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4. The tumour suppressor role of LKB1 in human cancer

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Abstract. Germ-line *LKB1* mutations are responsible for Peutz-Jeghers syndrome (PJS) and somatic mutations are involved in the development of some sporadic tumours, in particular lung cancer. The gene encodes the LKB1 protein with serine-threonine kinase activity, which is involved in the regulation of the cell energetic checkpoint through the phosphorylation and activation of AMP-dependent kinase (AMPK) and other substrates. LKB1 is also involved in other important cell processes such as the control of cell polarity in various types of tissues. Here we provide an overview of the alterations of LKB1 in human cancer, Lkb1-mice models and what is currently known about the biological function, substrates and binding partners of LKB1 protein, with a special focus on cancer development.

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Introduction

The human *LKB1* gene, also known as *STK11*, spans 23 kb and is made up of nine coding exons and a final non-coding exon. *LKB1* codes for an mRNA of ~2.4 kb transcribed in a telomere-to-centromere direction and for a protein of 433 amino acids and approximately 48 kDa. LKB1 is widely expressed in embryonic and adult tissues, albeit with some differences. In mouse, early embryos have high levels of Lkb1 expression in embryonic and extra-embryonic tissues and, along with progressive embryonic development, Lkb1 protein concentrates in the heart, esophagus, pancreas, kidney, colon, lung, small intestine and stomach (1, 2). In adult tissues, high levels of LKB1 protein can be found in most epithelia, in the follicles and corpus luteum of the ovary, the seminiferous tubules of the testis, in myocytes from skeletal muscle, and in glial cells (2-3). The *LKB1* gene gives rise to at least two transcripts ($LKB1_L$ and $LKB1_S$) from an alternative splice site at exon 9 (4). Both isoforms are widely expressed in rodent and human tissues, although $LKB1_S$ is particularly abundant in haploid spermatids in the testis. *LKB1* maps to 19p13.3, a chromosomal region to which the autosomal dominant Peutz-Jeghers syndrome (PJS) (OMIM 175200) was first linked by genetic analysis. Individuals with PJS typically exhibit mucocutaneous melanin pigmentation and suffer from hamartomatous polyps in the gastrointestinal tract and an increased risk of developing cancer (5-6). Following the linkage of PJS to chromosome 19p13, the identification of germ-line-inactivating mutations at *LKB1* in these patients was definitive in the conclusion that *LKB1* is the gene causing the syndrome (7-8).

The LKB1 protein kinase

LKB1 is a multi-tasking tumour suppressor serine-threonine kinase (9-10) and is a member of the CAMK (Calcium/ Calmodulin Regulated Kinase-Like) family of kinases. LKB1 orthologues include *Xenopus laevis* egg and embryonic kinase 1 (XEEK1)(11), mouse Lkb1 (12), *Caenorhabditis elegans* partitioning-defective gene 4 (Par-4)(13) and *Drosophila* lkb1 (dLKB1) (14). Par-4 and dLKB1 share 26% and 44% overall identity with human LKB1, respectively, and share 42% and 66% identity with the LKB1 kinase domain, respectively.

Post-translational modifications

Phosphorylation is a post-translational modification that alters the chemical properties of a protein allowing it to recognize, bind, activate, or

deactivate substrate. Mouse Lkb1 is autophosphorylated on the residues Thr185, Thr189, Thr336 and, Ser404, and phosphorylated by upstream kinases on Ser31, Ser325, Thr366, and Ser431 (15-18). The residues Thr366, Ser404, and Ser431 in the mouse sequence correspond to residues Thr363, Ser402 and Ser428 respectively, of human LKB1 (19) (Figure 1).

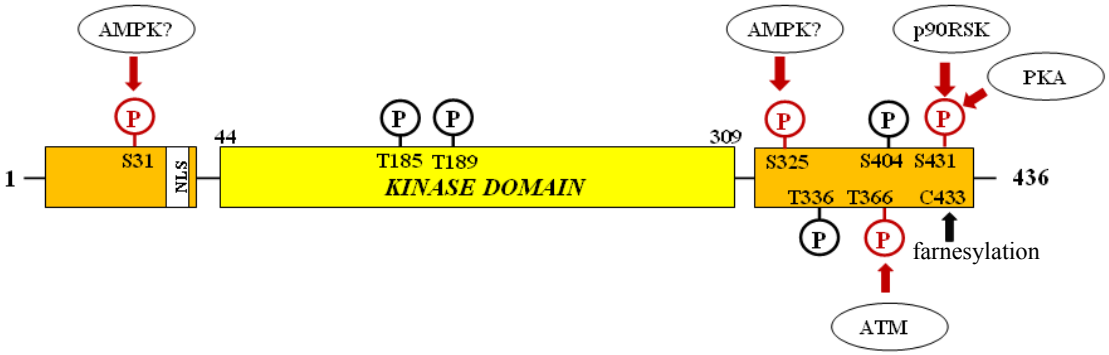

Figure 1. Post-translational modifications of murine LKB1 tumour suppressor kinase. The kinase domain is depicted in yellow, noncatalytic domains are depicted in orange and the nuclear localization sequence (NLS) is depicted in white. Autophosphorylation sites are depicted in black and sites phosphorylated by upstream kinases are depicted in red *(modified from Alessi et al. 2006).*

Currently there is no evidence that phosphorylation of LKB1 is necessary for its activity (15-16), thus the function of LKB1 may be indirectly regulated. LKB1 is phosphorylated at Ser431 by cAMP-dependent protein kinase A (PKA) and p90 ribosomal S6 kinase (RSK) (15-16). An earlier study suggested that phosphorylation at Ser431 is required for LKB1 mediated growth arrest (16). However, a recent study reported that the LKB1 mutants S431A, S431E, and the splice variant $LKB1_S$, that lacks Ser431, retained the ability to cause cell cycle arrest (20), indicating that phosphorylation on Ser431 is not required for this function. A role for phosphorylation of Ser431 has been implicated in axon differentiation (21), and neuronal polarization (22). In *Xenopus,* the LKB1 homologue XEEK1, is also phosphorylated by protein kinase A (PKA), and increased PKA activity is important for maintaining the oocytes at the G2 phase of cell cycle (15). However, phosphorylation of Ser431 is not essential for LKB1 catalytic activity, since mutagenesis of this residue does not alter the ability of LKB1 to autophosphorylate, or activate TP53 (16), AMP-activated protein kinase (AMPK) or brain-specific kinases 1 and 2 (BRSK1 and BRSK2 respectively) (20). Neither the cellular localization nor prenylation of LKB1 is affected by phosphorylation of Ser431. Phosphorylation at Ser31, Ser325 and Thr366 are not necessary for the catalytic activity of LKB1 since mutations to alanine to prevent phosphorylation, or to glutamic acid to mimic phosphorylation, or

addition of phosphatases did not affect LKB1 autophosphorylation, and its ability to activate TP53 (17). On the other hand, LKB1 autophosphorylation at Thr336, is important for LKB1-mediated growth arrest in G361 cells. Activation of ataxia telangiectasia (ATM) by ionizing radiation, leads to double strand breaks, and phosphorylation of LKB1 on Thr366 (18) (Figure 1). Phosphorylation at this site also plays a role in LKB1 mediated cell cycle arrest. The significance of LKB1 autophosphorylation at Thr185, Thr189, Ser404 is currently not known. Moreover, the upstream kinase responsible for phosphorylating LKB1 at S31 and S325 is not known, but has been proposed to be AMPK, a proline-directed kinase (23), where in LKB1, a proline residue follows Ser325 (17).

A recent study indicates that LKB1 is acetylated on a minimum of nine lysine residues (24), with acetylation at Lys48 being the most significant. Sirtuin 1 (SIRT1), a NAD-dependent protein deacetylase, deacetylates LKB1 at Lys48, resulting in an increased LKB1 interaction with STRAD. This in turn shifts the LKB1 cytoplasmic to nuclear distribution ratio in favour of the cytoplasm, ultimately increasing LKB1 catalytic activity. Mutations of Lys48 to Arg, to mimic effects of deacetylation, resulted in similar effects as overexpression of SIRT1. Overexpression of SIRT1, and consequently deacetylation of LKB1 Lys48, resulted in increased levels of phospho-Ser428- and phospho-Thr336- LKB1. Mutations of Lys48Arg displayed similar effects. As discussed above, phosphorylation of these residues (human Ser428 and Thr336) is required for LKB1 growth suppression function.

LKB1 catalytically deficient mutants: Oncogenic mutants

A previous study from the Marignani laboratory demonstrated a novel function for LKB1 catalytically deficient mutants (25). Since these LKB1 mutants are present in epithelial-derived cancers and PJS, it is important to understand the biological activity of these mutants within the context of disease. Marignani and colleagues discovered that LKB1 catalytically deficient mutants, such as SL26 and D194A, display oncogenic properties, whereby LKB1 mutants act as oncogenes or LKB1 mutants enhance expression of an oncogene (25). In a series of experiments, LKB1 catalytic deficient mutants (referred to as oncogenic mutants) enhanced the expression of the oncogene cyclin D1, which is required for cells to progress from G_0 phase to G_1 phase of the cell cycle. This study shows that the introduction of LKB1 oncogenic mutants into colorectal $DLD1^{TP53-/-,P21WAF-/-}$ cells resulted in elevated gene and protein levels of cyclin E, RB and cyclin D1, three proteins required for the progression of cells through S-phase of cell cycle. The cell cycle profile of these cells were unaltered following expression of ectopic TP53 or $P21^{WAF}$,

suggesting LKB1 is capable of arresting cells in G_1 independent of TP53 and $P21^{WAF}$ expression and LKB1 catalytic activity is required for this arrest, since LKB1 oncogenic mutants did not induce the G_1 arrest.

In this same study (25), chromatin immunoprecipitation experiments were conducted to demonstrate that endogenous LKB1, but more importantly LKB1 oncogenic mutants, were recruited to response elements within the endogenous cyclin D1 promoter, namely the AP1, Sp1 and CRE binding protein/Ets sites, but not other sites, such as the E2F site. The authors postulate that in cancers where *LKB1* is mutated, LKB1 oncogenic mutants are recruited to the promoter of cyclin D1, resulting in increased cyclin D1 expression, cell cycle progression and, cell division; perpetuating the uncontrolled cell growth characteristic of hamartomatous polyps. These findings represent the first characterized function for *LKB1* oncogenic mutants. Interestingly, mutants of another tumour suppressor, *TP53,* have also been shown to exert oncogenic properties, and are therefore often referred to as 'gain-of-function' mutants (26). Future studies of mutant tumour suppressor proteins may lead to the discovery of other gain-of-function mutants, thereby providing insight into how mutants of tumour suppressor proteins may function in the context of disease.

LKB1 interacting partners

After the discovery of *LKB1* as the gene responsible for PJS, the discovery of LKB1 signalling partners was pursued. LKB1 interacts with numerous proteins, highlighting the multifunctionality of this protein (Figure 2), as discussed below.

Brahma-related gene 1 (BRG1)

In an effort to identify a binding partner for LKB1, Marignani and colleagues (27) developed an *in vitro* expression cloning strategy. A HeLa cell cDNA library was *in vitro* transcribed and translated (TnT) in the presence of radiolabelled methionine. The TnT products were incubated with recombinant GST-LKB1 and GST fusion proteins, and subjected to a pull-down using GSH beads. A single TnT pool containing a 35-kDa protein that bound specifically to LKB1 was further identified by sib-selection as Brahma-related gene 1 (*BRG1*). BRG1 is a member of the mammalian SWI-SNF chromatin remodelling complex and contains ATP-dependent helicase activity required for chromatin remodelling.

To confirm the LKB1-BRG1 interaction *in vivo*, immunoprecipitation (IP) of LKB1 from Saos-2 cells was conducted, and BRG1 was identified in the IP complex, indicating endogenous LKB1 and endogenous BRG1 interact

in vivo. To identify the interface between LKB1 and BRG1, a series of LKB1 and BRG1 truncations were generated. LKB1 was found to bind exclusively to the helicase domain of BRG1 while the N-terminus of LKB1 was found to be sufficient for LKB1 to bind the BRG1 helicase domain. Next, it was confirmed that the oncogenic mutant SL26 interacted with BRG1, indicating kinase activity is not required for this interaction. Functional studies determined that the association of LKB1 with BRG1 in the presence of DNA increases the ATPase activity of BRG1 approximately 6-fold compared to BRG1 alone. SL26 increased BRG1 ATPase function, indicating kinase activity of LKB1 is not required for BRG1-ATPase activity. Since LKB1 was previously shown to induce G_1 growth arrest (28) and since BRG1 is involved in the retinoblastoma (RB)-dependent growth arrest pathway (29-30), Marignani and colleagues determined that LKB1 co-expressed with BRG1, induced growth arrest at the same level as BRG1 alone. However, co-expression of SL26 and BRG1 lead to cell growth and proliferation (27), suggesting that LKB1 is required for BRG1-mediated growth arrest, and LKB1 kinase activity is required for this function.

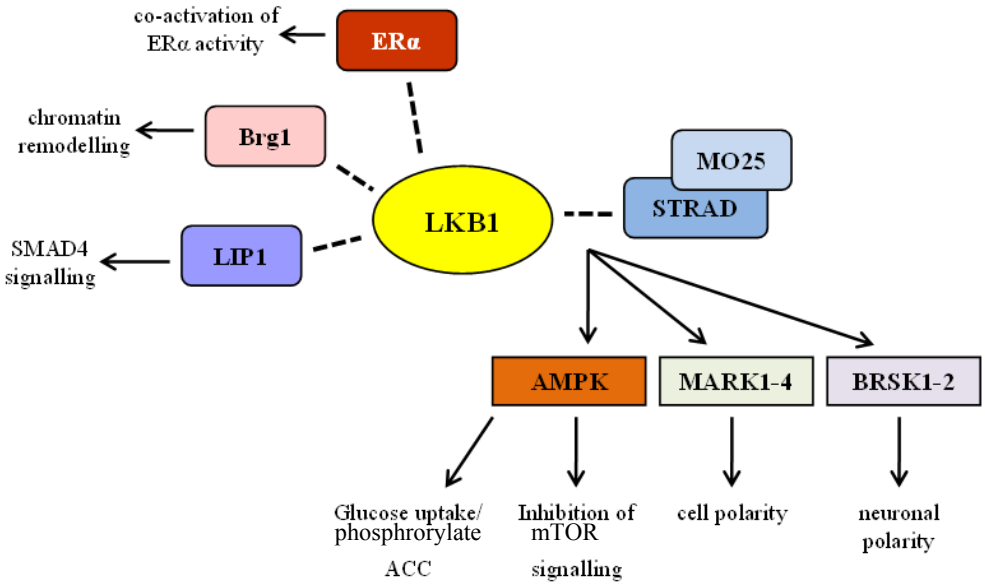

Figure 2. LKB1 interacting partner. A schematic representation of LKB1 interacting partners and signalling pathways. Dashed lines (---) indicate interacting partners, solid arrows (→) indicate phosphorylation, dotted arrows (-->) indicate downstream signalling effects.

LKB1 interacting protein 1 (LIP1)

Smith and colleagues (31) used a yeast-two-hybrid screen to identify potential interacting partners for LKB1 and found the novel protein LKB1 interacting partner-1 (LIP1). LIP1 is approximately 1,100 amino acids, with a molecular weight of about 121 kDa and was found to be ubiquitously

expressed in a wide variety of tissues. Co-immunoprecipitation experiments demonstrated that LKB1 and LIP1 interact in COS cells and LKB1 kinase activity was not required for this interaction since D194A catalytic deficient mutant co-immunoprecipitated along with LIP1. Kinase assays using the immunoprecipitated complexes showed that levels of LIP1 phosphorylation did not increase in the presence of LKB1, although low levels of LIP1 phosphorylation were seen. The authors suggest this is likely due to co-immunoprecipitation of an unidentified kinase, since similar levels of phosphorylated LIP1 were observed in the absence of LKB1. Thus LIP1 was determined not to be a substrate for LKB1. Immunofluorescent microscopy demonstrated that the LIP1 localized to the cytoplasm in punctuate structures, suggesting LIP1 may be localizing to a particular organelle. However, ectopic expression of LKB1 and LIP1 led to the re-localization of LKB1 to the cytoplasm, co-localizing with LIP1. This study also showed that LIP1 co-immunoprecipitates with SMAD4, a TGFβ-regulated transcription factor. LKB1 does not directly interact with SMAD4; suggesting LIP1 acts as an adaptor between LKB1 and SMAD4.

Having shown that LIP1 facilitates in the re-localization of LKB1, the authors suggest that LIP1 may play a role in regulating LKB1 function by contributing to LKB1 subcellular localization, allowing LKB1 to act upon both nuclear and cytoplasmic substrates. In summary, Smith and colleagues identified another LKB1 interacting partner, which regulates LKB1 function through partial control of LKB1 subcellular location.

STRAD and MO25

Another series of studies, using a combination of yeast two-hybrid analysis and affinity purification experiments, have shown that LKB1 exists in a ternary complex with STE20-related adaptor (STRAD) (32) and mouse protein 25 (MO25) (33) in mammalian cells. STRAD is homologous to the STE20 family of kinases but lacks several key catalytic amino acid residues within its kinase domain (residues 58-401) (34), namely the glycine in the glycine-rich loop (subdomain I), the lysine residue of VAIK (subdomain II), the catalytic Asp residue of HRD (subdomain VIb), a conserved Asn residue (subdomain VIb) as well as the DFG motif in subdomain VII (35), thus is referred to as a pseudokinase (35, 36). Van Aalten and colleagues (35) reported the structure of STRADα in complex with MO25α, forming a heterodimer. STRADα was previously determined to bind to MO25α through its C-terminal Trp-Glu-Phe residues (WEF motif) (33), however, recent structural analysis confirms extensive interface with MO25α through seven structurally similar α-helical repeats that form a horseshoe shaped concave surface (36). Although STRADα lacks the prerequisite residue for catalytic

activity, STRADα adopts an active conformation when bound to ATP. While the affinity of STRADα for ATP is enhanced by MO25α, the binding of both MO25α and ATP to STRADα, are essential for the activation of LKB1 (36). LKB1-STRADα-MO25α heterotrimeric complexes (Figure 3) can be purified from mammalian cells, with each component in approximately equal stoichiometry (33). When in complex with STRADα and MO25α, LKB1 becomes catalytically active, thus unlike most serine-threonine kinases LKB1 does not require the phosphorylation of its conserved threonine in the T-loop for activity (32-35). Individually, STRADα and MO25α are expressed in both the cytoplasm and nucleus; however when co-expressed, both are located exclusively in the cytoplasm. Expression of LKB1 with STRADα and MO25α results in extensive re-localization of LKB1 to the cytoplasm (32-33). Most oncogenic mutants of LKB1 that have been identified in human cancers are unable to interact with STRADα and MO25α (37), thus the majority of these mutants remain localized in the nucleus. The interaction of STRADα and MO25α with LKB1 increases the catalytic activity of LKB1 approximately 10-fold (32). As previously mentioned, LKB1 induces a G_1 cell cycle arrest, while LKB1 oncogenic mutants cannot (28,38); this difference may be attributed to the fact that LKB1 oncogenic mutants do not interact with STRADα-MO25α, since the majority of mutations are localized to the kinase domain of LKB1. STRADα interaction is required for LKB1-induced G_1 arrest, since siRNA-mediated knockdown of STRADα expression prevented LKB1 from inducing G_1 arrest (32). LKB1 can also form a complex with heat shock protein 90 (Hsp90) and the Cdc37 kinase-specific targeting subunit for Hsp90 (39). It is likely that these are two separate complexes, since STRADα-MO25α immunoprecipitate with LKB1, but not with Cdc37 or Hsp90 (19). The authors of this study suggest that Hsp90 and Cdc37 may facilitate in the formation of the LKB1-STRADα-MO25α complex (39).

A more complex role for STRADα has recently been identified. A series of experiments by Dorfman and colleagues (34) demonstrated that STRADα plays an important role in the nucleo-cytoplasmic shuttling of LKB1. The authors showed that STRADα prevents LKB1 from binding to importinα/β, preventing LKB1 from entering the nucleus. Furthermore, STRADα exits the nucleus in a CRM1- (exportin1) mediated fashion. LKB1 is exported from the nucleus in complex with STRADα. LKB1 alone is not exported by CRM1. STRADβ can prevent the binding of LKB1 to importinα/β but cannot facilitate nuclear export of LKB1 through CRM1, indicating functional differences for STRADα and STRADβ. STRADα when in complex with LKB1 can also be exported from the nucleus by exportin7; this is the first protein identified as cargo for both CRM1 and exportin7. Both STRADα and

MO25α can passively diffuse in and out of the nucleus through nuclear pores. Due to the presence of various STRAD isoforms and splice variants, it is possible that when LKB1 is in complex with different STRAD isoforms, LKB1 localization will vary, providing another means of control of LKB1 function. This was recently demonstrated by Marignani and colleagues (40) that identified eleven different STRADα isoforms isolated from human colorectal cancer cell lines, each of which differently interact with LKB1 and MO25α. Levels of LKB1 activity and localization also varied with the different STRADα isoforms. The authors of the study postulate that in PJS where LKB1 expression is normal, the syndrome manifests due to the presence of a STRADα isoforms that render LKB1 catalytically deficient (40).

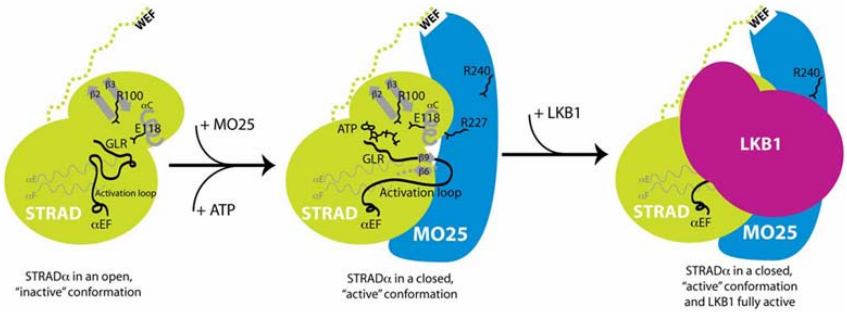

Figure 3. Schematic representation of how STRADα/MO25α may interact to activate LKB1. The model is based on known mutagenesis and structural data detailed in Zeqiraj et al. 2009. Binding of either ATP and/or MO25a to STRADα induces STRAD*α* to adopt a closed conformation, leading to the assembly of a fully active LKB1 complex. *From Zeqiraj et al, PloS Biology, 2009.*

Other interacting partners of LKB1

In addition to the proteins described above, LKB1 has been reported to regulate the expression of transcriptions factors. LKB1 associates with LIM domain only 4 (LMO4), an adaptor that assembles transcription factors such as GATA-6 (41). LKB1 kinase activity enhances GATA-6 mediated transactivation in HeLa cells. Furthermore this study demonstrated that, in contrast to previous reports, LKB1 enhances the promoter activity of P21 ^{WAF1} in the presence of LIMO4, GATA-6 and LDB1, in a TP53 independent manner (41). LKB1 along with its downstream kinase, the salt inducible kinase (SIK), regulates another transcription factor, cyclin AMP response element binding protein (CREB) by phosphorylating and repressing its coactivator, transducer of regulated CREB activity (TORC) (42). It has been reported that Lkb1 null fibroblasts from mice exhibit high expression levels of signal transducer and activator of transcription 3 (Stat3)-regulated genes such as,

matrix metalloproteinase 2 (Mmp2), Mmp9, vascular endothelial growth factor (Vegf), insulin-like growth factor binding protein 5 (IGFBP5) and Cyclooxygenase-2 (COX-2) (43-44). This has been attributed to the suppression of the oncogenic transcription factor Stat3 by Lkb1 and its kinase deficient mutants, which interact with Stat3 and reduce its association with target promoters (46). An alternative mechanism for the enhanced expression of COX-2 in PJS hamartomas (47), has been ascribed to the regulation of the transcription factor polyomavirus enhancer activator 3 (PEA3) by LKB1. LKB1 associates with PEA3, induces its phosphorylation, and targets it to the ubiquitination and proteosome-dependent degradation pathway (48). Hence LKB1 plays a role in gene transcription through the regulating of transcription factors.

LKB1 gene inactivation in human cancer

There is no doubt that *LKB1* is an important tumour suppressor gene involved in cancer development. Its genetic inactivation is not only the cause of PJS but also a critical step in the development of some forms of sporadic tumours. Since PJS patients have an increased risk of developing several types of malignancies, mutations at *LKB1* have been sought in a wide variety of sporadic tumours. Tumour-specific *LKB1* alterations have been identified in many tumour types, although their frequency is apparently low (49). Intriguingly, in lung tumours of the non-small-cell lung cancer (NSCLC) type, especially adenocarcinomas, *LKB1*-inactivating mutations are commonly detected, occurring in from one-third to a half of cases (50-51). The type and pattern of mutations in *LKB1* in the PJS and in lung cancer have been extensively reviewed elsewhere (49,52). These observations are fully consistent with the high frequency of loss of heterozygosity (LOH) at chromosome 19p13, reported in lung primary tumours and cancer cell lines (53). In tumours, frequent LOH at a particular chromosomal region constitutes a classic hallmark for pinpointing the location of a tumour suppressor gene. Thus, it can be concluded that LKB1 is one of the tumour suppressor genes targeted by the high frequency of LOH at chromosome 19p in lung cancer. More recently, another tumour suppressor on chromosome 19p13.2, *BRG1* (also called *SMARCA4*), has been found to be frequently inactivated by somatic mutations in lung cancer cell lines (54), indicating that LOH at chromosome 19p in lung cancer targets two distinct tumour suppressors (55). The reasons for the differences in the frequency of *LKB1* mutations between lung tumours and other tumour types are not yet understood and are puzzling because lung cancer is not among the commonest tumours arising in PJS. There are several possible explanations for these differences, and these have been discussed elsewhere (49).

The pattern of mutations in *LKB1* in tumours of sporadic origin and in those arising in PJS is that of a classic tumour-suppressor gene. First of all, mutations are homozygous, as predicted by Knudson's *two hit* hypothesis (56). Usually, mutations or small deletions/insertions arise in one of the alleles while the remaining allele is lost by LOH. PJS patients are born with an *LKB1* alteration (first hit) and the remaining allele is altered (second hit) during tumour development. In contrast, in tumours of sporadic origin both alleles arise during tumour development. *LKB1* mutations are scattered throughout exons 1 to 8, but no mutations in exon 9 have so far been identified. There is a large proportion of nonsense, indel, frameshift and intronic mutations in splicing-conserved sites, as well as large deletions. These changes predict the generation of truncated and, therefore, completely inactivated LKB1. Some missense mutations, which lead to aminoacid changes, have also been found, especially in PJS patients. Most of these are substitutions of highly conserved aminoacids in the kinase domain and abrogate the activity of LKB1 (52).

In lung cancer, *LKB1* mutations occur preferentially in smokers and are found concomitantly with alterations at other cancer genes such as *TP53*, *PIK3CA*, *MYC*, *CDKN2A* and *KRAS*, but not with *EGFR* mutations (57-58). Moreover, *LKB1* inactivation is less frequent in lung adenocarcinomas from patients of Asian origin (57-59), which exhibit a significantly higher frequency of *EGFR* alterations than found in lung adenocarcinomas arising in patients from western countries.

LKB1 mouse models and cancer

Genetic mouse models have provided insight into molecular mechanisms by which LKB1 elicits its tumour suppressor function. The first *Lkb1* mouse model was developed by Mäkelä and colleagues in 2001, targeted disruption of *Lkb1* resulted in embryonic lethality at midgestation characterized by neural tube defects, and abnormal vasculature due to aberrant regulation of vascular endothelial growth factor (Vegf) expression (60). A second *Lkb1* gene knockout mouse model generated by Taketo and colleagues (61) characterized hamartomatous tumours in heterozygous $Lkb1^{+/-}$ mice that were histologically similar to PJS hamartomas. Analysis of the gastrointestinal hamartomas confirmed expression of wild-type *Lkb1* and targeted *Lkb1* allele, thus suggesting that the initiation and development of polyps was due to Lkb1 haploinsufficiency and not due to *Lkb1* loss of heterozygosity (61). In addition to gastrointestinal hamartomas, greater than seventy per cent of the male $Lkb1^{+/-}$ mice developed hepatocellular carcinomas (HCC) compared to approximately twenty per cent of the female $Lkb1^{+/-}$ mice (62). Furthermore, histopathology confirmed the presence of trabecular, pseudoglandular and

sarcomatous forms of HCC, that shared histological characteristics with human HCC and displayed biallelic inactivation of *Lkb1*(62).

Since knockout mouse models of the *Lkb1* gene were found to be embryonic lethal, DePinho and colleagues developed a mouse model that carried a conditional *Lkb1* allele (Figure 4) (43). Similar to previous models, conditional $Lkb1^{-/-}$ mice were embryonic lethal, while conditional $Lkb1^{+/-}$ mice developed polyps that were histologically similar to PJS polyps. Like the polyps from people with PJS (63-64), polyps from conditional $Lkb1^{+/-}$ mice were not positive for activating *ras* mutations however, $Lkb1^{-/-}$ mouse embryonic fibroblasts (MEFs) were sensitive to Ras-induced senescence and activated *ras*-mediated transformation (43). Inconsistent with previous *Lkb1* knockout models (60-61), 25% of the conditional $Lkb1^{+/-}$ mice displayed LOH at the *Lkb1* locus. The reason for the discrepancy in *Lkb1* LOH between *Lkb1* knockout and conditional *Lkb1* mouse models is not known.

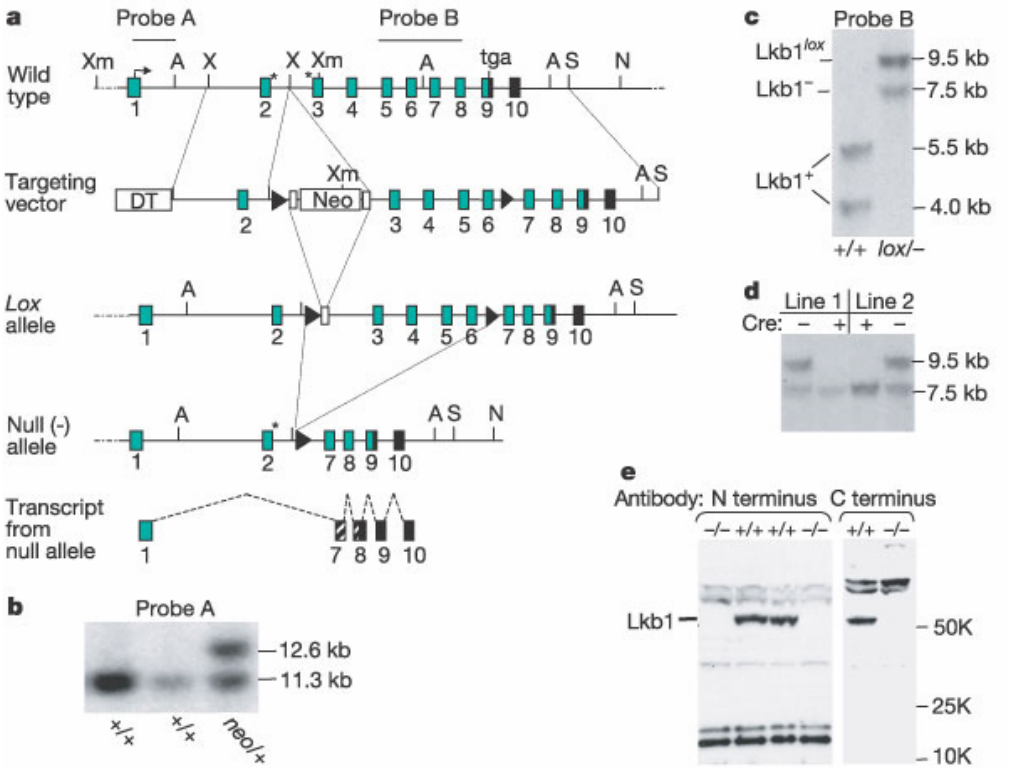

Figure 4. Targeting strategy and analysis of *Lkb1*. **a**) *Lkb1* genomic structure and recombinant alleles. The 3' untranslated region (black bars), and noncanonical splice sequences (asterisks) are indicated. The transcript from the *Lkb1* null allele eliminates exons 2–6, resulting in a translational frameshift (crosshatched bars). **b**) Southern analysis of DNA from ES cell clones showing targeting of the *Lkb1* locus. **c**) Southern analysis of the *lox*, null (-), and wild-type (+) alleles. **d**) Southern analysis of DNA from $Lkb1^{lox/-}$ MEFs, untreated (-) or infected with a Cre retrovirus (+). **e**) Western analysis of $Lkb1^{lox/-}$ (-/-) and wild-type (+/+) MEFs after Cre expression, using antibodies to the amino (left) and carboxy (right) termini of Lkb1. *From Bardeesy et al, Nature 2002*.

In 2005, Sakamoto and colleagues (65) generated mice conditional for expression of *Lkb1* by replacing exons 5-7 of the *Lkb1* gene with a cDNA cassette encoding exons V-VIII and IXb, flanked by *loxP* Cre excision sequence (Figure 5) (65). Surprisingly, male *Lkb1*$^{fl/fl}$ mice were sterile however a plausible explanation was made available several years later with the discovery of an alternative splice variant of Lkb1 located at exon IXa, referred to as *Lkb1* short form (*Lkb1*$_S$). *Lkb1*$_S$ was found to be highly expressed in testis whereby male mice lacking Lkb1$_S$ were found to be sterile (66) and presented with defects in spermatogenesis (67).

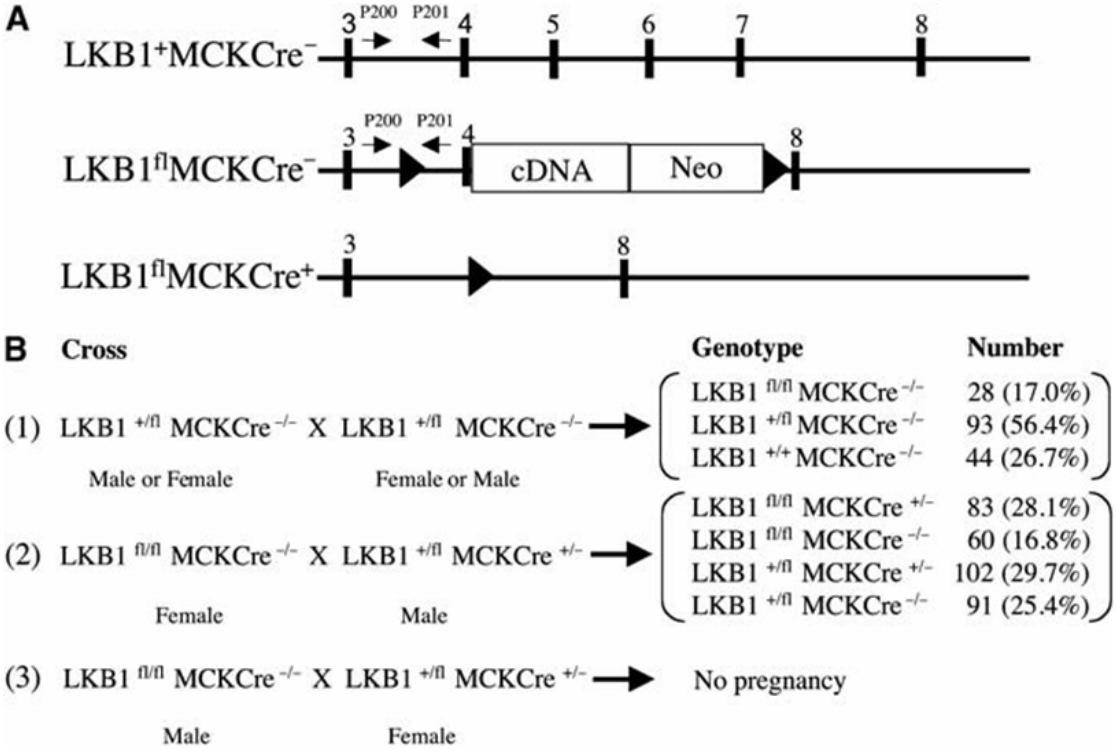

Figure 5. Generation of LKB1-deficient mice. a) Diagram illustrating the positions of exons 3–8 (▉) in the wild-type Lkb^{+}Cre^{-} allele. In the Lkb1^{fl}Cre^{-} allele, exon 4 of the Lkb gene is flanked with loxP Cre recombinase excision sites (▲), and exons 5–7 encoding the catalytic domain of Lkb1 are replaced with a cDNA construct encoding the remainder of the Lkb1 sequence, as well as a neomycin (Neo) selection gene. The expression of the neomycin gene is driven by the Lkb1 promoter and it is made as fusion mRNA with Lkb1. Its translation is directed by an internal ribosome entry site. In the Lkb1^{fl}Cre^{+} allele, exons 4–7 of the *Lkb1* gene are deleted through action of the Cre recombinase, there by ablating functional expression of LKB1. The positions of the PCR primers used to genotype the mice are indicated with arrows. b) Breeding strategy employed to generate Lkb1$^{fl/fl}$ and Lkb1-muscle-deficient (Lkb1$^{fl/fl}$Cre$^{+/-}$) mice, where MCKCre denotes transgenic mice expressing the Cre recombinase under the muscle creatine kinase promoter. The number and percentage of each genotype obtained are indicated. *From Sakamoto et al, EMBO J, 2005.*

The strategy used by the Alessi's laboratory (65) to generate a floxed *Lkb* allele, resulted in no expression of testicular $Lkb1_S$, thus explaining the sterility of these male mice. In addition to male sterility, protein expression in $Lkb1^{fl/fl}$ mice was found to be five- to ten-fold reduced compared to $Lkb1^{+/+}$, indicative of a hypomorphic phenotype for homozygous Lkb1 mice, whereas $Lkb1^{+/fl}$ mice expressed two-fold less *Lkb1* than $Lkb1^{+/+}$ mice (65). When $Lkb1^{fl/fl}$ mice were crossed with mice transgenic for Cre-recombinase under the muscle creatine kinase promoter (MCKCre), resulting progeny had *Lkb1* excised from skeletal and heart muscles prior to birth (68). AMPKα2 activation was evaluated in *Lkb1* lacking skeletal muscle and found to be significantly lower than AMPKα2 activity in skeletal muscle from wild-type or $Lkb1^{+/fl}$ mice (65), despite comparable AMPKα2 expression levels. Interestingly, AMPKα1 activity was modestly increased by 2-fold in Lkb1-deficient muscle. These observations were explained by the possible contamination of nonmuscle cells in the sample preparations (65). Finally, since AMPK activation during muscle contraction regulates the balance of energy, ADP:ATP and AMP:ATP ratios were evaluated in contracting tibialis anterior and extensor digitorum longus muscles. In muscles lacking expression of *Lkb1*, ADP:ATP and AMP:ATP ratios were significantly elevated compared to wild-type, $Lkb1^{+/fl}$, or hypomorphic $Lkb1^{fl/fl}$ mice. Overall, the work from Alessi and colleagues (65) confirms a critical role for Lkb1 in AMPK-mediated activation of skeletal muscle and energy balance.

The loss of LKB1 expression in pancreatic cancer is well described in the literature with neoplasms ranging from ductal adenocarcinomas, to intraductal papillary mucinous neoplasia and serous cytadenomas (69-70). Conditional ablation of Lkb1 expression in mice was conducted to determine the functional role of Lkb1 in the development of the pancreas (71). Genetic cross between conditional null allele of *Lkb1* and transgenic Pdx1-Cre (expressed in pancreatic progenitors between E8-E12) resulted in initially normal development, however shortly after birth, these mice exhibited increased weight gain, elevated serum amylase, steatorrhea and by 10 weeks, all mice became increasingly cachetic and therefore euthanized (71). Analysis of the abdominal cavity revealed complete replacement of pancreatic tissues with cystic masses that closely resembled serous cystadenoma observed in PJS. The penetrance of pancreatic disease was complete in these mice compared to heterozygous Lkb1-Pdx1-Cre or wild-type mice (71). These findings confirm a functional role for Lkb1 in pancreas development. Since polarized organization of acina is necessary for directional secretion of zymogen from the pancreatic ductal network and given that LKB1 is reported to be involved in mediating cell polarity (13-14, 72), as it is discussed later, acinar development was evaluated in the Lkb1-Pdx1-Cre mice (71). There

was a loss of acinar polarity in pancreatic tissues from Lkb1-Pdx1-Cre at E18.5, as determined by loss of basal positioning of nuclei, lateralization of microvilli and a reduction/loss of tight and/or adherents junctions, was observed. Overall, the authors of this study establish a physiological role for LKB1 in pancreatic cellular polarity and the maintenance of ductal intregrity.

LKB1 and AMPK: Cellular energetic metabolism and the cancer connection

As a tumour suppressor, the restitution of wild type LKB1 has the ability to suppress the growth of tumour cells that lack the LKB1 protein (28, 73). The molecular mechanisms underlying the tumour-suppressor function of LKB1 are still not completely understood, although it seems plausible that it depends on its serine-threonine kinase activity. The best characterized LKB1 substrate is the AMP-activated protein kinase (AMPK), a so-called 'metabolic master switch'. An increase in the intracellular AMP:ATP ratio triggers the phosphorylation and activation of AMPK, which is allosterically activated by AMP and subsequently phosphorylated at Thr172 by upstream kinases such as LKB1 (74). Activation of AMPK modulates the activity of multiple downstream targets in normalizing ATP levels, e.g. stimulating fatty acid oxidation to generate more ATP and simultaneously inhibiting ATP-consuming processes including fatty acid and protein synthesis (75). Enzymes essential for free fatty acid synthesis, including fatty acid synthase and acetyl CoA carboxylase (ACC), can be inhibited by AMPK by virtue of direct phosphorylation and/or regulation of transcription.

The upstream kinase that phosphorylates and activates AMPK (AMPKK) was a long-sought protein. The first evidence suggesting that LKB1 is an AMPKK came from the work by Hong et al. (76), who searched for kinases that triggered the phosphorylation/activation of Snf1, the homolog of AMPK in yeast. They identified three yeast kinases, Pak1p, Tos3p and Elm1p, that activate Snf1 kinase *in vivo*. Moreover, Tos3p was found to be able to phosphorylate and activate mammalian AMPK, suggesting functional conservation of the upstream kinases between yeast and mammals. Finally, it was shown that LKB1, which is related to Tosp3, is able to phosphorylate and activate AMPK *in vitro* and could be the long-sought mammalian AMPKK. Almost simultaneously it was described that LKB1, in a complex with STRAD alpha/beta and MO25 alpha/beta, activates AMPK via phosphorylation of Thr172. The heterotrimeric complex was essential for the proficient activation of AMPK *in vitro* and *in vivo* (74, 77). In further support of the AMPKK activity of LKB1, the AMPK-activating drugs 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) and phenformin

does not activate AMPK in the cervical cancer-derived and *LKB1*-mutated HeLa cells or in lung cancer-derived *LKB1*-mutant cells, but activation can be restored by stably expressing wild type, but not catalytically inactive, LKB1 (33, 78). Finally, the comparison of the expression profiles among lung tumours according to *LKB1* mutation status revealed that *LKB1*-mutant tumours has differential levels of many transcripts, including components of the PTEN/PI3K pathway as well as enzymes that participate in AMP metabolism, such as AMPD3 (adenosine monophosphate deaminase, isoform E) and APRT (adeninephosphoribosyl transferase) (79), further linking LKB1 function with that of AMPK.

How LKB1 and AMPK inactivation contribute to cancer development is still not completely clear. Abrogation of energetic checkpoints may be required for a cancer cell to ensure the maintenance of cell survival and growth in an environment with limited access to nutrients or oxygen. In a normal cell, these circumstances will trigger cessation of cell growth or apoptosis due to the compromised availability of ATP. Another link between the LKB1-AMPK pathway and cancer development is the tuberin protein, which is the product of the tuberous sclerosis complex 2 gene (*TSC2*), which represses of mTOR when activated by AMPK (80-81). Germ-line mutations of *TSC1* or *TSC2* genes cause the tuberous sclerosis syndrome, which has similarities with PJS, including the presence of hamartomas (82). Wild type LKB1 has been shown to be required for modulating AMPK activity and that of AMPK-downstream targets, including mTOR, in a variety of energetically depleted cancer cells (78, 80).

In addition to cancer, LKB1 may be important in type II diabetes mellitus, a metabolic disorder characterized by resistance to the actions of insulin to stimulate skeletal muscle glucose disposal. The involvement of LKB1 in type II diabetes may arise from its function in the activation of AMPK. Deletion of LKB1 in the liver of adult mice abolished almost all AMPK activity and resulted in hyperglycemia with increased gluconeogenic and lipogenic gene expression (83). Furthermore, metformin, a drug widely prescribed to type II diabetes patients, lowers blood glucose concentrations by indirectly promoting AMPK activity (84). The effectiveness of metformin depends on the presence of LKB1 (83). Among the drugs that activate AMPK are AICAR and the biguanides, metformin and phenformin. All of these require LKB1 activity, and so therapies based on AMPK activators would be effective only in LKB1 wild type tissues. A recent population-based case-control study showed that type II diabetic patients treated with the AMPK activator metformin were less likely to get cancer (85), suggesting that AMPK activators could also be used in cancer prevention. Although the role of these compounds in cancer prevention looks

promising, their effectiveness in patients who have already developed cancer may not be so clear.

More recently, LKB1 and AMPK activity have also been related to SIRT1, a NAD-dependent protein deacetylase that regulates energetic homeostasis in response to nutrient availability. SIRT1 overexpression diminished lysine acetylation in specific lysine residues of LKB1 and concurrently increased its activity, cytoplasmic/nuclear ratio, association with the LKB1 activator STRAD and, as expected, increased AMPK and ACC phosphorylation (86). It has also been reported that resveratrol, a polyphenol that protects against metabolic disease by activating SIRT1, increased LKB1 phosphorylation at Ser(428) and, thus, AMPK activity. These effects are abolished by pharmacological and genetic inhibition of SIRT1 (24). Taken together, these findings suggest that SIRT1 functions as a novel upstream regulator for LKB1/AMPK signalling.

Finally, the LKB1-AMPK signalling pathway has also been functionally associated with the *BRAF* oncogene. In melanoma cells, AMPK activation is suppressed by activated BRAF, V600E mutation. Conversely, downregulation of BRAF signalling triggers activation of AMPK. In these cells LKB1 was found to be phosphorylated by ERK and Rsk, two kinases downstream of BRAF, which compromised the ability of LKB1 to bind and activate AMPK (87).

The role of LKB1 in the regulation of cell polarity

The identification of AMPK as a key substrate of LKB1 kinase activity provides definitive evidence that the abrogation of energetic checkpoints, probably with the purpose of maintaining energetically costly processes such as DNA replication and cell division, is an obligatory event for cancer development. However, LKB1 functions as a master upstream protein kinase of many AMPK-related kinases (88), indicating that LKB1 modulates other important cell processes. Some of these AMPK-related kinases are the MAP/microtubule affinity-regulating kinases (MARK1, MARK2, MARK3 and MARK4) involved in microtubule stabilization, which may be related to the proposed role of LKB1 in cell polarity. The substrates of LKB1, their downstream targets and associated biological roles are summarized in Figure 2.

Watts et al. (13) provided the first hint about the role of LKB1 in the control of cell polarity. They observed that the maternally expressed *par* genes were asymmetrically distributed in the first cell cycle of *Caenorhabditis elegans* embryogenesis. Establishing asymmetries at this early stage is essential for determining the subsequent developmental fates of

the daughter cells. They found that the *par4* gene of *C. elegans* encodes a putative serine-threonine kinase that was similar to LKB1 and to the *Xenopus* egg and embryo kinase, XEEK1. In support of this, the protein encoded by the homolog of *par4* in Drosophila, lkb1, has also been implicated in embryonic polarity, since it is required for the early anterior-posterior (A-P) polarity of the oocyte and for the repolarization of the oocyte cytoskeleton that defines the embryonic A-P axis (14). Finally, in mammalian development, it has been reported that Lkb1 is asymmetrically localized to the animal pole of the mouse oocyte and that it associates with the microtubules at metaphase during oocyte maturation, indicating that Lkb1 protein participates in the polarization of the mouse oocyte and in the regulation of the asymmetry of meiotic divisions during oogenesis (89).

LKB1 has also been associated with the polarization of adult somatic cells of a number of cell types. In human epithelial intestinal cells it has been observed that the inducible expression of STRAD activates LKB1, which leads the individual cells to the rapid remodeling of the actin cytoskeleton whereby, in the absence of cell-cell contacts, they form an apical brush border and redistribute junctional proteins in a dotted circle at the periphery of the brush border. This implies that LKB1 can induce complete polarity in intestinal epithelial cells (72). In addition, LKB1 is required for axon differentiation and specification during neuronal polarization in the mammalian cerebral cortex through the phosphorylation and activation of brain-specific kinase, BRSK (21-22). Finally, immunostaining of LKB1 in a variety of normal human tissues has revealed apical accumulation of this protein in several types of epithelial cells (3). LKB1 has also been shown to be essential for NSCLC polarity and to colocalize at the leading edge of the cell with the small rho GTPase cdc42 and its downstream binding partner, p21-activated kinase (PAK), two key components of the polarity pathway (90). The exact mechanism and downstream targets that mediate the role of LKB1 in the control of cell polarity are still under debate. Some observations suggest AMPK and its target non-muscle myosin regulatory light chain (MRLC; also known as MLC2), which is directly phosphorylated by AMPK at an important regulatory site (91). Since the microtubule affinity-regulating kinases (MARK1, MARK2, MARK3 and MARK4) are among the AMPK-related kinases phosphorylated by LKB1 they may also mediate the role of LKB1 in cell polarity. The MARK family phosphorylates microtubule-associated proteins (tau/MAP2/MAP4), causing detachment from microtubules, and their disassembly (92-94). Interestingly, aberrant phosphorylation of the microtubule-associated protein tau is one of the pathological features of neuronal degeneration in Alzheimer's disease, which points towards LKB1 being a candidate target for novel therapies against this neurodegenerative disease.

LKB1 in regulation of hormone receptor signalling

Clinical evidence suggests a role for the tumour suppressor kinase LKB1 in the development of breast carcinoma (95-97). Early evidence in the literature suggests that a loss of LKB1 expression leads to papillary breast carcinoma (98). More recently, a study that evaluated the expression of LKB1 in 85 cases of breast cancers, the authors found that in a subset of high-grade *in situ* and invasive mammary carcinomas, the expression of LKB1 was completely lost as determined by immunohistochemistry (96). In a separate study, 116 patients with confirmed breast carcinoma were evaluated for LKB1 protein expression, of which expression was low in approximately one third of the patients compared to control population of women. The authors of this study concluded that low expression of LKB1 correlated with higher histological grade, tumour size, and presence of lymph node status (95). Both studies conclude that LKB1 expression profile may serve as a prognostic marker for breast carcinoma, however further investigation is warranted. These recent findings suggest a role for LKB1 in breast cancer however the molecular mechanism by which this occurs is not fully understood.

Recent work from the Marignani lab (98) highlight the discovery of a novel function for LKB1, coactivator of estrogen receptor alpha (ERα) signalling. In a series of experiments, LKB1 was discovered to bind to ERα, thereby enhancing ERα-mediated transcription. Furthermore, it was determined that LKB1 catalytic activity contributes to enhanced ERα-mediated transcription and that LKB1 is recruited to the promoters of ERα-responsive genes. Clinical evidence suggests a role for LKB1 in breast cancer since low expression of LKB1 is correlated with poor clinical outcomes. This said the molecular mechanisms by which LKB1 is involved in mammary gland development and/or malignancies are not known. The significance of this work is the demonstration, for the first time, of a functional link between LKB1 and ERα, placing LKB1 in a coactivator role for ERα signalling, similar to the other tumour suppressors such as RB (99-102), $P21^{WAF/CIP}$ (103-104), TSC2 (105) and BRG1 (106), that are reported to have coactivator function for hormone receptors. This discovery broadens the scientific scope of LKB1 and lays the ground work for further investigation into the role of LKB1 in mammary gland development and tumourigenesis.

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