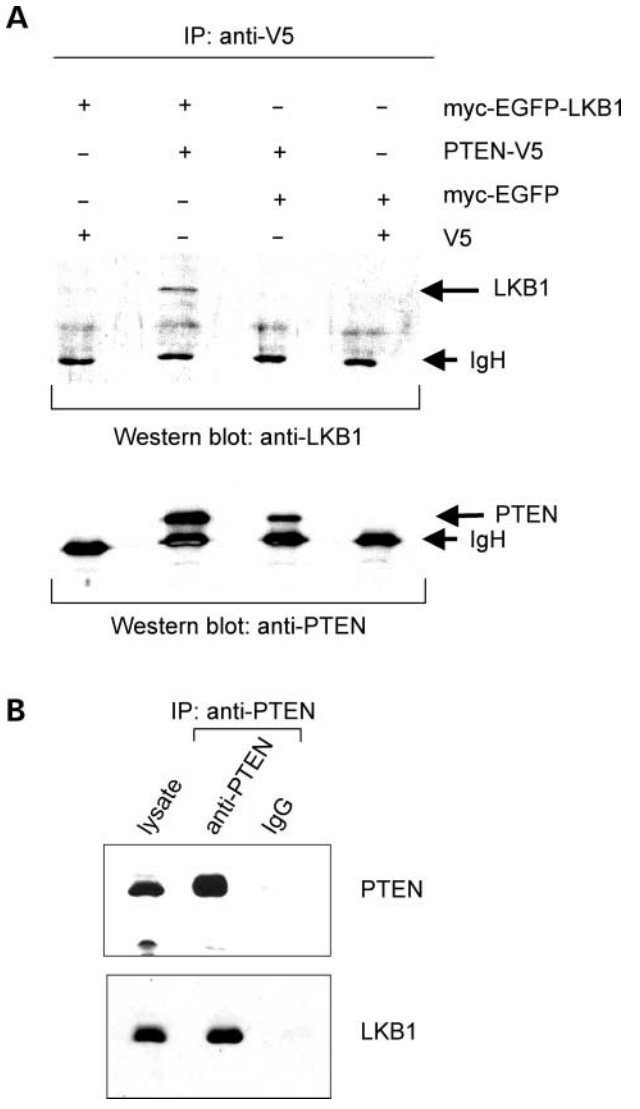

Figure 2. Biochemical evidence for the interaction of LKB1 with PTEN. (**A**) Exogenously expressed LKB1 and PTEN interact in COS7 cells. The two proteins were transiently co-expressed from plasmids pcDNA3–myc-EGFP and pcDNA–V5. 500 μg of each lysate were immunoprecipitated with an anti-V5 antibody (Invitrogen). Immunoprecipitates were subjected to immunoblot analysis with anti-LKB1 antibodies (top) or with anti-PTEN antibodies (bottom). The upper arrow ('LKB1') indicates the band corresponding to the fusion protein myc-EGFP–LKB1 coprecipitated with the fusion protein PTEN–V5 ('PTEN'). IgH indicates the antibody heavy chain. The micrographs are representative of an experiment done in triplicate. (**B**) Endogenous LKB1 and PTEN interact in human small intestinal tissue. 5 mg of a total protein lysate from normal human small intestine were immunoprecipitated with anti-PTEN antibodies. Immunoprecipitates were submitted to immunoblot analysis with anti-PTEN (top) and anti-LKB1 antibodies (bottom). Lysate, 50 μg of the input lysate.

When overexpressed, an HA-tagged LKB1 localizes predominantly in the nucleus (78% in COS7 and 64% in U2OS) (Fig. 3A, D and E). In contrast, the Myc-EGFP–PTEN fusion protein, referred to as GFP–PTEN, shows a more cytoplasmic localization (59% in COS7 and 87% in U2OS cells) (Fig. 3A–C). These results are in accordance with previously published analyses (16,35–38). Because of the previous-described difference in subcellular localization of the two proteins, we tested the consequences of the LKB1 and PTEN interaction on the localization of both proteins upon co-expression. Interestingly, co-expression of GFP–PTEN and HA-LKB1 resulted in the accumulation of LKB1 in the cytoplasm. Upon co-expression, the number of cells showing mainly cytoplasmic localization of HA-LKB1 increased from 22 to 58% in COS7 cells and from 36 to 60% in U2OS cells ($P < 0.0001$) (Fig. 3D and E). In contrast, co-expression of epitope-tagged LKB1 had less dramatic effects on the cellular localization of GFP–PTEN (Fig. 3B and C), although we did note a subtle cytoplasmic relocalization of PTEN in COS7 cells. The cytoplasmic relocalization of epitope-tagged LKB1 is specific to PTEN co-expression, as HA-LKB1 remained predominantly nuclear when the GFP tag by itself was co-expressed (Fig. 3D and E). The C-terminal deletion mutant of PTEN (PTEN314), which still interacted with LKB1 in the two-hybrid assay (Fig. 1B), produced a cytoplasmic relocalization of LKB1 that was not significantly different from that affected by wild-type PTEN ($P = 0.1$; Fig. 3D and E). These results show that PTEN influences the subcellular localization of LKB1, possibly by retaining it in the cytoplasm.

Next, we determined the influence of PTEN on the localization of two known LKB1 mutants: D176N and G163D, of which we have shown the latter to be defective for interaction with PTEN in the yeast two-hybrid system (Fig. 1A). D176N is known to have a nucleo-cytoplasmic distribution similar to that of wild-type LKB1, whereas G163D is predominantly nuclear (Fig. 3F). Interestingly, PTEN could only affect the localization of D176N ($P < 0.001$) but not that of the non-interacting mutant G163D (Fig. 3F).

LKB1 phosphorylates PTEN *in vitro*

To explore the functional consequences of the interaction of LKB1 and PTEN, we first examined whether PTEN is an LKB1 phosphatase. Although we cannot formally rule out this possibility, we were not able to find any evidence for it (data not shown). Next, we carried out *in vitro* kinase assays to assess whether LKB1 phosphorylates PTEN. We used purified PTEN expressed as a thioredoxin fusion protein in bacteria as a substrate for wild-type LKB1, or for the kinase-dead LKB1 mutant D176N, immunoprecipitated from transfected HEK293 cells. We found that immunoprecipitated LKB1 phosphorylates recombinant PTEN, but not the thioredoxin tag alone (Fig. 4; compare lanes 1 and 2). This phosphorylation is due to LKB1 rather than another co-immunoprecipitating kinase activity as the immunoprecipitate of the kinase-defective mutant D176N fails to phosphorylate the recombinant PTEN (Fig. 4; compare lanes 1 and 3).

Phosphorylation of PTEN by LKB1 depends on a cluster of residues preceding and encompassing S385

To pinpoint the residues of PTEN that are phosphorylated by LKB1, and because the C-terminus of PTEN contains many potential phosphorylation sites, we split PTEN into two fragments (amino acids 1–314 and amino acids 314–403). We found that only the C-terminal fragment (PTEN314-403) is phosphorylated (Fig. 5) (data not shown). Within this

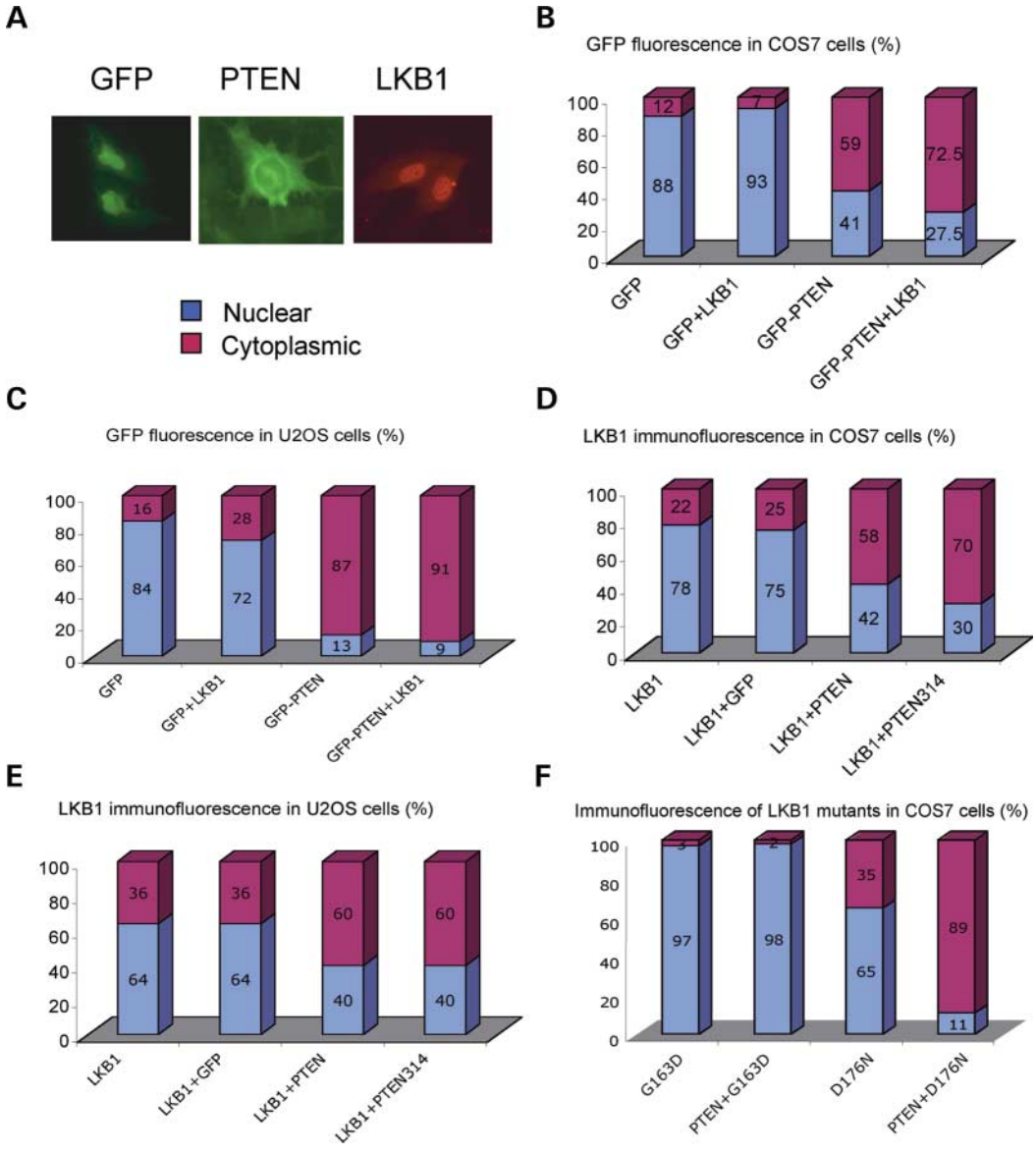

Figure 3. PTEN influences the subcellular localization of LKB1. (**A**) Typical examples of the subcellular localization patterns of transiently expressed myc-EGFP (GFP), myc-EGFP–PTEN (GFP–PTEN) and HA-LKB1 (LKB1). (**B**) and (**C**) The effects of LKB1 co-expression on the localization of PTEN in COS7 and U2OS cells, respectively. Results are indicated as the percentage of cells showing either mostly cytoplasmic or mostly nuclear fluorescence (see color-coded legend). The effects of PTEN co-expression are statistically highly significant ($P < 0.0001$). (**D**) and (**E**) The effects of overexpression of wild-type or C-terminally truncated PTEN on the localization of LKB1 in COS7 and U2OS cells, respectively, as determined by immunofluorescence with an antibody against the HA tag. Statistical comparison showed that there is no significant difference between wild-type PTEN and PTEN314 ($P = 0.10$) on the localization of LKB1. (**F**) Influence of PTEN overexpression on the localization of LKB1 point mutants G163D and D176N in COS7 cells. Statistically wild-type PTEN has the same effect on D176N as on wild-type LKB1 ($P < 0.0001$) but not on the mutant G163D ($P > 0.05$). Equal amounts of both LKB1 and PTEN expression vectors were cotransfected and about 120 labeled cells were counted. Individual panels represent separate experiments with replicates. Thus, absolute numbers for a given sample may vary between panels. Most bars represent averages of multiple independent replicates, but error bars have been omitted for clarity (standard errors were typically $\leq$5%).

C-terminal fragment, we then decided to focus on a cluster of serine and threonine residues between S360 and S385 (Fig. 5A), which includes predicted and demonstrated phosphorylation sites (39–41). We mutated the corresponding codons in sets and individually and determined phosphorylation of the mutants as described earlier for the wild-type fragment. Experiments summarized in Figure 5A established that all single mutants and the multiple mutants A6 and even A3, which combines S380A, T382A and T383A, could still be phosphorylated. In contrast, phosphorylation was abolished when S385A was combined with any of the single mutants between S380 and T383 (mutants A4, A2A, A2B and A2C). Thus, S385 becomes critical whenever one of the potential phosphorylation sites between S380 and T383 is mutated. These results point to these four serine/threonine residues as the most likely phosphorylation sites, but also indicate a relatively complex mechanism that may involve distinct recognition and possibly cooperative phosphorylation steps.

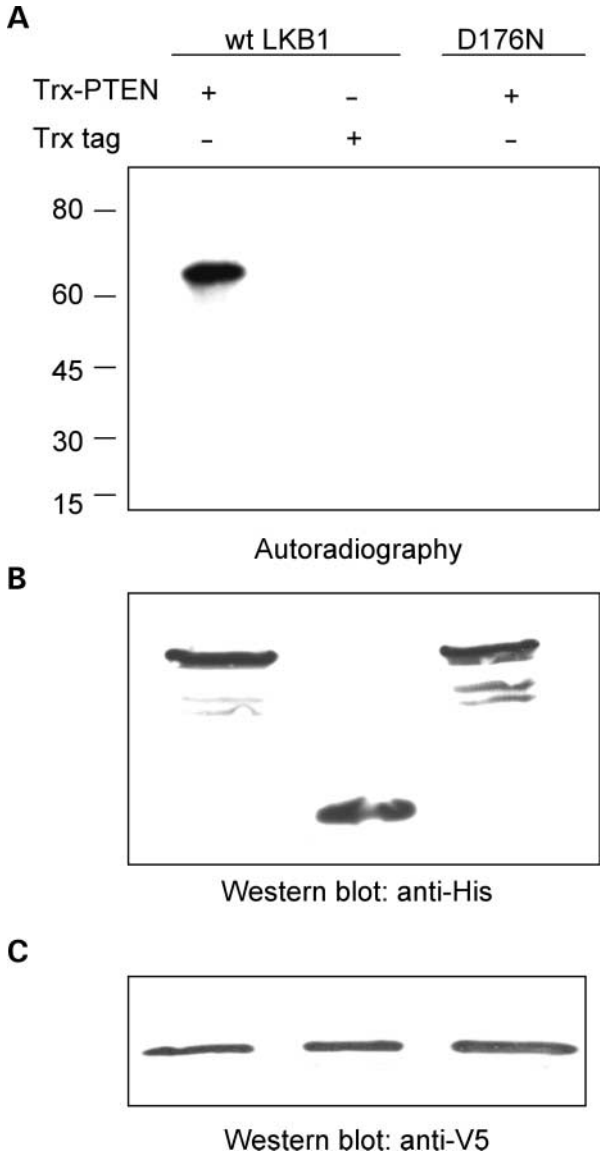

Figure 4. Recombinant PTEN is phosphorylated by LKB1 *in vitro*. (**A**) Autoradiography of *in vitro* kinase assay performed with recombinant substrates and LKB1 immunoprecipitated from HEK293 cells transfected with plasmid pcDNA-LKB1-V5. Substrates were a thioredoxin fusion protein of PTEN (Trx-PTEN) or the thioredoxin tag (Trx tag) alone. Kinases were either the wild-type LKB1 (wt) or a kinase-defective LKB1 point mutant (D176N). (**B**) and (**C**) Immunoblots illustrating that equal amounts of both substrate (revealed with an anti-His6 tag antibody) and kinase (anti-V5) were used.

DISCUSSION

We have demonstrated a functional link between LKB1 and PTEN, whose genes are both mutated in different but related autosomal dominant hamartomatous polyposis syndromes. Although *LKB1* is mutated in PJS, germline mutations in *PTEN* are the genetic cause not only for CD but also for another hamartomatous polyposis syndrome, the BRRS. The *PTEN* tumor suppressor gene encodes the first identified phosphatase that is frequently mutated or deleted somatically in various human cancers (26,28). PTEN can dephosphorylate phospho-tyrosine, -serine and -threonine peptides *in vitro* (30). However, the predominant enzymatic activity of PTEN appears to be the dephosphorylation of the phosphoinositide PIP3, a product of phosphatidylinositol-3-kinase (PI3K), which is required for the phosphorylation and activation of PKB/Akt, a survival factor protecting many cell types against apoptosis (23,24,30,42–44).

Our finding that PTEN is an LKB1-interactor is particularly interesting as it provides the first direct evidence that these two tumor suppressors are involved in a common pathway. This is consistent with several previous observations: (i) The expression patterns of both genes overlap significantly (7); (ii) a cDNA microarray study with the *Lkb1*-deficient lung cancer cell line A549 revealed an upregulation of PTEN upon transient re-expression of LKB1 (45); (iii) the lethal phenotypes of *Lkb1*−/− and *Pten*−/− mice share some features (46); (iv) both types of null homozygous mice die at a similar stage of embryonic development and homozygous mutations in both genes induce dysregulation of VEGF (10,47); (v) growth-suppressive effects of LKB1 or PTEN overexpression are limited to LKB1 or PTEN-deficient cells, respectively (8,48,49).

The kinase domain of LKB1 is sufficient for binding to PTEN, but kinase activity *per se* is not essential. Some LKB1 mutants are still able to interact with PTEN despite having an impaired kinase activity (K78I and D176N). The other tested LKB1 mutants (L67P, G163D and W308C) fail to bind to PTEN. Among these, G163D retains some kinase activity. It is tantalizing to speculate that the pathogenicity of this mutant is due, at least in part, to its decreased affinity for PTEN and that wild-type LKB1 function depends on the both interaction and phosphorylation of PTEN. Although the C2 domain of PTEN is sufficient to interact with LKB1, mutations in the phosphatase domain abolish the interaction. The importance of the phosphatase domain is further underlined by the observation that a truncated PTEN mutant lacking the C2 domain (PTEN314) still promotes the subcellular relocalization of LKB1 (discussed subsequently). Although the very C-terminus of PTEN is sufficient to serve as a substrate for phosphorylation by LKB1, we speculate that it is not part of the primary interaction surface and that the catalytic interaction is favored by the *in vitro* conditions of the kinase assay. Taken together, all of these results indicate a complex interplay involving multiple domains of PTEN. While our data are consistent with the findings of the crystal structure of PTEN that reveal extensive intramolecular interactions between independently folding phosphatase and C2 domains (24), further studies will be necessary to clarify these observations.

One consequence of the interaction is that PTEN overexpression leads to increased cytoplasmic accumulation of LKB1. Unlike the kinase activity, the ability to interact with PTEN correlates with the subcellular localization of LKB1 mutants. Most of the kinase-defective LKB1 mutants are concentrated in the nucleus (9,35) and are unable to interact with PTEN. In contrast, those LKB1 mutants that are partially cytoplasmic (D176N and K78I) (9,35) also interact with PTEN.

Although we found no evidence that LKB1 is a substrate for PTEN, this possibility remains to be further explored. Indeed, many phosphorylation sites have been identified in LKB1 (50), including sites that are phosphorylated by PKA (13)

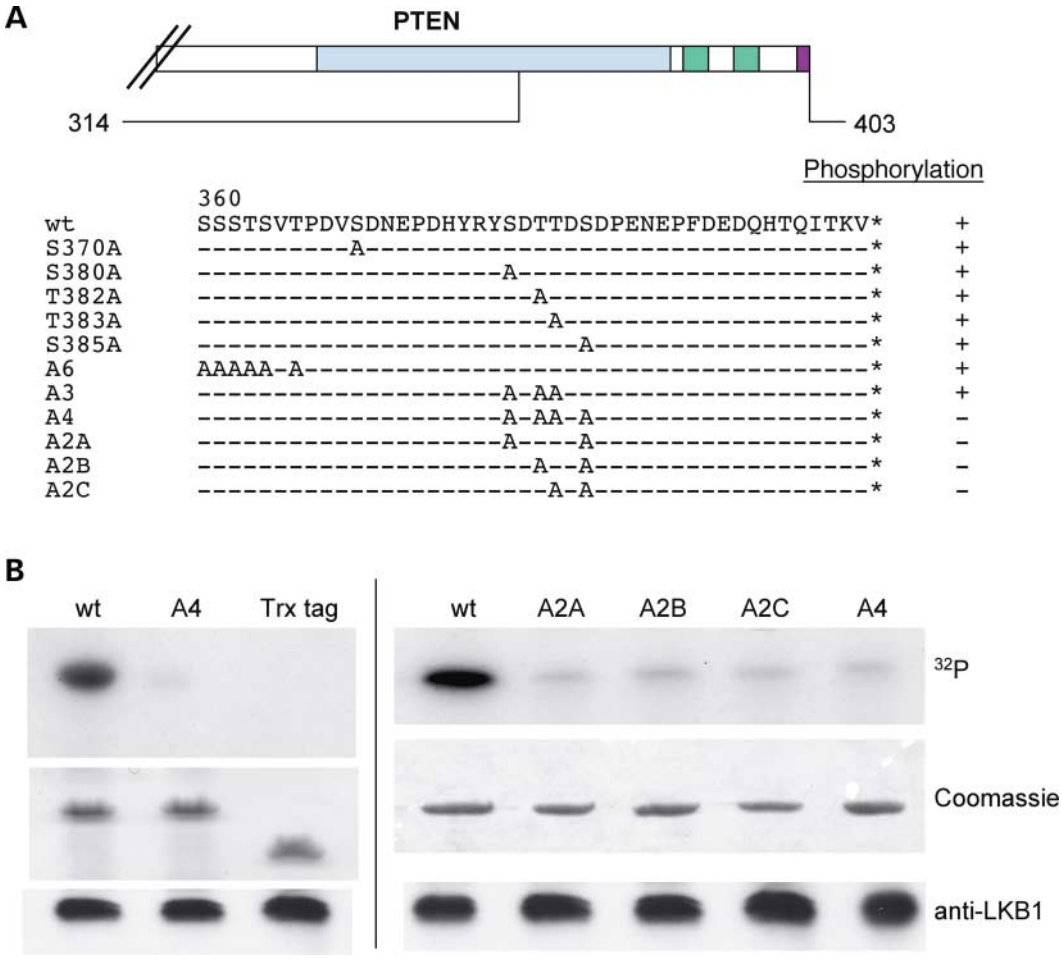

Figure 5. Mapping the PTEN phosphorylation site(s) of LKB1. (**A**) Schematic representation of the C-terminal 89 residues of PTEN that can be phosphorylated by LKB1 *in vitro.* The details of the mutants are shown below the sequence of amino acid 360–403. (**B**) The results for all tested mutants are summarized. *In vitro* kinase assays were performed as described in the legend to Figure 4 and in Materials and Methods, except that the substrate was a thioredoxin fusion protein encompassing only the 89 C-terminal amino acids (314–403) of PTEN. The left panel is a control experiment demonstrating that the thioredoxin (Trx) tag of the substrate and the A4 mutant are not phosphorylated by LKB1. Top, middle and bottom panels show the autoradiography (phosphorylated substrate), the Coomassie-stained gel (total substrate) and the LKB1 immunoblot (kinase), respectively.

and one or several of these sites could be targets for PTEN in its protein phosphatase mode.

Recent analyses identified PTEN residues T366, S370, S380, T382, T383 and S385 as *in vivo* phosphorylation sites (39–41). Phosphorylation of residues S380, T382 and T383 by casein kinase II (CK2) affects PTEN stability and activity (39,40). The same kinase is responsible for phosphorylation of residues S370 and S385, whereas another yet unidentified kinase is likely to phosphorylate T366 (41). We provide evidence suggesting that LKB1 phosphorylates PTEN at residue S385 in combination either with S380, T382 or T383. Thus, the mode of phosphorylation of PTEN by LKB1 is different from that by CK2. Interestingly, the tumor suppressor PTEN can inhibit cell migration and this activity depends both on its protein phosphatase activity and its C2 domain (51). This activity of the C2 domain is controlled by the phosphorylation state of the T383. When phosphorylated by an unknown kinase, the C2 domain loses its inhibitory effect on cell migration. LKB1 might be the kinase that phosphorylates T383 on the basis of our *in vitro* phosphorylation data and the fact that both lipid and protein phosphatase domains of PTEN are required for the interaction. It will be interesting to test the role of LKB1 in PTEN-regulated cell migration.

In mammalian cell culture, PTEN has been shown to regulate cell proliferation and survival via its lipid phosphatase activity (48,52). It is unlikely that LKB1 has an effect on cell proliferation and survival through the regulation of the PTEN lipid phosphatase activity. This statement is based on the following observations: (i) The PTEN construct that we found in our yeast two-hybrid screen lacks the first fourteen amino acids that are required for the binding of PIP2; (ii) the phosphorylation of AKT is not up-regulated in polyps of PJS patients and heterozygote *Lkb1*+/− mice; (iii) LKB1 re-expressed in transfected *Lkb1*-deficient cells does not affect the levels of phosphorylated AKT (53,54).

Several other proteins have been shown to interact with LKB1. LKB1 interacts with and phosphorylates p53 (16,21,55). Germline mutations of p53 cause the autosomal dominant Li-Fraumeni syndrome (OMIM no. 151623), a cancer syndrome associated with breast cancer, soft tissue

sarcomas, brain tumors, osteosarcoma, leukemia and adrenocortical carcinoma. However, patients with Li-Fraumeni syndrome do not develop hamartomatous polyposis. Moreover, *p53*−/− mice do not develop small intestinal polyps during their life span, and eventually die of other types of cancers. In addition, the phenotypes of either *Lkb1*−/− or *Pten*−/− mice are completely different from that of *p53*−/− mice (56). Taken together, these data suggest that p53 may not be a physiological substrate of LKB1 for the onset of PJS although it may play a role later in the development of cancer in PJS patients (57).

It has been suggested that loss of the tumor suppressor LKB1 may result in cellular transformation as a consequence of disrupted epithelial polarity (20). In this context, it is noteworthy that the LKB1 complexes with the two accessory subunits, STRADα/β and MO25α/β (21), could play a role in activating AMP-activated kinase (AMPK) in mammalian cells (58). Moreover, STRAD activates LKB1, which has been shown to be responsible for cell polarization. The paradox is that STRAD is not expressed in intestinal epithelial cancer cell lines (20) nor are there human ESTs or tags corresponding to STRAD in gastrointestinal tissues. This suggests that STRAD might not be expressed in gastrointestinal tissues where the phenotype of PJS is the most expressed as hamartomatous polyps and/or cancer, notably in the small intestine, colorectum and stomach (1). Gene expression studies using MO25α as a probe have not been done on gastrointestinal tract tissues (except liver and pancreas where it is expressed) (21). Thus, although STRAD and MO25α have been demonstrated to interact with LKB1 in several tissues and cell lines, this remains to be demonstrated for the human gastrointestinal tract. It is also important to point out that polyps in PJS patients are not only the consequence of proliferation of the intestinal epithelium but also of smooth muscle cells displaying a characteristic pattern called tree-like branches of smooth muscle, which is an arborizing network of smooth muscle bundles and the most widely used criterion for establishing the diagnosis of PJS polyps.

On the basis of its biochemical interaction with the LIP1–SMAD4 complex, LKB1 has previously been linked to yet another polyposis syndrome, the Juvenile polyposis syndrome (PJI) (18). Moreover, LKB1 was found to activate *TSC2*, the gene mutated in Tuberous sclerosis, another hamartomatous syndrome, through the AMP-dependent protein kinase (AMPK) (59). Our finding of PTEN as an LKB1 interacting protein and substrate provides new evidence for functional connections among several hamartomatous polyposis syndromes, namely Peutz-Jeghers syndrome, Cowden syndrome, BRRS, PJI and Tuberous sclerosis.

MATERIALS AND METHODS

Plasmids and mutants

All yeast two-hybrid constructs containing the full-length cDNA of human LKB1 and its deletion mutants were expressed from plasmids pGBT9 and/or pGBDU (Clontech). The inserts were amplified using Pfu DNA polymerase (Promega) and cloned between *Eco*RI/*Pst*I sites. For expression in mammalian cells, LKB1 and PTEN coding sequences were cloned into pcDNA3-myc-EGFP (pcDNA3 modified to include EGFP with an N-terminal myc-tag) (60) or pcDNA3-V5 (Invitrogen) or pcDNA-HA. For bacterial expression, *PTEN* derivatives encompassing codons 1–314 and 314–403 were subcloned into pET-32Ek/LIC (Novagen) between *Eco*RI/*Xho*I sites. Missense mutations of *LKB1* and *PTEN* were generated with the Quick-change mutagenesis kit (Stratagene). The sequences of all of the constructs used in this study were verified.

Yeast two-hybrid selection

The yeast two-hybrid selection with a human testis cDNA MatchMaker library (Clontech) was performed in the yeast strain Y190 (Clontech) using a fusion of the GAL4 DBD with the N-terminal 313 amino acids of LKB1 (expressed from plasmid pGBT9-LKB1-313) as a bait. In subsequent two-hybrid assays, yeast strain PJ69-4A (Clontech) was also used either interchangeably or for certain bait constructs that displayed auto-activation in Y190. As judged by Western blot analysis, different bait and prey fusion proteins were expressed at similar levels (data not shown).

Cell culture and immunoprecipitation

COS7, U2OS or HEK293 cells (ATCC) were grown at 37°C in 5% CO_2 and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% of penicillin–streptomycin (100 IU/ml and 100 μg/ml, respectively). Cells were transfected with plasmids pcDNA3–myc-EGFP–LKB1 and pcDNA3–PTEN–V5 using lipofectin (Life Technologies). Two days later, cells were lysed in 500 μl of ice-cold lysis buffer [50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Triton X-100 containing a protease inhibitor cocktail (Roche)] and centrifuged for 20 min at 10 000*g* and 4°C. The supernatant was recovered and precleared for 4 h with protein G agarose beads (Roche). Subsequently, monoclonal antibodies to either the myc (Santa Cruz Biotechnology) or V5 (Invitrogen) tags were incubated with the cell extracts, and 1 h later the protein G-agarose beads (Roche) were added and incubated over night at +4°C with shaking. The agarose beads were pelleted by centrifugation, washed three times with 1 ml of washing buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Triton X-100) and resuspended in SDS gel-loading buffer (62 mM Tris–HCl pH 7.4, 2% SDS, 10% glycerol, 5% β-mercaptoethanol) and boiled for 3 min. After centrifugation the supernatant was subjected to SDS gel electrophoresis on a 10% SDS–PAGE gel. Proteins were electro-transferred to nitrocellulose membranes (Hybond, Amersham Pharmacia). Blots were incubated with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20. To immunoprecipitate endogenous proteins with antibodies against PTEN, a commercial total protein extract from human small intestine (Biochain) was used. For immunoblotting, antibodies to LKB1 or to PTEN (both from Santa Cruz Biotechnology) were diluted 1:1000. Horseradish peroxidase-conjugated donkey anti-goat, mouse anti-goat (Sigma), anti-mouse antibodies (Santa Cruz Biotechnology) or goat anti-mouse Kappa light chain antibodies (Bethyl)

were used. The ECL chemiluminescence system (Amersham-Pharmacia) was used for detection.

Immunofluorescence

COS7 or U2OS cells were grown on coverslips and fixed with 3% PFA 24 h after transfection. HA-tagged proteins were detected using an anti-HA monoclonal antibody at 1:200 (Santa Cruz Biotechnology) and Rhodamine-coupled anti-mouse secondary antibody (Santa Cruz Biotechnology). Alternatively, EGFP fusion proteins were used. DNA staining was carried out with DAPI (Sigma).

Bacterial expression and purification of PTEN and mutant derivatives

For expression of wild-type and mutant derivatives of PTEN fused to a His6-thioredoxin tag (Trx-PTEN), *Escherichia coli* BL21 transformants were grown in 250 ml of L-broth at 37°C to mid-log phase (A600 nm = 0.6). Isopropyl β-D-thiogalactopyranoside was added at a concentration of 0.2 mM the culture was incubated for an additional 6 h at 25°C. Thereafter, all of the procedures were performed at 4°C. The pellets were resuspended in 5 ml of ice-cold lysis buffer containing 50 mM NaH_2PO_4 pH 8.0, 500 mM NaCl, 5 mM imidazole, 5 mM β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF), and bacteriolysis was performed by sonication until the cell suspension became transparent. After the addition of 50 μl of Tween-20, the lysate was incubated on ice for 20 min and then centrifuged at 14 000*g* for 15 min. The supernatant was mixed with 250 μl of Ni-NTA-agarose (Qiagen) for 45 min at 4°C. The beads were washed three times with 1 ml of the wash buffer containing 50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl and 20 mM imidazole, and eluted three times with 250 μl of the elution buffer containing 50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl and 250 mM imidazole. The buffer of the eluted solution was then replaced with 500 μl of TED buffer containing 20 mM Tris–HCl pH 8.0, 300 mM NaCl, 2 mM EDTA, 2 mM DTT and 1 mM PMSF by using a Microcon column (Millipore Corporation, Bedfort, MA, USA) and reduced to a volume of 50 μl. The purified protein was stored in the presence of 10% (v/v) glycerol at −80°C until use. Protein concentrations and the integrity of fusion proteins were determined by 10% SDS–PAGE and by comparison with known concentrations of BSA using Coomassie blue staining and with western blot using the anti-His tag antibodies (Sigma).

In vitro LKB1 kinase assays

V5-tagged wild-type and D176N mutant LKB1 were transiently expressed in HEK293 cells. Immunoprecipitation was carried out with anti-V5 antibodies as described earlier except that the immunoprecipitates were washed six times with 1 ml [twice each with: lysis buffer, washing buffer and kinase buffer (20 mM Tris–HCl pH 7.5, 150 mM KCl, 0.3 μM ATP, 5 mM $MgCl_2$, 0.5 mM dithiothreitol)]. One microgram of Trx-PTEN fusion protein was incubated with 50 μl of protein G Sepharose beads with immunoprecipitates of wild-type and mutant (D176N) LKB1 for 30 min at 30°C in kinase buffer (20 mM Tris–HCl pH 7.5, 150 mM KCl, 0.3 μM ATP, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 50 μl final volume) containing 2 μCi of γ-[^{32}P]ATP per sample. The reactions were stopped by adding SDS–PAGE sample buffer and boiling, followed by SDS–PAGE and autoradiography.

Statistical analysis

Comparative analyses of subcellular localizations were done with Fisher's exact test and Student's *t*-test. A *P*-value of <0.05 was considered statistically significant.

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