THE *LKB1* TUMOR SUPPRESSOR

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CONTENTS

ABBREVIATIONS

Aa	amino acid(s)
AMP	adenosine monophosphate
AMPK	AMP activated protein kinase
ARNT	aryl hydrocarbon receptor nuclear translocator
ATP	adenosine triphosphate
BMP	bone morphogenetic protein
CaMK	calmodulin-dependent protein kinase
cAMP	cyclic adenosine 3´, 5´-monophosphate
cDNA	complementary deoxyribonucleic acid
CD	Cowden Disease
CGH	comparative genomic hybridization
CMV	Cytomegalo Virus
CNS	central nervous system
C-terminus	carboxy terminus
DAB	3,3'-diaminobenzidine
DNA	deoxyribonucleic acid
DMEM	Dulbecco´s modified Eagle´s medium
E	embryonic day
EBV	Epstein-Barr Virus
ELISA	enzyme-linked immunosorbent assay
ES	embryonic stem cell
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
flk-1	fetal liver kinase 1 (VEGF receptor 2)
flt-1	fms-like tyrosine kinase 1 (VEGF receptor 1)
HA	hemagglutinin
HIF-1	hypoxia inducible factor-1
HSV	Herpes Simplex Virus
Ig	immunoglobulin
IU	international unit
JP	Juvenile Polyposis
kb	kilobase
kDa	kilodalton
LOH	Loss of heterozygosity
MEF	mouse embryonic fibroblasts
mRNA	messenger ribonucleic acid
N-terminus	amino terminus
ORF	open reading frame
PCR	polymerase chain reaction
PI-3'-kinase	phosphatidyl inositol 3 '-kinase
PJS	Peutz-Jeghers syndrome
PKA	protein kinase A
PKB	protein kinase B/Akt

Abbreviations

p53	p53 tumor suppressor (transcription factor)
p16	p16 tumor suppressor (cyclin dependent kinase inhibitor)
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SCTAT	sex cord tumor with annular tubules
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMAD4	cytoplasmic mediator of TGF-ß and BMP signaling
SMC	smooth muscle cell
tk	thymidine kinase
TUNEL	terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (apoptosis detection)
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau tumor suppressor
Xeek1	Xenopus egg and embryo kinase-1

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications and unpublished data presented in the results.

I   Ylikorkala A[*], Avizienyte E[*], Tomlinson IPM[*], Tiainen M, Roth S, Loukola A, Hemminki A, Johansson M, Sistonen P, Markie D, Neale K, Phillips R, Zauber.P, Twama T, Sampson J, Jarvinen H, Makela TP, Aaltonen LA. Mutations and impaired function of *LKB1* in familial and non-familial Peutz-Jeghers syndrome and a sporadic testicular cancer. ***Hum. Mol. Gen.*** 8: 45-51, 1999

II Tiainen M, Ylikorkala A, Makela TP. Growth suppression by Lkb1 is mediated by a G(1) cell cycle arrest. ***Proc. Natl. Acad. Sci. U S A*** 96: 9248-51, 1999

III Luukko K, Ylikorkala A, Tiainen M, Makela TP. Expression of *LKB1* and *PTEN* tumor suppressor genes during mouse embryonic development. ***Mech. Dev.*** 83:187-90, 1999

IV Ylikorkala A[*], Rossi DJ[*], Korsisaari N, Luukko K, Alitalo K, Henkemeyer M, Mäkelä TP. Vascular Abnormalities and Deregulation of *VEGF* in *Lkb1*-Deficient Mice. ***Science*** 293:1323-6, 2001

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ABSTRACT

Cancer is considered a disease of the genome triggered by environmental factors. Most cancers occur sporadically without an apparent family history, but inherited cancer susceptibility syndromes are thought to account for 1-2% of cases. Recent advances in human genetics have revealed multiple tumor suppressor genes that underlie inherited cancer syndromes. Although these syndromes are rare conditions, tumor suppressor genes play a key role also in the development of sporadic cancers.

Peutz-Jeghers syndrome is an inherited disease characterized by pigmentation of the mucous membranes, hamartomatous polyps in the gastrointestinal tract and an elevated risk of cancer. The cancers affect a variety of organs, but especially high risk has been demonstrated for gastrointestinal malignancies and pancreatic carcinoma.

Peutz-Jeghers syndrome is caused by germline mutations in the *LKB1* serine/threonine kinase gene. Most serine /threonine kinases are intracellular proteins that typically form signal transduction cascades translating various signals into a coordinated transcriptional response in the nucleus. While *LKB1* mutations are found in Peutz-Jeghers syndrome the mechanism how these mutations predispose to cancer is not known.

In this study we have demonstrated that *LKB1* gene mutations in Peutz-Jeghers syndrome disrupt Lkb1 kinase activity. We also show that *LKB1* mRNA expression and Lkb1 kinase activity are downregulated in tumor cell lines suggesting that *LKB1* mRNA downregulation is a mechanism to inactivate Lkb1 function in cancer. We have also demonstrated that Lkb1 kinase activity inhibits cell proliferation by inducing a G_1 cell cycle arrest. Therefore, demonstrating that *LKB1* is a gatekeeper gene directly controlling cell proliferation.

To study the *in vivo* functions of Lkb1 kinase we have generated mice lacking Lkb1. Mice lacking Lkb1 die during midgestation, thus demonstrating that Lkb1 is essential for mammalian development. Moreover, severe vascular abnormalities were observed in embryonic and extraembryonic compartments and in further studies the vascular endothelial growth factor *VEGF* mRNA expression was shown to be deregulated in both compartments. VEGF expression was also shown to be upregulated in fibroblasts derived from the *Lkb1* mutant embryos thereby placing Lkb1 in the VEGF signaling pathway.

This study has demonstrated that Lkb1 regulates both cell proliferation and angiogenesis, both of which are key steps in cancer development. Finally, this study may provide a rationale for the increased risk of cancer incidence among Peutz-Jeghers syndrome patients and helps to develop novel cancer therapies based on the identified mechanisms.

INTRODUCTION

Cancer is a Disease of the Genome

Cancer is one of the leading causes of morbidity and mortality in developed societies (Peto, 2001). Epidemiological studies have shown that smoking, diet, radiation and certain virus infections increase cancer risk. Many of these risk factors contain chemical carcinogens or physical agents capable of damaging DNA resulting in genomic lesions. Thus, cancer is considered a disease of the genome triggered by environmental factors. Although most cancers occur sporadically without an apparent family history, up to 10% of cancers are thought to have familial predisposition. Rare inherited cancer susceptibility syndromes account only for 1-2% of cases. However, similar genetic alterations have also been suggested to play critical role in common sporadic cancers.

Genes in Cancer Development

Cancer is a disease characterized by clonal expansion of cells that progressively shift from normal cells to invasive cancer cells via intermediate steps (Fearon and Vogelstein, 1990). Cells require certain characteristics to adopt malignant phenotype. This multistep process requires alterations in the function of many genes.

Genes involved in the development of cancer are commonly divided into oncogenes and tumor suppressors. Both groups control key cellular properties required for cancer development. These properties can be grouped into six separate characteristics including sustained positive growth signaling, insensitivity to negative growth signaling, tissue invasion, cell immortalization, induction of angiogenesis and evasion from programmed cell death (*Table1*) (Hanahan and Weinberg, 2000). Each genetic event, which either activates oncogenes or inactivates tumor suppressors gives cells a growth advantage over normal cell population and leads to sequential conversion of normal cells into cancer cells.

Table 1. Characteristics of malignant cells (Hanahan and Weinberg 2001)

List of key characteristics that normal cells have to adopt to become malignant and capable of developing metastatic cancer.

Self-sufficiency in growth signaling
Insensitivity to anti-growth signaling
Tissue invasion and metastasis
Limitless replicative potential
Sustained angiogenesis
Evasion of apoptosis

Oncogenes

The first oncogenes (e.g. *src*, *Ha-ras)* were originally discovered in the DNA of retrovirus genomes having the capacity to induce malignant transformation of cultured cells. Subsequently, the same oncogenes were found also in human cancer cells (Bishop, 1981) (Parada et al., 1982). Surprisingly, even normal cells were reported to carry genes that were related to oncogenes. These genes were termed proto-oncogenes to distinguish them from oncogenes found in viruses and cancer cells. Proto-oncogenes were found to have an important function in normal cell signaling regulating cell growth and differentiation (Cantley et al., 1991). However, proto-oncogenes could become cancerous oncogenes by genomic alterations enhancing their function.

In fact, gain-of function mutations, over-expression, chromosomal translocations and gene amplifications of proto-oncogenes are frequently observed in human neoplasia (Bishop, 1991).

Tumor Suppressors

The first evidence for the presence of tumor suppressor genes came from epidemiological studies of the familial and non-familial forms of a rare ocular tumor - retinoblastoma. Knudson proposed that both copies of a given tumor suppressor gene are inactivated in cancer (Knudson, 1971). This "two-hit" hypothesis suggested that familial retinoblastoma patients had inherited one defective allele of a gene predisposing to retinoblastoma. The other allele of this gene would then be somatically mutated leading to tumor development. In the non-familial cases both alleles had to be somatically inactivated demanding more time. This explained the later occurrence of retinoblastoma in sporadic cases. Later, the *Rb* gene was identified and mutations in both alleles of this gene were reported in retinoblastomas, supporting the Knudsons hypothesis (Lee et al., 1987).

Gatekeepers and Caretakers

Tumor suppressors can be further divided into gatekeepers and caretakers based on gene function (Kinzler and Vogelstein, 1997). Gatekeepers prevent neoplasia directly by controlling cell growth, either by regulating proliferation or by promoting cell death. Although multiple gatekeeper genes have been identified, only one gatekeeper is thought to be active in a given cell type and inactivation of this gatekeeper is rate limiting for tumor development. Thus, when both alleles of a gatekeeper gene are inactivated in target tissue, cells escape from normal growth control.

In contrast, caretaker genes prevent malignant transformation indirectly by maintaining genomic integrity. This class of tumor suppressors contains genes involved in DNA repair and replication. Mutations in caretaker genes result in genomic instability and in increased mutation rate. Consequently, gatekeepers are prone to become inactivated and oncogenes activated due to the genomic instability.

Landscaper genes have been suggested to be a third category of tumor suppressors, in which the genetic defect is not in the neoplastic cell population itself but rather in the adjacent stromal cells. As a result, cells associated with the abnormal stroma develop malignancy due to an abnormal intercellular signaling (Kinzler and Vogelstein, 1998).

REVIEW OF THE LITERATURE

1. Cancer Syndromes are Caused by Tumor Suppressor Gene Mutations

Linkage studies and positional cloning have revealed multiple tumor suppressor genes that underlie inherited cancer syndromes (*Table 2*). Although inherited cancer syndromes are rare conditions, tumor suppressor gene inactivation has a major role also in the development of sporadic cancers (Weinberg, 1995).

According to Knudson´s "two hit" model tumor development requires inactivation of both alleles of a tumor suppressor gene (Knudson, 1971). Indeed, loss of heterozygosity (LOH) of a tumor suppressor gene is frequently detected in cancers. However, some recent reports suggest that haploinsufficiency caused by losing only one tumor suppressor allele is sufficient for tumor development (Fero et al., 1998; Roberts et al., 2000; Smits et al., 2000; Wetmore et al., 2000; Xu et al., 2000; Zurawel et al., 2000).

In addition to inactivating mutations in tumor suppressors, some cancer syndromes arise due to activating mutations in proto-oncogenes. A particularly interesting mechanism underlies familial melanoma, where mutations in *CDK4* gene render cell cycle regulator cdk4 (cyclin dependent kinase 4) resistant to its inhibitors thus increasing cdk4 activity and promoting cell division (FitzGerald et al., 1996; Whelan et al., 1995).

Table 2. Cancer syndromes caused by tumor suppressor mutations

A list of cancer syndromes that are caused by inactivating mutations in tumor suppressor genes. Proposed category indicates whether the tumor suppressor gene has a caretaker or a gatekeeper function.

Disease	Affected gene	Protein function	Proposed category	Tumor spectrum in affected patients
Ataxia-telangiectasia	*ATM*	Protein kinase, maintains genomic integrity	Caretaker	Breast cancer, leukemia and lymphoma
Bannayan-Riley-Ruvalcaba syndrome	*PTEN*	Phosphatase that inhibit PI 3-kinase –Akt pathway. Regulates cell cycle, apoptosis and angiogenesis	Gatekeeper	Breast and thyroid cancer, intestinal hamartomas
Basal cell nevus	*PTCH*	Receptor for sonic hedgehog pathway	Gatekeeper	Basal cell carcinoma, medulloblastoma
Bloom´s syndrome	*BLM*	DNA helicase, maintains genomic integrity	Caretaker	Leukemias, lymphomas and multiple carcinomas

Carney complex	PRKAR1A	Protein kinase A regulatory subunit	Unknown	Cardiac and other myxomas, endocrine tumors and melanotic schwannomas.
Cockayne syndrome	XPB, XPD and XPG	Required for DNA excision repair	Caretaker	Melanoma, basal cell carcinoma
Cowden syndrome (CS),	PTEN	Phosphatase that inhibit PI 3-kinase –Akt pathway. Regulates cell cycle, aopotosis and angiogenesis	Gatekeeper	Breast and thyroid cancer, intestinal hamartomas
Familial Adenomatous Polyposis (FAP)	APC	Sequesters ß-Catenin in the cytoplasm	Gatekeeper	Cancer in GI-tract, osteomas, medulloblastoma
Familial breast and ovarian cancer	BRCA1, BRCA2	Maintains genomic integrity by repairing DNA doublestrand breaks	Caretaker	Breast and ovarian cancers, BRCA2 also in pancreatic and prostate cancer
Familial gastric cancer	E-Cadherin	Interacts with ß-Catenin and regulates cell adhesion	Gatekeeper	Gastric cancer
Familial melanoma/dysplastic nevus	CDKN2 (P14, p16, p19ARF)	p14 and p16 are CDK inhibitors, block cell cycle, p19ARF regulates p53 via MDM2	Gatekeeper	Melanoma, pancreatic, bladder and esophageal cancer, leukemia
Fanconi anemia	FANCA, FANCC,FANCD, FANCE, FANCF, FANCG	Maintain genomic integrity	Caretaker	Leukemia and squamous cell carcinomas
Hereditary Non-Polyposis Colorectal Carcinoma (HNPCC)	MSH2, MSH3, MSH6, PMS1, PMS2, MLH1	DNA mismatch repair	Caretaker	Colorectal, gastric, endometrial, ovarian, hepatobiliary and urinary tract cancers, glioblastoma
Li Fraumeni	p53	Transcription factor, Regulates cell cycle, apoptosis and angiogenesis.	Gatekeeper	Multiple sarcomas, brain tumors, breast cancer, leukemia
Multiple endocrine neoplasia type I	MENI	Interacts with the AP-1 transcription factor JunD and represses JunD-activated transcription	Unknown	Gastrinoma, insulinoma, parathyroid tumors
Multiple exostoses	EXT1, EXT2, EXT3	Heparan sulfate polymerase activity	Unknown	Exostoses, chondrosarcoma
Nijmegen breakage syndrome	NBS1	Maintains genomic integrity by repairing DNA doublestrand breaks	Caretaker	Lymphomas
Neurofibromatosis	NF1,NF2	Regulates Ras signaling and cytoskeleton	Gatekeeper	Neurofibromas, gliomas, astrocytomas, meningeomas, scwannomas
Paraganglioma	SDHD	Small subunit of cytochrome b in mitochondrial complex II	Unknown	Nonchromaffin paragangliomas
Peutz-Jeghers syndrome (PJS)	LKB1	Serine/threonine kinase. Angiogenesis and cell cycle regulation	Gatekeeper	Cancer in GI-tract, pancreas, breast, ovary and testis
Retinoblastoma	Rb	Cell cycle control	Gatekeeper	Retinoblastoma, osteosarcoma

Rothmund-Thomson syndrome	*RECQ4*	DNA helicase, maintains genomic integrity	Caretaker	Osteosarcoma
Tuberous sclerosis	*TSC1, TSC2*	Regulates cell proliferation, growth and adhesion via small GTPases	Gatekeeper	Renal cell carcinoma, angiomyolipoma, astrocytoma, hamartomas
Werner syndrome	*WRN*	DNA helicase and exonuclease maintaining genomic integrity	Caretaker	Soft tissue sarcomas, meningeomas, thyroid carcinomas, melanomas
Willms tumor	*WT1*	Zinc finger transcription factor with multiple target genes	Gatekeeper	Nefroblastoma
von Hippel-Lindau	*VHL*	Component of ubiquitin-ligase complex, regulates cell cycle, adhesion and angiogenesis	Gatekeeper	Clear renal cell carcinoma, feochromocytomas, hemangioblastomas, colorectal cancer
Xeroderma pigmentosum	*XPA, XPB, XPC, XPD, XPE, XPF, XPG*	DNA excision repair	Caretaker	Melanoma, basal cell carcinoma

2. Peutz-Jeghers Syndrome (PJS)

Peutz-Jeghers syndrome (PJS) is an autosomally dominantly inherited disease characterized by pigmentation of the mucous membranes, hamartomatous polyps in the gastrointestinal tract and an elevated risk of cancer (Hemminki, 1999; Tomlinson and Houston, 1998).

The first two patients with signs of PJS were described in the late 19th century. One died of small bowel obstruction and the other developed breast cancer (Hutchinson, 1896). Later, Peutz described a family with a history of polyposis and aberrant mucocutaneous pigmentation (Peutz, 1921) and in 1940s Jeghers reported observations of 12 patients with polyposis and pigmentation linking them together as a clinical entity (Jeghers 1944, 1949).

The incidence of PJS has been estimated to be between 1:8300 to 1:29000 live births (Finan and Ray, 1989; Mallory and Stough, 1987), but some investigators have estimated it to be less common (Spiegelman, 1994; Hemminki, 1999). Peutz-Jeghers patients commonly have a family history of polyposis and pigmentation, but 10-20% of cases lack an apparent family history and are caused by sporadic *de novo* mutations. Reliable cases of incomplete penetrance have not been reported suggesting a complete penetrance of PJS (Amos et al., 1997; Hemminki et al., 1997; Mehenni et al., 1997; Nakagawa et al., 1998).

2.1 Symptoms of PJS

The earliest presenting sign of PJS is the characteristic pigmentation of the oral area, buccal mucosa, vulva, fingers and toes. The pigmentation is commonly first noted on the lower lip. The vast majority of PJS patients exhibit pigment macules, but marked differences in localization and intensity between patients and families has been documented (Westerman, 1997). In older age, pigmentation may diminish or even disappear. The mechanism of the pigmentation is poorly understood, although one electron microscopic study demonstrates a block in the melanosome transfer from the melanocytes to keratinocytes suggesting melanocyte dysfunction (Yamada et al., 1981).

In addition to the pigment macules the symptoms caused by gastrointestinal polyposis can lead to the diagnosis of PJS. The polyps commonly cause occlusion, intussusception, abdominal pain, bleeding and a prolapse of a rectal polyp (Burdick and Prior, 1982; Foley et al., 1988; Utsunomiya et al., 1975). The majority of cases are diagnosed before the 3rd decade of life (Utsunomiya et al., 1975), but in some patients the polyposis causes only mild symptoms and the disease manifests in old age (Laughlin, 1991).

2.2 Polyposis in PJS

Gastrointestinal polyps are commonly classified into adenomatous, hyperplastic, and hamartomatous polyps. Adenomatous polyps are masses of mucosal epithelium originating from aberrant proliferation in intestinal crypts. The majority of colorectal cancers develop stepwise from pre-existing adenomatous polyps, thus establishing adenomas as premalignant lesions (Fearon and Vogelstein, 1990; Vogelstein and Kinzler, 1993). Hyperplastic polyps are histologically well-differentiated polypoid mucosal protrusions, but progression to cancer is less common than in adenomatous polyps (Daibo et al., 1987; Jass et al., 1992).

Hamartomatous polyps consist of multiple well-differentiated tissues

endogenous to the site of polyp. Originally, hamartomas were thought to be benign lesions, but recent studies have indicated that hamartomas may progress to carcinoma via intermediate steps mimicking the stepwise transformation of adenoma to carcinoma (De Facq et al., 1995; Defago et al., 1996; Entius et al., 1997; Gruber et al., 1998; Hizawa et al., 1993; Perzin and Bridge, 1982; Spigelman et al., 1989).

The diagnostic feature of PJS is the development of specific hamartomatous polyps in the gastrointestinal tract. PJS-associated hamartomatous polyps are pedunculated, usually relatively large, and often cystic. Macroscopically they may resemble adenomas. Polyps are frequently found in small intestine (70-90%) of the patients, but they can also be detected in colon and rectum (50%) and in stomach (25%) (Burdick and Prior, 1982; Foley et al., 1988; Utsunomiya et al., 1975). In addition to the gastrointestinal tract, hamartomatous polyps have occasionally been reported in nasal and oral cavities, esophagus, in respiratory and urinary tracts and in breasts in PJS patients. (Burdick and Prior, 1982; De Facq et al., 1995; Jancu, 1971; Keating et al., 1987; Sommerhaug and Mason, 1970).

Histologically PJS polyps are characterized by well differentiated, but dysorganized glandular epithelium with numerous clear goblet cells (Rosai, 1996). A diagnostic feature of the hamartomatous polyps in PJS is a dominant, well-developed smooth muscle component originating from the polyp stalk. This smooth muscle infiltration can be observed even in the periphery of the polyp by immunohistochemistry with antibodies against smooth muscle actin and desmin (Fulcheri et al., 1991). In addition to hamartomatous polyps,

hyperplastic and adenomatous polyps are occasionally detected in PJS patients.

2.3 Increased Risk of Cancer in PJS

Early studies of PJS failed to demonstrate increased cancer risk among PJS patients. A study of 21 PJS cases concluded that malignant transformation of hamartomatous polyps rarely occurs (Dormandy, 1957). Similarly, a 10-year follow-up of a large PJS family did not provide evidence for an increased cancer incidence in PJS (Burdick et al., 1963). However, 19 years later after a 27-year follow-up, two breast cancers, one jejunal adenocarcinoma arising from a polyp and three benign ovarian tumors were detected in the same family. (Burdick and Prior, 1982).

The first evidence of the increased cancer incidence in PJS patients came from a study, which reported four gastric, three duodenal, one ileal and three colorectal cancers in 321 PJS patients (Dozois et al., 1969). Later, 28 cancers and increased mortality were reported in a large study of 222 Japanese PJS patients (Utsunomiya et al., 1975). However, a 33-year follow-up study of 48 PJS patients detected only one gastrointestinal cancer and failed to demonstrate decreased survival questioning the possible premalignant potential of PJS (Linos et al., 1981).

The most convincing evidence supporting the increased risk of cancer in PJS came from a 12-year follow-up of 31 familial PJS patients. Fifteen histologically verified cancers were reported including four gastrointestinal carcinomas, ten non-gastrointestinal carcinomas and one myeloma (Giardiello et al., 1987). The cancer risk was estimated 18-fold higher than in general

population. Moreover, Boardman et al. have suggested that female PJS patients are at even higher risk than male patients due to particularly high risk of breast and gynecologic cancers (Boardman et al., 1998).

A recent meta-analysis has summarized data collected from six publications including 210 patients. The relative risk of all cancers among PJS patients was estimated 15.2 fold higher than in general population and the cumulative risk of developing malignancy by the age of 64 was 94% (Giardiello et al., 2000). The relative risks calculated for individual types of cancers presented in *Table 3* demonstrate high relative risk for gastrointestinal and pancreatic cancers. The high cumulative risk of ovarian, uterine and breast cancers suggests that female PJS patients are at higher risk of developing malignancy than men.

Table 3. *The incidence of cancer in PJS Patients (Giardello et al., 2000)*

The meta-analysis included data from publications where all PJS diagnoses were based on typical clinical and histopathologic findings. The cancer diagnoses were also confirmed by histology. Malignancies were most commonly seen in breast, colon, pancreas and stomach. There was especially high relative risk for developing small intestinal cancer, which otherwise is a rare malignancy.

Carcinoma	Number of cases reported	Relative risk (Observed/Expected)	Cumulative risk from age 15 to 64
Small intestine	6	520	13%
Stomach	10	213	29%
Pancreas	6	132	36%
Colon	15	84	39%
Esophagus	1	57	0.5%
Ovary	4	27	21%
Lung	5	17	15%
Uterus	2	16.0	9%
Breast	11	15.2	54%
Testes	1	4.5 *	9%
Cervix**	3	1.5 *	10%
All cases	66	15.2	93%

*) statistically not significant **) Most cases of adenoma malignum not included

2.4 Characteristic Ovarian and Uterine Tumors in PJS

Sex-cord tumor with annular tubules (SCTAT) is a rare benign neoplasm almost exclusively seen in PJS patients. SCTAT tumor is commonly calcified, small, multifocal and bilateral. Histologically it has features of granulosa cell and sertoli cell tumor and focal differentiation of both cell types may occur. Occasionally, these tumors are hormonally active resulting in symptoms associated with hyperestrogenism (Herruzo et al., 1990; Scully, 1970; Young et al., 1983; Young et al., 1982).

Adenoma malignum or minimal deviation adenocarcinoma is a rare well-differentiated form of cervical adenocarcinoma seen in PJS patients. It has been estimated that 10% of all adenoma malignum cases occur in PJS patients (Gilks et al., 1989; Szyfelbein et al., 1984) and that it commonly co-exists with ovarian tumors (Srivatsa et al., 1994; Young and Scully, 1988).

2.5 Polyposis Syndromes Related to PJS

Cowden disease (CD) and Juvenile polyposis (JP) are dominantly inherited polyposis syndromes similar to Peutz-Jeghers syndrome, which exhibit genetic heterogeneity. PJS is distinguished from these syndromes by the extensive infiltration of smooth muscle in the polyps.

Patients with Cowden disease exhibit cutaneous hamartomas and trichilemmomas, hamartomatous polyps in gastrointestinal tract and increased risk of breast, thyroid and skin cancer (Hanssen and Fryns, 1995; Weinstock and Kawanishi, 1978). Mutations in *PTEN* gene have been shown to underlie Cowden disease (Liaw et al., 1997; Lynch et al., 1997; Nelen et al., 1997). *PTEN* gene encodes for a phosphatase that regulates negatively the PI3'K/PKB/Akt signaling pathway by dephosphorylating phosphoinositoles (Maehama and Dixon, 1998; Myers et al., 1998; Stambolic et al., 1998). One study suggests that PTEN also dephosphorylates focal adhesion kinase (FAK) regulating cell adhesion and motility (Tamura et al., 1998), but the tumor suppressive function of PTEN has been shown to be dependent only on its lipid phosphatase activity (Myers et al., 1998)

Juvenile polyposis (JP) is characterized by distinct type of hamartomatous polyps in the colon (Desai et al., 1995). Occasionally adenomatous and mixed histology polyps are found in JP patients. Histologically the polyps exhibit dilated glandular structures lined by mucus secreting epithelium with thick lamina propria. Although juvenile polyps rarely undergo malignant transformation, patients with JP are at higher risk of gastrointestinal cancer (Giardiello et al., 1991; Giardiello and Offerhaus, 1995; Jarvinen and Franssila, 1984; Sassatelli et al., 1993). JP is caused by mutations in *SMAD4*, *BMPR1* and *PTEN* genes demonstrating genetic heterogeneity between these cancer syndromes (Howe et al., 2001; Howe et al., 1998; Olschwang et al., 1998). *BMPR1* encodes for a bone morphogenetic protein receptor 1, and *SMAD4* for a cytoplasmic mediator of the bone morphogenetic protein and transforming growth factor-beta signaling. This suggests that the BMP signaling pathway is involved in the pathogenesis of JP.

3. Mutations in *LKB1* Gene Underlie PJS

3.1 PJS locus and *LKB1* gene

The PJS susceptibility locus was identified by assuming that hamartomas are clonal expansions that exhibit loss of heterozygosity (LOH) according to Knudson´s "two-hit" model. Indeed, small chromosomal deletions were found in the short arm of chromosome 19 by comparative genomic hybridization (CGH) followed by demonstration of linkage to 19p13.3 (Hemminki et al., 1997). Other groups confirmed this finding (Amos et al., 1997; Nakagawa et al., 1998). In addition, Olschwang et al. reported three families, and Mehenni et al. one family, that were not linked to 19p13.3, suggesting the presence of another PJS locus (Mehenni et al., 1997; Olschwang et al., 1998). Furthermore, Mehenni et al. demonstrated linkage to alternative locus at 19q13.4 in a large Indian family that did not show linkage to 19p13.3 (Mehenni et al., 1997).

Subsequently, germline mutations were identified in PJS patients in *LKB1* (*STK11*) serine/threonine kinase gene located in 19p13.3 region (Hemminki et al., 1998; Jenne et al., 1998). *LKB1* gene consists of 10 exons, the first being partially coding, and 10th exon non-coding. The *LKB1* mRNA is ubiquitously expressed, but particularly high expression has been reported in testis and fetal liver (Hemminki et al., 1998; Jenne et al., 1998).

3.2 Germline Mutations in PJS

Surprisingly, considerable variation in the *LKB1* mutation frequency has been reported. The first reports detected germline *LKB1* mutations in vast majority of PJS patients (Gruber et al., 1998; Hemminki et al., 1998; Jenne et al., 1998). Later studies, however, have found coding region or splice mutations only in 55% to 77% of PJS patients (Mehenni et al., 1998; Nakagawa et al., 1998; Olschwang et al., 2001; Resta et al., 1998; Wang et al., 1999; Westerman et al., 1999; Ylikorkala et al., 1999). Some studies have suggested that *LKB1* mutations are even more uncommon (Boardman et al., 2000; Jiang et al., 1999; Yoon et al., 2000). However, it is likely that these studies have underestimated the *LKB1* mutation frequency due to low sensitivity mutation screening methods.

The majority of *LKB1* mutations are small deletions or point mutations predicted to truncate Lkb1 protein and compromise its function, but many mutations cause only single amino acid substitutions, small in-frame deletions or minor truncations (*Figure 1*).

3.3 *LKB1* Mutations in PJS Polyps

In addition to the chromosomal losses in CGH analysis, Hemminki et al. demonstrated LOH near the *LKB1* locus by PCR in three PJS polyps. LOH was found to be restricted to areas having high number of clear goblet cells suggesting that they might be the primary clonal neoplastic cell population. No allelic imbalance was detected in the stromal tissues of the polyps suggesting that the smooth muscle hyperproliferation in PJS polyps is not a clonal event. (Hemminki et al., 1997). Other studies have reported variable frequencies of LOH in the PJS polyps. While Gruber et al. demonstrated LOH by PCR in majority of polyps (Gruber et al., 1998). Entius and Miyaki suggested that LOH of *LKB1* might be less frequent in PJS polyps (Entius et al.,

2001; Miyaki et al., 2000). Alternatively, promoter methylation or small missense mutations, could be responsible for inactivating *LKB1*. Interestingly, Rowan studied LOH negative PJS polyp by *in situ* hybridization and demonstrated *LKB1* mRNA expression throughout the polyp, suggesting that some polyps retain *LKB1* mRNA expression (Rowan et al., 2000). This is supported by our data showing that *LKB1* mRNA and protein expression and kinase activity are not compromised in hamartomatous polyps arising in mice modeling PJS (Rossi et al., unpublished).

The molecular analysis of carcinomas in PJS patients has not been extensive due to limited material, but interestingly all cancers have been reported to LOH of *LKB1* (Entius et al., 2001; Su et al., 1999). This could suggest that while polyps may arise in heterozygous state, cancer development requires biallelic inactivation of *LKB1*

3.4 *LKB1* Mutations in Sporadic Cancers

Hundreds of sporadic tumors and cell lines have been studied to evaluate the role of *LKB1* mutations in sporadic tumorigenesis. Although chromosomal loss at 19p13.3 is commonly observed in cancers only limited numbers of somatic mutations in the remaining *LKB1* allele have been demonstrated. Colorectal cancer is among the most extensively studied tumor type. Most studies have shown that biallelic inactivation of *LKB1* is rare in colorectal carcinomas (Avizienyte et al., 1998; Launonen et al., 2000; Nakagawa et al., 1999; Resta et al.,

1998; Wang et al., 1998). A notable exception to this is the high frequency of LOH with accompanying missense mutations in the remaining allele in Korean patients with left sided colon cancer (Dong et al., 1998). However, in more detailed analysis 6 of 7 mutations were shown not to disrupt Lkb1 autocatalytic activity (Launonen et al., 2000).

Biallelic mutations have not been demonstrated in breast cancer, CNS tumors, adenoma malignum and SCTAT tumors (Bignell et al., 1998; Connolly et al., 2000; Forster et al., 2000; Sobottka et al., 2000). However, a few cases of biallelic *LKB1* mutations have been described in lung adenocarcinoma, ovarian cancer and melanoma (Avizienyte et al., 1999; Rowan et al., 1999; Wang et al., 1999). In 5% of sporadic pancreatic and biliary adenocarcinomas truncating mutations in the remaining *LKB1* allele are accompanied with LOH (Su et al., 1999), arguing that somatic *LKB1* mutations might have a role in the development of pancreatic and biliary cancers.

Since somatic mutations in sporadic cancers have been relatively rare, it has been suggested that alternative mechanisms may be important for inactivating *LKB1*. This has been supported by demonstrating *LKB1* promoter methylation and m*RNA* down regulation in primary tumors and cell lines (Esteller et al., 2000; Tiainen et al., 1999; Trojan et al., 2000).

Type of Mutation	Figure	Origin of Mutation	Reference
wild type			
Y49D		melanoma cell line	Rowan A et al. 1999
Y60X		PJS	Olschwang S et al. 2001
L67P		PJS	Hemminki A et al. 1998
Q100X		PJS	Westerman AM et al. 1999
K108R		PJS	Wang ZJ et al. 1999
Y118X		PJS	Olschwang S et al. 2001
C132X		PJS	Olschwang S et al. 2001
G135R		melanoma	Rowan A et al. 1999
F157S		PJS	Ylikorkala A et al. 1999
D162N		PJS	Westerman AM et al. 1999
G163D		PJS	Westerman AM et al. 1999
G163D		testicular cancer	Avizienyte E et al. 1998.
L164M		PJS	Westerman AM et al. 1999
G171S		colorectal cancer	Dong SM et al. 1998
D176N		PJS	Mehenni H et al.1998
N181Y		PJS	Ylikorkala A et al. 1999
L182P		PJS	Olschwang S et al. 2001
D194N		PJS	Westerman AM et al. 1999
D194V		lung cancer	Avizienyte E et al. 1999
D194Y		melanoma	Guldberg P et al.1999
E199K		colorectal cancer	Dong SM et al. 1998
D208N		colorectal cancer	Dong SM et al. 1998
G215D		colorectal cancer	Dong SM et al. 1998
S232P		PJS	Yoon KA et al. 2000
G242W		PJS	Olschwang S et.al 2001
G242V		PJS	Olschwang S et al. 2001
G251S		PJS	Resta N et al. 1999
E256S		PJS	Yoon KA et al. 2000
H272Y		PJS	Boardman LA et al. 2000
P281L		ovarian cancer	Nishioka Y et al. 1999
P281L		colorectal cancer	Dong SM et al. 1998
R297K		PJS	Westerman AM et al. 1999
R297S		PJS	Boardman LA et al. 2000
R304W		PJS	Resta N et al. 1999
W308C		PJS	Mehenni H et al.1998
P314H		colorectal cancer	Resta N et al. 1999
P324L		gastric carcinoma	Park WS et al. 1998
P324L		PJS	Yoon KA et al. 2000
F354L		colorectal cancer	Dong SM et al. 1998
T367M		colorectal cancer	Dong SM et al. 1998
51-56		PJS	Mehenni H et al.1998
52		PJS	Olschwang S et al. 2001
107-109		PJS	Wang ZJ et al.1999
137-140		PJS	Ylikorkala A et al. 1999
175-176		PJS	Resta N et al. 1999
247		PJS	Nakagawa H et al. 1998
303-306		PJS	Hemminki A et al. 1998
330-334		PJS	Gruber SB et al. 1998
98-155		PJS	Hemminki A et al. 1998
156-307		PJS	Jenne et al.1998
416 stop		PJS	Wang ZJ et al.1999
frameshift 359		PJS	Westerman AM et al. 1999
frameshift 342		PJS	Yoon KA et al. 2000
frameshift 319		PJS	Olschwang S et.al 2001
frameshift 316		PJS	Westerman AM et al. 1999

frameshift 312		Pancreatic cancer	Su et al. 1999
308 stop		PJS	Ylikorkala A et al. 1999
frameshift 307		PJS	Hemminki A et al. 1998
Frameshift 306		PJS	Westerman AM et al. 1999
Frameshift 305		PJS	Ylikorkala A et al. 1999
Frameshift 302		PJS	Mehenni H et al.1998

Figure 1.**Small mutations reported in PJS patients, sporadic cancers and cell lines**

High frequency of mutations in the kinase domain (grey).

4. *LKB1* Gene is Predicted to Encode a Serine/Threonine Kinase

The characteristic feature of serine/threonine kinases is a catalytic kinase domain composed of eleven subdomains. The kinase domain binds substrate proteins and ATP in the presence of a divalent cation (Mg^{2+}, Mn^{2+}), and transfers the -phosphate group of the ATP to a serine or threonine residue of the substrate. Hundreds of serine/threonine kinases have been identified in eukaryotes, the majority of which are implicated in signal transduction. Typically, kinases form cascades phosphorylating their target proteins to achieve amplified signal with high specificity. As a result, extracellular signals (such as growth factors and hormones), nutritional and enviromental stress signals, and cell cycle checkpoints are translated into coordinated transcriptional responses in the nucleus.

Serine/threonine kinases are subdivided into major groups based on the degree of homology in the kinase domain (Hanks et al., 1988). Lkb1 is placed into the calmodulin dependent kinase (CaMK) group, where it exhibits weak homology to AMP activated protein kinase (AMPK). AMPK is composed of a catalytic subunit and two regulatory subunits (Beri et al., 1994; Carling et al., 1994) and it is activated allosterically by elevated AMP levels during metabolic stress (Ferrer et al., 1985; Harwood et al., 1984).

4.1 Lkb1 Kinase in Other Species

The conservation of genes and their function between species allows scientists to use others species as model organisms to study the function of genes *in vivo*. Model organisms have been used successfully to identify novel pathways that control cell growth, differentiation and programmed cell death.

Lkb1 kinase has been highly conserved during evolution and some information is available on Lkb1 homologues in mice, Xenopus, and *C.elegans*. The Drosophila homologue has been identified only by sequence. Smith et al. characterized the mouse *Lkb1,* and found it to be localized on mouse chromosome 10. The genomic structure of mouse *Lkb1* is similar to the human *LKB1* consisting of ten exons (Smith et al., 1999). The human and mouse Lkb1 proteins share 96.2% identity in the kinase domain and 89.7% identity overall.

The *Xenopus* Lkb1, termed Xeek1, was identified in a screen to identify novel kinases homologous to cell cycle kinase Cdc2 (Su et al., 1996). In fact, Xeek1 was the first Lkb1 kinase family member to be identified and it was identified well before *LKB1* was linked to the Peutz-Jeghers syndrome. Human Lkb1 and Xeek1 share 93% identity in the kinase domain and 82% overall identity (Jenne et al., 1998). XEEK1 has been shown to co-immunoprecipitate and phosphorylate an unknown 155 kDa protein. Moreover, the XEEK1 C-terminus is phosphorylated by PKA, but the significance of this modification is unknown.

A kinase (Par-4) homologous to Lkb1 was identified from nematode *C.elegans*.

Par-4, along with five other Par genes (Par-1 to Par-6), were required for cell polarization and asymmetric cleavage in *C.elegans* embryos (Kemphues et al., 1988; Watts et al., 1996). Par-4 gene was subsequently cloned and shown to encode a serine/threonine kinase homologous to Lkb1 and Xeek1 (Watts et al., 2000). The sub-cellular distribution of Par-3/Par-6 protein complex was altered in Par-2, Par-4 and Par-5 mutant embryos, thus establishing a genetic link between these genes (Hung and Kemphues, 1999). Recently, mammalian homologues have been identified for Par-3 and Par-6 (Izumi et al., 1998), and they have been shown to form a functional unit with atypical protein kinase C and cdc42 or Rac1 that control cell polarity (Johansson et al., 2000; Lin et al., 2000; Qiu et al., 2000). This suggests that Par genes form a pathway that may be functionally conserved from nematodes to man.
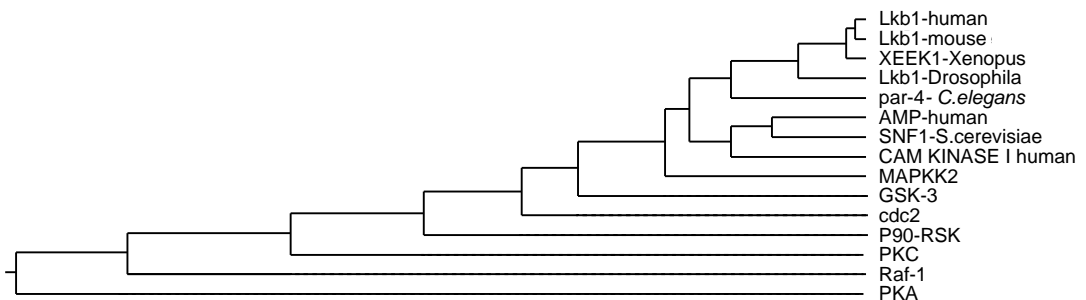
Figure 2. **Relationship of Lkb1 to other kinases**

Lkb1 kinases are conserved during evolution. The human Lkb1 has homologues in Mouse (Lkb1), *Xenopus* (Xeek1), *Drosophila* (dLkb1) and *C.elegans* (Par-4). The closest Lkb1 relative in humans is the AMP activated kinase (AMPK), which belongs to the CAM kinase group. However, AMPK shares more homology with the SNF-1 stress kinase in yeast demonstrating that Lkb1 kinases are a distinct kinase group.

AIMS OF THE STUDY

Mutations in *LKB1* serine/threonine kinase gene are found in Peutz-Jeghers syndrome patients, but the functions of Lkb1 kinase and how *LKB1* mutations predispose to cancer is not known.

1. Characterize the protein encoded by the *LKB1* gene including generation of a functional assay.

2. Study the functional consequences of *LKB1* mutations with the functional assay.

3. Explore Lkb1 expression and function in tumor cell lines

4. Study the functional effects of reintroduction of Lkb1 into tumor cell line

5. Study the expression patterns of the *Lkb1* and *Pten* tumor suppressor genes during mouse development.

6. Study functions of *Lkb1 in vivo* by creating mice lacking *Lkb1*.

MATERIALS AND METHDOS

Detailed description of materials and methods are in original publications.

cDNA Sequencing (Study I)

RNA was converted to cDNA in RT reaction using random priming method with M-MLV reverse transcriptase and RNase inhibitor. The coding region of *LKB1* was amplified by standard PCR protocol.

Exon Sequencing (Study I)

33 PJS patients were available for the mutation screening in *LKB1*. The diagnosis was based on the presence of histopathologically confirmed intestinal Peutz-Jeghers polyposis. Other clinical data, such as information on mucocutaneous pigmentation, and clinical features of family members were not available for all patients. Of the 33 patients, 20 had a family history of PJS and 8 were sporadic. In 5 cases family data was not available. Mucocutaneous pigmentation was documented in almost all cases (17 of 18) where the information about the pigmentation was available. Isolation of DNA was performed using standard procedures and EBV-immortalized cell lines were prepared from PJS patients' blood samples using standard methods. Mutation screening was performed by genomic sequencing of the nine coding *LKB1* exons. Primers were designed to cover all exonic coding sequences as well as splice acceptor and donor sites.

Genotype and Linkage Analysis (Study I)

Microsatellite markers D19S180, D19S880, D19S891 and D19S254 were used to test for linkage to 19q13.4 in six families negative for *LKB1* germline mutation. Multipoint linkage analyses were performed using the program GENEHUNTER. Marker allele sizes and frequencies were obtained from Centre d´Etudes du Polymorphisme Humain (CEPH; http://www.cephb.fr) and from the Genome Database (http://gdbwww.gdb.org). A dominant mode of inheritance with 85% penetrance was assumed. The disease allele frequency was set to 0.0002.

Southern Blotting (Study I)

8 µg of genomic DNA was digested with EcoRI and TaqI restriction enzymes, resolved by 0.8% agarose gel electrophoresis and blotted onto membrane. The probes were PCR amplified from the *LKB1* cDNA and genomic DNA. Hybridizations were carried out using standard protocols.

LKB1 Expression Constructs (Study I)

The coding region of wild type or mutant *LKB1* was PCR amplified and the PCR fragment was digested and subcloned into EcoRI and SalI sites of pCI-Neo based vectors containing N-terminal hemagglutinin (HA) or Myc epitope tags. All *LKB1* alleles were confirmed by sequencing.

Cell Culture and Transfections (Study I, II)

G361 (melanoma), HeLa S3 (cervical carcinoma), SW480 (colorectal adenocarcinoma), U2OS (osteosarcoma), and NIH3T3 (fibroblast) cells were grown in DMEM with 10% FCS, L-glutamine, and penicillin/streptomycin. The cells were transfected using the calcium phosphate transfection method. For determining the colony forming ability, the transfected cells were subjected to 2-3 mg/ml G418 selection for 16 to 20 days. For counting, the colonies were fixed with 5% trichloroacetic acid and stained with 3% Giemsa stain.

Metabolic Labeling of Cellular Proteins (Study I)

48 hours after transfection cells were starved for 1 hour in media lacking cysteine and methionine followed by a metabolic labeling with 200 μCi/ml of a mixture of S^{35}cysteine and S^{35}methionine for 2 hours. Subsequently cells were lysed and subjected to immunoprecipitation. Immunoprecipitates were washed and subjected to SDS-PAGE analysis followed by fluorography.

Immunoprecipitation and Kinase Assays (Study I, II)

48 h after transfection cells were collected and lysed followed by an overnight immunoprecipitation with 12CA5 anti-HA, 9E10 anti-myc or a specific polyclonal antiserum raised against a 15 amino acid C-terminal peptide of human Lkb1. Control immunoprecipitations were performed with anti-Lkb1 preincubated with the antigenic peptide. Subsequently immunoprecipitates were washed and incubated at 30°C for 30 min in kinase buffer containing 10 μCi of P^{32} ATP. The reactions were stopped by adding boiling SDS-PAGE sample buffer and analyzed on 10% SDS-PAGE gel.

Northern Blotting (StudyII)

Clontech Multiple Tissue Northern (MTN™) Blot (#7757-1) was hybridized according to standard protocols.

Western Blotting (Study I, II, IV)

20 μg-40 μg of protein from cell lysates were analyzed by SDS-PAGE and western blotting according to standard techniques using anti-HA, anti-Myc, polyclonal anti-Lkb1 or anti-Glut-1 antibodies and detected by enhanced chemiluminescence.

Immunofluorescence (Study II)

Cells were seeded on coverslips and fixed with 3.5% paraformaldehyde 48 hours post-transfection. Double immunofluorescence was performed with polyclonal anti-Lkb1 and monoclonal anti-ß-galactosidase, which were detected with rhodamin-conjugated anti-rabbit and fluorescein-conjugated anti-mouse secondary antibody, respectively. The nuclei were visualized with Hoechst 33342.

Flow Cytometry Analysis (Study II)

G361 cells were co-transfected with *LKB1* plasmids and pCMV/CD20 selection plasmid. The cells were treated with nocodazole to induce a G2/M phase

block. Subsequently cells were detached, incubated with anti-CD20-FITC and fixed with 80% ethanol. Propidium iodide was used to stain the nuclei. The cell cycle distribution of CD20 positive and negative cells was analyzed with a Coulter EPICS flow cytometer. Percentages of cells in G1, S and G2/M phases were determined with CellFIT cell cycle analysis program.

Lkb1 Deficient Mice
(Study IV)

Two independent targeting strategies were used. The first (figure 3) resulted in a deletion of genomic sequences encompassing exons 2 to 7 (of 10 total); the second (figure 4), based on Cre/LoxP methodology, resulted in the inversion of these sequences. In both cases, we used a 6.3 kb NsiI-HindIII (5') fragment and 2.0 kb BamHI-BamHI (3') fragment of genomic sequence. Positive (PGK-Neomycin) and negative (PGK-HSV-tk) selection markers were used in both strategies. The target vectors were electroporated into ES cells and correctly targeted clones were identified by Southern blotting with 5' and 3' external probes. ES cell clones were injected into C57BL/6 blastocysts. Several chimeric offspring were found to transmit targeted alleles in the germline. Germline inactivation of *Lkb1* utilizing the LoxP/Cre-technique was achieved by crossing targeted animals to PGK-Cre mice (Lallemand et al., 1998). Stable inversion of the targeted sequences was then achieved by breeding the PGK-Cre transgene out of subsequent generations. $Lkb1^{-/-}$ animals obtained with both strategies were found to have identical phenotypes and both were used in this study.
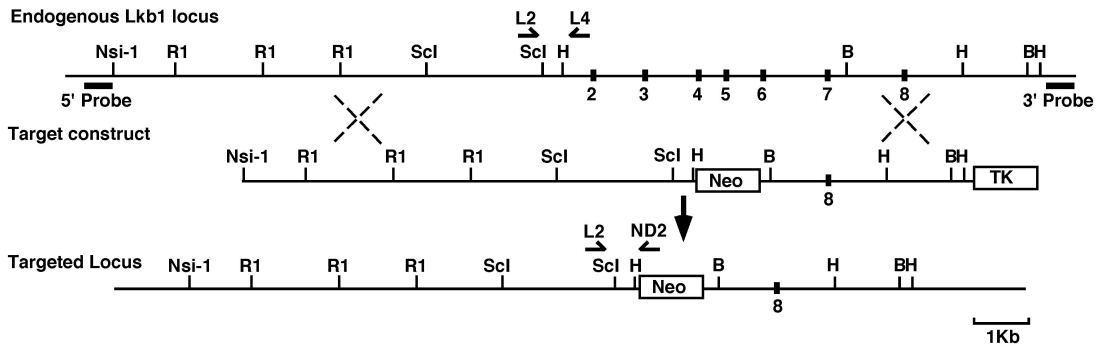
Figure 3.

Targeting strategy designed to replace exons 2-7 of the mouse *Lkb1* locus by PGK-Neo cassette.
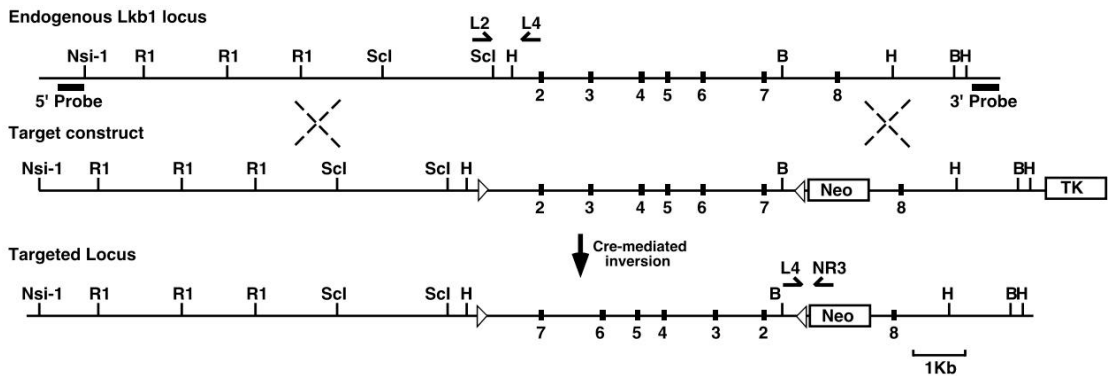
Figure 4.

Cre-recombinase mediated targeting strategy designed to place Lox-P sites in 1st and 7th introns. Lox-P sites result in the inversion of the exons 2-7 and creation of non-functional allele in the presence of Cre recombinase.

Embryo Cultures (Study IV)

E9.5 embryos were minced and cultured on 48-well plates in DMEM containing 15% FCS. For the VEGF analysis, 25,000 MEFs from individual embryos in passage 3 were plated on 24-well plates and cultured in normoxic (21% O_2) or hypoxic (1% O_2) conditions for 24 hours. Conditioned medium was removed and VEGF concentration was analyzed by ELISA. Remaining cells were lysed immediately in SDS sample buffer, and subjected to SDS-PAGE analysis.

TUNEL Labeling (Study IV)

Paraformaldehyde-fixed and paraffin-embedded 7-µm tissue sections were used to study cell death in embryonic tissues. Terminal deoxytransferase-mediated deoxy-uridine nick end-labeling (TUNEL) analysis was performed according to manufacturer´s instructions and counterstained with Hoechst 33342.

In situ Hybridization (Study III, IV)

A mouse *Lkb1* open reading frame in pGEM-T or a PCR fragment of human PTEN (96% identity to mouse sequence) open reading frame were used for *in vitro* transcription of ^{35}S-UTP-labeled antisense and sense probes. *In situ* hybridizations were performed according to Wilkinson and Green (Wilkinson et al., 1990) with modifications (Luukko et al., 1996). The probes for *Vegf*, *flk-1*, *flt-1* are described previously (Kaipainen et al., 1993).

Whole-mount *in situ* Hybridization (Study IV)

Embryos were fixed in 4% paraformaldehyde, bleached in 7% H_2O_2-93% methanol, treated with proteinase K and post-fixed in 4% paraformaldehyde/0,2% glutaraldehyde. Hybridization with digoxygenin-UTP-labeled antisense probe was followed by RNaseA/RNaseT1 treatment. Embryos were incubated with alkaline phosphatase conjugated anti-digoxigenin antibody.

Whole-mount Immunostainings (Study IV)

Embryos were fixed in 4% paraformaldehyde, bleached in 5% H_2O_2-95% methanol and blocked. After incubation with antibodies against CD31 and smooth muscle actin the embryos were treated with peroxidase-conjugated secondary antibodies and developed in 3,3'-diaminobenzidine (DAB).

RESULTS AND DISCUSSON

The Prevalence of *LKB1* Mutations in PJS (Study I)

To investigate the prevalence and nature of germline mutations in PJS families and in non-familial PJS cases, we performed germline mutation analyses of *LKB1* in 33 unrelated PJS patients.

In direct genomic sequencing 18 different mutations were detected. Fifteen mutations compromised the *LKB1* open reading frame, and involved small truncations and point mutations, and three changed splice donor or acceptor sites. Samples which did not exhibit mutations in genomic sequencing were subsequently scrutinized for large genomic rearrangements by RT-PCR or Southern blotting analysis. Southern analysis of genomic DNA revealed aberrant bands suggesting that an approximately 2 Kb deletion may have occurred in one *LKB1* allele in one sample.

The prevalence of observed *LKB1* mutations in familial cases was 12/20 (60%), and 4/8 in sporadic cases (50%). In the whole series 19 (58%) mutations in *LKB1* were observed in 33 PJS patients. The proportion of mutation-positive PJS individuals in our study was significantly lower than in the initial reports (Gruber et al., 1998; Hemminki et al., 1998; Jenne et al., 1998), but it is in accordance with recent reports with more material. This suggests that a second PJS locus might exist (Mehenni et al., 1998; Nakagawa et al., 1998; Olschwang et al., 2001; Resta et al., 1998; Wang et al., 1999; Westerman et al., 1999; Ylikorkala et al., 1999).

Linkage to 19q13.4 (Study I)

In our series, 10 mutation-negative patients failed to demonstrate linkage to 19p13.3. To study linkage to the possible minor PJS locus in 19q13.4 (Mehenni et al., 1997), we used microsatellite markers D19S180, D19S880, D19S891 and D19S254 in 6 families where no *LKB1* genetic defect or linkage to 19p13.3 was found. LOD scores ranging from –10.16 to 0.54 with these markers suggested no linkage to 19q13.4, excluding it as an alternative locus among these patients.

LKB1 Gene Encodes a Protein Kinase (Study I)

To characterize the protein encoded by *LKB1*, we cloned the *LKB1* open reading frame (ORF) into a mammalian expression vector and transiently expressed HA-epitope-tagged Lkb1 in the human osteosarcoma cell line U2OS. Cells were metabolically labeled using S^{35}cysteine and S^{35}methionine and analyzed on SDS-PAGE. Lkb1 protein was observed to migrate at 60 kDa, which is higher than its predicted molecular weight (48 kDa). This suggests that Lkb1 protein could be covalently modified *in vivo*.

To study the catalytic activity of Lkb1, HA-tagged Lkb1 was immunoprecipitated from cellular lysates an in vitro kinase was carried out. In contrast to the Lkb1 homologue in Xenopus, which did not exhibit autocatalytic activity, a prominent phosphorylated band was noted at 60 kDa in Lkb1 immunoprecipitates. This band was presumed to represent Lkb1 autophosphorylation. Alternatively, the

immunoprecipitates could contain another kinase capable of phosphorylating Lkb1.

Moreover, a 115 kDa protein was observed to co-immunoprecipitate with Lkb1 in S^{35} labeled lysates. This band could represent an endogenous Lkb1 interacting protein or a substrate. However, phosphorylation of this 115 kDa protein was not observed.

Mutations in PJS Patients Impair Lkb1 Activity

By measuring Lkb1 kinase activity we compared wild type Lkb1 to three mutants with minor predicted changes identified in Peutz-Jeghers syndrome. The mutants used included a C-terminal truncation (308 stop), 4 amino acid in-frame deletion (303-306) and a one amino acid substitution (L67P) (*Figure 1*). PJS-associated mutations encoded a stable Lkb1 protein, but in contrast to the wild type, the PJS-mutatnts did not exhibit detectable kinase activity. Furthermore, a sporadic testicular cancer-derived mutation (G163D) was included in the studies (*Figure 1*). In contrast to the undetectable activity in the PJS-derived mutations, the testicular cancer-derived mutant had retained small amount of activity, although it was significantly reduced compared to the wild-type Lkb1. These results demonstrated that PJS- and cancer-derived mutations inactivate or severely compromise Lkb1 kinase activity.

The Subcellular Localization of Lkb1 (Study II)

The subcellular localization of Lkb1 was studied in G361 cells transiently transfected with *LKB1* expression constructs by immunofluoresence analysis. Lkb1 was detected predominantly in the nucleus, but in a significant fraction (ca. 30%) of cells, Lkb1 was found mainly in the cytoplasm. Similar Lkb1 localization pattern has been reported by other groups (Collins et al., 2000; Nezu et al., 1999). In addition to nuclear and cytoplasmic localization, Lkb1 has been demonstrated to associate with plasma membrane (Collins et al. 2000; Sapkota at al., 2001). The distinct distribution of Lkb1 in the cells may suggest that Lkb1 function is regulated by altering its subcellular localization. Interestingly, Nezu et al. have proposed that Lkb1 may be inactivated by nuclear sequestration; thus, these authors have demonstrated full autocatalytic activity, but exclusively nuclear localization, of the (303-306) deletion (Nezu et al., 1999). However, other groups, including ours, have not been able to demonstrate measurable kinase activity in the 303-306 mutant (Marignani et al., 2001). In fact, our results suggest that nuclear localization is a common feature of all catalytically inactive Lkb1 alleles, including 303-306 (Tiainen et al., unpublished).

In addition to the Lkb1 kinase activity the nuclear localization requires a nuclear localization signal sequence (NLS) localized on the N-terminus of Lkb1 (Smith et al., 1999).

LKB1 mRNA Expression and Activity in Tumor Cells (Study II)

In order to study *LKB1* mRNA expression in human tumors, we performed northern blotting analysis on a panel of human tumor cell lines originating from various tissues. HeLa S3 cells (cervical adenocarcinoma) had undetectable, and G361 cells (melanoma)

exhibited severely reduced levels of *LKB1* mRNA, suggesting that mRNA downregulation could be a mechanism to impair Lkb1 function in human tumors.

We also analyzed Lkb1 activity in HeLa S3 and G361 cell lines by immunoprecipitating Lkb1 with a specific polyclonal antiserum followed by in vitro kinase assay. Lkb1 activity was undetectable in HeLa S3 cells, and markedly reduced in G361 cells, in accordance to their *LKB1* mRNA levels. Thus, measuring endogenous Lkb1 kinase activity provides a method to assess the function of Lkb1 in tumors and to detect *LKB1* gene inactivation.

Lkb1 Induces Cell Cycle Arrest (Study II)

To investigate the possibility that *LKB1* mRNA and kinase activity downregulation in HeLa S3 and G361 provides a growth advantage to these cells. G361 and HeLa S3 cells were transfected with an expression vector encoding both Lkb1 and a neomycin resistance gene, or a vector encoding the selection marker only. The transfections were subsequently subjected to G418 selection. A strongly reduced number of colonies was detected in *LKB1* transfections compared to the vector-only transfections in both G361 and HeLa S3 cells, indicating that re-expression of Lkb1 resulted in growth suppression in these cells.

The growth suppression by ectopic Lkb1 was limited to cells with undetectable or low endogenous levels of Lkb1 (such as G361 and HeLa S3 cells). Thus, cells resistant to growth inhibition by ectopic Lkb1 may have acquired mutations, which prohibit suppression by Lkb1. Alternatively, a regulatory subunit might be needed for full Lkb1 activity.

We also investigated whether Lkb1 kinase activity was necessary for growth inhibition and took advantage of three *LKB1* mutant alleles (303-306, 308 stop and G163D) that impair Lkb1 kinase activity. All three naturally occurring mutant *LKB1* alleles were unable to suppress growth of G361 cells. Indicating that Lkb1 kinase activity is required for the growth suppression.

The cell cycle changes in G361 cells transfected with either wild-type or mutant Lkb1 were studied using flow cytometry (FACS). Proliferating cells were arrested in mitosis with the microtubule-stabilizing agent nocodazole to reveal cells blocked in G_1. The G_1 fraction of wild-type *LKB1* transfected cells was 36% compared to 18% of 303-306 mutant transfected cells, indicating that *LKB1* growth suppression result from G_1 cell cycle arrest. The observation that Lkb1 induces cell cycle arrest provides evidence that *LKB1* tumor suppressor has a gatekeeper function.

The mechanism of the *LKB1*-mediated cell cycle arrest has been further studied by Marignani et al., who has demonstrated that Lkb1 protein associates with Brg1 chromatin remodeling protein and stimulates its ATPase activity *in vitro*. However, the increase in Brg1 ATPase activity was not dependent on Lkb1 kinase activity, since the inactive 303-306 mutant was also capable of increasing Brg1 activity. On the other hand, the Brg1-dependent growth arrest and flat cell morphology was shown to require Lkb1 kinase activity. This controversy might suggests that Lkb1 kinase activity may not be mediating all Lkb1 functions (Marignani et al., 2001).

In addition to cell cycle control, it has been suggested that Lkb1 might have role in programmed cell death. Karuman et al. have demonstrated that Lkb1 and p53 proteins interact and that Lkb1

translocates into mitochondria followed by apoptotic stimulus. Moreover, expression of a C-terminal truncation of Lkb1 was capable of inducing a p53-dependent cell death (Karuman et al., 2001). However, similar C-terminal truncations have been reported in cancer prone PJS patients (*Figure 1*), arguing that these mechanisms do not provide explanation for the increased cancer risk in PJS. Despite this controversy, other groups have also connected Lkb1 to p53 by demonstrating that Lkb1 phosphorylates p53 *in vitro* (Sapkota et al., 2001, Vaahtomeri et al. unpublished).

Similar to the Xenopus Lkb1 (Xeek1), mammalian Lkb1 contains a cAMP-dependent protein kinase A (PKA) consensus phosphorylation site (serine 431) on its C-terminus. This site has been shown to be phosphorylated by p90 ribosomal S6 kinase (p90RSK) and (PKA) *in vivo*. The functional relevance of this modification was studied by showing that a mutant lacking this phosphorylation site (S431A) was not able to suppress G361 melanoma cell growth. Interestingly, the S431A allele retains full kinase activity, thus separating Lkb1 kinase activity and growth suppression (Sapkota et al., 2001).

The C-terminus of Lkb1 has been reported to contain a prenylation sequence that is actively farnesylated *in vivo*. The functional relevance of this modification is still unclear. Abrogation of this motif did not alter the localization, activity or growth suppressive properties of Lkb1 (Collins et al., 2000; Sapkota et al., 2001). Moreover, a putative PKB/Akt phosphorylation site at threonine 336 has also been identified in the Lkb1 C-terminus (Sapkota et al., 2001) and the role of this potential regulatory element is currently under evaluation.

Expression of *Lkb1* and *Pten* During Mouse Development (Study III)

Peutz-Jeghers syndrome (PJS) and Cowden disease (CD) are conditions that share some clinical features, such as hamartomatous polyposis and increased risk of cancer. Due to the similarities of PJS and CD, *PTEN* and *LKB1* could function in the same cells and in the same signaling pathway. To analyze potential co-localization of *PTEN* and *LKB1* and their possible roles in embryonic development, we studied their expression during mouse embryonic development by RNA *in situ* hybridization.

Hybridization of *Lkb1* and *Pten* probes to embryonic day 7 – 11 (E7.0-E11.0) embryos revealed a high ubiquitous expression of both mRNAs in all extraembryonic and embryonic tissues. In later stages of development, the expression of both *Pten* and *Lkb1* became more pronounced in lung epithelium and mesenchyme, thyroid gland, thymus, salivary gland, kidney epithelium and urinary bladder epithelium. The embryonic liver contained relatively more *Lkb1* than *Pten* expression.

In the gastrointestinal tract, *Lkb1* and *Pten* signal was detected from the early stages of development. Later, an intense expression became restricted to the mucosal epithelium of the small intestine, colon and rectum. Both transcripts were also seen in the epithelium of the oral cavity, esophagus and stomach.

In the E15.0 embryo, the central nervous system (CNS) had relatively more expression of *Pten*, but later prominent expression of both genes was observed in the (CNS) and in peripheral nerve ganglia. *Lkb1* and *Pten* mRNAs were also present

at high levels in the epithelium lining the nasal cavity.

Additionally, intense *Lkb1* expression was noted in seminiferous tubules of the testis containing spermatogonia and Sertoli cells.

In summary, expression of *Lkb1* and *Pten* mRNA were observed in tissues and organs affected in Peutz-Jeghers syndrome and Cowden disease. In addition, similar expression patterns of *Lkb1* and *Pten* suggests that these genes may interact functionally during embryonic development.

In later studies, similar *LKB1* mRNA expression pattern has been reported in human fetal tissues. In adult human and mouse tissues high *LKB1* expression has been detected in testis, esophagus, and in crypts of the colonic villi (Rowan et al. 2000; Luukko et al. unpublished).

Mice Lacking *Lkb1* Die During Midgestation (Study IV)

To study the function of *Lkb1 in vivo*, we generated *Lkb1*-deficient mice by introducing an inactivating *Lkb1* allele into murine embryonic stem (ES) cells by homologous recombination (*Fig3, 4*).

In intercrosses of *Lkb1* heterozygous (*Lkb1$^{+/-}$*) mice, both *Lkb1$^{+/+}$* (n=87) and *Lkb1$^{+/-}$* (n=177) animals were observed at expected frequencies, while no *Lkb1$^{-/-}$* animals were obtained, demonstrating that *Lkb1$^{-/-}$* mice die during development. Analysis of *Lkb1$^{-/-}$* embryos throughout embryonic development revealed no abnormalities prior to embryonic day 7.5 (E7.5), and the majority of embryos appeared to develop normally up to E8.0.

Macroscopic analysis of *Lkb1$^{-/-}$* embryos beyond E8.25 revealed multiple abnormalities, including a failure of the embryo to turn, a defect in neural tube closure, and a hypoplastic or absent first branchial arch.

Whole-mount *in situ* hybridization was used to study the integrity of various developmental lineages in the *Lkb1$^{-/-}$* embryos at E8.5 and E9.5. The expression of the mesodermal marker brachyury showed that notochord developed normally along the anterior/posterior axis in the mutant embryos, but the defective somites in *Lkb1$^{-/-}$* embryos failed to express Engrailed1 at E9.5. No pronounced changes in the expression of *Wnt3A*, *Fgf8* or *Krox-20* were noted, suggesting that there was normal development of the mesoderm of the tail bud, forebrain and primitive streak, and normal segmentation of the hindbrain. No viable embryos were observed after E11.0, indicating that *Lkb1* is essential for embryonic development.

Severe Vascular Defects in *Lkb1$^{-/-}$* Embryos (Study IV)

Mutant embryos at E9.25 had a translucent appearance, suggesting the possibility of vascular defects. The embryonic vasculature was visualised by PECAM-1 immunostaining. Both mutant and wild-type embryos developed a paired dorsal aorta at E8.5, but the mutant aorta was thin and discontinuous particularly in the anterior part of the vessel. At E9.5, the lumen of the mutant aorta remained thin, with intersomitic branches terminating prematurely in the mesenchyme. Abnormalities were also observed in vascular smooth muscle cells (VSMCs) in E9.5 embryos. *Lkb1$^{-/-}$* embryos showed a complete absence of VSMC staining in the dorsal aorta and somites. In addition, an unusual, strong ectopic smooth muscle actin signal was detected in head folds of mutant embryos, which did not appear to

contribute to the supportive vascular structures.

Apoptosis in Lkb1^{-/-} Embryos (Study IV)

Sections of E9.5 *Lkb1^{-/-}* embryos often revealed large cystic degenerations near the dorsal aorta occasionally containing embryonic blood cells. Additionally, the surrounding cephalic mesenchyme had a lower cell density and fewer developing capillaries than controls. The decreased cell density was due to increased cell death in the mesenchyme as determined by TUNEL. This phenotype was limited to the E9.5 embryos; the E8.5 embryos showed similar number of TUNEL positive nuclei regardless of genotype suggesting that the increased cell death in the mesenchyme was secondary to the vascular defects.

Vascular Defects in *Lkb1^{-/-}* Yolk Sacs and Placentas (Study IV)

Analysis of extraembryonic tissues at E9.5 revealed that the mutant yolk sacs failed to develop large vitelline vessels and an extensive capillary network. Moreover, the vitelline artery was completely atretic in *Lkb1^{-/-}* yolk sacs, effectively disconnecting the embryo from the yolk sac (vitelline) circulation.

Mutant placentas at E9.5 were oedematous, hemorrhagic, and small in diameter and the invasion of embryonic blood vessels into the placenta did not occur in the mutant. *In situ* hybridizations with a VEGF receptor probes (*flk-1, flt-1)*, confirmed the lack of fetal blood vessels in the rudimentary labyrinth layer.

Deregulation of VEGF Expression in *Lkb1^{-/-}* Mice (Study IV)

To investigate the mechanism underlying the vascular defects we analysed the expression of vascular endothelial growth factor (*VEGF*), which is a key regulator of embryonic vascular development. *VEGF* mRNA was found to be deregulated in both the embryonic and extraembryonic compartments at E8.5 and E9.5. The *Lkb1^{-/-}* placentas exhibited markedly diminished *VEGF* mRNA expression particularly in the trophoblast giant cells, whereas the *Lkb1^{-/-}* embryos expressed abnormally elevated levels of *VEGF* in several tissues. This demonstrated that *Lkb1* can both positively and negatively regulate VEGF expression in a tissue-specific manner.

VHL is a component of a SCF-like E3 ubiquitin-protein ligase complex that regulates VEGF expression by targeting the Hypoxia Inducible Factor 1a (HIF-1a) to proteosome-mediated degradation (Maxwell et al., 1999; Cockman et al., 2000; Ohh et al., 2000). Interestingly, disruption of the murine *VHL* tumor suppressor gene results in a similar down-regulation of *VEGF* in the extraembryonic tissues (Gnarra et al., 1997), while the expression of *VEGF* in *VHL^{-/-}* embryos has not been reported. *VHL* loss in other systems has been found to lead to up regulation of *VEGF* (Mukhopadhyay et al., 1997; Siemeister et al., 1996).

VEGF Expression in *Lkb1^{-/-}* MEFs (Study IV)

To study the VEFG expression in individual cells wild-type and mutant mouse embryonic fibroblasts (MEFs) from littermate embryos were isolated and subjected to hypoxia to induce VEGF

expression. VEGF levels were measured from the cell culture media by ELISA. Analysis of VEGF levels in cell culture media revealed that the mutant MEFs produced significantly higher levels of VEGF than controls in hypoxic conditions demonstrating that Lkb1 regulates VEGF expression.

To study the role of Lkb1 in hypoxia responses we monitored the HIF-1 dependent induction of glucose transporter 1 (Glut1) in hypoxia. HIF-1 is a transcription factor consisting of HIF-1 and ARNT proteins forming a heterodimeric complex to activate target gene transcription (Wang et al., 1995; Jiang et al., 1996; Wood et al., 1996). In hypoxic conditions HIF-1 translocates into nucleus and binds to the hypoxia responsive elements (HRE) at promoters and activates transcription of Glut1, erythropoetin, VEGF and several enzymes involved in the glycolytic pathway (Semenza et al., 1994; Forsythe et al.,

1996; Kallio et al., 1998). Comparable induction of Glut1 in wild-type and mutant MEFs suggested that the HIF-1 pathway was intact in *Lkb1* mutant cells. Moreover, VEGF secretion was increased in normoxic conditions in the mutant MEFs arguing that VEGF deregulation was not hypoxia-dependent.

In order to explore the mechanism by which *Lkb1* regulates VEGF expression we studied the activation of p42/44[MAPK] and p38 kinases implicated in VEGF regulation (Milanini et al., 1998; Pal et al., 1997; Xiong et al., 2001). Comparable levels of activated p42/44[MAPK] and p38 kinases were observed in *Lkb1* mutant and wild-type MEFs suggesting that Lkb1 regulates VEGF expression by alternative mechanisms.

These results show that loss of Lkb1 function leads to increased basal and induced expression of VEGF in fibroblasts thereby placing Lkb1 in the VEGF signaling pathway.

CONCLUDING REMARKS

Cancer is a disease, which is characterized by malignant transformation and clonal expansion of cells. Tumor suppressor genes and oncogenes control this process. The *LKB1* tumor suppressor gene mutated in Peutz-Jeghers syndrome encodes a serine/threonine kinase.

This work describes two separate functions for the Lkb1 kinase. The observation that Lkb1 activity induces cell cycle arrest demonstrates that Lkb1 has a gatekeeper function, suggesting that *LKB1* mutations provide a growth advantage to cells that promote transformation and tumor development. On the other hand, Lkb1 was shown to regulate VEGF expression in Lkb1 deficient mice. This surprising finding suggests that decreased Lkb1 activity may convert cells to an angiogenic state promoting the recruitment of new bloodvessels into the tumor.

Angiogenesis plays a key role in the development of cancer and metastasis. New blood vessels feed the expanding tumor and provide a route for metastasis. Large parts of the tumor are deprived of oxygen due to an insufficient blood supply (Helmlinger et al., 1997). This activates the expression of genes that facilitate cells to adapt to hypoxia. Thus, tumor switches to an angiogenic state to meet the metabolic needs of the rapidly growing cells.

Besides direct growth control some tumor suppressors regulate angiogenesis by controlling the expression of angiogenic mediators. The observation that PTEN, VHL and p53 are able to inactivate HIF-1, suggests that the HIF-1 transcription factor is crucial for the tumor suppressor-mediated angiogenesis. Consequently, inactivation of these tumor suppressors result in the activation of HIF-1 leading to sustained expression of VEGF (Maxwell et al., 1999; Ravi et al., 2000; Zundel et al., 2000). The observation that HIF-1 mediated hypoxia response, p42/44^{MAPK} and p38 kinase pathways were unaffected in Lkb1 deficient cells suggest, that Lkb1 may regulate VEGF expression by an alternative less well-characterized mechanism. Studying the mechanism how Lkb1 regulates VEGF levels may increase our understanding on the angiogenesis process and lead to novel therapies targeting VEGF.

The study of Peutz-Jeghers syndrome has been restrained due to the rarity of the disease and limited material. Using Lkb1 heterozygous mice as a model for PJS could circumvent this problem. In fact, recent findings suggest that these mice mimic the PJS-associated polyposis providing a valuable tool to study the Lkb1-dependent tumorigenesis *in vivo*. These studies may provide insight on the role of Lkb1-dependent cell cycle control and VEGF deregulation in the development of Peutz-Jeghers syndrome and cancer.

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