Minireview

LKB1, a protein kinase regulating cell proliferation and polarity

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Abstract LKB1 is a serine-threonine protein kinase mutated in patients with an autosomal dominantly inherited cancer syndrome predisposing to multiple benign and malignant tumours, termed Peutz–Jeghers syndrome. Since its discovery in 1998, much research has focused on identification and characterisation of its cellular roles and analysing how LKB1 might be regulated. In this review we discuss exciting recent advances indicating that LKB1 functions as a tumour suppressor perhaps by controlling cell polarity. We also outline the current understanding of the molecular mechanisms by which LKB1 is regulated in vivo, through interaction with other proteins as well as by protein phosphorylation and prenylation.

Key words: Tumor suppressor; Cancer; Cell growth; Peutz–Jeghers syndrome

1. Introduction

Peutz–Jeghers syndrome (PJS) was first described in 1922 by a Dutch physician, Dr Johannes Peutz [1], and further characterised in the 1940s by Dr Harold Jeghers [2,3]. It is an autosomal dominantly inherited disorder exemplified by patients developing multiple hamartomatous polyps (benign overgrowth of differentiated tissues) in the gastrointestinal tract as well as pigmentation of the mucous membranes. PJS patients also have a markedly increased risk of developing malignant tumours mostly in the intestine, stomach and pancreas but also in other tissues including breast, cervix, lung, ovary, testis and uterus [4–6]. It is estimated that 93% of PJS patients will develop malignant tumour(s) at an average age of 43 years [7]. The reported estimates of the incidence frequency of PJS vary significantly. One report estimated the frequency to be 1:8300 live births [8], while another study put the estimate at 1:120 000 [4]. In the UK, there are estimated to be around 2000 people with PJS, indicating the incidence frequency is 1:30 000 (Association of International Cancer Research, http://www.aicr.org.uk/cancer_syndromes.htm). Although the majority of PJS patients have a family history of this condition, 10–20% of the cases are apparently caused by de novo LKB1 germline mutations [9].

In 1997, linkage analysis of multiple hamartomas derived from PJS patients suggested that the causative locus for this disorder was located at chromosome 19p13.3 [10]. In 1998, two groups reported that the gene mutated in PJS families was a previously uncharacterised serine-threonine protein kinase, termed LKB1 [11] or STK11 [12]. The LKB1 nomenclature is now normally employed and arose as a code name for the PJS causative gene. The LKB1 gene spans 23 kb and is composed of 10 exons, nine of which are coding, and the gene is transcribed in the telomere to centromere direction [12]. The human LKB1 protein kinase comprises 433 residues (436 amino acids for mouse LKB1), and its catalytic domain (residues 44–309) is poorly related to other protein kinases. The N-terminal and C-terminal non-catalytic regions of LKB1 are not related to any other proteins and possess no identifiable functional domains. LKB1 is ubiquitously expressed in all foetal and adult tissues examined [11,12]. To date, 75 mutations in LKB1 have been identified to our knowledge in PJS patients, and the types of mutations found are summarised in Fig. 1. The majority of these mutations would be expected to impair LKB1 catalytic activity, but a number of mutations only affect the C-terminal non-catalytic region (Fig. 1). Patients with sporadic cancers have also been screened for mutations in the LKB1 gene, and although their frequency is relatively rare, 24 mutations have been identified, which are listed in Fig. 1. Recently, it has been suggested that sporadic mutations in LKB1 are found in one third of lung adenocarcinomas [13].

2. Overexpression studies

An important early finding was that overexpression of wild type LKB1 in two cancer cell lines (HeLa and G361) which do not express endogenous LKB1, suppressed the growth of these cells by inducing a G1 cell cycle arrest [14]. Catalytically inactive LKB1 mutants including some of those isolated from PJS patients failed to suppress cell growth. This was the first indication that LKB1 functioned as a tumour suppressor and that serine-threonine protein kinase catalytic activity was required for this function. One study indicated that overexpression of LKB1 in G361 melanoma cells induced the expression of the $p21^{WAF1/CIP1}$ inhibitor of the cyclin-dependent kinases,

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Abbreviations: ATM, ataxia-telangiectasia mutated kinase; Brg1, Brahma related gene-1; COX-2, cyclooxygenase-2; ERK, extracellular regulated kinase; Hsp90, heat shock protein 90; LIP1, LKB1 interacting protein-1; PAR, partitioning defective; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PJS, Peutz–Jeghers Syndrome; PKA, cyclic AMP dependent protein kinase; RSK, p90 ribosomal S6 kinase; STRAD, STE20 related adapter protein; VEGF, vascular endothelial growth factor

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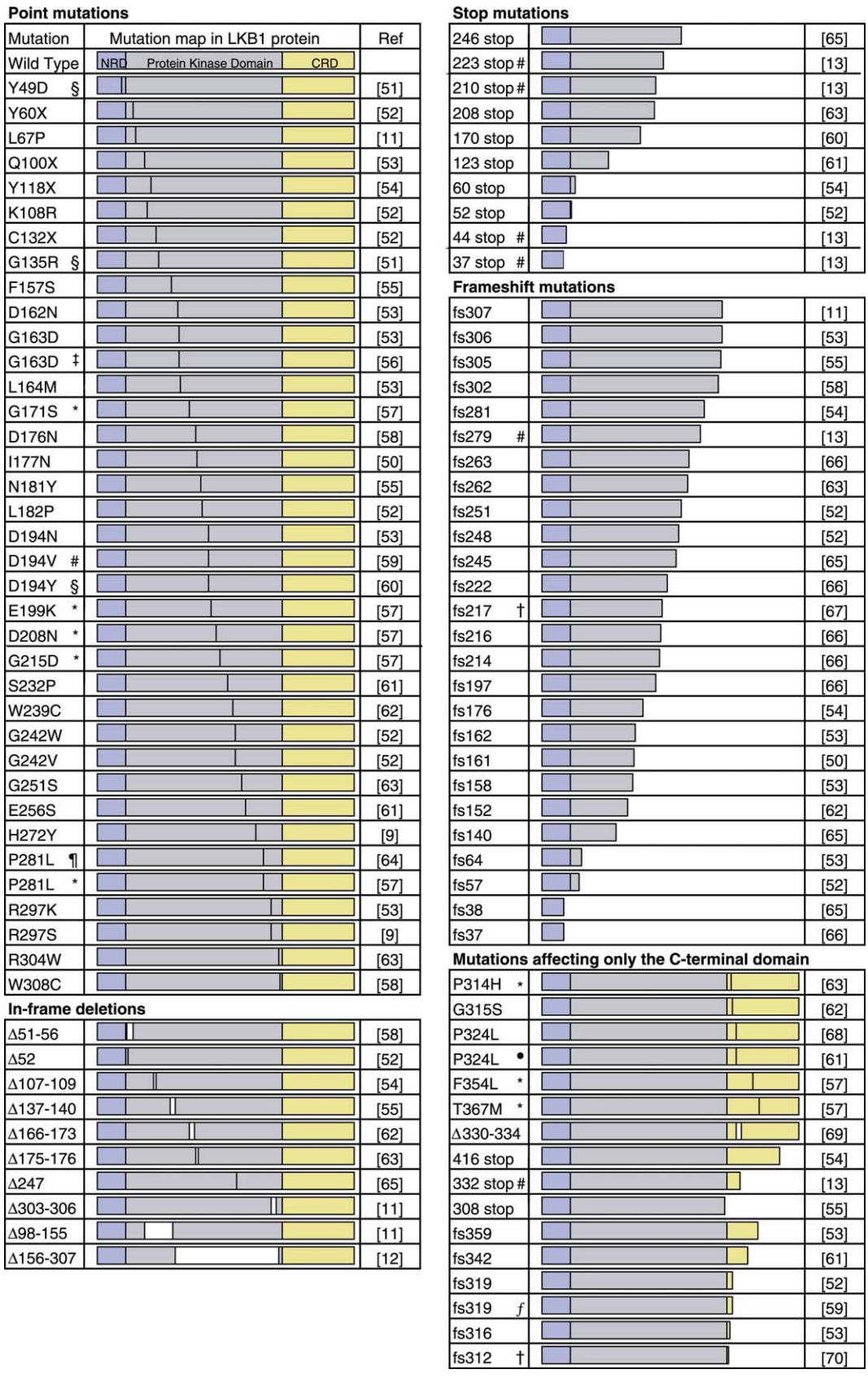

Fig. 1. Mutations identified in the LKB1 gene in patients with PJS and sporadic cancer and schematic representation of their predicted effects on the primary structure of LKB1; Refs. [9,11–13,50–70]. Unless otherwise marked, the mutations are derived from PJS patients. The mutations marked with symbols are derived as follows: * colorectal cancer, # lung cancer, † pancreatic cancer, § melanoma cancer, ‡ testicular cancer, ¶ ovarian cancer, ● gastric carcinoma, ƒ cervical cancer. Abbreviations: fs, frameshift; NRD, N-terminal Regulatory Domain; CRD, C-terminal Regulatory Domain.

which could account for the ability of LKB1 to induce a G1 cell cycle arrest [15]. In another study, microarray analysis revealed that overexpression of LKB1 in A549 cells induced expression of several p53 responsive genes, implicating the p53 pathway being controlled by LKB1 [16]. Overexpression of LKB1 in A549 cells also resulted in a significant increase in the tumour suppressor PTEN mRNA expression. PTEN functions as a lipid phosphatase metabolising the phosphatidylinositol 3,4,5-trisphosphate (PIP3) second messenger which controls proliferation and survival of cells [17]. Thus, lack of LKB1 could result in reduced expression of PTEN, thereby increasing PIP3 levels and enhancing proliferation and survival of these cells [16]. Interestingly, PTEN is also mutated in an autosomal dominantly inherited cancer disease termed Cowden's syndrome, which is phenotypically similar to PJS [18], further suggesting a link between PTEN and LKB1. In future work it would be important to study the levels of PTEN and PIP3 in tumours from PJS patients.

Much work has also focused on analysing the cellular localisation of LKB1, mainly employing overexpression approaches. These studies revealed that LKB1 was mainly localised in the nucleus, although a small fraction was reproducibly found in the cytoplasm [15,19,20]. LKB1 possesses a nuclear localisation signal at its N-terminal non-catalytic region (residues 38–43) and mutation of this motif results in LKB1 being located throughout the cell [19,21]. Interestingly, a mutant of LKB1 lacking the nuclear localisation signal still retains ability to suppress cell growth [15], suggesting that the cytosolic pool of LKB1 may play an important role in mediating its tumour suppressor properties. In addition, at least one mutant of LKB1 isolated from a PJS patient, termed SL26, which lacks three amino acids at the C-terminus of the kinase domain, still retains normal in vitro catalytic activity, but is unable to suppress cell growth and localises exclusively in the cell nucleus [15]. This further suggests that cytosolic location of LKB1 is important for its cell growth suppression properties.

3. Mouse knockouts

Several groups have reported that targetted disruption of both LKB1 alleles leads to embryonic lethality at midgestation, indicating that LKB1 plays an important role in embryogenesis [22–24]. $LKB1^{-/-}$ embryos displayed no apparent abnormalities until E8.0. Beyond E8.25, the embryos revealed multiple irregularities, including failure of the embryo to turn, a defect in neural tube closure, abnormal development of the aorta and hypoplastic first branchial arch. No viable $LKB1^{-/-}$ embryos were found after E11.0. Extraembryonic development was also severely affected in $LKB1^{-/-}$ embryos, with the placenta displaying defective labyrinth layer development and the foetal vessels failing to invade the placenta [22]. Vascular endothelial growth factor (VEGF) mRNA levels in $LKB1^{-/-}$ embryos at E8.5 and E9.5 were observed to be elevated, and primary mouse embryonic fibroblasts derived from $LKB1^{-/-}$ embryos at E8.5 displayed abnormally high basal and hypoxia-induced levels of VEGF mRNA, indicating that LKB1 might regulate VEGF production and vascular development [22]. Interestingly, the *Xenopus* counterpart of LKB1, termed XEEK1, is also highly expressed in oocytes, eggs and early embryos, further suggesting a role of LKB1 in embryogenesis [25].

$LKB1^{+/-}$ heterozygous mice were viable and displayed no obvious phenotype at birth and early adult life. However, by the age of 45 weeks most $LKB1^{+/-}$ animals developed polyps in the gastrointestinal tract, predominantly in the glandular stomach. Histologically, these hamartomatous polyps were similar to those found in PJS patients [23,24,26,27]. It should be noted, however, that the location of polyp development in $LKB1^{+/-}$ mice, predominantly in glandular stomach, was distinct from that of PJS patients, who develop polyps mainly in the small intestine. Differences in preferential sites for intestinal tumour formation in mice and humans have also been observed with adenomatous polyposis coli knockout mice [28].

A major question concerning the mechanism of tumour formation in the $LKB1^{+/-}$ animals was whether gastrointestinal polyps still expressed LKB1. Unfortunately the results obtained from such studies have yielded conflicting conclusions. Three groups, who analysed a total of 10 polyps for LKB1 mRNA expression and eight polyps for LKB1 protein expression, reported that the levels were similar to those seen in normal mucosa cells taken from $LKB1^{+/-}$ mice and were roughly half the amount compared to that observed in the wild type ($LKB1^{+/+}$) gastric mucosa or glandular stomach cells [23,24,26]. No LKB1 mutations were identified in any of the polyps analysed from $LKB1^{+/-}$ mice. These results suggested that haploinsufficiency in $LKB1^{+/-}$ animals is sufficient to induce polyposis [23,24,26]. However, a fourth study reported that three out of 12 polyps isolated from $LKB1^{+/-}$ mice showed loss of the wild type LKB1 allele [27]. Moreover, the LKB1 protein was not detected in four out of eight polyps from $LKB1^{+/-}$ mice that retained a wild type LKB1 allele, suggesting epigenetic gene inactivation had occurred [27]. Thus, these results suggest that the loss of LKB1 expression is required to induce polyp formation.

The incidence of intestinal or extraintestinal cancers has not been reported in the majority of $LKB1^{+/-}$ mice, possibly because the mice die before the onset of carcinomas and metastasis, as a result of intestinal obstruction and/or bleeding from polyps [24]. However, a recent report indicated that a significant number of $LKB1^{+/-}$ mice over the age of 50 weeks developed hepatocellular carcinoma [29]. Interestingly, no expression of LKB1 mRNA and protein was observed in these hepatocellular carcinomas, indicating that complete loss of LKB1 expression is required for the development of carcinomas [29].

Molecular analysis of the polyps from $LKB1^{+/-}$ mice also revealed that the levels of cyclooxygenase-2 (COX-2) protein were significantly increased [26]. Induction of COX-2 is implicated in promotion of tumour formation and progression [30]. A marked increase in the levels of activated extracellular regulated kinase (ERK) 1/ERK2 was also noted in the polyps of $LKB1^{+/-}$ mice, which could be responsible for inducing elevated COX-2 levels, as the expression of this gene is known to be regulated by ERK1/ERK2 [26]. However, the mechanism by which LKB1 could regulate ERK1/ERK2 and/or COX-2 expression has not been investigated.

4. Role of LKB1 in cell polarity

Putative counterparts of mammalian LKB1 have been reported in *Caenorhabditis elegans* [31] and *Drosophila* [32]. The *C. elegans* homologue, termed PAR-4, has 42% amino acid

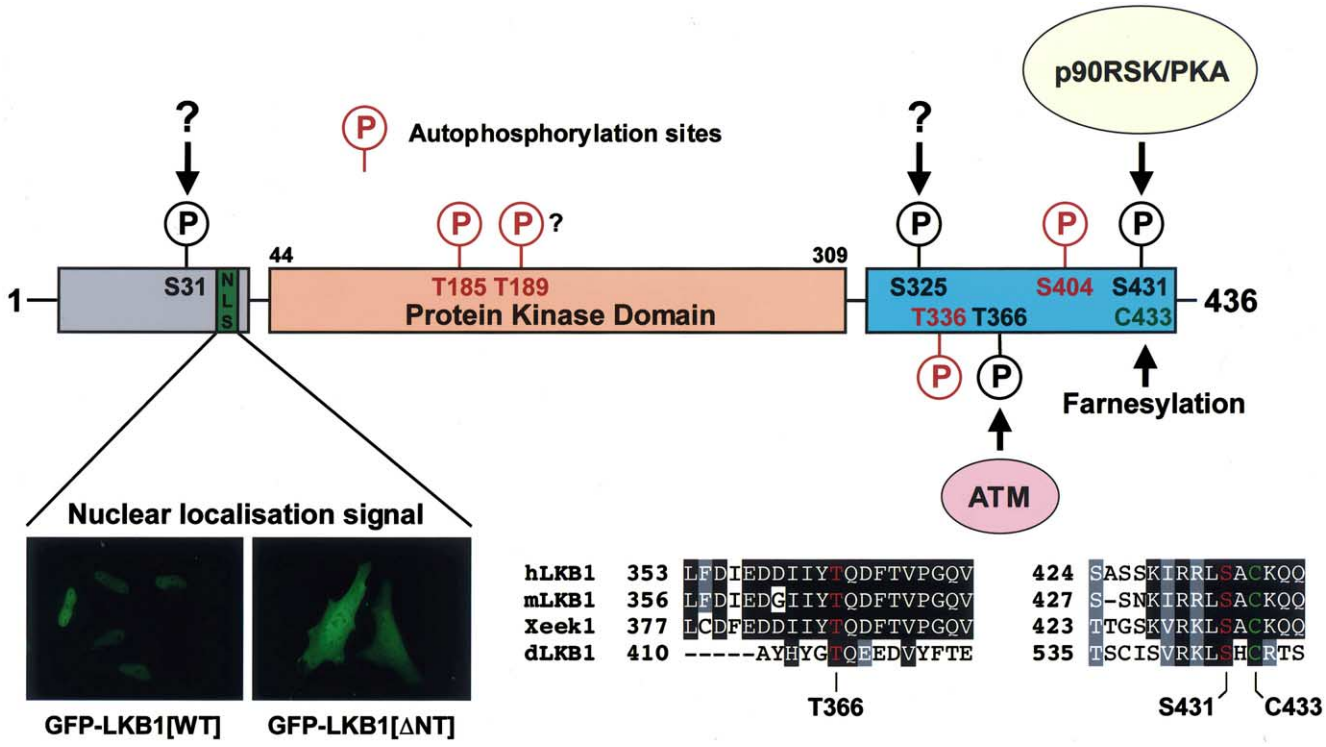

Fig. 2. Location of the phosphorylation and prenylation sites on mouse LKB1. The identity of the upstream protein kinases phosphorylating LKB1 is indicated. It should be noted that it is controversial whether Thr189 represents a site of autophosphorylation [21]. The Ser31, Thr185 and Thr189, Ser325 and Ser336 are identical in number in human and mouse LKB1. In human LKB1 Thr366 is Thr363, Ser404 is Thr402, Ser431 is Ser428 and Cys433 is Cys430. NLS is nuclear localisation signal.

identity within the kinase domain to human LKB1 but only displays 26% overall identity to the human LKB1 protein, as the non-catalytic regions of these proteins are poorly conserved. PAR-4 was identified as a member of the maternally expressed PAR (partitioning defective) gene family, which are required for establishing cell polarity during the first cycle of *C. elegans* embryogenesis, determining the subsequent developmental fates of the daughter cells [33]. Maternal effect lethal mutations in PAR-4 have been shown to affect several aspects of cell polarity [34]. Interestingly, mutations in the PAR-4 gene lead to similar phenotypes as mutations in the PAR-1 gene, which also encodes a serine-threonine kinase [35]. This suggests that PAR-1 and PAR-4 could function on the same signalling network. Recent findings suggest that the *Drosophila* homologue of human LKB1, which possesses 44% overall identity to human LKB1, with 66% identity within the kinase domain, also regulates cell polarity, as it is required for establishing the polarity of the anterior–posterior embryonic axis [32]. As observed in *C. elegans*, *Drosophila* PAR-1 and LKB1 mutants also display similar phenotypes [36,37]. Moreover, *Drosophila* LKB1 can suppress the polarity phenotype of PAR-1 mutants when overexpressed, suggesting that *Drosophila* LKB1 functions downstream of PAR-1 [32]. Consistent with this notion, *Drosophila* LKB1 was shown to be phosphorylated by PAR-1 [32]. The finding that LKB1 regulates cell polarity is important as it suggests that hamartoma formation in PJS patients could result in inappropriate overgrowths of differentiated cells, which have lost their ability to regulate their polarity as a consequence of LKB1 inactivation.

5. Posttranslational modifications

It has been reported that eight residues on LKB1 are phosphorylated (Fig. 2). Ser31, Ser325, Thr366 and Ser431 are phosphorylated by distinct upstream kinases whereas Thr185, Thr189, Thr336 and Thr402 comprise autophosphorylation sites. It should be noted that Thr185 and Thr402 sites were mapped in human LKB1 [38], whereas the other residues were mapped in mouse LKB1 [21,39] (Fig. 2). Mutation of any of these sites of phosphorylation, either to Ala to abolish phosphorylation or to Glu to mimic phosphorylation, had no effect on LKB1 in vitro catalytic activity or its in vivo cellular localisation [21,39]. Mutation of Ser431 to either Ala or Glu prevented LKB1 from suppressing the growth of G361 cells in a colony formation assay, suggesting that phosphorylation of this residue is essential for LKB1 to inhibit cell growth [39]. Mutation of Thr336, the major autophosphorylation site on LKB1, to Glu but not Ala prevented LKB1 from inhibiting G361 cell growth, suggesting that phosphorylation of this residue may inhibit LKB1 tumour suppressor function [21]. In contrast, mutation of Ser31, Ser325 or Thr366 had no major effect on the ability of LKB1 to suppress G361 cell growth [21].

The Thr336, Thr366 and Ser431 phosphorylation sites and the residues surrounding these are highly conserved in *Drosophila*, *Xenopus* and mammalian LKB1 but not in *C. elegans* LKB1 (Fig. 2). Genetic and pharmacological evidence strongly indicates that endogenously expressed LKB1 is phosphorylated at Ser431 by the p90 ribosomal S6 protein kinase (RSK) and the cyclic AMP-activated protein kinase (PKA), in

response to agonists which trigger the activation of these kinases [39,40]. Therefore, RSK and PKA may regulate cell growth through phosphorylation of LKB1. Phosphorylation of LKB1 at Thr366 is triggered only following exposure of cells to ionising radiation, and it is likely that the DNA damage-activated ataxia-telangiectasia mutated (ATM) kinase mediates this phosphorylation in vivo [41]. This latter observation provides the first evidence that LKB1 may be controlled by ATM and could thus play a role in mediating DNA damage responses in cells. The upstream kinases phosphorylating LKB1 at Ser31 and Ser325 have not yet been characterised.

Collins et al. [40] were the first to notice that mammalian LKB1 terminated in the amino acid sequence Cys-Lys-Gln-Gln, which lies in the consensus sequence for protein prenylation [42,43]. This motif is conserved in LKB1 homologues in *Xenopus*, *Drosophila* but not *C. elegans*. LKB1 expressed in 293 cells was shown to be prenylated by labelling with [^{14}C]mevalonic acid, and mutation of the Cys residue in the prenylation motif (Cys433) abolished prenylation [39,40]. Mass spectrometry analysis revealed that the form of prenylation on LKB1 was farnesylation rather than geranylgeranylation [39]. Recent genetic analysis in *Drosophila* indicated that both phosphorylation of the Ser431 residue by PKA and the prenylation of Cys433 are essential for LKB1 to regulate cell polarity [32]. However, the mechanism by which prenylation controls LKB1 is unclear, as the LKB1[C433A] mutant possessed normal in vitro catalytic activity, was able to suppress G361 cell growth [39] and displayed indistinguishable cellular localisation to wild type LKB1 [21]. Cys433 is located two residues away from Ser431, the site of RSK and PKA modification, but mutation of Ser431 to either Ala or Glu did not affect farnesylation of Cys433 and mutation of Cys433 to Ala also had no effect on phosphorylation of LKB1 at Ser431 in response to agonists that activate RSK and PKA [39].

6. LKB1 interacting proteins

In order to elucidate the mechanisms by which LKB1 functions in cells or is regulated in vivo, many groups have focussed on identifying proteins that interact with LKB1, and numerous proteins have been reported to bind this enzyme. The LKB1 interacting protein-1 (LIP1) was identified in a yeast two hybrid screen of a mouse embryonic cDNA library, using mouse LKB1 as the bait [44], and this interaction was also confirmed in mammalian cells using coimmunoprecipitation experiments. LIP1 is not a substrate of LKB1, but instead was proposed to anchor LKB1 to the cytoplasm [44]. LKB1 was also reported to interact with the Brahma-related gene-1 protein (Brg1), a member of the SWI1–SNF2 complex. Brg1 possesses an ATP-dependent helicase activity that is necessary for the chromatin remodelling function of the SWI1–SNF2 complex and is required for the transcription of many genes [45]. The N-terminal region of LKB1 (residues 1–146) mediated the binding to the helicase domain of Brg1. Although Brg1 was not phosphorylated by LKB1, the in vitro Brg1 ATPase activity was increased by binding to LKB1, and Brg1-induced cell cycle arrest was reported to require LKB1 [45]. LKB1 is highly expressed in apoptotic epithelial cells which express the tumour suppressor p53 and LKB1 was found to be coimmunoprecipitated with p53 from fibrosarcoma HT1080 cell lysates, suggesting that LKB1 physically associated with p53 or its associated proteins [46]. Furthermore, it was shown that overexpression of LKB1 in HT1080 cells induced apoptosis in a p53-dependent manner and that LKB1 kinase activity was required for this. In apoptotic cells, LKB1 was also suggested to translocate to mitochondria, and overexpression of a catalytically inactive mutant of LKB1 inhibited apoptosis induced by agents that disrupt microtubules but not by compounds that induce DNA damage. Based on these observations, it was suggested that LKB1 might function as a sensor for microtubule integrity [46]. Endogenous LKB1 immunoprecipitated from a variety of mammalian cell lines was also found to be complexed with heat shock protein 90 (Hsp90) and the Cdc37 kinase specific targetting subunit for Hsp90 [47]. As is the case for other protein kinases complexed to Cdc37 and Hsp90, such as the Raf and IKK protein kinases, the kinase domain of LKB1 mediated the interaction with this chaperone complex. Treatment of cells with Hsp90 inhibitors induced the degradation of cellular LKB1, which was prevented by proteasome inhibitors, suggesting that the binding of LKB1 to Cdc37 and Hsp90 is required for the stabilisation of LKB1 [47]. Hsp90 inhibitors are being considered as antitumour agents but the finding that these will destabilise LKB1 suggests that such compounds could also have the potential to induce tumours. Human and *Drosophila* LKB1 have also been found to interact with and phosphorylate the activator of G protein signalling-3 protein. It has been proposed that through this mechanism, LKB1 could modulate G protein coupled signalling pathways as well as potentially impinging on pathways that affect cell polarity [48].

Recently endogenous LKB1 was discovered to interact through its catalytic domain with a novel STE20-related protein termed STRADα [38]. STRADα possesses a STE20-like protein kinase domain, but lacks two key motifs in subdomains VIb and VII of its catalytic domain, indispensable for catalytic activity. This explains why STRADα is catalytically inactive and has therefore been classified as a pseudokinase. Interestingly, binding of STRADα to LKB1 enhanced LKB1 in vitro activity about five-fold [38]. Moreover, STRADα is directly phosphorylated by LKB1 both in vitro and in vivo. The LKB1 phosphorylation sites on STRADα have been mapped to Thr329 and Thr419 but the role that this phosphorylation plays in regulating LKB1 function is unknown [38]. STRADα, like LIP1, also functions to anchor LKB1 in the cytoplasm. Importantly, the SL26 LKB1 mutant was found to be incapable of binding to STRADα, which might account for the exclusive nuclear localisation and inability of this mutant to suppress cell growth [38]. The importance of binding of STRADα to LKB1 was also demonstrated by the finding that RNAi depletion of STRADα in G361 cells inhibited LKB1 from inducing a G1 cell cycle arrest [38].

7. Conclusions and perspective

Five years have now elapsed since LKB1 was originally identified. Although considerable progress has been made mainly from genetic analysis in humans, mice, and model organisms, indicating that LKB1 regulates cell proliferation and polarity, we still only have a limited understanding of the detailed molecular mechanism by which these effects are mediated. A major challenge for future research will be to identify the cellular substrates which LKB1 phosphorylates, and identify how phosphorylation of these provoke the effects

that LKB1 has on cell polarity and proliferation. This is likely to provide significant new insights into the intrinsic cellular mechanisms that control growth and polarity. LKB1 is unlikely to represent a drug target itself, as many tumours lack expression of LKB1, and moreover, drugs that inhibited LKB1 would be expected to induce tumours. However, if substrates of LKB1 could be identified which were enzymes, then drugs that mimicked the effect of LKB1 on these could potentially inhibit cell growth. Such compounds might not only be useful for the treatment of PJS but might also be employed for inhibiting the growth of other types of cancers. Further investigation is also required to understand how LKB1 is regulated by interaction with other proteins and by protein phosphorylation and prenylation. It should be noted that many of the sites of phosphorylation and prenylation are located in the C-terminal non-catalytic region of LKB1 (Fig. 2), and numerous mutations that affect only this region of LKB1 have been identified in tumours (Fig. 1). Thus, the C-terminal region of LKB1 is likely to play important roles, although these have yet to be defined. Apparently, a small but nevertheless significant number of inherited forms of PJS found in certain families do not exhibit mutations in the LKB1 gene [49,50], indicating that there could be other causative loci for PJS. Identifying these genes is of crucial importance as these are likely to lie in the same signalling network as LKB1. Although genetic analysis to date has excluded that LIP1 is mutated in these PJS patients [49], it would be very interesting to verify whether mutations in the genes encoding STRADα or any of the upstream kinases that phosphorylate LKB1 could result in PJS.

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