LKB1 tumor suppression in Peutz-Jeghers syndrome

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“A high-powered mutant never even considered for mass-production. Too weird to live, too rare to die."

- Hunter S. Thompson

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ABREVIATIONS

AMP	adenosine monophosphate
AMPK	amp activated protein kinase
APC	adenomatous polyposis coli
ATP	adenosine triphosphate
BMP4	bone morphogenic protein 4
BRSK	BR serine/threonine kinase 1
CDK7	cyclin-dependent kinase 7
COX-2	cyclooxygenase-2
CPG	change In polyposis grade
DHPLC	denaturing high-performance liquid chromatography
D-MEM	Dulbecco's modified eagle media
ECL	enhanced chemiluminescence
EGFR	epithelial growth factor receptor
FAP	familial adenomatous polyposis
FCS	fetal calf serum
FGF	fibroblast growth factor
GI-tract	gastrointestinal tract
GST	glutathione S-transferase
HCC	hepatocellular carcinomas
HGF	hepatocyte growth factor
IL-6	interleukin 6
K14	keratin 14
KRAS	Kirsten rat sarcoma viral oncogene
LOH	loss of heterozygosity
LSB	Laemmli lysis buffer
MARK	microtubule affinity-regulating kinase
MEF	mouse embryonic fibroblast
MLPA	multiplex ligation-dependent probe amplification
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MVD	microvessel density
PAGE	polyacrylamide gel electrophoresis
par-1	partitioning gene-4
PCR	polymerase chain reaction
PGE2	prostaglandin E-2
PI3K	phosphatidylinositol 3-kinase
PJS	Peutz-Jeghers syndrome
RB1	retinoblastoma-1
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
SIK	salt-inducible protein kinase
siRNA	short interfering ribonucleic acid
SM22	smooth muscle gene 22 / Transgelin
SMA	smooth muscle actin
SMC	smooth muscle cell
STRAD	STE20-related adaptor

Tagln	Transgelin / SM22
TCF	transcription factor
TGFß	transforming growth factor ß
TGFßRII	TGFß receptor 2
Tris	hydroxymethylaminoethane
TSC	tuberous sclerosis complex
TUNEL	Terminal uridine nick-end labeling
VEGF	vascular endothelial growth factor
wt	wild type

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals. In addition, some unpublished data is presented.

I Mutation analysis of three genes encoding novel LKB1 interacting proteins, BRG1, STRADα, and MO25α, in Peutz-Jeghers Syndrome

Alhopuro P, Katajisto P, Lehtonen R, Ylisaukko-oja SK, Näätsaari L, Karhu A, Westerman AM, Wilson JHP, de Rooij FWM, Vogel T, Moeslein G, Tomlinson IP, Aaltonen LA, Mäkelä TP, Launonen V.

British Journal of Cancer, 2005 Mar 28;92(6):1126-9.

II Suppression of Peutz-Jeghers polyposis by inhibition of cyclooxygenase-2

Udd L, Katajisto P, Rossi DJ, Lepistö A, Lahesmaa AM, Ylikorkala A, Järvinen HJ, Ristimäki AP, Mäkelä TP.

Gastroenterology, 2004 Oct;127(4):1030-7.

III LKB1 Signaling in Mesenchymal Cells Required for Suppression of Gastrointestinal Polyposis

Katajisto P, Vaahtomeri K, Ekman N, Ventelä E, Ristimäki A, Bardeesy N, Feil R, DePinho RA, Mäkelä TP.

Nature Genetics, 2008 Apr;40(4):455-9.

ABSTRACT

Peutz-Jeghers syndrome (PJS) is a condition characterized by gastrointestinal polyposis and increased risk of cancer. At the onset of this study, mutations in the *LKB1* serine/threonine kinase had been identified in approximately half of the studied PJS patients, indicating that *LKB1* is a tumor suppressor underlying at least a subset of PJS cases. Mice with targeted heterozygous deletion of *Lkb1* develop gastrointestinal polyps remarkably similar to PJS polyps corroborating the tumor suppressor function of *LKB1/Lkb1*. Most importantly, the polyps in $Lkb1^{+/-}$ mouse contain a notable stromal compartment with a core of smooth muscle cells that is the defining characteristic of a PJS polyp. In addition, the PJS and mouse polyps frequently exhibit elevated cyclooxygenase-2 (COX-2), a feature also noted in other gastrointestinal polyposis syndromes. Despite the aforementioned characterizations, the molecular mechanism driving PJS polyp development has not been identified.

As only a subset of PJS patients have been reported to harbor *LKB1* mutations, mutations in other loci might represent an alternative cause of PJS. To characterize the genetic background of PJS, mutation analysis of three genes encoding LKB1 interacting proteins, *BRG1*, *STRADα*, and *MO25α*, was performed in PJS patients without identified *LKB1* mutations. No disease causing mutations were detected in these genes. The exclusion of the studied candidate genes with these criteria, and the concurrent increase in frequency of detected *LKB1* mutations does not support involvement of other loci in PJS.

As COX-2 inhibition has been effective in suppression of familial adenomatous polyposis, the elevation of COX-2 in PJS polyps suggested that COX-2 inhibition could decrease PJS polyposis. This was investigated by genetic and pharmacological interventions that targeted COX-2 in the $Lkb1^{+/-}$ mice. In comparison to PJS modeling $Lkb1^{+/-}$ mice, the polyp burden in the $Lkb1^{+/-}$ mice with either mono- or biallelic deletion of *COX-2* was dramatically reduced. Furthermore, a selective COX-2 inhibitor celecoxib efficiently suppressed the tumor burden in $Lkb1^{+/-}$ mice. COX-2 inhibition reduced particularly the incidence of the large (>5mm) polyps whereas the number of smaller polyps was unaffected, suggesting that COX-2 expression is not required for polyp initiation but contributes significantly to tumor growth. These results prompted a pilot clinical trial with PJS patients receiving 200mg of celecoxib twice a day for 6 months. A subset of PJS patients (2/6) responded favorably to the celecoxib treatment, and a significant reduction in polyposis severity was also noted when the polyp grading data of all patients were combined. Together the data establish the polyposis promoting role of COX-2 in Peutz-Jeghers polyposis, and suggest that COX-2 inhibition is efficient in suppression of PJS polyposis.

Polyp development is commonly thought to result from mutations in epithelial cells. However, the characteristic stroma of PJS polyps together with the suggestion that polyps can

retain a functional copy of *Lkb1* raised the possibility of non-epithelial origin of tumorigenesis in PJS. To investigate this possibility, murine *Lkb1* was deleted tissue specifically in smooth muscle cells. Both monoallelic and biallelic deletions resulted in the development of gastrointestinal polyps indistinguishable from PJS polyps. Moreover, multiplicity of tumors in the smooth muscle specific biallelic deletion was only moderately (1.8-fold) higher than in the monoallelic deletion, demonstrating that *Lkb1* LOH in stromal cells is not required for polyp development. The *Lkb1* deficient mesenchymal cells produced less TGFß, and p-Smad2 staining was reduced in epithelial cells adjacent to *Lkb1* deleted mesenchyme. These results identified impaired TGFß signaling from mesenchyme to epithelium as a possible mechanism for polyp development. Consistently, the epithelial proliferation in tumors coincided with the reduced p-Smad2 staining and with *Lkb1* deficiency of the adjacent stroma. The TGFß signaling defect was importantly also noted in polyps from PJS patients, supporting the role of stroma as a major contributor to PJS polyposis by generation of a permissive microenvironment for epithelial expansion.

The results of this thesis contribute to the notion that *LKB1* is the only PJS gene substantiating the findings from the *Lkb1* heterozygous mouse model of PJS. From the viewpoint of therapy development, the results provide proof of principle for the efficacy of COX-2 chemoprevention in PJS. Furthermore, the discovery of the stromal-derived mechanism of *LKB1* tumor suppression forms a basis for the development of novel therapeutic interventions targeting mesenchymal signaling in PJS polyposis and possibly other cancers with loss of *LKB1*.

REVIEW OF THE LITERATURE

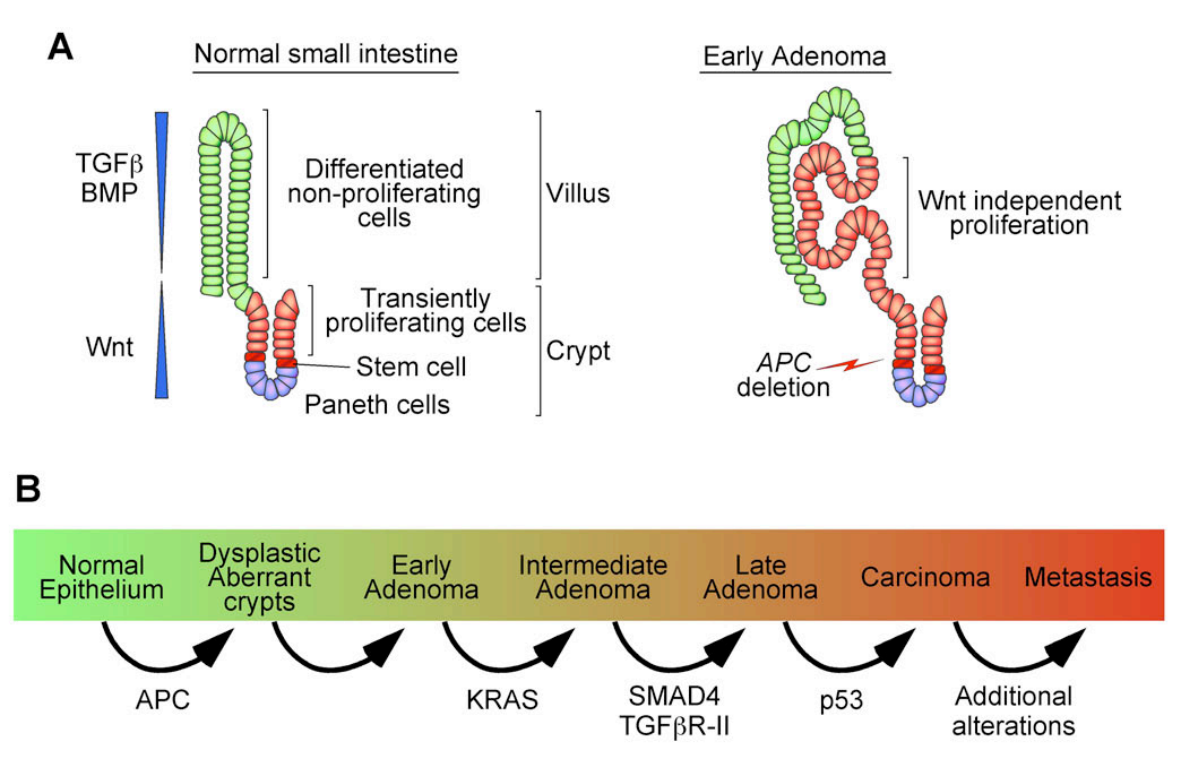
Tumor suppressors

Tumorigenesis is generally considered to be a consequence of mutations that will cause a cell to multiply pathologically. This view was first based on chromosomal abnormalities often noted in cancer cells, but it was the discovery of the cellular proto-oncogenes and oncogenes (Stehelin et al. 1976) that explained how rearrangements of DNA could result in defective growth control and cancer. However, many familial cancer syndromes were hard to explain by activated oncogenes, and the fundamental role of growth regulation was regarded too important to be controlled only by positive control of the oncogenes.

The first evidence for negative growth regulators, or “anti-oncogenes” that were later termed tumor suppressors, came from studies on the childhood eye tumor retinoblastoma. These tumors occur in sporadic and familial forms. While sporadic tumors appear unilaterally, that is in one eye, the tumors in familial cases are present bilaterally and appear multifocal even within a single eye. Combining this difference with his ground braking work on the kinetics of tumor appearance in the hereditary and sporadic cases, Alfred Knudson concluded that both forms could be explained by loss of a single gene (Knudson 1971). According to his "two-hit" hypothesis individuals with familial cases inherit one mutated allele of this *RB1* gene and require a single additional mutation event, or a "second hit", to begin tumorigenesis. Correspondingly, the sporadic retinoblastomas are delayed compared to familial cases, as the stochastic mutation events have to inactivate both alleles of the first identified tumor suppressor *RB1*.

Studies on many other cases of cancer have since demonstrated that progression of cancer is a multistep process, commonly involving sequential malfunctions in both oncogenes and tumor suppressors (Hahn et al. 2002). Studies on gastrointestinal polyposis syndromes, particularly on Familial Adenomatous Polyposis (FAP) have been most revealing in this regard. Gastrointestinal tumors in patients with FAP develop due to hereditary mutations in the *Adenomatous polyposis coli* (*APC*) tumor suppressor gene (Groden et al. 1991; Kinzler et al. 1991). Functioning according to Knudson’s “two-hit” hypothesis, a second sporadic mutation of *APC* is noted in the tumor cells of epithelial origin (Nakamura et al. 1991). However, while loss of *APC* lays the groundwork for clonal expansion, development and progression of adenomas requires an additional oncogenic mutation, most commonly of *KRAS* (Vogelstein et al. 1988) (Box 1). These adenomatous polyps develop in FAP by the hundreds, but still only some of them progress to cancer. For this to happen, the epithelial cells typically gain additional inactivating mutations of tumor suppressors *SMAD4* and *p53* that allows for the accelerated clonal expansion and transformation required for cancer development (reviewed in Fodde et al. 2001). Importantly, loss of *APC* is noted in approximately 85% of both

familial and sporadic colorectal cancers disregarding their stage (Kinzler et al. 1996). This demonstrates that the very first "nudge" toward cancer, caused by the loss of a tumor suppressor identified from hereditary polyposis is of utmost importance for cancer in general.



Box 1. Model for the sequence of events occurring in colorectal cancer development.

A. Schematic figure of factors involved with homeostasis maintenance and cancer initiation in the small intestine. Normally the balance of cellular proliferation and differentiation of the gastrointestinal epithelium is maintained by Wnt, Transforming growth factor beta (TGFß) and Bone morphogenetic protein (BMP) signaling. Wnt signaling is required for the rapid proliferation (red cells) to compensate for continuous shedding of differentiated cells (green cells) from the villus tip. Deregulated Wnt pathway is the hallmark of Familial adenomatous polyposis (FAP) where mutations in *APC* lead to inability to degrade Wnt mediator ß-catenin (reviewed in Fodde et al. 2001). As a result, $APC^{-/-}$ cells do not respond to reduction in Wnt signals that occurs while cells migrate towards the villus tip. This leads to development of an adenoma due to sustained Tcf/Lef mediated transcription of multiple genes involved in driving the cell cycle and maintaining an undifferentiated, proliferating state of cells.

B. Development of colorectal cancer requires multiple genetic alterations. Deletions of *APC* constitute the first step towards cancer in both FAP and in 85% of non-familial colorectal cancers (1 hereditary and 1 sporadic mutation in FAP, and 2 sporadic mutations in non-familial cancer). Loss of *APC* leads to genomic instability and typically to activation of *KRAS* oncogene and to deletions of members of the TGFß pathway, *SMAD4* or *TGFßR-II* to drive tumor progression. Malignant transformation is frequently associated with mutations in the *p53* tumor suppressor gene. Modified from Radtke et al. 2006.

Peutz-Jeghers Syndrome

Peutz-Jeghers Syndrome (PJS) is a rare hereditary polyposis syndrome that was first described by Peutz in 1921 (Peutz 1921) and Jeghers in 1949 (Jeghers et al. 1949). The main characteristics of PJS are mucocutaneus pigmentation and gastrointestinal polyposis. While the pigmentation of the lips and mouth (sometimes also of hands and genitalia) can be quite distinct at infancy, it commonly fades with age. Diagnosis of PJS is made when the patient meets two of the following criteria: two or more gastrointestinal polyps, mucocutaneous pigmentation or family history of PJS (Vasen 2000).

The most common cause of symptoms and thus diagnosis of PJS are the gastrointestinal polyps that manifest in adolescence by abdominal pain, bleeding, obstructions or intussusceptions (Vasen 2000). Polyps are most commonly reported in the small intestine where their occurrence is 80% among PJS patients (Allen et al. 2003). However, polyps are found throughout the gastrointestinal tract with occurrences of approximately 40% in stomach, colon and rectum (Utsunomiya et al. 1975; Allen et al. 2003) (Fig. 1). In accordance with their hamartomatous nature, the PJS polyps are benign and display a pedunculated appearance with apparently near-normal cellular composition according to basic histological analysis. The most prominent feature is the arborizing smooth muscle core that also serves as the defining attribute of a PJS polyp (Fig. 1).

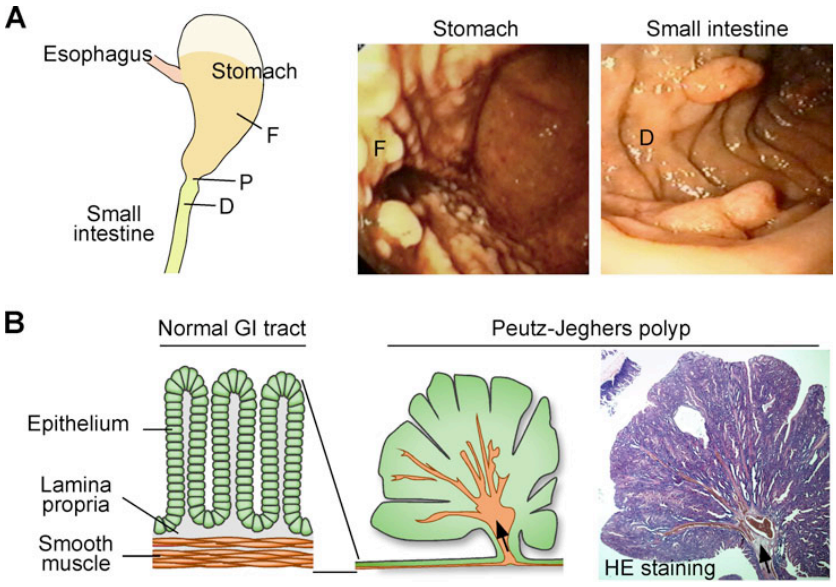


Figure 1. Gastrointestinal polyposis in Peutz-Jeghers syndrome.

A. Polyps are observed in the stomach and small intestine by gastroscopy. The schematic picture on the left points the typical localization of tumors. F, fundus; P, pylorus; D, duodenum. **B.** Normal cellular composition of the gastrointestinal tract contains epithelium surrounded by connective tissue of lamina propria and layers of smooth muscle. In a histological HE-staining, PJS polyps distinctively display a branching smooth muscle core (black arrow) together with lamina propria and non-dysplastic epithelium. Modified from Katajisto et al. 2007.

As there currently is no noninvasive therapy for PJS polyposis, the management is restricted to surgery. Patients are often forced to undergo multiple laparotomies in order to rid themselves of symptomatic polyps. Surveillance recommendations propose upper gastrointestinal endoscopy and colonoscopy every 2-3 years to facilitate the removal of clinically relevant polyps prior to complications (Giardiello et al. 2006; Zbuk et al. 2007). Even though the surveillance of polyposis particularly in the small intestine will benefit from advances in the capsule and double-balloon endoscopy, development of therapies reducing polyp growth is desirable, as they would reduce the need of surgical interventions.

In addition to gastrointestinal hamartomatous polyps, PJS patients have an increased risk of cancer (Giardiello et al. 1987; Spigelman et al. 1989; Gruber et al. 1998; Lim et al. 2004; Hearle et al. 2006; Mehenni et al. 2006) with 4-15 fold relative risk of any cancer as compared to the general population (Giardiello et al. 2000; Hearle et al. 2006; Mehenni et al. 2006). While gastrointestinal cancers are most frequent with 90-270 fold relative risk (Giardiello et al. 2000; Hearle et al. 2006; Mehenni et al. 2006), increased risk of pancreatic, lung, breast, and gynecologic cancers have also been reported (Lim et al. 2004; Hearle et al. 2006). Whether the coinciding locale of the benign hamartomatous polyps in PJS predisposes to the dramatically high incidence of gastrointestinal cancers has remained an open question. Dysplasia is occasionally noted in PJS polyps suggesting that such "hamartoma-carcinoma" sequence may exist (Bosman 1999).

Genetics of PJS – discovery of *LKB1*

Genetic linkage studies demonstrated that inactivating mutations in the serine-threonine kinase *LKB1* (*STK11*) are found in PJS (Hemminki et al. 1997; Hemminki et al. 1998; Jenne et al. 1998). This distinguished *LKB1* as the first identified tumor suppressor kinase. The *LKB1* gene spans 23kb and has 10 exons. Even though *LKB1* mutations are found with comparable frequencies in both sporadic and familial PJS cases (Ylikorkala et al. 1999; Boardman et al. 2000; Scott et al. 2002), the overall detection frequency of *LKB1* inactivating mutations has varied between 21-70% in studies relying on analysis of the coding region and splice site mutations. (Nakagawa et al. 1998; Wang et al. 1999; Westerman et al. 1999; Ylikorkala et al. 1999; Boardman et al. 2000; Olschwang et al. 2001; Scott et al. 2002; Lim et al. 2003; Schumacher et al. 2005; Mehenni et al. 2007). Together with reports on PJS families unlinked to *LKB1* (Mehenni et al. 1997; Olschwang et al. 2001), this relatively low rate of detected mutations suggested the possible existence of other PJS loci. However, studies on 19q13.4, that demonstrates genetic linkage to some PJS families, have not revealed mutations in the subsequently screened genes (Marneros et al. 2001; Buchet-Poyau et al. 2002; Hearle et al. 2004) and so far other PJS loci have not been identified.

LKB1 interacting proteins

LKB1 is a kinase of 433 amino acids that shares little relation with any particular kinase family of the human kinome (Manning et al. 2002). It forms a trimeric complex with a pseudokinase STRAD and the scaffolding protein MO25 (Baas et al. 2003; Boudeau et al. 2003; Brajenovic et al. 2004). There are two functionally redundant isoforms (α and ß) of both STRAD and MO25, and the trimeric complex is tethered to the cytoplasm (Baas et al. 2003; Boudeau et al. 2003). This is where LKB1 also appears to mediate its known cellular functions, including the ability to suppress growth (Tiainen et al. 2002). Importantly, formation of the trimeric complex is required for full kinase activity of LKB1 (Baas et al. 2003; Boudeau et al. 2003). Some of the point mutations of LKB1 found in PJS disrupt the complex formation in addition to kinase activity (Boudeau et al. 2004), suggesting a role for STRAD and MO25 in molecular pathogenesis of PJS.

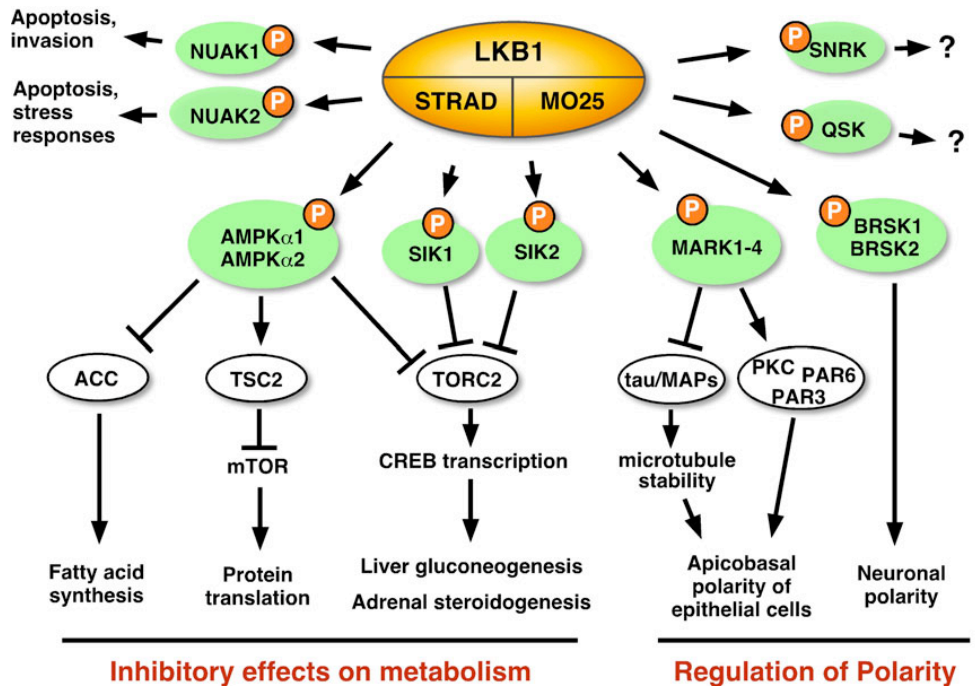


Figure 2. LKB1 signaling pathways mediated by the 14 substrate kinases.

Following the discovery of AMPK kinase family substrates, LKB1 has been implicated in various aspects of cellular metabolism and polarity control. Functions of some LKB1 substrates appear to overlap, as is exemplified by repression of CREB mediated transcription via TORC2 phosphorylation by both SIKs and AMPKs. Whereas SIK1 and SIK2 are inactivated through hormonal induction of cAMP and PKA (Katoh et al. 2004; Screaton et al. 2004; Shaw et al. 2005), AMPK inactivates TORC2 in response to elevated AMP at least in cultured hepatocytes (Koo et al. 2005). Thus this pathway integrates cellular (AMPK) and hormonal (SIK) inputs to negatively regulate transcriptional events promoting synthesis of gluconeogenic enzymes (Shaw et al. 2005). See text for more detailed introduction to the relevance of the other pathways in PJS. Modified from Katajisto et al. 2007.

Another LKB1 interactor, BRG1, was identified with LKB1-GST pull down from an *in vitro* translated HeLa cell cDNA library (Marignani et al. 2001). BRG1 is interesting regarding the LKB1 tumor suppressor function as *Brg1* heterozygous mice form subcutaneous tumors (Hendricks et al. 2004), and loss of BRG1 is a prognostic indicator of non-small cell lung cancer (Fukuoka et al. 2004); the lung cancer type also associated with loss of LKB1 (see below). BRG1 is the essential catalytic helicase of the SWI/SNF chromatin remodeling complex, and is involved with epigenetic regulation of gene expression (Xi et al. 2008) important in development, differentiation and carcinogenesis. Upon association with BRG1, LKB1 has been proposed to stimulate BRG1 and to have a role in BRG1 dependent growth arrest (Marignani et al. 2001).

Facilitated by the increased activity of STRAD:MO25 bound LKB1, the first physiological substrate of LKB1 was identified to be the AMP-activated protein kinase (AMPK) (Hawley et al. 2003; Woods et al. 2003; Shaw et al. 2004). The number of physiological substrates quickly extended to 14 serine/threonine kinases referred to as the AMPK-related kinases or AMPK subfamily due to sequence similarity of their kinase domains (Lizcano et al. 2004; Jaleel et al. 2005). LKB1 phosphorylates the "T-loop" threonine in the kinase domain of the substrates, and this phosphorylation is required for their full activity. Via the substrates, LKB1 is implicated to regulate cellular energy management and polarity, and possibly other pathways as functions of some substrates are poorly characterized (Fig. 2).

LKB1 regulates cellular energy levels and protein synthesis

Depletion of cellular ATP is reflected as an increase of free AMP, which leads to activation of AMPK (Kahn et al. 2005). For AMPK to be active, the trimeric AMPK complex needs to bind AMP in order to allow the activating phosphorylation by LKB1 (Hawley et al. 2003). AMPK therefore functions as the cellular energy sensor, and upon activation it shuts down lipid and glycogen synthesis and promotes fatty acid oxidation and glycolysis to restore cellular energy levels (Fig. 2) (reviewed in Carling 2005). In addition, AMPK also targets both transcription (Foretz et al. 1998; Koo et al. 2005) and translation (Bolster et al. 2002) to inhibit cell growth and proliferation (Carling 2005). To inhibit translation, AMPK phosphorylates and activates TSC2, which suppresses the mTOR pathway activation (Corradetti et al. 2004; Shaw et al. 2004). Interestingly, TSC2 is a tumor suppressor mutated in tuberous sclerosis (Consortium 1993; van Slegtenhorst et al. 1997) that is characterized by various features including hamartomatous tumors in diverse organs (Jozwiak 2006). This similarity in etiology between TSC and PJS suggests a possible role for deregulated AMPK-mTOR pathway in PJS type polyp development.

Regulation of polarity by LKB1

The involvement of LKB1 in polarity control was first noted in *C. elegans* where two of the identified mutants failing in asymmetric cell divisions were the orthologues of mammalian *LKB1* and *MARK* kinases (Fig. 2) termed *par-4* and *par-1* respectively (Kemphues et al. 1988). Supporting a role for LKB1 also in mammalian polarity control, co-expression of LKB1 and STRAD in epithelial cancer cells resulted in a formation of an actin cap and in a directed endosomal transportation resembling polarization (Baas et al. 2004). Besides the four MARK kinases, the LKB1 substrates BRSK1 and BRSK2 (SAD-A and SAD-B) (Fig. 2) have also been implicated in polarity regulation as their double knockout leads to reduced axon growth and mislocalization of axon and dendritic markers in mouse (Crump et al. 2001; Kishi et al. 2005). This neuronal polarity regulation by BRSKs is dependent on activation by LKB1 (Barnes et al. 2007).

Suggesting that disruption of cellular polarity might also play a role in tumorigenesis following *LKB1* deletion, LKB1 depletion by RNAi in lung cancer cell lines causes cell polarity defects including aberrant Golgi positioning, reduced lamellipodia formation, and aberrant morphology (Zhang et al. 2008). Based on the general relevance of polarity in tumorigenesis and cancer, it has also been speculated that PJS polyposis may be a result of polarity defects of the gastrointestinal epithelium (Jansen et al. 2006; de Leng et al. 2007).

LKB1 in the control of cell proliferation

Polyp formation in PJS could be due to the increased cell proliferation or decreased apoptosis resulting in imbalance of renewal and death of cells. Several studies indicate that re-introduction of LKB1 into various cancer cell lines lacking its expression induces a G1 cell cycle arrest (Tiainen et al. 1999; Sapkota et al. 2001; Shen et al. 2002; Jimenez et al. 2003; Qiu et al. 2006), and in some studies the noted G1 arrest correlates with the induction of cell cycle regulator p21WAF/CIP (Shen et al. 2002; Tiainen et al. 2002). Moreover, the LKB1 induced growth arrest is dependent on LKB1 kinase activity as several mutants failed to inhibit cell growth (Tiainen et al. 1999).

The Wnt signaling pathway is intimately involved with gastrointestinal proliferation and tumorigenesis (reviewed in Radtke et al. 2006) (see Box 1). LKB1 has been reported to suppress Wnt signals by phosphorylation of MARK3/PAR1A to inhibit Wnt mediator disheveled (Spicer et al. 2003). In addition, LKB1 has been reported to modulate Wnt-signaling both positively (Ossipova et al. 2003) and negatively (Lin-Marq et al. 2005) via direct regulation of GSK3ß, a kinase targeting ß-catenin for degradation. Consequently, the relationship of Lkb1 with the Wnt pathway is unclear.

Similarly to Wnt signaling, the role of LKB1 in apoptosis is currently ambiguous. Lkb1 has been reported to mediate p53-dependent apoptosis by physically interacting with p53 (Karuman et al. 2001). On the other hand, LKB1 deficient cells are sensitized to apoptosis

(Shaw et al. 2004). Furthermore, the LKB1 substrates NUAK1 and NUAK2 (Fig. 2) have been linked to apoptosis indirectly (reviewed in Alessi et al. 2006).

Taken together, several LKB1 mediated signaling pathways with potential implications in tumorigenesis been identified. The relevance of these pathways in PJS pathology has however not been addressed, and it is unclear whether they or other yet undiscovered pathways underlie PJS tumorigenesis.

Mouse model of PJS

The identification of *LKB1* enabled the generation of mice with targeted mutations at the *Lkb1* locus in an attempt to create a mouse model for PJS, and to study this novel tumor suppressor in a physiological setting. Homozygous inactivation of *Lkb1* demonstrated that the gene is essential for embryogenesis (Ylikorkala et al. 2001; Bardeesy et al. 2002; Jishage et al. 2002; Miyoshi et al. 2002). *Lkb1*$^{-/-}$ mouse embryos do not survive beyond embryonic day E10.5, and display severe defects in their vasculature demonstrating a critical function of *Lkb1* in murine angiogenesis possibly through regulation of vascular endothelial growth factor VEGF (Ylikorkala et al. 2001).

Analysis of heterozygous *Lkb1*$^{+/-}$ mice confirmed the tumor suppressor function of *Lkb1* as the heterozygous mice exhibited widespread gastrointestinal polyposis (Bardeesy et al. 2002; Jishage et al. 2002; Miyoshi et al. 2002; Rossi et al. 2002). Polyps are detected at 5 months, and cause premature lethality by gastrointestinal obstruction from 8 months onwards. Importantly, the polyps were remarkably similar to hamartomas of PJS patients both macroscopically and histologically, indicating that the *Lkb1*$^{+/-}$ mouse represents a mouse model of PJS (Bardeesy et al. 2002; Jishage et al. 2002; Miyoshi et al. 2002; Rossi et al. 2002).

The generation of a PJS mouse model has enabled a more detailed analysis of the molecular mechanisms involved in PJS-type polyposis. From the viewpoint of therapy development, the two discoveries on the mTOR and COX-2 pathways in the *Lkb1*$^{+/-}$ mouse polyps are particularly interesting. The *in vitro* discovered LKB1-AMPK-TSC2-mTOR link (see above and Fig. 2) implied that increased mTOR activity might participate to tumor development in PJS. Supporting this notion, increase in phosphorylation of mTOR effectors S6K1, S6 and 4E-BP was noted in the polyps of *Lkb1*$^{+/-}$ mice (Shaw et al. 2004). These results suggest that inhibition of mTOR by rapamycin might suppress polyposis.

Induction of COX-2 was noticed already by one of the original studies describing the PJS mouse model (Rossi et al. 2002). COX enzymes catalyze the rate-limiting step in the synthesis of prostaglandins, which are bioactive lipid molecules mediating a large variety of tissue-specific effects (reviewed in Cha et al. 2007). While the isoform 1 of COX (COX-1) is constitutively expressed and catalyses production of prostaglandin PGI_2 (Craven et al. 1983; Smith et al. 1982; Lawson et al. 1987) that is important for homeostasis maintenance of

gastric mucosal surface (McAdam et al. 1999), COX-2 is inducible by inflammatory stimuli and synthesizes most importantly PGE_2 (Herschman et al. 1995) (Fig. 3).

COX-2 induction has also been noted in PJS patient polyps (Rossi et al. 2002; De Leng et al. 2003; McGarrity et al. 2003; Wei et al. 2003; Takeda et al. 2004) and carcinomas (De Leng et al. 2003; Wei et al. 2003). COX-2 is also induced in sporadic intestinal adenomas and adenocarcinomas (Eberhart et al. 1994), and in FAP polyps as COX-2 is a downstream target of ß-catenin/Tcf-signaling that is activated following *APC* mutations (reviewed in Wang et al. 2005) (Fig. 3). *Lkb1*$^{+/-}$ polyps demonstrate a modest increase of β-catenin, but it is strictly localized to the cell membrane of the epithelial cells (Rossi et al. 2002) suggesting that deregulated Wnt signaling is not the cause of Cox-2 induction in *Lkb1*$^{+/-}$ polyps. However, pharmacological COX inhibition has been effective in polyp suppression both in FAP patients (Giardiello et al. 1993) and in FAP mouse models (Oshima et al. 1996), indicating the important role of COX-2 induction in FAP polyposis. This chemopreventive effect in FAP together with the noted COX-2 induction in *Lkb1*$^{+/-}$ polyps suggests that COX-2 may also contribute to PJS type polyposis.

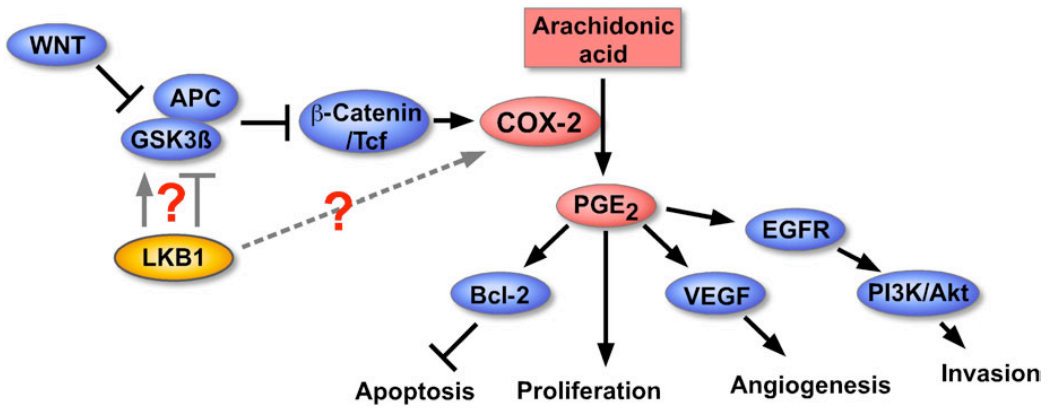


Figure 3. Roles of COX-2 in cancer.

COX-2 derived PGE_2 is a mediator of multiple molecular mechanisms that contribute to carcinogenesis. These include inhibition of apoptosis, increase of proliferation and induced angiogenesis. Whether mutations of LKB1 cause induction of COX-2 via deregulation of ß-catenin (as is the case in FAP due to APC mutations), or via another yet unidentified pathway is unclear. Modified from Wang et al. 2005.

Loss of heterozygosity of *LKB1* and polyp development

According to the classical tumor suppressor definition both alleles of the tumor suppressor gene must be inactivated for oncogenic effects to take place. According to this dogma, loss of heterozygosity (LOH) of the tumor suppressor gene marks the cells driving familial tumorigenesis. Surprisingly, subset of the murine hamartomatous polyps have a functional copy of *Lkb1* in both epithelial and stromal cells (Jishage et al. 2002; Miyoshi et al. 2002; Rossi et al. 2002), suggesting that haploinsufficiency of *Lkb1* may be sufficient for polyp formation. This is consistent with the observation of protein levels and kinase activity being similarly decreased in both normal heterozygote tissues as well as in polyps (Miyoshi et al. 2002; Rossi et al. 2002).

Interestingly, a study on human PJS polyps utilizing fluorescent resonance energy transfer demonstrated the presence of the wild-type *LKB1* allele also in hamartomatous PJS polyps (Hernan et al. 2004). More recently, it has even been proposed that PJS polyps in fact are simple mucosal prolapses resulting from asymmetrical stem cell division induced by *Lkb1* haploinsufficiency, and omitting requirement of a "second hit" (Jansen et al. 2006; de Leng et al. 2007). On the other hand, previous analyses of human PJS polyps found evidence for LOH of *LKB1* in many of the analyzed polyps (Gruber et al. 1998; Hemminki et al. 1998; Miyaki et al. 2000; Entius et al. 2001), and evidence for *Lkb1* inactivation in some of the mouse tumors has also been reported (Bardeesy et al.). Consequently, the requirement of *LKB1/Lkb1* LOH for PJS-type polyp development is unclear.

LKB1 and cancer in PJS

The increased risk of multiple cancer types in PJS patients suggests that inactivation of *LKB1* promotes cancer. In studies comparing the frequency of *LKB1* LOH in polyps and carcinomas of PJS patients, LOH has been detected in average in 41% of polyps and in 85% of carcinomas demonstrating a 2.2-fold higher incidence of *LKB1* LOH in carcinomas (Wang et al. 1999; Miyaki et al. 2000; Entius et al. 2001; De Leng et al. 2003). Moreover, several studies on PJS related cancers including liver, colorectal, breast, pancreas and sex cord tumors report the biallelic loss of *LKB1* (Connolly et al. 2000; Entius et al. 2001; Sato et al. 2001; Kim et al. 2004; Nakanishi et al. 2004).

Many of the studies using various *Lkb1*$^{+/-}$ mice have not observed increased cancer incidence, but this may be due to the relatively early age (8-12 months) at which the majority of mice develop fatal large hamartomas occluding the gastrointestinal tract. Attempts to accelerate the development of carcinomas have been made using mice with loss of the p53 tumor suppressor, but while hamartomas in these mice develop somewhat earlier (Takeda et al. 2005; Wei et al. 2005), the tumors do not display signs of progression nor LOH of *Lkb1*.

Supporting the notion that malignancies do not have time to develop in *Lkb1*$^{+/-}$ mice, analysis of aged *Lkb1*$^{+/-}$ mice specimen that did not succumb to occlusions from large polyps revealed that over 70% of *Lkb1*$^{+/-}$ mice that survived until 50 weeks of age had hepatocellular carcinomas (HCC) (Nakau et al. 2002). All analyzed HCCs demonstrated *Lkb1* LOH, never noted in the hamartomas in this same line of mice (Miyoshi et al. 2002; Nakau et al. 2002). Recently another study demonstrated that also spontaneous endometrial adenocarcinomas develop with long latency in *Lkb1*$^{+/-}$ mice and demonstrate *Lkb1* LOH (Contreras et al. 2008). In summary, accumulating data from PJS and the *Lkb1*$^{+/-}$ mouse model support the notion that biallelic *LKB1* deletion can promote cancer.

LKB1 and sporadic cancer

Despite the link of *LKB1* with malignancies in PJS patients, *LKB1* mutations appear to be uncommon in most sporadic cancers (Resta et al. 1998; Connolly et al. 2000; Sobottka et al. 2000; Trojan et al. 2000; Sato et al. 2001; Forbes et al. 2006). A notable exception is that up to 34% of primary lung adenocarcinomas and 27% of lung squamous cell carcinomas harbor somatic *LKB1* gene mutations (Sanchez-Cespedes et al. 2002; Conde et al. 2007; Ji et al. 2007; Matsumoto et al. 2007). An even higher portion of *Lkb1* mutations was discovered in cell lines derived from lung adenocarcinomas with frequencies of 54-42% (Carretero et al. 2004; Ikediobi et al. 2006; Matsumoto et al. 2007). Taken together, the above studies warrant further attention towards *LKB1* with close to 20 000 deaths yearly in the US alone from lung cancers harboring (at least) *LKB1* loss presuming a 25% frequency of *LKB1* loss in just lung adenocarcinomas (http://www.cdc.gov/cancer/lung/statistics/).

Interestingly the human lung adenocarcinomas with *LKB1* mutations also carry *Ras* mutations significantly more often than expected by random (Matsumoto et al. 2007). Paradoxically, evidence for genetic interaction between *Ras* and *Lkb1* first came from transformation assays demonstrating that *Lkb1*$^{-/-}$ MEFs were in fact resistant to *Ras* induced transformation (Bardeesy et al. 2002). However, conditional activation of *Ras* and deletion of *Lkb1* in the mouse lung demonstrated that *Lkb1* loss cooperates with *Kras* in driving lung cancer (Ji et al. 2007). In this setting, deletion of *Lkb1* increased both cancer initiation and metastasis, and showed the strongest Kras cooperation of the assayed tumor suppressors, including p53. Underlining the tissue specific relationship of LKB1 and RAS activation, *RAS* mutations in the PJS polyps are rare (Entius et al. 1997).

Conditional targeting of mouse *Lkb1* also enabled studies on biallelic *Lkb1* loss in other tissues than the lung. Biallelic *Lkb1* deletion induced by adenoviral-Cre in the endometrial epithelium caused adenocarcinomas mimicking the spontaneously developing cancers in *Lkb1*$^{+/-}$ mice (Contreras et al. 2008). Similarly, epidermal (K14-Cre) loss of *Lkb1* caused squamous cell carcinomas of the skin in a subset of mice with long latency, and sensitized mice significantly to carcinogen-induced tumorigenesis (Gurumurthy et al. 2008). In a third study targeting epithelial cells the biallelic loss of *Lkb1* in the pancreatic epithelium resulted

in polarity defects and pancreatic serous cystadenomas (Hezel et al. 2008). Contrasting the epithelial *Lkb1* deletions, loss of *Lkb1* in skeletal muscle or cardiac muscle did not lead to cancer (Sakamoto et al. 2005; Sakamoto et al. 2005). In addition to supporting the association of epithelial *Lkb1* loss and carcinogenesis, these results demonstrate the tissue dependent response to *Lkb1* deficiency. The results are also interesting considering the complex histology of the PJS polyps that contain both gastrointestinal epithelial cells and smooth muscle cells. The effect of *Lkb1* loss in these cell types has not yet been addressed.

AIMS OF THE STUDY

LKB1 has been implicated in the molecular pathogenesis of PJS and sporadic cancer. This study was undertaken to investigate the mechanism of *LKB1* tumor suppression in PJS polyposis.

In order to understand the molecular pathogenesis underlying PJS, the first aim of the study was to investigate the possibility of mutations in loci other than *LKB1*. Identification of such a second PJS gene could also enlighten how *LKB1* mediates its tumor suppressor function.

Based on the observation of increased COX-2 expression in the PJS polyps the second aim was to investigate the therapeutic potential of COX-2 inhibition in PJS in order to understand the relevance of COX-2 induction in PJS and to develop the first PJS therapy strategy.

PJS polyps are distinctive in their histology and contain a notable amount of stromal cells. Together with the possibility that *LKB1* is haploinsufficient regarding its tumor suppressor function, this raises the possibility of non-epithelial origin of tumorigenesis. By deleting *Lkb1* tissue specifically in the smooth muscle, the third aim of this study was to explore the function of stromal Lkb1 and the role of the smooth muscle component in the PJS polyp development.

RESULTS AND DISCUSSION

No evidence for PJS loci besides *LKB1* (I)

Apart from *LKB1*, the studies on chromosomal regions that exhibit genetic linkage with PJS have not revealed mutations in the screened candidate genes (Marneros et al. 2001; Buchet-Poyau et al. 2002; Hearle et al. 2004). To understand the full genetic background of PJS, patients that did not harbor identified mutations of *LKB1* were screened for mutations in the functionally related LKB1 interactors STRAD and MO25, and LKB1 interactor BRG1.

STRADα and *MO25α* did not harbor any mutations in the coding regions, and the polymorphisms noted in the non-coding regions were *in silico* predicted not to affect splicing. Supporting this result, another study undertaken during the course of this thesis work demonstrated that in addition to *STRADα* investigated here, also *STRADß* is void of mutations in PJS patients (de Leng et al. 2005). These results support the notion that mutations in LKB1 binding partners *STRAD* and *MO25* do not cause PJS.

Only one novel silent mutation (Pro372Pro, 980A>C) in the coding region of *BRG1* of one patient was found to discern the PJS patients from healthy subjects. Even though this mutation was predicted to alter acceptor splice site *in silico*, RT-PCR analysis did not reveal any alternate transcripts. Thus no evidence was found for mutations of *BRG1* participating in the molecular pathogenesis of PJS.

During the progress of this work, also the four MARK kinases have been investigated and were found not to harbor mutations in the PJS patients without identified mutations in *LKB1* (de Leng et al. 2007). On the other hand, frequency of *LKB1* mutations in PJS has been further defined to account for 68-94% of the PJS cases (Table 1) as the use of multiplex ligation-dependent probe amplification (MLPA) has enabled the identification of large deletions that are undetectable with classic sequencing strategies. Demonstrating the significance of previously undetected *LKB1* deletions, a study noting mutation frequency of 52% in *LKB1* by sequencing, detected 94% mutation frequency with the additional use of MLPA (Aretz et al. 2005). The average *LKB1* mutation frequency of 81% in the 241 PJS families currently analyzed with MLPA, demonstrates that technical inadequacies in the earlier reports account for the lower reported (52% average) frequencies. Furthermore, even higher frequency of *LKB1* mutations is probable considering the possibility of intronic mutations and yet unscreened deletions. Together with lack of evidence for a "second PJS gene" this suggests that over 94% of all PJS cases harbor *LKB1* inactivating alterations.

Table 1. Detected frequencies of *LKB1* mutations in PJS.

For each study the number of families with detected mutations and the number of all analyzed families (mut/all) are shown. The frequency of *LKB1* mutations in PJS is calculated accordingly for each report (Frequency). Studies using conventional sequencing based methods or MLPA are presented separately, as the reports using the latter are likely to represent more accurate estimates (see text)

Reference	mut/all	Frequency
Sequencing		
(Nakagawa et al. 1998)	10/15	67%
(Wang et al. 1999)	7/12	58%
(Westerman et al. 1999)	12/19	63%
(Ylikorkala et al. 1999)	19/33	58%
(Jiang et al. 1999)	1/10	10%
(Boardman et al. 2000)	6/28	21%
(Olschwang et al. 2001)	24/34	70%
(Scott et al. 2002)	6/14	46%
(Lim et al. 2003)	17/33	52%
(Schumacher et al. 2005)	27/41	66%
(Mehenni et al. 2007)	27/46	59%
MLPA		
(Aretz et al. 2005)	67/71	94%
(Chow et al. 2006)	24/33	73%
(Volikos et al. 2006)	59/76	80%
(Hearle et al. 2006)	25/38	68%
(de Leng et al. 2007)	21/23	91%

To directly investigate on LKB1 activity in PJS patients that have been screened by both sequencing (Ylikorkala et al. 1999) and MLPA (Volikos et al. 2006) but have not been identified to carry *LKB1* mutations, a lymphoblast cell line derived from such patient was analyzed by LKB1 kinase assay. In this assay *LKB1* was immunoprecipitated and subsequently the kinase activity of the precipitate was quantified. Interestingly, this method demonstrated reduced LKB1 activity in all of the assayed (n=3) PJS patient cell lines including the one without an identified *LKB1* mutation (Fig. 4, unpublished). This result supports the notion that LKB1 underlies most if not all PJS cases, and suggests that the patients without identified *LKB1* mutations may for example harbor heritable epigenetic changes of *LKB1* similarly to what has been noted in models of other tumorigenesis predisposing conditions (Linhart et al. 2007). Furthermore, the used kinase assay might provide means for LKB1 activity based PJS diagnosis, but further studies with larger material are needed to validate the robustness of the assay.

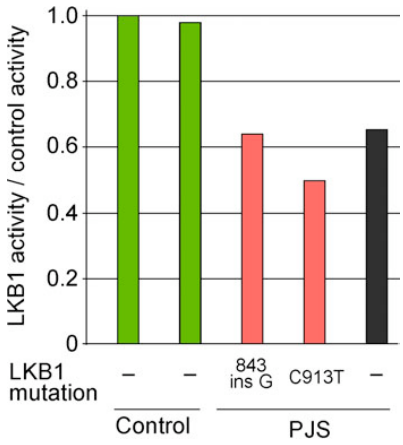


Figure 4. Detection of reduced LKB1 kinase activity in lymphoblast cell lines from PJS patients.

In comparison to control samples (green), PJS samples with and without identified *LKB1* mutations (red and black respectively) exhibit lower LKB1 kinase activity. All measurements are normalized according to CDK7 kinase activities of the cell lines.

COX-2 expression is an early, but not required event in *Lkb1*$^{+/-}$ polyp development (II)

While multiple reports have demonstrated COX-2 expression of polyps from *Lkb1*$^{+/-}$ mice and PJS patients (Rossi et al. 2002; De Leng et al. 2003; McGarrity et al. 2003; Wei et al. 2003; Takeda et al. 2004), it has not been addressed whether COX-2 expression represents a late feature of PJS type polyps or an early event possibly contributing to the polyp initiation. To address this, a panel of polyps ranging in diameter from the smallest polyps distinguishable in a nonfixed mouse stomach to polyps commonly found in terminal animals was collected from *Lkb1*$^{+/-}$ mice, and analyzed for COX-2 protein levels. All polyp size categories (1mm, 2mm, 5mm) demonstrated induced COX-2 in a subset (43%, 57%, 63% respectively) of polyps. The observation of high COX-2 already in the smaller polyps indicates that COX-2 induction is a common early event in development of PJS type hamartomatous polyps, and possibly has an impact on further polyp development. However, in concordance with previous studies (Rossi et al. 2002; Takeda et al. 2004) the lack of COX-2 in a subset of polyps demonstrates that COX-2 is not required for polyp initiation and development. Furthermore, studies on other hamartomatous polyps with mutations in the Smad4, Cdx2 or BMPR1 have demonstrated that COX-2 induction is a common event occurring with hamartoma growth (Takeda et al. 2004; Kurland et al. 2007).

Genetic inactivation of *Cox-2* reduces polyp burden (II)

To study the relevance of COX-2 in *Lkb1*$^{+/-}$ polyposis, mice heterozygous for *Lkb1* and hetero- or homozygous for inactivating allele of the *Cox-2* gene were generated. Whereas the number of small polyps was similar in *Lkb1*$^{+/-}$*;Cox-2*$^{+/-}$ and *Lkb1*$^{+/-}$*;Cox-2*$^{-/-}$ mice as compared to *Lkb1*$^{+/-}$ mice at 8,5 months of age, the number of large polyps was significantly reduced in both *Lkb1*$^{+/-}$*;Cox-2*$^{+/-}$ and *Lkb1*$^{+/-}$*;Cox-2*$^{-/-}$ mice. This reduction in number of large polyps was reflected as a significant reduction also in total polyp burden of *Lkb1*$^{+/-}$*;Cox-2*$^{+/-}$ and *Lkb1*$^{+/-}$*;Cox-2*$^{-/-}$ mice demonstrating a significant role for COX-2 expression in pathogenesis of PJS-type polyposis in *Lkb1*$^{+/-}$ mice.

Pharmaceutical COX-2 inhibition by celecoxib hinders tumor growth (II)

The noted efficacy of genetic COX-2 ablation suggested that also pharmacologic intervention of COX-2 could reduce polyposis. As COX-2 is a target of multiple drugs including widely used NSAIDs such as aspirin and ibuprofen, pharmacologic inhibition of COX-2 activity is possible with simple oral administration of approved drugs. However, the aforementioned drugs also inhibit activity of COX-1 and cause adverse effects such as dyspepsia and peptic ulceration in the gastrointestinal tract (reviewed in Lanas et al. 2006). The newer COX-2 inhibitors selectively inhibit COX-2 while the mucosa protective function of COX-1 is maintained. Therefore the polyp growth-reducing efficacy of COX-2 inhibition was studied with a selective COX-2 inhibitor celecoxib in two cohorts of *Lkb1*$^{+/-}$ mice with treatments starting either before (3,5 months) or after (6,5 months) polyposis onset. In comparison to untreated *Lkb1*$^{+/-}$ mice, *Lkb1*$^{+/-}$ mice that had received 1500 ppm of celecoxib from age of 3,5 months until analysis at 10 months demonstrated 86% reduction ($P < 0{,}001$) in polyp burden. Polyp burden was, as with the genetic intervention, reduced due to decrease in number of large polyps, particularly due to the total lack of polyps > 5mm in diameter whereas polyp quantity of the smallest size category was unaffected. Similarly, the cohort treated for a shorter 3,5 month period starting after polyposis onset had significantly fewer large polyps (47% reduction, $P < 0{,}05$) while again the number of small polyps was not changed. In the *APC*^{min} mouse, celecoxib reduced tumor burden to similar extent (85% reduction), but it also reduced the tumor multiplicity of all tumor sizes by up to 70% (Jacoby et al. 2000) suggesting a more direct role for COX-2 in development of FAP polyps. Taken together, the results suggest that inhibition of COX-2 suppresses *Lkb1*$^{+/-}$ tumor growth without an influence on polyp initiation, and demonstrate that pharmacologic intervention with celecoxib effectively reduces polyp burden in the mouse model of PJS.

COX-2 inhibition results in decreased vascularity of *Lkb1*$^{+/-}$ polyps (II)

Suggested mechanisms for tumor prevention by COX-2 inhibition include effects on cell proliferation, apoptosis, and angiogenesis (Cha et al. 2007). Apoptotic index of *Lkb1*$^{+/-}$ polyps was unaltered in the celecoxib treated mice, likewise to histological appearance indicative of unchanged proliferation. In contrast, the microvessel density (MVD) of celecoxib treated tumors was reduced by 54% ($P<0.01$) in comparison to untreated polyps of the same size category. These results suggest that COX-2 induces angiogenesis in *Lkb1*$^{+/-}$ mice possibly via up-regulated VEGF and FGF as is noted in FAP (Seno et al. 2002). Furthermore, the results suggest that reduced angiogenesis by COX-2 inhibition may be the polyp growth suppressing mechanism of celecoxib in *Lkb1*$^{+/-}$ mice.

Celecoxib reduces polyposis severity in PJS patients (II)

The elevated COX-2 in PJS patient polyps (Rossi et al. 2002) together with the encouraging results from the PJS mouse model prompted an investigation on therapeutic potential of COX-2 inhibition in PJS. In a pilot clinical trial, 8 PJS patients received 2 x 200mg of celecoxib daily for 6 months. Video recorded gastroscopies before and after celecoxib were used to address polyposis grade in the stomach and duodenum, and revealed gastric polyps in all subjects. The multitude of observed gastric polyps (II, figure 4) suggests that previous reports on low incidence of gastric polyps compared to small intestine (Allen et al. 2003) may simply reflect a higher incidence of symptoms due to intestinal polyps rather than polyp distribution. Polyposis severity was significantly reduced after celecoxib in 2 out of the 6 patients with gastroscopy recordings allowing grading. The overall grade of polyposis of all 6 patients was also reduced ($P<0.01$), and it is interesting to note that the most dramatic effect was observed in the patient with the highest polyp burden prior to treatment. Despite the short treatment period, these results demonstrate the efficacy of pharmaceutical COX-2 inhibition by celecoxib in PJS polyposis suppression in at least a subset of patients. However, selective COX-2 inhibitors have been associated with cardiovascular events in long-term follow-ups undertaken during this thesis work (Graham et al. 2005). The balance of benefits to risk for celecoxib chemoprevention in general population with normal risk of gastrointestinal tumors has therefore been regarded unfavorable (Rostom et al. 2007). Whether the benefits of selective COX-2 inhibition for PJS patients with their high occurrence of gastrointestinal tumors warrants COX-2 chemoprevention, calls for larger clinical trials to fully establish the therapeutic efficacy of COX-2 inhibition in this disease.

Deletion of *Lkb1* in smooth muscle cells (III)

Influenced by the well-defined mechanism of colorectal carcinogenesis starting from epithelial mutations of the *APC* tumor suppressor (reviewed in Fodde et al. 2001), gastrointestinal tumorigenesis in general has been considered to follow similar epithelially originating path. Contrasting *APC* mutations in FAP, studies on *LKB1/Lkb1* deletions in PJS-type polyps have not elucidated the tissue type(s) critical for polyp development, as data on LOH of *LKB1/Lkb1* has been ambiguous. To investigate whether smooth muscle cells (SMCs) that form the distinctive stromal "core" of PJS polyps actively contribute to polyp development, *Lkb1* was deleted tissue specifically in mouse SMCs by the use of conditional $Lkb1^{lox}$ allele (Bardeesy et al. 2002) and a *SM-CreERT2(ki)* allele (Kuhbandner et al. 2000) expressing tamoxifen inducible Cre-recombinase under the *SM22/Transgelin* (*Tagln*) promoter in SMCs. The *SM-CreERT2(ki)* allele is here forth referred to as $Tagln^{Cre}$ according to the official gene symbol.

The specificity of *Lkb1* deletion was assessed with LCM-PCR from mice at 3 months (1,5 months after the recombination induced by the tamoxifen injections) demonstrating the recombined $Lkb1^{-}$ allele only in samples containing smooth muscle cells. The 50% efficiency

of *Lkb1* recombination noted in SMCs at 8 months indicates that either the recombined SMCs are sustained for at least 6,5 months or that the used strategy targets a SMC progenitor cell. The latter option is unlikely as SM22/Tagln expression is a marker of myogenic differentiation (Chen et al. 2006).

Lkb1 heterozygosity of the smooth muscle predisposes to polyposis (III)

Mesenchymally targeted $Lkb1^{lox/+};Tagln^{Cre/+}$mice exhibited signs of gastrointestinal obstruction and premature lethality from 11 months onwards. As expected, also the $Lkb1^{+/-}$ heterozygous mice showed similar symptoms, but with shorter latency. In necropsy, terminal $Lkb1^{lox/+};Tagln^{Cre/+}$ animals exhibited widespread gastric polyposis comparable to $Lkb1^{+/-}$ mice. Strikingly, the polyps in $Lkb1^{lox/+};Tagln^{Cre/+}$mice were histologically indistinguishable from $Lkb1^{+/-}$ polyps notwithstanding their smaller average size. The noted size difference may either reflect the later (6 weeks postnatal) and partial recombination of *Lkb1* in smooth muscle cells, or slower development of purely stroma-driven polyps. Taken together, these results demonstrate that monoallelic inactivation of *Lkb1* in SMCs predisposes to polyposis and phenocopies the $Lkb1^{+/-}$ mouse model of PJS.

LOH of *Lkb1* is not required for polyp development (III)

Embryonic lethality of biallelic *Lkb1* inactivation (Ylikorkala et al. 2001) has previously prevented direct investigations on causality of *Lkb1* LOH in polyp development. The use of the conditional strategy to delete *Lkb1* facilitated analysis of animals with *Lkb1* nullizygous cells. Biallelic deletion of *Lkb1* did not appear to influence the short-term viability of smooth muscle cells. This was demonstrated by the normal histology of the $Lkb1^{lox/lox};Tagln^{Cre/+}$ gastrointestinal tract that exhibited the recombined $Lkb1^{-}$ allele as determined by PCR genotyping 6 weeks after tamoxifen induced deletion. Consistent with this result, mice with *Lkb1* knockout skeletal or cardiac muscle cells are viable but targeted cells are deficient in AMPK activation following contraction or ischemia (Sakamoto et al. 2005; Sakamoto et al. 2005). Furthermore, the mice with biallelic *Lkb1* deletion in the smooth muscle did not exhibit compromised survival during the 11-month follow-up.

When sacrificed at 11 months of age the $Lkb1^{lox/lox};Tagln^{Cre/+}$ mice exhibited gastrointestinal polyposis similar to $Lkb1^{lox/+};Tagln^{Cre/+}$. The polyps and unaffected GI-tract were histologically indistinguishable between the genotypes indicating that biallelic deletion of *Lkb1* does not cause progression of polyps or spontaneous cancer. This result is consistent with the results from other studies on mesenchymally targeted *Lkb1* mutations (Sakamoto et al. 2005; Sakamoto et al. 2005), and highlights the different responses to *Lkb1* loss in the mesenchyme and the epithelium where biallelic deletion has resulted in carcinogenesis (Contreras et al. 2008; Gurumurthy et al. 2008; Hezel et al. 2008).

Importantly, the size, quantity, and penetrance of the polyps were only moderately higher in the biallelic deletion (Fig. 5). This small difference in polyposis severity is in strong disagreement with the requirement of *Lkb1* LOH for polyp development. Otherwise the noted 1.8-fold increase in polyp number (unpublished) with biallelic deletion would require more than half of the cells in monoallelic deletion to undergo stochastic somatic mutations. This finding is also consistent with identification of both monoallelic and biallelic inactivation in polyps in both mice (Bardeesy et al. 2002) and Peutz-Jeghers patients (Entius et al. 2001). However, whereas mesenchymal deletion of *Lkb1* in the gastrointestinal tract causes benign hamartomatous polyps, the development of cancer after epithelial *Lkb1* deletions in other tissues (Contreras et al. 2008; Gurumurthy et al. 2008; Hezel et al. 2008) insinuates that the malignant GI-tract cancers in PJS may require epithelial *LKB1* loss, and possibly represent a different entity to PJS polyps. This issue waits for studies addressing the phenotypes of mono- and biallelic deletions of *Lkb1* in the gastrointestinal epithelium.

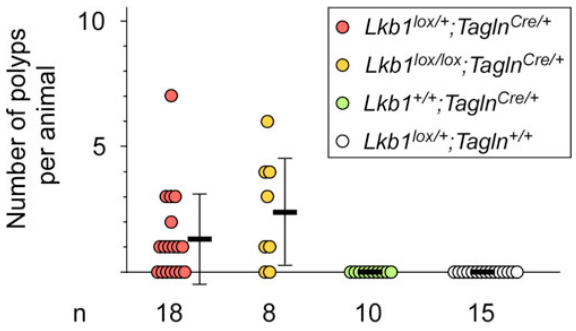


Figure 5. Number of gastric polyps in 11-month-old mice with mono- and biallelic deletions of *Lkb1* in the smooth muscle. Each circle represents an experimental animal. Number of analyzed mice (n), and average number of macroscopically detectable polyps (black bars, average +/- s.d.) is presented.

Epithelial deregulation of mTOR signaling is not the primary defect causing PJS polyps (III)

The discovery of the LKB1-AMPK-TSC2-mTOR signaling link (Corradetti et al. 2004) (see Figure 2) and activation of mTOR effectors in $Lkb1^{+/-}$ polyps (Shaw et al. 2004) has led to a proposal that LKB1 functions as a tumor suppressor via TSC2 mediated mTOR inhibition. Involvement of this pathway in the identified smooth muscle driven tumorigenesis was addressed by immunohistochemistry. Surprisingly, mTOR effector ribosomal S6 protein was similarly activated in the epithelium of both $Lkb1^{lox/+};Tagln^{Cre/+}$ and $Lkb1^{lox+/-}$ polyps, indicating that mTOR activation at least in the $Lkb1^{lox/+};Tagln^{Cre/+}$ polyp epithelium is a secondary event in polyp development as the epithelium in these polyps does not harbor *Lkb1* mutations.

Though the mechanism how mesenchymal *Lkb1* deletions lead to epithelial mTOR activation is unresolved, the results from $Lkb1^{lox/+};Tagln^{Cre/+}$ mice oppose the proposal of mTOR

activation via compromised LKB1-AMPK-TSC2 signaling as the primary cause of PJS polyposis. Suggesting that mTOR activation in *Lkb1*$^{+/-}$ polyps might occur via different pathways, cell lines derived from *Lkb1*$^{-/-}$ squamous cell carcinomas exhibit Lkb1 dependent growth arrest that is not abrogated by introduction of dominant negative AMPK despite the noted increase in mTOR pathway activity (Gurumurthy et al. 2008). Indirect support for this notion also comes from *Tsc1*$^{+/-}$ and *Tsc2*$^{+/-}$ mice that do not develop hamartomas in the gastrointestinal tract (Kobayashi et al. 1999; Onda et al. 1999; Kobayashi et al. 2001; Kwiatkowski et al. 2002). However, inhibition of mTOR with rapamycin was recently reported to suppress polyp burden of *Lkb1*$^{+/-}$ mice by reducing the number of large (>8mm) polyps (Wei et al. 2008). The noted effect of rapamycin particularly on large polyps is reminiscent of the effect noted with COX-2 inhibition in this thesis. This suggests that similarly to celecoxib, also rapamycin treatment might target processes that are important for polyp growth, but secondary to tumor initiation caused by *Lkb1* deficiency.

Reduced mesenchymal TGFß signaling may cause PJS polyp development (III)

The comparable composition of stroma and epithelium in both *Lkb1*$^{lox/+}$*;Tagln*$^{Cre/+}$ and *Lkb1*$^{+/-}$ polyps suggested that similar mesenchymal response to *Lkb1* heterozygosity might underlie epithelial proliferation and tumorigenesis in both genotypes. Indeed, the immunohistochemical analysis of the myogenic stromal cells revealed altered ratio of myofibroblasts (SMA+, desmin–) and SMCs (SMA+, desmin+) to favor myofibroblasts in both polyp types compared to normal gastric tissue. LCM-PCR detected the recombined *Lkb1* allele exclusively from SMA+ stromal cells indicating that the myofibroblasts arise due to Lkb1 deficiency and designate *Lkb1* deletions in *Lkb1*$^{lox/+}$*;Tagln*$^{Cre/+}$ polyps. Correspondingly, the SMA– cells located distally to myofibroblasts were negative for *Lkb1* deletions. The propensity of myofibroblasts to express COX-2 (Mifflin et al. 2002) could also promote PJS polyp development. However, while COX-2 inhibition reduced average polyp size, it had no effect on the number of small polyps in the study II indicating that COX-2 is not the alteration of cellular microenvironment that underlies tumor initiation.

The noted alteration in myofibroblast:SMC-ratio indicated a disturbance in the myogenic differentiation normally involving TGFß signaling (Hirschi et al. 1998). Activity of TGFß signaling was therefore addressed by analysis of Smad2 signal transducer phosphorylation (p-Smad2). Stromal p-Smad2 was specifically reduced in the SMA+ cells suggesting that Lkb1 deficiency results in reduced TGFß signaling. This was confirmed with primary cultures of mouse embryonic fibroblasts demonstrating that *Lkb1* deletion leads to decrease in TGFß production. Furthermore, reduction in TGFß production correlated with the allelic dosage of *Lkb1*, and demonstrated the haploinsufficiency of *Lkb1* in this regard.

For mesenchymal *Lkb1* deletion to evoke a tumorigenic response in the epithelium, the interaction between the two tissue types must be altered. The noted reduction in

mesenchymally produced TGFß is interesting in this setting, as in addition to myogenic differentiation TGFß signaling controls epithelial proliferation (Kurokowa et al. 1987; Russell et al. 1988). Importantly, reduction in Smad2 activation was noted also in the epithelial cells adjacent to the SMA+ stroma. Furthermore, epithelial proliferation marked by Ki-67 positive nuclei coincided with the reduced p-Smad2 suggesting that reduced TGFß signaling from the *Lkb1* heterozygote mesenchyme results in epithelial proliferation and may underlie polyp development (Fig. 6).

In order to obtain evidence for possible defects in mesenchymal-epithelial TGFß signaling in the human disease, Peutz-Jeghers patient polyps were also stained for SMA and p-SMAD2. SMA+ cells constituted most of the PJS polyp stroma, but SMA– stromal cells were noted distally to SMA+ core of the polyps. Strikingly, the SMA+ stroma and the adjacent epithelial cells exhibited reduced p-SMAD2 in comparison to areas with SMA– stroma. Considering the results from the $Lkb1^{lox/+};Tagln^{Cre/+}$ mouse, these results support the role of stroma as a major contributor to PJS polyposis by generation of a permissive microenvironment for epithelial expansion.

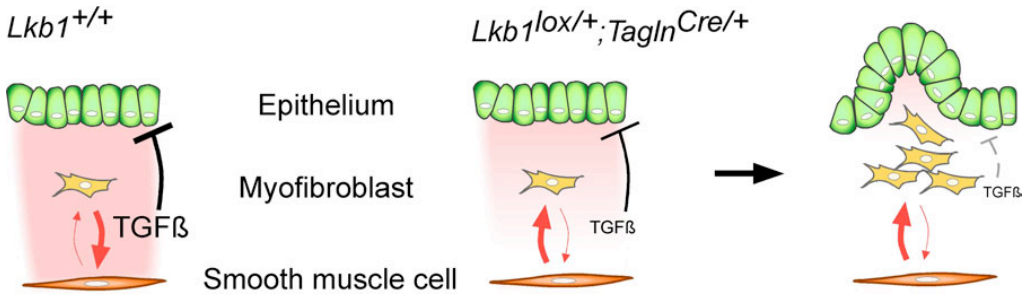


Figure 6. Proposed model of PJS-type polyp development. In $Lkb1^{+/+}$ tissues, mesenchymally produced TGFß (pink) drives myogenic differentiation toward mature smooth muscle cells (red arrows) and inhibits epithelial proliferation (black line). In the $Lkb1^{lox/+};Tagln^{Cre/+}$ mouse model of PJS smooth muscle specific deletion results in reduced mesenchymal TGFß production. This is reflected in the balance of myogenic differentiation to possibly favor dedifferentiation of smooth muscle cells, and in impaired mesenchyme-to-epithelium signaling. As a result, the number of myofibroblasts increases and leads to further reduction of mesenchymal TGFß and in hyperproliferation of the epithelium and polyp development.

Landscaper tumor suppressor function of *LKB1* (III)

The surprising results regarding *Lkb1* haploinsufficiency and mesenchymal functions make *Lkb1* hard to categorize as a tumor suppressor. According to the original proposal by Kinzler and Vogelstein, tumor suppressors represent two principal modes of action termed as *gatekeepers* and *caretakers* (Kinzler et al. 1997). Of the two, gatekeepers have maybe the more intuitive function, as they directly inhibit tumor cell proliferation or promote cell death.

With examples like *Rb*, *APC* and *P53* gatekeepers are well appreciated for their importance in cancer biology.

Coinciding with the cancer-promoting propensity of mutations the second group of tumor suppressors, the caretakers, function indirectly by maintaining the genomic integrity and consequently their loss promotes genomic instability that eventually results either in oncogene activation or inactivation of gatekeepers. ATM and ATR, the master controllers of DNA damage response, are prototypic caretakers.

Intrinsic to the definition of gatekeepers and caretakers, their mutations increase the oncogenic potential of the mutated cell itself (Kinzler et al. 1998). However, smooth muscle specific deletion of *Lkb1* caused tumorigenic growth of epithelial cells in addition to the targeted mesenchyme. The sufficiency of the two tumor suppressor classes was similarly challenged by the discovery of clonally expanded stroma in Juvenile polyposis (JP) polyps (Jacoby et al. 1997). Stroma of JP polyps exhibited LOH of the *SMAD4* gene, whereas no LOH was found in the predominant epithelial cells of the polyps (Howe et al. 1998). This led Kinzler and Vogelstein to propose the existence of a third type of tumor suppressor, the *landscaper* (Kinzler et al. 1998). Mutations of a landscaper were proposed to alter the cellular microenvironment thereby influencing neighboring cells and inducing, or at least supporting their oncogenic potential. However, the molecular mechanism of tumorigenesis driven by stromal *SMAD4* loss was not characterized, and epithelial cells of JP polyps were later demonstrated do harbor *SMAD4* mutations (Woodford-Richens et al. 2000). Consequently, the whole existence of landscapers was questioned (Playford 2001).

SMAD4 is an intracellular signal transducer of TGFß signaling; a pathway that the results of this study suggest to mediate mesenchymal Lkb1 tumor suppression. Recent investigation on murine *Smad4* demonstrated that in fact also *Smad4* is haploinsufficient in tumor suppression of mouse polyps (Alberici et al. 2006) possibly explaining the previous contradictory reports on *SMAD4* LOH (Howe et al. 1998; Woodford-Richens et al. 2000). It is interesting to note that including the results of this study, current evidence supporting the landscaper hypothesis comes from mouse models with defects in TGFß superfamily signaling from mesenchyme to epithelia. More specifically, deletion of mouse TGFßRII in fibroblasts leads to gastric squamous cell carcinoma (Bhowmick et al. 2004), and tissue specific deletion of murine Smad4 in T-cells results in gastrointestinal cancer (Kim et al. 2006). Furthermore, blocking of stromal BMP4 signals with transgenic expression of noggin in epithelial cells leads to adenoma-like lesions (Haramis et al. 2004). In these studies, several deregulated cytokines and growth factors (HGF, FGF, IL-6) were implicated in aberrant signaling with the epithelium. None of them were altered in $Lkb1^{lox/+};Tagln^{Cre/+}$ mice, indicating that even though deregulated TGFß superfamily signaling may link all these models demonstrating mesenchymal induction of epithelial tumorigenesis, specific mechanisms probably vary.

The observation of *Lkb1* haploinsufficiency in TGFß regulation is important, as it provides a rational for polyp development without *Lkb1* LOH and for infrequency of sporadic PJS type

polyps (Burkart et al. 2007). As *Lkb1* deletions cause a reduction in mesenchymally produced TGFß, the effect of a sporadic *LKB1* mutation in a single cell is likely to result only in a minute alteration of the cellular microenvironment. Hereditary *Lkb1* mutations on the other hand reduce TGFß production of all mesenchymal cells lining the whole gastrointestinal epithelium, and thus profoundly modulate mesenchymal signaling that normally keeps epithelial proliferation at bay (Halttunen et al. 1996). In addition, the effect is likely to be stronger with biallelic loss of *Lkb1* as TGFß production in $Lkb1^{-/-}$ cells was lower than in $Lkb1^{+/-}$ cells. Accordingly, polyposis in $Lkb1^{lox/lox};Tagln^{Cre/+}$ mice was more sever than in $Lkb1^{lox/+};Tagln^{Cre/+}$ mice, but the increase was too small to indicate a requirement for *Lkb1* LOH.

The mechanism of LKB1 mediated TGFß regulation is of great interest. Possible involvement of the known LKB1 substrates needs to be investigated in this regard for example by the means of RNA interference. Moreover, comparative expression profiling of normal gastrointestinal tissues, and polyps resulting from mesenchymal *Lkb1* deletion or "conventional" $Lkb1^{+/-}$ heterozygosity, could in addition to mesenchymal TGFß regulation elucidate what are the epithelial transcriptional responses critical for proliferation and polyp development.

CONCLUSIONS

Peutz-Jeghers syndrome is a disease characterized by a devastating gastrointestinal polyposis and increased cancer risk. Lack of non-invasive therapy forces PJS patients to undergo typically multiple laparotomies due to polyposis related complications.

This thesis work further defined the genetic background of this disease by excluding three PJS candidate loci whose products interact with the known PJS gene, *LKB1*. Results support the role of LKB1 as the only PJS culprit and thereby direct future studies on PJS. The results highlight the relevance of the findings in the *Lkb1* heterozygote mouse model, as the model is likely to represent most if not all cases of PJS.

Earlier observation on COX-2 induction of PJS polyps together with the dramatic results in other hereditary GI polyposis syndromes obtained by pharmaceutical COX-2 inhibition held the promise of a pharmaceutical therapy for PJS patients. The encouraging results from both the PJS mouse model and limited set of patients in the pilot clinical trial call for further studies in order to evaluate the applicability of COX-2 inhibition in PJS. Currently use of selective COX-2 inhibitors is however undesirable as they have been noted to increase the risk of cardiovascular episodes. Ongoing development of replacements for the inhibitor used here may allow for risk-free suppression of PJS polyp growth in the future.

LKB1 has been suggested to be haploinsufficient as a tumor suppressor, and previous studies on *LKB1* loss of heterozygosity have not pinpointed the cell type driving polyp development in PJS. The development of polyps following mesenchymal *Lkb1* deletion demonstrates that *Lkb1* is an atypical tumor suppressor controlling epithelial growth from the mesenchyme. Furthermore, the comparable polyposis following monoallelic and biallelic deletions of *Lkb1* indicates that *Lkb1* indeed is haploinsufficient in gastrointestinal tumor suppression. The observation of decreased TGFß signaling after mesenchymal *Lkb1* deletion links this mouse model with other mouse models supporting the landscaper tumor suppressor function, and suggests a mechanism for stromal-derived *LKB1* tumor suppression.

Considering the goal of therapy development for PJS, the results provide proof of principle for effectiveness of COX-2 inhibition in PJS, and serve as the basis for the development of therapeutic interventions targeting mesenchymal signaling in PJS polyposis and LKB1 related cancer.

MATERIALS AND METHODS

Mutation screening

DNA prepping from whole blood samples of PJS patients without identified *LKB1* mutation was performed using standard methods. For *BRG1*, 34 exons and their flanking intronic regions were analyzed by PCR and denaturing high-performance liquid chromatography (DHPLC) with automated instrumentation (Agilent Technologies). Samples demonstrating different DHPLC patterns from control samples were sequenced using ABI 3730 sequencer. The mutation screening of STRADα and MO25α was performed by genomic sequencing. Sequences of the used primers are available from the author upon request. Splicing predictions were conducted using NetGene2 splice site prediction web server (http://genome.cbs.dtu.dk/services/NetGene2/).

Patients and clinical study protocol

Patients invited to participate gave written informed consent prior to the beginning of the study. The study protocol was approved by the ethical board of the department of surgery at the University of Helsinki Central Hospital. It consisted of a gastroscopy prior to and following a six-month celecoxib treatment (2 x 200 mg/day). Grading of tumor burden before and after treatment was based on gastroscopy video recordings. Following the initial gastroscopies, all patients complied with the treatment regimen according to questionnaires made one month following onset and at the second gastroscopy.

Gastroscopy procedures

Gastroduodenoscopies were conducted by two endoscopists using forward-viewing video endoscopes (Olympus GIF Q 140, 145 or 160), on outpatient basis. When required, midazolam was used for sedation. During the initial endoscopy, clinically significant polyps (>0.5 cm in diameter) were removed before video recording from the second part of duodenum backwards up to the gastro-oesophageal junction. Five independent evaluators analyzed the video material from the gastroscopies.

Mouse husbandry and drug administration

All experimental mice were of mixed background. The mice had free access to food and water before and throughout these studies. Animal health was monitored according to the recommendations of the Animal Committee of the University of Helsinki. For the pharmacological interventions celecoxib powder (Searle-Pharmacia, Skokie, IL) was mixed at a concentration of 1500 parts per million with rodent chow. Tamoxifen was administered by intraperitoneal injections of 1 mg of 4OH-tamoxifen (Sigma) in to mice at 1.4–1.5 months of age, and repeated on two consecutive days.

Mouse genotyping

The two strategies of targeted inactivation and genotyping of the murine *Lkb1* gene have been described previously (Ylikorkala et al. 2001; Bardeesy et al. 2002). The generation and genotyping of mice with *SM22-CreERt*^{2}*(KI)* allele has been described previously (Kuhbandner et al. 2000). The *Cox-2* deficient strain (Dinchuk et al. 1995) was obtained from Jackson Laboratories (Bar Harbor, ME), and the genotyping strategy was kindly provided by Professor S. K. Dey (University of Vanderbilt Medical Center, Nashville, TN).

Laser Capture Microdissection (LCM) for recombination measurements was conducted with Arcturus Autopix LCM-system, and DNA was purified according to manufacturers instructions.

Recombination frequency of Lkb1 was measured by realtime-PCR (ABI Prism 7500, Applied Biosystems) using primer pairs for non-recombined ($Lkb1^{lox}$) or recombined ($Lkb1^{-}$) alleles. For this, a standard sample with a 1:1 mix of non-recombined ($Lkb1^{lox/lox}$) and recombined (adeno-Cre infected $Lkb1^{lox/lox}$) DNA was used to normalize PCR efficiency of the primer pairs according to 50% recombination. Subsequently the assay was validated using a series of DNA mixes prepared as above and DNA from tissue samples were similarly analyzed.

Macroscopic assessment of polyps

The small intestine and stomach of sacrificed animals were inflated with 2-4% PFA, then fixed with 4% PFA and 1% PFA for 1h and o/n respectively. The diameter of the polyps was measured using a digital micrometer and dissection microscope. Tissues were embedded and sectioned according to standard procedures. The PJS patient tissue samples were treated according to protocols of Department of Pathology, HUSLAB, Helsinki University Central Hospital (HUCH).

Image analysis

Quantitations of stainings was carried out with ImageJ software. Blue channel (counter stain) was omitted from RGB images to perform densitometric analysis for staining of selected areas or in nuclei automatically detected from images after thresholding.

Histochemistry

The following antibodies were used: mouse CD31, PharMingen 01950D; mouse and human SMA, Sigma a-5691; mouse desmin, Novocastra clone DE-R-11; mouse Ki-67, Dako M7249; mouse and human Phospho-SMAD2 ser465/467, Chemicon AB3849; Phospho-ACC, Cell signaling technology #3661; phospho-S6 Cell signaling technology #2211. DakoCytomation ARK (K3954) kit for mouse primary antibodies was used according to manufacturers instructions with monoclonal antibodies raised in mouse. For CD31 immunohistochemistry the Tyramide Signal Amplification kit (Perkin-Elmer, Boston, MA) was used to enhance signal. Other Immunostainings were performed according to standard procedures. Stainings were developed with 3, 3' Diaminobenzidine (DAB) and slides were

counterstained with hematoxylin. TUNEL assay was performed with the ApopTag peroxidase *in situ* apoptosis detection kit (Chemicon/ Serologicals, Temecula, CA) according to manufacturers instructions.

Cell culture

Mouse embryonic fibroblasts (MEFs) were collected from E9.5 embryos that were minced into small fragments and cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 25mM glucose, glutamine, penicillin, and streptomycin. Lymphoblast cell lines were prepared using standard EBV transfection methods and grown using 10% horse serum supplemented RPMI growth media.

Immunoblotting and kinase assays

Protein lysates from tissue samples were prepared in 1x Laemmli lysis buffer with FastPrep homogenizer (QBiogene, Carlsbad, CA). Lysates from cultured cells were either prepared in ELB or Laemmli lysis buffers. Protein concentrations were determined using spectrophotometry. Western blotting analysis using antibodies against COX-2 (160126, Cayman Chemical), actin (AC-40, Sigma) and p-S6 was performed as previously described (Rossi et al. 2002). LKB1 immunoprecipitation and kinase assays were performed as described (Ylikorkala et al. 1999).

TGFß measurements

Mink lung epithelial cells stably expressing the luciferase gene under a fragment of the PAI-1 promoter were kindly provided by Dr. Daniel B. Rifkin (New York University School of Medicine, New York, NY). These cells were used to assay TGFß activity in media of cultured cells as previously described (Abe et al. 1994).

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