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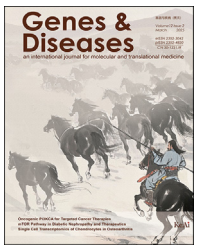

REVIEW ARTICLE

Insights into targeting LKB1 in tumorigenesis

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Abstract Genetic alterations to serine-threonine kinase 11 (*STK11*) have been implicated in Peutz-Jeghers syndrome and tumorigenesis. Further exploration of the context-specific roles of liver kinase B1 (LKB1; encoded by *STK11*) observed that it regulates AMP-activated protein kinase (AMPK) and AMPK-related kinases. Given that both migration and proliferation are enhanced with the loss of LKB1 activity combined with the prevalence of *STK11* genetic alterations in cancer biopsies, LKB1 was marked as a tumor suppressor. However, the role of LKB1 in tumorigenesis is paradoxical as LKB1 activates autophagy and reactive oxygen species scavenging while dampening anoikis, which contribute to cancer cell survival. Due to the pro-tumorigenic properties of LKB1, targeting LKB1 pathways is now relevant for cancer treatment. With the recent successes of targeting LKB1 signaling in research and clinical settings, and enhanced cytotoxicity of chemical compounds in LKB1-deficient tumors, there is now a need for LKB1 inhibitors. However, validating LKB1 inhibitors is challenging as LKB1 adaptor proteins, nucleocytoplasmic shuttling, and splice variants all manipulate LKB1 activity. Furthermore, STE-20-related kinase adaptor protein (STRAD) and mouse protein 25 dictate LKB1 cellular localization and kinase activity. For these reasons, prior to assessing the efficacy and potency of pharmacological candidates, the functional status of LKB1 needs to be defined. Therefore, to improve the understanding of LKB1 in physiology and oncology, this review highlights the role of LKB1 in tumorigenesis and addresses the therapeutic relevancy of LKB1 inhibitors.

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Introduction

Genetic screening patients diagnosed with Peutz-Jeghers syndrome (PJS) first linked serine-threonine kinase 11 (*STK11*), hereafter liver kinase B1 (*LKB1*) mutations to disease.[1] Since linking *LKB1* mutations to PJS, *LKB1* inactivation has induced tumor formation in numerous animal studies,[2] and sporadic *LKB1* mutations are frequently detected in solid tumors.[3,4] Given the overwhelming evidence that genetic inactivation of *LKB1* induces tumor growth[5–7] and overexpression decreases microvessel density,[8] tumor burden,[9] and cell proliferation,[10] *LKB1* is a widely accepted tumor suppressor. Contrary to these findings, some investigations conclude that LKB1 contributes to tumorigenesis through activating reactive oxygen species (ROS) scavenging, DNA repair machinery, and autophagy.[11–13]

The paradoxical role of LKB1 in tumor biology in conjunction with the pre-clinical success of pharmacological agents targeting LKB1 pathways[14–16] suggests a need for more precise modeling of LKB1 signaling in physiology and tumor biology. Generating LKB1-specific modulators is problematic because, in some contexts, agonists of LKB1 signaling are therapeutic,[17] whereas other reports have linked these agonists to aggressive tumor phenotypes.[18,19] Alternatively, due to the label of tumor suppressor, few have tried to synthesize LKB1 antagonists. Given that current strategies targeting LKB1 pathways are indirect, and few pharmacological agents become eligible for market approval,[20] developing LKB1-specific modulators with minimal cytotoxicity is of utmost importance. Therefore, this review addresses the biochemical processes dictating LKB1 activity in cell biology and pathology and the therapeutic relevancy of targeting LKB1 activity.

Linking *LKB1* mutations to disease

LKB1 mutations in familial PJS

PJS is an autosomal dominant disorder characterized by hyperpigmented macules, benign gastrointestinal hamartomatous polyps, and an increased risk of malignancy.[21–23] Hemminki et al in 1997 were the first to use comparative genomic hybridization and loss-of-heterozygosity analyses to localize a gene implicated in PJS. Comparative genomic hybridization assessed copy number variations in DNA extracted from 16 hamartomatous polyps while a loss-of-heterozygosity analysis using previously identified microsatellite markers of 19p (Dl9S886, D19S894, D19S413, and Dl9S565)[24] as a reference revealed that PJS patients had variable chromosome 19p sequence lengths.[25] Linkage analysis assessed the distances between the 19p microsatellite markers of 12 PJS families, which identified a susceptible locus between D19S886 and D19S883. Database searches and solution hybridization of complementary DNA (cDNA) identified 27 transcripts between D19S886 and D19S883, and *LKB1* was among them.[1] In some PJS patients, the *LKB1* PCR products were truncated suggesting genetic deletions.[25] Yet, for many PJS patients, the PCR products were not aberrant.[26,27] Instead, these patients contained point mutations or frameshift mutations that produced a stop codon prematurely, disrupted intron splice acceptor sites, or shifted the reading frame.[28,29] Only 1 of the 12 PJS families did not show *LKB1* sequence variation, which may have been due to incomplete sequencing, insensitivity of the mutation detecting methods, or large genomic deletions that were not detected by PCR.[1,30] Alternatively, unidentified mutations may induce PJS as an analysis of 34 PJS families detected *LKB1* germline mutations in only 70% of cases.[31] Indeed, linkage analysis linked chromosome 19q markers to a minority of PJS families,[32] but despite the identification of a possible second locus on chromosome 19q, *LKB1* remains the only gene linked to PJS.

LKB1 mutations in tumorigenesis

Although heterozygous germline mutations in *LKB1* are implicated in PJS, the two-hit hypothesis suggests that a second allele must be genetically altered to induce tumor formation,[33] which explains why PJS increases cancer frequency but does not guarantee malignancy.[34] Subsequent investigations revealed that LKB1 was the first kinase in which inactivating its kinase activity increased the risk of tumorigenesis.[27] Indeed, sequencing biopsies of intestinal polyps and pancreatic cancer confirmed that PJS patients are more susceptible to tumor formation as only the cancerous tissues contained additional somatic mutations to the remaining *LKB1* allele.[35] In addition to pancreatic cancer, *LKB1* somatic mutations, structural variants, amplifications, and deletions have been described in many cancer types including lung adenocarcinoma,[36] hepatocellular carcinoma,[37] head and neck squamous cell carcinoma[38] as well as cancers of the colon,[39] cervix,[4] and breast[40] (Fig. 1).[41]

Despite the incidence of *LKB1* mutations being relatively uncommon in most cancers,[42] genomic analyses routinely detect *LKB1* mutants in approximately 10%—30% of non-small cell lung cancer patients.[43] In fact, the only mutations in lung adenocarcinoma with a greater incidence than *LKB1* are mutations to Kristen rat sarcoma viral oncogene homolog (*KRAS*) and tumor protein p53 (*TP53*).[44] In many cases, genomic investigations found that *LKB1* mutations coincided and synergized with other mutations.[45] For instance, approximately half of $KRAS^{G12D}$ driven lung cancers inactivate *LKB1*.[46] In addition to *KRAS*, *LKB1* is often co-mutated with *TP53*[47] and *KEAP1*.[48] From a clinical standpoint, understanding the synergy of *LKB1* co-mutants may predict patient prognosis as the overall survival of patients with *LKB1*/*KRAS* co-mutant tumors is poor compared with other *LKB1* co-mutant tumors.[47] In fact, *LKB1* mutations are prognostic biomarkers that may influence treatment regimens as immune checkpoint blockade

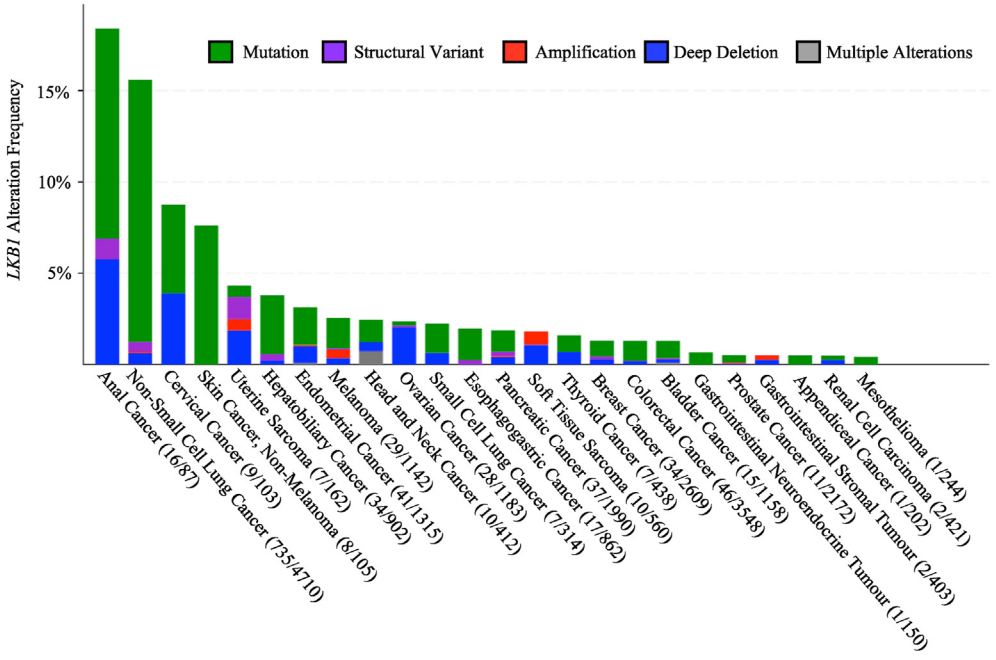

Figure 1 Prevalence of *LKB1* genetic alterations in human cancers. The alteration frequency including mutations, structural variants, amplifications, deep deletions, and multiple alterations of *LKB1* in tumors. Listed in descending prevalence are *LKB1* genetic alterations with the number of genetic alterations and total specimens tested in brackets. The data was obtained using cBioPortal for Cancer Genomics of the pan cancer study entitled MSK MetTropism (MSK, *Cell* 2021[41]).

inhibitors are less effective in *LKB1*/*KRAS* mutant tumors.[48] A potential explanation is that *LKB1* inactivation up-regulates DNA methyltransferases to silence stimulator of interferon genes, which is associated with poor immune responses and aggressive tumor phenotypes.[49]

AMPK and AMPK-related kinases (ARKs) dictate the role of LKB1 in tumorigenesis

Searching for LKB1 substrates responsible for its tumor suppressing functions revealed that 5′ adenosine monophosphate-activated protein kinase (AMPK) and AMPK-related kinases (ARKs) are phosphorylated by LKB1.[50,51] Indeed, most mammalian tissues rely on LKB1 to activate AMPK during oxidative and metabolic stress[52] to regulate a broad scope of cellular processes including cell division,[53] metabolism,[54] growth,[55] polarity,[56] energy expenditure,[57] transcription,[58] DNA repair,[59] and apoptosis.[60] As such, in many patient specimens and animal models, the loss of LKB1-AMPK signaling alters cell growth and repair mechanisms triggering cancer development.[61–63] Furthermore, AMPK agonists and pharmacological compounds that activate AMPK, such as cafestol and β-ionone disrupt tumor progression.[64,65] Alternatively, LKB1-AMPK signaling enhances tumor cell survival through NADPH-mediated ROS scavenging,[66] blocking tumor cell anoikis during basement membrane detachment,[67] and activating autophagy to sustain metabolism in tumor cells to mediate proliferation.[68]

In addition to AMPK, ARKs such as microtubule affinity-regulated kinases (MARKs), salt inducible kinases (SIKs), NUAK family kinases (NUAKs), and sucrose non-fermenting-related kinase (SNRK) have been linked to tumor suppressing or promoting functions of LKB1. For instance, gene expression signatures were similar between *LKB1*- and *SIK*-deficient lung tumors indicating that these proteins both regulate tumorigenesis through the same pathways.[69] In fact, KRAS-dependent non-small cell lung cancer mouse models revealed that the conditional knockout of *SIK1* and *SIK3* accelerated tumor growth.[70] LKB1-SIK signaling also maintains the activity of the oncogenic transcription factor MEF2C (myocyte-specific enhancer factor 2C) in acute myeloid leukemia linking LKB1-SIK activity to cancer.[71] Furthermore, LKB1-NUAK1 activity augments tumor invasion by promoting cell detachment and activating cytoskeletal motor proteins.[72] Elevated *NUAK1* expression is also associated with poor pancreatic ductal adenocarcinoma survival as inactivating NUAK1 reduces pancreatic cancer proliferation.[73] Alternatively, LKB1-MARK signaling suppresses high-grade ovarian cancer development by reducing angiogenesis and cell growth.[74] Finally, SNRK decreases β-catenin protein levels and activity in colon cancer, which decreases tumor cell proliferation.[75] Therefore, there is a duality to both LKB1-AMPK and LKB1-ARK signaling in tumorigenesis as different downstream pathways either augment or antagonize tumor progression.

Animal models linking LKB1 to disease

In vivo investigations that analyze LKB1-AMPK activity on cell polarity and migration rely on administering tamoxifen to tissue-specific Cre-Lox transgenic mouse models because germline knockouts of *LKB1*, *AMPK*, or their respective homologs are lethal in *Caenorhabditis elegans*[76], *Drosophila melanogaster*,[77] and murine models.[55] Tissue-specific Cre-Lox *LKB1* murine knockout models have identified many pathologies driven by mutational inactivation of *LKB1*

Table 1 Non-tumorigenic and tumorigenic Cre-Lox *LKB1* knockout mouse models.

	Experimental mouse model	*LKB1* knockout tissue	Phenotype	Reference
Non-tumorigenic	Kidney-specific-CadherinCre:LKB1$^{flox/flox}$	Kidney	Decreased expression of regulators of metabolism, kidney disease, dedifferentiated tubule epithelial cells, and dilated tubules	86
	Rosa26-Cre^{ER}:LKB1$^{flox/flox}$	All tissues	Decreased expression of regulators of metabolism, embryonic lethality, reduced body weight, hyperglycemia, glucose intolerant	57
	Myosin heavy chain αCre:LKB1$^{flox/flox}$	Heart	Increased expression of collagen I/III, spontaneous atrial fibrillation, atrial remodelling, left ventricular hypertrophy, fibrosis, and death within 6 months of birth	82
	Muscle creatine kinaseCre:LKB1$^{flox/flox}$	Skeletal and cardiac muscle	Myopathy, atrial dilation, decreased body weight, decreased fast twitch skeletal muscle, skeletal muscle atrophy, and loss of hindlimb function	85
	Leucine-rich repeat-containing G-protein coupled receptor 5GFP-Cre/+:LKB1$^{flox/flox}$:R26$^{Td/+}$	Intestinal stem cells	Restricted intestinal stem cells differentiation to secretory lineages	90
	Pro-opiomelanocortin-Cre:LKB1$^{flox/flox}$	Pituitary gland	Altered expression of metabolic genes in the liver, diabetes, insulin resistance, and glucose intolerance.	84
	Pancreatic and duodenal homeobox 1-Cre^{ERtam}:LKB1$^{flox/flox}$	Pancreas	Glucose tolerance and resistance to hyperglycemia	91
	Transthyretin-CRE^{ERT2}:LKB1$^{flox/flox}$	Liver	Increased liver regeneration and hepatocyte proliferation	55
	Tyrosine kinase with immunoglobulin-like and epidermal growth factor-like domains 1-CRE:LKB1$^{flox/flox}$	Endothelium	Embryonic lethal and vascular defects	92
	Empty spiracles homeobox1-Cre:LKB1$^{flox/flox}$	Cortical neurons	Disrupted axon initiation, axon specification, neuronal proliferation, and neural development	58
	MX dynamin-like GTPase 1-Cre:LKB1$^{flox/flox}$	Haematopoietic stem cells	Depletion of Haematopoietic stem cells	93
Tumorigenic	Keratin 14-Cre:LKB1$^{flox/flox}$	Epidermis	Spontaneous squamous cell carcinoma	94
	Small proline rich protein 2F-Cre:LKB1$^{flox/flox}$	Endometrial	Death and diffuse malignant endometrial cancers sensitive to mTOR inhibitors	89
	Cluster of differentiation 19-Cre:LKB1$^{flox/flox}$	B-lymphocytes	Death and B-cell lymphoma	95
	AhER-Cre:LKB1$^{flox/flox}$:PTEN$^{flox/flox}$	Epithelium and Urothelium	Bladder cancer and signs of hypoxia, EMT, and increased proliferation in the urothelium	96
	W-Myc:W-Cre:LKB1$^{flox/flox}$	Epithelium and Urothelium	Loss of mammary gland epithelial integrity; LKB1 deletion in combination with oncogenic C-Myc induced mammary tumorigenesis	97
	Ah-Cre:LKB1$^{flox/flox}$	Prostate	Prostate hyperplasia, prostate intraepithelial neoplasia, bulbourethral gland cysts, and hyperplasia of the urethra	98

Table 1 (*continued*)

	Experimental mouse model	*LKB1* knockout tissue	Phenotype	Reference
	Pancreatic and duodenal homeobox 1-Cre:$Kras^{G12D}$:$LKB1^{flox/flox}$	Pancreas	Pancreatic ductal adenocarcinoma	99
	$Kras^{LSL-G12D/+}$: $LKB1^{flox/flox}$	Lung	Adenocarcinoma to squamous cell carcinoma transdifferentiation. Resistant to $KRAS^{G12C}$ inhibitors	100
	$LKB1^{flox/flox}$/PTEN $^{flox/flox}$	Lung	Squamous cell carcinoma	101

including cardiac defects,[78] metabolic disorders,[79] cachexia,[80] kidney disease,[81] hormonal imbalances,[82] and tumorigenesis[2,83–85] (Table 1).

Non-tumorigenic *LKB1* knockout models have historically been utilized to assess the role of LKB1 on organ/tissue development. For instance, deleting *LKB1* in somatic testicular cells produced irregular seminiferous tubules lined with only Sertoli cells, which lacked polarity, junctional complexes, and focal adhesion kinase activity.[86] In the developing brain, LKB1 regulates neural cell migration and the development of the cerebral cortex. *LKB1* inactivation in green fluorescent protein-labeled neurons within embryonic mice increased migration to the intermediate zone but decreased migration to the middle and upper cortical plate of the cerebral cortex.[87] Generating hindbrain *LKB1* knockout mice by crossing floxed *LKB1* mice with *Atoh1*-cre mice revealed that *LKB1* deletion decreased migration of cerebellar granule cells leading to structural abnormalities of the cerebellum.[88] Mechanistically, LKB1 activates glycogen synthase kinase 3β at the leading edge of migrating neurons, and as a result, adenomatous polyposis coli stabilizes microtubules at the leading edge, which promotes centrosome forward movement.[89] *AMPKB1* null mice developed using *AMPKB1* gene trap embryonic stem cells have demonstrated that AMPK is essential for mammalian brain development.[90] However, another group utilized *AMPKA*-null cortical neurons and found that neural polarization may not require AMPK.[91] Regardless, due to the overwhelming evidence suggesting the contrary, LKB1-AMPK signaling is essential to neuronal cell polarity, migration, and embryo development.

Given the function of LKB1-AMPK as an energy sensor, *LKB1* knockout mouse models have linked LKB1 inactivation to metabolic diseases. Indeed, female mice with POMC neuron-specific *LKB1* deletion developed phenotypes resembling type 2 diabetes including insulin resistance and glucose intolerance.[92] Mass spectrometry analysis of newly synthesized proteins in mice with $LKB1^{-/-}$ liver revealed that phenotypes resembling type 2 diabetes correlate with increased expression of proteins implicated in fatty acid synthesis and fatty liver.[93] Likewise, mice with liver-specific *LKB1* deletion developed hyperglycemia post Cre injection and PCR revealed that *LKB1* loss increased the expression of genes that regulate gluconeogenesis.[94] LKB1-AMPK signaling is also important for hormonal stimulation of glucose uptake as *LKB1* knockdown can suppress testosterone-dependent AMPK activation and glucose transport in adipocytes.[95] In addition to pathology, muscular dysfunction is observed in the absence of LKB1 activity. Skeletal muscle-specific $LKB1^{-/-}$ mice demonstrated reduced AMPK activation and glucose transport as well as elevated AMP:ATP ratios following contraction.[96]

LKB1-AMPK activity down-regulates genes important for lipogenesis and cholesterol synthesis including fatty acid synthase, acetyl-CoA carboxylase, and sterol regulatory element—binding protein 1.[94] Indeed, up-regulating AMPK activity decreased atherosclerosis and hepatic steatosis in diet-induced insulin-resistant mice by antagonizing sterol regulatory element—binding proteins.[97] Given that lipogenic enzymes induce ferroptosis, an iron-dependent non-apoptotic cell death, and lipid hydroperoxide accumulation, LKB1-AMPK protects cells from metabolic damage.[98] The LKB1-AMPK pathway is essential to survival as viability was significantly reduced in *LKB1*-deficient zebrafish larvae during nutrient deprivation due to autophagy ablation.[99]

There have been numerous animal models linking *LKB1* inactivation to spontaneous tumor formation. For example, mice with pancreatic-specific *LKB1* inactivation developed serous cystadenomas with evidence that their pancreatic acinar cells had impaired integrity, loss of basal nuclear positioning, and absence of cell—cell junctions.[100] Impairing the LKB1-AMPK-mTORC1 (mechanistic target of rapamycin complex 1) pathway through the conditional knockout of *LKB1* in an osteogenic mouse model induced tumor formation and bone formation and increased osteoblast differentiation and cell invasion into medullary cavities of bones.[101] In fact, mouse models have linked LKB1 inactivation to cancers of the liver,[102] bladder,[103] prostate,[104] *etc*. In support of these findings, activating the LKB1-AMPK axis attenuates epithelial—mesenchymal transition and/or metastasis in renal cell carcinoma,[105] hepatocellular carcinoma,[106] and cancer of the colon,[107] breast,[108,109] and lung.[110]

As previously discussed, LKB1/KRAS mutant lung cancer mouse models demonstrated that LKB1/KRAS-deficient tumors are more likely to develop metastases compared with other KRAS-linked mutations. Co-mutations to LKB1 and KRAS often result in mixed tumor histology linking LKB1 to cancer plasticity such as adeno-to-squamous cell-to-large cell carcinoma transdifferentiation.[111] In LKB1/KRAS mice, the adeno-to-squamous cell carcinoma transdifferentiation is mediated by the loss of lysyl oxidase, which up-regulates p63, an oncogene linked to squamous cell survival.[112] The clinical significance of LKB1 loss in cancer plasticity is that $KRAS^{G12D}$ and *STK11* co-mutations have a squamous cell carcinoma gene signature linked to resistance against KRAS inhibitors.[113] The role of LKB1 in lung cancer

transdifferentiation is not limited to KRAS as LKB1 and PTEN co-mutations in adenocarcinoma acquire squamous cell carcinoma properties.[114] As such, further investigations into the role of LKB1 in cancer plasticity will expand the current understanding of LKB1 in tumorigenesis.

Although many animal models provide evidence for the tumor suppressing functions of LKB1, there is now evidence for the contrary. Using ovarian cancer models, LKB1-AMPK activation increased tumor metastasis whereas siRNA knockdown of *AMPK* impaired peritoneal dissemination and metastasis.[115] In fact, female NOD/SCID mice injected with *LKB1* knockout epithelial ovarian cancer cells had reduced tumor burden and metastasis through an AMPK-independent mechanism.[116] Given these conflicting reports, more work is needed to characterize the role of LKB1 in the presence or absence of AMPK signaling. This work should aim to uncover why LKB1 may increase ovarian carcinoma invasion while dampening the invasion of other cancers.

Linking LKB1 expression to disease

LKB1 genetic structure

Jenne et al in 1998 sequenced the PJS gene susceptibility region using D19S886 microsatellite markers and chromosome 19-specific cosmid libraries in Genbank as a reference.[117,118] Restriction digests and southern-blot analysis first mapped the D19S886 locus, which was followed by several rounds of cosmid walking (primer generation, PCR, and sequencing) revealing that human *LKB1* was approximately 190 kbp from D19S886. The exon-intron structure of *LKB1* was subsequently sequenced using the reverse transcribed 1302 bp cDNA sequence of *LKB1*[119] as a template and primer for both PCR and sequencing. *LKB1* is comprised of 10 exons, spanning approximately 23 kbp on chromosome 19p13.3. However, only nine coding exons (exons 1—9) are transcribed in a telomere to centromere direction to produce mature LKB1 (Fig. 2A).[28] Both human and mouse LKB1 contain an N-terminal PRRKRA (aa 38—43) motif similar to a single basic type nuclear localization signal[120] and mutating this sequence resulted in the cytoplasmic accumulation of LKB1.[121] Other than the serine-threonine kinase motif (aa 44—309) that is poorly related to other protein kinases, there are no other functional domains on flanking N-terminal or C-terminal regions[29] (Fig. 2B).

LKB1 methylation promotes tumorigenesis

Hypermethylation of the *LKB1* promoter (Fig. 2A) is linked to tumorigenesis,[122] environmental health hazards, such as smoking,[123] advanced stages of the tumor-node-metastasis staging, and shorter patient survival times according to Kaplan—Meier survival analyses.[124] Methylation-specific PCR has detected *LKB1* methylation in PJS[125] as well as renal cell carcinoma,[124] melanoma,[126] and colon cancer.[122] Interestingly, LKB1 controls the expression of numerous genes through methylation as assessing an Illumina 450K microarray of lung adenocarcinoma uploaded to The Cancer Genome Atlas (TCGA) found that LKB1 loss-of-function decreased β-values representing a global reduction of CpG methylation. Indeed, methylation of 33.7% of CpG sites was decreased in *LKB1*-deficient tumors. In fact, *DNMT1* (DNA methyltransferase 1) expression is reduced by *LKB1* loss.[127]

MicroRNAs targeting LKB1 are linked to tumorigenesis

The TCGA has been utilized to identify microRNAs (miRs) including miR-17,[128] −30b,[129] −34a,[130] −93,[129] −100,[131] −106a-5p,[132] −144,[133] −195,[134] and −451[135] (Fig. 2A) that antagonize *LKB1* expression. For instance, there is an inverse relationship between *LKB1* and *miR-100* expression in head and neck cancer samples[131] and *LKB1* and *miR-106a-5p* in lung adenocarcinoma.[132] miR targeting software (TargetScan, miRBD, miRTArbase, and miRWalk) have assessed sequence alignment between *miR-17* and *LKB1* and a retrospective analysis confirmed an inverse relationship between *miR-17* and *LKB1* expression, which was verified using TCGA analyses.[128] Nanostring nCounter technology generated a miR prediction model by quantifying miR counts using digital readouts of miR fluorescent probes that hybridize to *LKB1*. These techniques discovered that both miR-93 and miR-30b down-regulate *LKB1*.[129] Given the evidence that increasing the expression of *LKB1*-targeting miRs contributes to tumorigenesis by down-regulating *LKB1*, targeting miRs may be a therapeutic option for tumors with low LKB1 activity that retain wild-type *LKB1* alleles. However, there is evidence that miR-17∼92 targeting of LKB1 increases tumor cytotoxicity of biguanide treatments suggesting that miR targeting of LKB1 may be beneficial in cancers reliant on LKB1 activity.[136]

LKB1 mutations linked to PJS and tumorigenesis

While genomic analyses discovered the genetic structure of *LKB1* and contributions of *LKB1* mutations to pathology, computational algorithms in combination with constitutively updated genomic/proteomic databanks have further realized the importance of LKB1 in disease.[137] Computational assessments of *LKB1* have accurately predicted disease loci and detected low-frequency mutations absent in sequenced patient cohorts,[138] and in conjunction with online databases, are routinely utilized as a reference to approximate *LKB1* gene/protein structure,[139] regulatory elements,[140] and protein function.[141]

Initial applications of sequenced *LKB1* discerned specific genomic alterations that resulted in PJS phenotypes.[142] Mutant *LKB1* contained deletions within introns 3, 5, and 7, deletions within coding exons 4 and 5, and inversion of exons 6 and 7. Compared with wild-type alleles, genomic deletions and rearrangements of *LKB1* sequences identified in PJS patients produced truncated polypeptides, which were later revealed to lack significant proportions of the kinase domain.[28] More specifically, LKB1 enzymatic activity is disrupted in D176N mutants suggesting D176 is in the catalytic core where its negative charge may function to stabilize nearby residues and the phosphate group during transfer.[143] LKB1 folding is most likely disrupted in LMGD (residues 50—53) deletion mutants because protein folding may generally accommodate deletions of surface residues[144] or whole domains[145] whereas LMGD deletions occur at the beginning of an internalized β-sheet. Aberrant LKB1

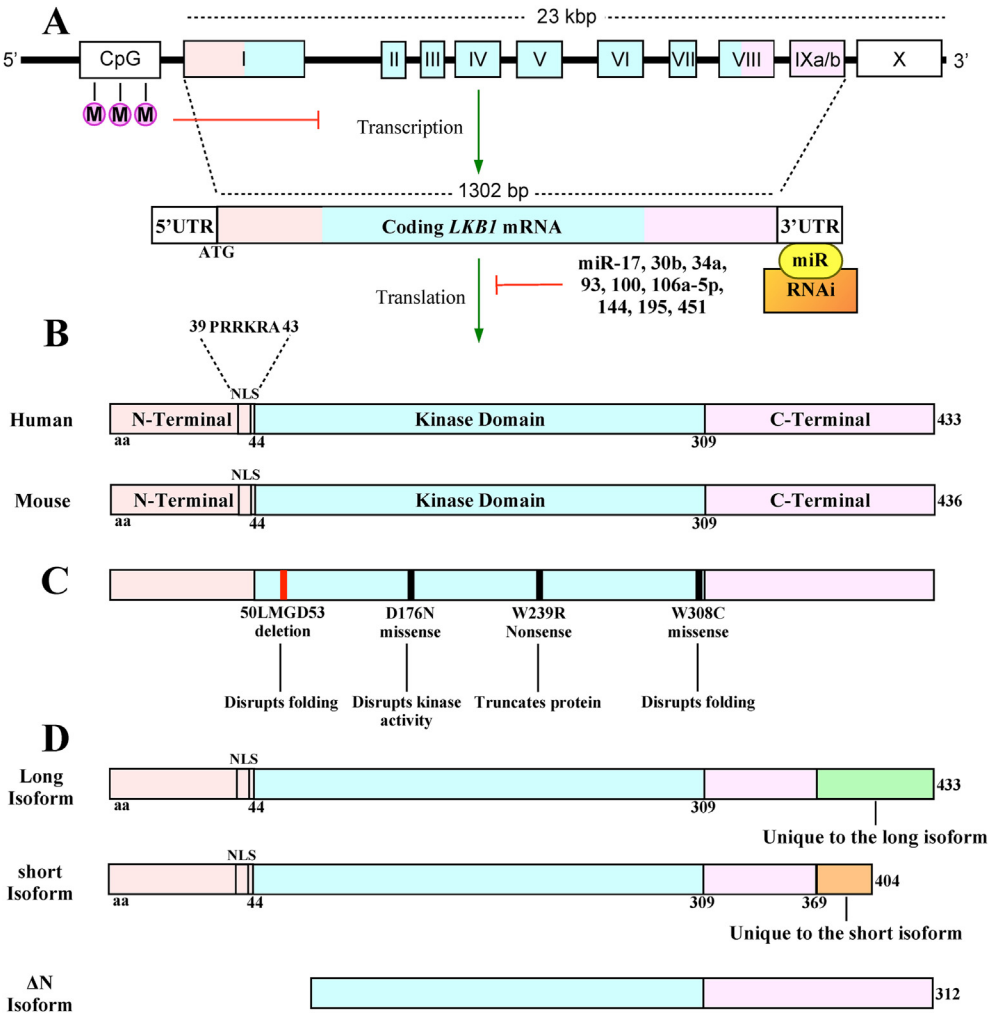

Figure 2 The regulation of *LKB1* DNA and mRNA. **(A)** The *LKB1* gene is 23 kbp containing 10 exons (I—X). Only exons I—IX are translated, exon I contains an ATG start codon, and exon IX has two termination signals making two alternative exon variants denoted as IXa and IXb. The *LKB1* mRNA is 1302 bp in length flanked by a 5′ and 3′ untranslated region (UTR) produced by part of exon I and the entirety of exon X, respectively. The methylation of CpG islands near the *LKB1* promoter suppresses *LKB1* transcription whereas miR-17, 30b, 34a, 93, 100, 106a-5p, 144, 195, or 451 binding to the 3′ UTR recruits RNA-induced silencing complex (RNAi) to cleave *LKB1* mRNA prior to translation. **(B)** Human and mouse LKB1 proteins are 433 and 436 amino acids (aa) long, respectively. The N-terminal domain (aa 1—43; beige) contains a nuclear localization signal (NLS) consisting of PRRKRA residues located between aa 39—43. The kinase domain (aa 44—309; aqua) is between the N-terminus and C-terminus (aa 309—; pink). The beige, aqua, and pink in the gene and mRNA correspond to the protein region. **(C)** Mutations identified in Peutz-Jeghers syndrome patients that disrupt LKB1 activity. 50 aa-LMGD-53 aa deletions disrupt protein folding, D176N missense mutation disrupts kinase activity, W239R nonsense mutation truncates the protein, and W308C missense mutation disrupts folding. Adapted from Mehenni et al, 1998.[135] **(D)** LKB1 is spliced to produce a long 433 amino acid (aa) or short 404 aa isoform. Although both long and short LKB1 isoform have an equivalent N-terminus domain, NLS, and kinase domain, they both have unique C-terminal regions (green and orange). The ΔN isoform (312 aa) is missing the N terminus and part of the kinase domain.

folding is observed in PJS patients containing the W308C missense mutation likely through mutant C308 forming a disulfide bridge with the proximal C158.[143] Finally, W239R and W308C destabilized LKB1, decreased ATP binding capacity, and disrupted kinase activity[137] by producing truncated proteins[138] (Fig. 2C).

LKB1 splicing

An RNase protection analysis has demonstrated that all mouse tissues express *LKB1* mRNA[146] whereas northern blots of human mRNA probed with an [α-^{32}P] dCTP-labelled *LKB1* cDNA fragments have revealed that *LKB1* is ubiquitously expressed in fetal and adult tissues.[28] Despite its ubiquitous expression, *LKB1* is alternatively spliced in different tissues, which may contribute to its function and, in some contexts, its pathology.[147] After designing antibodies specific to N-terminal epitopes of LKB1, co-immunoprecipitation and immunoblotting revealed two protein bands in some tissues.[148] As a result of variations in exon 9 splicing, several tissues produce both 50 kDa and 48 kDa long and short LKB1 isoforms, respectively.[147] Both isoforms

are widely expressed in humans, but most tissues preferentially express the 433 amino acid long isoform whereas the 404 amino acid short isoform is predominantly expressed in the testis.[149,150] Compared with the LKB1 long isoform, the short isoform lacks 63 C-terminal residues, which are replaced by a 39-residue sequence[148] (Fig. 2D). When *in vitro* kinase assays assessed LKB1 long and short isoform activity, there were no observable differences in LKB1-dependent kinase activation. Albeit, differences are observed in *in vivo* models.[148,149,151] Male mice lacking the short LKB1 isoform displayed irregular spermatogenesis and were sterile whereas female mice lacking the short LKB1 isoform were fertile.[148,151] Functional differences between LKB1 splice variants are not specific to mammals as *Drosophila melanogaster* expresses two different *LKB1* mRNAs where only the mRNA with a longer 5′ untranslated region is essential to spermatid morphogenesis.[149]

In addition to the LKB1 long and short splice isoforms and splice mutants implicated in PJS,[35,143] alternative splicing of exon 1 is observed in a human lung cancer cell line, NCI-H460. NCI-H460 cells express a 42 kDa LKB1 isoform (ΔN-LKB1) 312 amino acids in length missing the N-terminal nuclear localization signal and part of the kinase domain (Fig. 2D).[12] Although the kinase domain is incomplete, ΔN-LKB1 is restricted to the cytoplasm, increases the activity of downstream kinases, and functions in opposition to tumor suppressing wild-type LKB1. As such, inhibiting ΔN-LKB1 decreases the survival of NCI-H460 cells engrafted in nude mice.[12] In addition to ΔN-LKB1, next generation sequencing technology has detected nonsense, frameshift, and splice mutations in exon 1 of *LKB1* across a broad scope of tumors.[152] Given that deleting the splicing regulator *Rbm10* in *LKB1*-deficient mice had a moderate tumor suppressing effect,[153] *LKB1* splicing may prove to be a suitable target to antagonize tumor cell survival.[154]

Considerations prior to targeting LKB1 activity

Variations in LKB1 splicing

A decade passed between characterizing *LKB1* splice mutants in PJS patients and identifying physiological *LKB1* splice variants[1,148] and yet, despite success in detecting LKB1 splice sites,[155] challenges detecting splice variants using genomic studies and computational algorithms remain.[156] This is due to the numerous processes that regulate *LKB1* splicing. Indeed, mutations, cell and tissue types, species, and the species sex impact *LKB1* splicing and thus splicing is specific to each experimental model. Caution must be warranted when selecting an experimental model because the impact of alternative *LKB1* splice variants, if left undetected or omitted, may have drastic consequences on cell physiology and tumor pathology.[139] Furthermore, early investigations raised LKB1 antibodies against the C-terminus,[157] but based on the current understanding of LKB1 splice mutants, this is problematic as these antibodies only anneal to the long LKB1 isoform. Therefore, many historical investigations of LKB1 biology missed splicing variants, which may have drastically changed the outcomes regarding the role of LKB1 in physiology and disease.

The impact of STRAD and MO25 on LKB1 activity

LKB1 catalytic activity depends on the formation of a 1:1:1 heterotrimeric complex *in vivo*, with the pseudokinase STRAD and mouse protein 25 (MO25) where mutations in all these proteins have proven to disrupt LKB1-STRAD-MO25 activity.[158] In addition to mutations, miR-195 and miR-451 disrupt LKB1 signaling by targeting MO25.[134] Therefore, steady-state levels of MO25 regulate the functional status of LKB1 and is thus important to LKB1 biology and pathology. Alternatively, there have been numerous investigations focused on linking STRAD to pathology. Given that *LKB1* is directly linked to the majority of PJS cases, it was hypothesized that *STRAD* may be a second genetic locus implicated in the remaining PJS cases.[159] Thus, a loss-of-heterozygosity analysis screened for the presence of *STRAD* genetic deletions in PJS patients. In some cases, loss of heterozygosity was identified in a molecular marker near *STRAD*, yet sequencing discerned that all exons were intact suggesting STRAD is not linked to PJS.[160] However, after isolating several STRADα splice variants in colorectal cancer cell lines, the interest of *STRAD* splice variants in tumorigenesis is renewed. In fact, measuring the activity of purified LKB1 complexes using LKB1tide kinase assays revealed that each STRADα splice variant differentially impact LKB1 localization, kinase activity, and downstream substrate activation.[161] Thus, altering *STRADA* splicing may fine tune LKB1 signaling in tumorigenesis and serve as a potential therapeutic target.

Disrupting LKB1 activity may be achieved through several mechanisms including blocking LKB1 kinase activity, nucleocytoplasmic shuttling, and STRAD binding. Since STRAD binding is essential to LKB1 localization and protein stability *in vivo*,[162] STRAD allosteric inhibitors are in development to disrupt LKB1 signaling.[163] In fact, due to LKB1-independent STRAD activities, blocking STRAD instead of LKB1 may be more favorable. For instance, in LKB1-deficient lung cancer cells, STRAD facilitates cell invasion.[164] Therefore, blocking LKB1 activity may increase LKB1-independent STRAD signaling which may facilitate invasion.

The targeted delivery of LKB1 inhibitors

Although there is therapeutic merit in targeting LKB1 in cancer, there are some drawbacks delaying LKB1 inhibitor synthesis. First, LKB1 has historically been considered a tumor suppressor which could make targeting LKB1 in cancer treatment undesirable.[165] Another limitation is the targeted delivery of LKB1 inhibitors as there is risk of spontaneous tumor formation in non-cancerous tissues relying on LKB1.[166] For this reason, the ideal LKB1 inhibitor should be a small molecule signal transduction inhibitor capable of transversing across plasma and nuclear membranes specifically targeting LKB1 in tumor cells. Targeted therapies may be classified as "active" or "passive". Active targeting involves inhibitors designed to target specific markers up-regulated in diseased cells whereas passive targeting combines inhibitors with nanoparticles to enhance inhibitor accumulation at the target.[167] Since LKB1 is ubiquitously expressed in both non-cancerous and cancerous cells, LKB1 inhibitors would rely on passive

targeting. As such, prior to generating LKB1 inhibitors, more research is needed to limit off-target effects, which is a critical component for feasible clinical application. One option when designing LKB1 inhibitors is to utilize liposome nanocarriers to improve the delivery of LKB1 inhibitors to tumors. Mechanistically, liposomes are designed to utilize properties of the tumor microenvironment, such as the leaky tumor vasculature, that enhance permeability and retention in tumor microenvironments.[168] Other advantages of utilizing liposomes as the drug delivery system include the interaction between liposome and cell plasma membranes allowing direct release of inhibitors in target cells. Furthermore, the risk of patient adverse reactions is minimal due to the non-immunogenic, non-toxic, and biodegradable nature of liposomes.[169] Due to advancements in drug delivery systems to enhance targeting specificity, there are many prospective delivery methodologies that mitigate risks associated with LKB1 inactivation in non-cancerous tissues.

Targeting LKB1 activity

Pharmacological agents targeting the LKB1-AMPK pathway

As previously discussed, the tumor suppressing functions of LKB1 are derived from screening tumor biopsies and conditional *in vivo* knockout models. These investigations demonstrate that there are numerous neoplasms with mutations in the LKB1-AMPK signaling pathway[46] and these mutations are sufficient for spontaneous tumor formation in translational animal models.[170–172] As such, pharmaceutically activating LKB1-AMPK signaling using AMPK agonists, such as metformin and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), are under investigation as a potential therapeutic strategy for tumorigenesis. Unlike metformin and AICAR, A-769662, a small molecule AMPK agonist,[173] increased tumor cell proliferation under low nutrient conditions.[174] As such, despite extensive evidence linking LKB1 and AMPK to tumor suppressing processes, investigations implicating these proteins in tumor cell survival and metastasis are growing. Indeed, most screens of tumor biopsies report a shorter median overall survival for patients with *LKB1* mutant tumors citing increased disease progression, reduced time to treatment failure, and increased treatment failure frequency.[4,152,175–179]

Metformin activates AMPK through two mechanisms. The first relies on increasing AMP levels responsible for both activating and sustaining AMPK phosphorylation while the second involves promoting S428 LKB1 phosphorylation, which initiates LKB1 nuclear export and AMPK binding.[180,181] The therapeutic potential of metformin is highlighted by its success in treating type 2 diabetes and reducing cancer mortality in type 2 diabetic patients by 57%.[182] In addition to lowering cancer mortality in diabetic patients, meta-analyses of metformin clinical trials indicate that overall survival for a variety of cancers is improved.[183–185] Similar to metformin, AICAR disrupts tumor migration, induces apoptosis,[15] and has decreased lymphadenopathy in chronic lymphocyte leukemia clinical trials.[186] The anti-tumorigenic properties of metformin are attributed to *in vitro* investigations finding both fewer tumor cells and cells lacking the ability to migrate in the presence of metformin.[187,188] Alternatively, AICAR is metabolized to an AMP mimetic that binds to AMPKγ, which ultimately activates AMPK and disrupts mTOR.[16] LKB1-AMPK activation has also suppressed tumor growth and delayed tumor onset in *PTEN*-deficient mice. A possible explanation is that during *PTEN* deficiency, the mTOR kinase is hyperactivated to augment tumorigenesis and facilitate therapeutic resistance. Therefore, disrupting mTOR by up-regulating LKB1-AMPK signaling is an emerging tumor therapeutic strategy.[189]

Although clinical success has justified activating LKB1-AMPK signaling, there are several limitations regarding the use of metformin and AICAR as cancer therapeutics. First, due to poor oral bioavailability[190] and renal toxicity,[191] the therapeutic potential of AICAR is limited. Another limitation is the ambiguity of their mechanisms of action. Indeed, it is difficult to differentiate the AMPK-dependent and -independent effects as well as the role of LKB1.[192] In fact, there is evidence suggesting that AICAR activates AMPK through LKB1-independent mechanisms.[193] Therefore, the extent that the therapeutic properties may be attributed to LKB1-dependent AMPK activation is unknown. What is known is the therapeutic benefit of activating LKB1-AMPK has been replicated using agents that indirectly target the LKB1-AMPK axis. For instance, tankyrase inhibitors reduced mice lung tumor burden through increasing LKB1-AMPK activity as tankyrase1/2-dependent polyubiquitination of LKB1 disrupts LKB1-STRAD-MO25 complex formation (Fig. 3).[84]

The tumor promoting properties of LKB1

Despite the success of LKB1-AMPK agonists, *LKB1* inactivation also increase the median overall survival for some tumor types.[194–203] Given that LKB1 activity up-regulates stress response pathways and ROS scavenging in tumor cells, LKB1 deficient tumors are more susceptible to metabolic stressors. Mechanistically, LKB1 inactivation increases ROS levels making tumor cells more susceptible to ROS-mediated cytotoxicity. In fact, cell lines lacking LKB1 are more susceptible to oxidative stress-inducing therapies such as cisplatin and γ-irradiation.[204] Furthermore, LKB1-deficient NSCLC cell lines were more sensitive to tunicamycin and other endoplasmic reticulum stress activators.[205] Patient derived xenografts generated from LKB1-deficient lung cancer patients displayed increased tumor necrosis with an impaired ability to adapt to metabolic stress mediated by the anti-angiogenic vascular endothelial growth factor (VEGF) inhibitor bevacizumab.[206] In LKB1-deficient cells, chemical inhibitors that mediate metabolic stress including erlotinib and metformin have enhanced selectivity and cytotoxicity.[207–209] The efficacy of poly(-ADP-ribose) polymerase (PARP) inhibitors,[210] extracellular signal-regulated kinase (ERK) inhibitors,[211] and biguanide treatments[136] are also improved when LKB1 expression is down-regulated. For these reasons, screening patients for LKB1 deficiency may dictate treatment combinations to

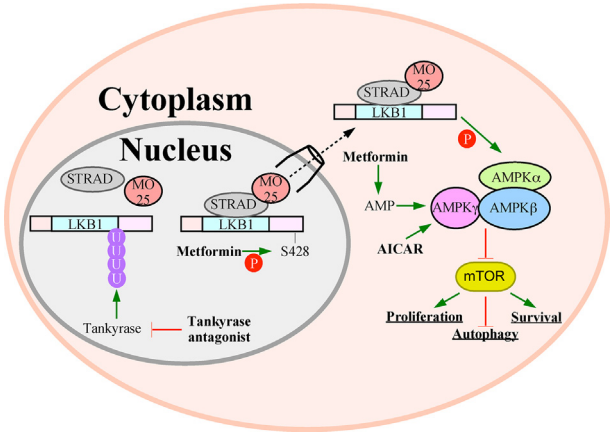

Figure 3 Tumor suppressing pathways of LKB1. The LKB1-AMPK-mTOR pathway suppresses tumor development. LKB1-AMPK disrupt mTOR activity leading to decreased survival and proliferation while increasing autophagy. Pharmacological compounds that activate the LKB1-AMPK axis and are proven to suppress tumorigenesis include a tankyrase antagonist, metformin, and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR). Tankyrase antagonists enhance LKB1-STRAD-MO25 complex forming by blocking tankyrase-dependent ubiquitination (U) of LKB1. Metformin increases S428 LKB1 phosphorylation and AMP levels both promoting AMPK activation. AICAR also functions as an AMP mimetic to increase AMPK activity.

improve patient survival while inactivating LKB1 in tumors enhances the efficacy of other cytotoxic compounds. Therefore, investigations should explore synthesizing novel LKB1 inhibitors as anti-tumorigenic agents.

A possible explanation of the pro-tumorigenic properties of the LKB1-AMPK axis involves mediating resistance to death via basement membrane detachment. AMPK protects tumor cells from anoikis through suppression of protein synthesis via mTOR inhibition.[67] For instance, *in vitro* spheroid models of ovarian cancer and breast cancer demonstrated that *LKB1* expression is essential for tumor cell growth in suspension. Both CRISPR/Cas9-dependent *LKB1* knockout in ovarian cancer cells and *LKB1*-specific siRNA silencing in breast cancer cells decreased tumor burden and metastatic potential.[116,212] However, the LKB1 effects in the ovarian cancer model were due to AMPK-independent mechanisms.[116] Another potential explanation for pro-tumorigenic LKB1-AMPK signaling is disrupting NADPH consuming processes such as fatty acid synthesis while promoting NADPH producing processing like fatty acid oxidation.[66] Since NADPH is essential to ROS scavenging, maintaining NADPH levels prolongs tumor cell survival in response to oxidative and metabolic stressors.[213]

Protection against anoikis and ROS represent significant physiological benefits and justifies why some tumors increase the LKB1-AMPK pathway. Indeed, hepatocellular carcinoma adapts to energy stress by activating LKB1 through skp2-dependent K63 polyubiquitination.[13] In addition to anoikis resistance and ROS scavenging, LKB1-AMPK

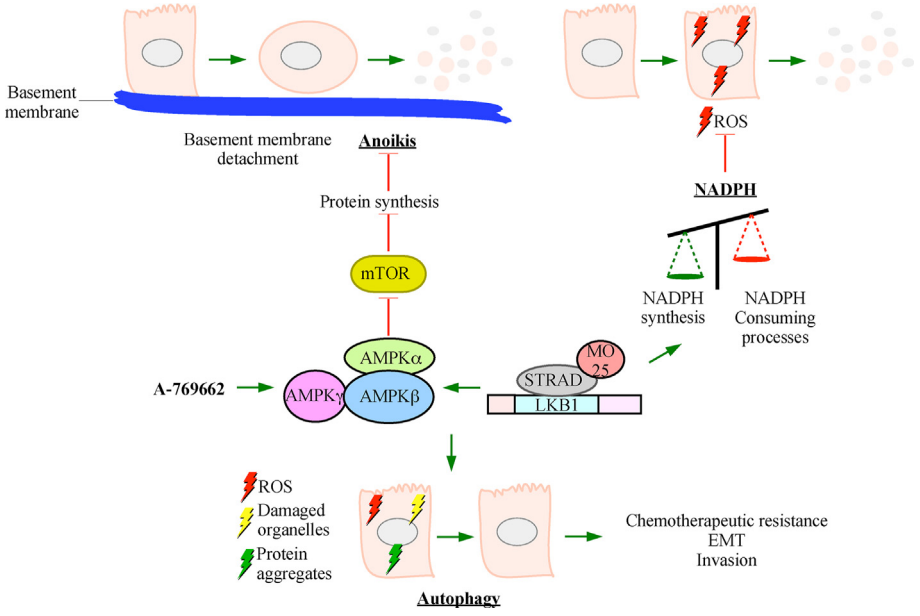

Figure 4 Tumor promoting roles of LKB1. LKB1-STRAD-MO25 activity can promote tumorigenesis by increasing reactive oxygen species (ROS) scavenging NADPH. Given that ROS damage tumor cells, increasing NADPH in tumors protects these cells from ROS-mediated damages. LKB1-AMPK signaling can enhance autophagy. Increasing autophagy in tumor cells leads to chemotherapeutic resistance, epithelial—mesenchymal transition (EMT), and invasion. Activating the LKB1-AMPK-mTOR pathway could protect tumor cells from anoikis, which is a form of cell death that occurs when cells detach from the basement membrane. A-769662 is an AMPK agonist that has increased tumorigenesis.

up-regulates tumor metabolism. Tumor cells undergo extensive metabolic reprograming to adapt to their energy needs as many tumors switch from oxidation phosphorylation to aerobic glycolysis—known as the Warburg effect.[214] In some instances, activating LKB1-AMPK in gastric cancer induced a metabolic shift that reversed the pro-tumorigenic Warburg effect[215]; however, LKB1-AMPK activate autophagy in response to metabolic and oxidative stress thus protecting cells from damage and apoptosis.[216] Given that spontaneous tumors form in autophagy-related gene knockout models,[217] autophagy suppresses tumor formation. Alternatively, tumors increase autophagic flux to protect against stress, chemotherapeutic agents, and promote invasion.[218–220] Therefore, autophagy activation may be important for the tumor promoting properties of LKB1-AMPK signaling (Fig. 4).

Concluding remarks

Genomic analyses first detected *LKB1* inactivation in pathology and recent improvements to these analyses and the development of next generation sequencing technologies pave the way for PJS and cancer diagnosis. For this reason, genomic medicine is the future of personalized therapy for pathologies induced by *LKB1* mutations.[43,221] However, since LKB1 activity is regulated by protein–protein interactions, lipid binding, post-translational modifications, alternative splicing, and cellular localization, investigating LKB1 on pathology is currently beyond the scope of genomic studies alone. As such, advancements in *in silico* investigative practices, computational algorithms, and molecular techniques are together discerning the role of LKB1 in biology and oncology.

It is important to further explore context-specific roles for LKB1 and its regulation, especially given its paradoxical function in cancer. Prior to the recognition of its pro-tumorigeneic signaling, LKB1 was long regarded as strictly a tumor suppressor. However, the discovery of pro-tumorigenic LKB1 signaling combined with LKB1-deficiency enhancing the efficacy of other chemical agents, there is now a need to synthesize LKB1-specific inhibitors. Although there are now some inhibitors that indirectly target LKB1 signaling, there are no known pharmacological compounds that specifically antagonize LKB1.

Despite the extensive effort from numerous investigators providing unique perspectives, significant work is still warranted as few studies characterize how their findings function in context with other LKB1 regulating processes. If LKB1 is to be used as a disease biomarker, the appropriate tools and methods must be implemented to differentiate expression versus functional status. Therefore, the goal of this work was to highlight LKB1 activity in physiology and oncology providing a rationale for targeting LKB1 in cancer.

Author contributions

Charles B. Trelford generated the first draft of the manuscript and generated the figures. Trevor G. Shepherd edited the manuscript and verified its scientific accuracy.

Data availability

The data for Fig. 1 was obtained using cBioPortal for Cancer Genomics of the pan cancer study entitled MSK MetTropism (MSK, *Cell* 2021).

Conflict of interests

None to disclose.

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