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ORIGINAL PAPER

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Peutz–Jeghers LKB1 mutants fail to activate GSK-3β, preventing it from inhibiting Wnt signaling

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Abstract Peutz–Jeghers syndrome (PJS) is caused by germline mutations in the *LKB1* gene, which encodes a serine-threonine kinase that regulates cell proliferation and polarity. This autosomal dominant disorder is characterized by mucocutaneous melanin pigmentation, multiple gastrointestinal hamartomatous polyposis and an increased risk of developing various neoplasms. To understand the molecular pathogenesis of PJS phenotypes, we used microarrays to analyze gene expression profiles in proliferating HeLa cells transduced with lentiviral vectors expressing wild type or mutant LKB1 proteins. We show that gene expression is differentially affected by mutations that impair the kinase activity (K78I) or alter the cellular localization of the LKB1 protein. However, both mutations abrogate the ability of LKB1 to up-regulate the transcription of several genes involved in Wnt signaling, including *DKK3*, *WNT5B* and *FZD2*. In addition—and in contrast to the wild type protein—these LKB1 mutants fail to activate the GSK-3β kinase, which otherwise phosphorylates β-catenin. The increase in β-catenin phosphorylation that occurs upon expression of wild-type LKB1 results in transcriptional inhibition of a canonical Wnt reporter gene. This suggests that pathogenic LKB1 mutations that lead to activation of the Wnt/β-catenin pathway could contribute to the cancer predisposition of PJS patients.

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Introduction

Peutz–Jeghers syndrome (PJS) is a dominantly inherited disorder associated with mucocutaneous pigmentation and gastrointestinal hamartomatous polyposis (OMIM no. 175200). More than 90% of PJS patients go on to develop malignancies, about half of which affect the gastrointestinal tract (Boardman et al. 1998; Giardiello et al. 2000). The gene responsible for the majority of PJS cases has been mapped to chromosome 19p13.3 and found to encode a serine/threonine protein kinase named LKB1 (alias STK11; Hemminki et al. 1997, 1998; Jenne et al. 1998). Functional analysis of LKB1 showed that most mutations observed in PJS patients result in loss of its kinase activity (Mehenni et al. 1998; Ylikorkala et al. 1999; Yoo et al. 2002; Boudeau et al. 2003b).

The *LKB1* gene has been conserved during evolution and is essential for viability. Orthologs have been identified in mouse (Smith et al. 1999), *Xenopus laevis* (*XEEK1*; Su et al. 1996), *Caenorhabditis elegans* (*par-4*; Watts et al. 2000), *Drosophila melnaogaster* (Martin and St. Johnston 2003) and yeast (Sutherland et al. 2003). Knockout studies in model organisms demonstrate that LKB1 plays an essential role in development and polarity. In *C. elegans* and *Drosophila melanogaster*, LKB1 is required to establish embryonic polarity (Watts et al. 2000; Martin and St. Johnston 2003) and mice homozygous for an inactivating LKB1 mutation die at mid-gestation with multiple developmental defects including abnormal vasculogenesis (Ylikorkala et al. 2001). In contrast, $Lkb1^{+/-}$ mice are viable and display no obvious phenotype at birth or in early adult life. However, such mice later develop gastrointestinal polyps; cells from these polyps express one normal *LKB1* allele, suggesting that polyposis arises from haploinsufficiency of *LKB1* rather than loss of heterozygosity

(LOH; Hemminki et al. 1997; Miyoshi et al. 2002). *LKB1* has therefore been defined as a context-dependent tumor suppressor gene: loss of its function in normal cells may result in the formation of benign tumors, whereas its loss in advanced neoplasms could promote malignant progression (Bardeesy et al. 2002).

In *Xenopus* LKB1 has recently been shown to function in the Wnt pathway, regulating GSK-3β activity during early development (Ossipova et al. 2003). In mammalian cells, LKB1 triggers phosphorylation of PAR1A, a kinase that also regulates the Wnt/β-catenin pathway (Spicer et al. 2003). Activation of this pathway is crucial for dorso-ventral axis specification and is regulated during embryonic development in lower vertebrates. Constitutive activation of the Wnt pathway is associated with malignant transformation in adult tissues (Miller 2002; Giles et al. 2003). Lastly LKB1 has been shown to activate *TSC2*, a gene that is mutated in tuberous sclerosis complex, which is an autosomal dominant syndrome characterized by the development of benign tumors (Corradetti et al. 2004).

LKB1 is mainly localized in the nucleus, but is also detected in the cytoplasm and at cell membranes (Smith et al. 1999; Collins et al. 2000; Sapkota et al. 2001). Several proteins have been shown to interact with LKB1, including BRG1 (Marignani et al. 2001), p53 (Karuman et al. 2001), LIP1 (Smith et al. 2001), AGS3 (Blumer et al. 2003) and STRADα (Baas et al. 2003). Both LIP1 and STRADα anchor LKB1 in the cytoplasm. The binding of STRADα to LKB1 also activates the capacity of the kinase to autophosphorylate, allowing complete polarization of intestinal epithelial cells (Boudeau et al. 2003a; Baas et al. 2004a). LKB1 has been shown to form a ternary complex with STRADα and MO25α, which regulates cell proliferation through activation of AMP-activated protein kinase (AMPK) by LKB1-dependent phosphorylation (Hawley et al. 2003; Hong et al. 2003; Woods et al. 2003). In spite of the identification of these LKB1-interacting proteins (reviewed in Boudeau et al. 2003c) carcinogenesis in PJS remains poorly understood. However, it has recently been suggested that loss of polarity in epithelial cells may lead to neoplastic transformation (Baas et al. 2004b).

In this study we investigated whether mutations that affect the kinase activity or the cellular localization of LKB1 have similar effects on LKB1 function. To this end, we compared gene expression profiles in HeLa cells expressing wild type or mutant LKB1. We show here that the mutations K78I (which eliminates the kinase activity) and SL26 (associated with nuclear accumulation of LKB1) differentially affected gene expression patterns when compared to wild-type LKB1. However, both mutants failed to induce dephosphorylation of GSK-3β at residue Ser9, thus maintaining the kinase in its inactive state. Because β-catenin is a substrate for GSK-3β and also regulates transcription of Wnt target genes, we also investigated the phosphorylation state of β-catenin. We found that the protein was only phosphorylated in cells expressing wild-type LKB1. Since phosphorylated β-catenin is likely to be ubiquitinylated and then degraded, this allows LKB1 to inhibit the expression of Wnt-responsive genes in A549 cells. We showed that this ability is dependent on the kinase activity of LKB1, since the K78I mutation has no effect on Wnt signaling. In contrast, expression of the SL26 form of LKB1 further activates expression of a Wnt reporter gene. Together our results suggest that PJS mutants may affect the regulation of Wnt/β-catenin signaling, a pathway which is known to be constitutively active in many human malignancies.

Materials and methods

Expression vectors and constructs

cDNAs encoding wild-type and mutant LKB1 fused to the coding sequence for enhanced yellow fluorescent protein (EYFP) were excised from the pcDNA3 vector by digestion with *Nhe*I (5′ end) and *Eco*RI (3′ end); the ends were blunted using Klenow enzyme (Roche) and the fragments were subcloned into pLOX/Ewgfp (Salmon et al. 2000) after digestion of the vector with *Sal*I and removal of overhangs. The ORFs are inserted downstream of the EF1alpha promoter and upstream of the post-transcriptional regulatory element isolated from woodchuck hepatitis virus (Zufferey et al. 1999).

Cell lines, transfections and lentiviral Infections

HeLa cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 10% v/v fetal calf serum (FCS; Invitrogen) in a 5% CO_2 atmosphere at 37°C. To produce infectious viral particles, 293T cells were seeded at a density 10^6 cells/plate and transfected with three plasmids expressing (1) the envelope protein of vesicular stomatitis virus (pMDG, 10 µg), (2) the packaging system (pR8.91, 15 µg), and (3) the gene of interest (pLOX/YFP, pLOX/YFP-LKB-WT, pLOX/YFP-LKB-K78I, or pLOX/YFP-LKB-SL26; 20 µg). Supernatants from three plates of transfected 293T cells were pooled, filtered through a Millipore filter (SLHV 033RS, pore diameter 0.45 µm), and used to infect one plate of HeLa cells seeded to a density of 10^6 cells/plate. Subsequently, HeLa cultures were expanded for preparation of RNA for hybridization to Affymetrix microarrays (see below).

FACS analysis

Infected cells were trypsinized and resuspended in PBS, and YFP fluorescence was directly measured by FACS. Cell cycle analysis was performed using

CycleTest Plus (Becton Dickinson) according the manufacturer's protocol.

Microarray experiments and quantitative RT-PCR

Total RNA was isolated from cell pellets using an RNA extraction kit (RNeasy, Qiagen) and treated with DNase (Qiagen) according to the manufacturer's protocols. Infections were performed in triplicate, each providing one RNA preparation. Two different RNA preparations per sample were then used to generate cRNAs. We generated a hybridization mixture containing 15 μg of biotinylated cRNA and hybridized it to human HG-U133A chips (Affymetrix). Each sample [null (non-transduced cells), YFP, YFP-LKB-WT, YFP-LKB-K78I and YFP-LKB-SL26) was analyzed in duplicate, i.e. for each condition two chips were hybridized and scanned, and the fluorescence signals were analyzed with the Affymetrix software MAS 5.0 and DMT 3.0. The software calculates "signal" values and provides 'detection' calls (present, marginal or absent) for each probe set. Detection calls are determined from statistical calculations of the difference in hybridization signals between perfect match oligonucleotides and their corresponding control mismatch sequence (probe set). To identify differentially expressed transcripts, pairwise comparison analyses were carried out with Affymetrix MAS 5.0. Each of the experimental samples was compared with each of the reference samples, resulting in pairwise comparisons. This approach, which is based on the Mann-Whitney pairwise comparison test, allows the ranking of results by concordance, as well as providing an estimate of the significance (*P*-value) of each identified change in gene expression (Hubbell et al. 2002; Liu et al. 2002). Pairwise analyses of the DNA microarray data obtained with cRNA probes derived from RNA prepared from non-transduced cells or cells expressing YFP or wild-type or mutant LKB1 proteins resulted in four comparison sets. Transcripts were considered to be differentially expressed if their levels changed in the same direction in all four comparisons, if the average median change was at least twofold, or if the *P* value for the median relative change was < 0.05. Results are expressed as the average relative change in the four comparisons.

Ribonucleic acid preparations from three independent experiments were used as templates for quantitative RT-PCR analysis. We carried out quantitative RT-PCRs according to the manufacturer's (Applied Biosystems) protocols using Taqman probes purchased from Eurogentec. All primers were designed with Primer Express Software (Applied Biosystems) and all amplified fragments corresponded to exon–exon junctions. Samples were analyzed in triplicate and the raw data consisted of PCR cycle numbers required to reach a fluorescence threshold (Ct). Raw Ct values were obtained using SDS 2.0 (Applied Biosystems). Relative expression level of target genes was normalized according to geNorm (Vandesompele et al. 2002) using *TBP* (TATA-binding protein) and *EEF1A1* (eukaryotic translation elongation factor α-1) as references to determine the normalization factor. All primer sequences are available on request.

Immunoblot assays

Aliquots (50 μg) of protein extracts were fractionated by SDS-PAGE on 10–15% polyacrylamide gels. Proteins were then electrophoretically transferred to nitrocellulose membranes, which were incubated in PBS containing blocking solution (5% non-fat dry milk and 0.05% Tween 20) for 1 h at room temperature. We then incubated the membranes either overnight at 4°C or for 1.5 h at room temperature in the blocking solution containing appropriate antibodies: anti-fibronectin [F 3648 (Sigma), 1:2,000 dilution], anti-IGFBP3 [sc-9028 (Santa Cruz), 1:1000 dilution], anti-HA (Babco, 1:1000 dilution), anti-β-catenin total [C 2206 (Sigma), 1:8,000 dilution], anti-phospho-β-catenin T41/S45 [9565 (Cell Signaling), 1:1,000 dilution], anti-phospho-GSK-3β S9 [9336 (Cell Signaling), 1:1,000 dilution], anti-GSK-3β [G7914 (Sigma), 1:2,000 dilution], anti-actin [MAB1501 (Chemicon), 1:2,000] and anti-cyclin D1 [CC12 (Oncogene), 1:100 dilution]. The secondary horseradish peroxidase-linked antibody to rabbit or mouse IgG was then added (diluted at 1:8,000 in the blocking solution) and incubated at room temperature for 1 h. We detected immunoreactivity with a commercially available detection system (Catalog No. 34075, Pierce), followed by exposure to Hyperfilm ECL (Amersham) for 1–10 min.

Luciferase assays

HeLa or A549 cells were plated in 6-well plates at 50% confluency and co-transfected with 0.3 μg of pLOX (pLOX/HA-LKB-WT, pLOX/HA-LKB-K78I, pLOX/HA-LKB-SL26 or pLOX/HA-LKB-D176N as indicated), 0.7 μg of luciferase reporter vector (firefly, TOPflash or FOPflash; a gift from Dr. R. Moon) and 0.2 ug of pRL-TK (*Renilla*, Promega) using FuGene Reagent (Roche) according the manufacturer's protocol. Firefly and Renilla luciferase activities were determined 24–48 h post transfection using the Dual Luciferase Reporter Assay System (Promega).

Results

Establishment of viable HeLa cells expressing LKB1 after lentiviral infection

In order to compare gene expression profiles of cells expressing wild type or mutant LKB1 proteins, we decided first to establish a cell line that does not express endogenous LKB1 and survives de novo expression of wild type LKB1. Overexpression of wild-type, but not

catalytically inactive, LKB1 results in a G1 cell cycle arrest in tumor cell lines that do not express the endogenous LKB1 gene (Tiainen et al. 1999; Karuman et al. 2001; Jimenez et al. 2003). Therefore, we tested the hypothesis that expression of low levels of LKB1 does not affect cell growth. For this purpose we used lentiviral vectors because (1) nearly 100% of infected cells express the exogenous protein, and (2) the level of protein expression depends on the promoter used to drive transcription of the gene of interest and on the number of integrated proviruses (which is usually between one and five copies; Naldini et al. 1996). Hence, we chose to express wild type (WT) or mutant (K78I and SL26) LKB1 proteins, driven by the EF1α promoter, from a lentiviral construct after infection of epithelial HeLa cells lacking endogenous LKB1 (no LKB1 expression is detectable at the mRNA or protein level; data not shown).

These two mutants were chosen for the following reasons. The LKB1-K78I mutant, unlike the WT protein, cannot autophosphorylate because of the mutation of the catalytic residue K78 (Su et al. 1996), and the K78I substitution is representative of the majority of loss-of-function LKB1 mutations found in PJS patients (reviewed in Boudeau et al. 2003c). The LKB1-SL26 mutant was identified in a PJS patient. It retains its ki-

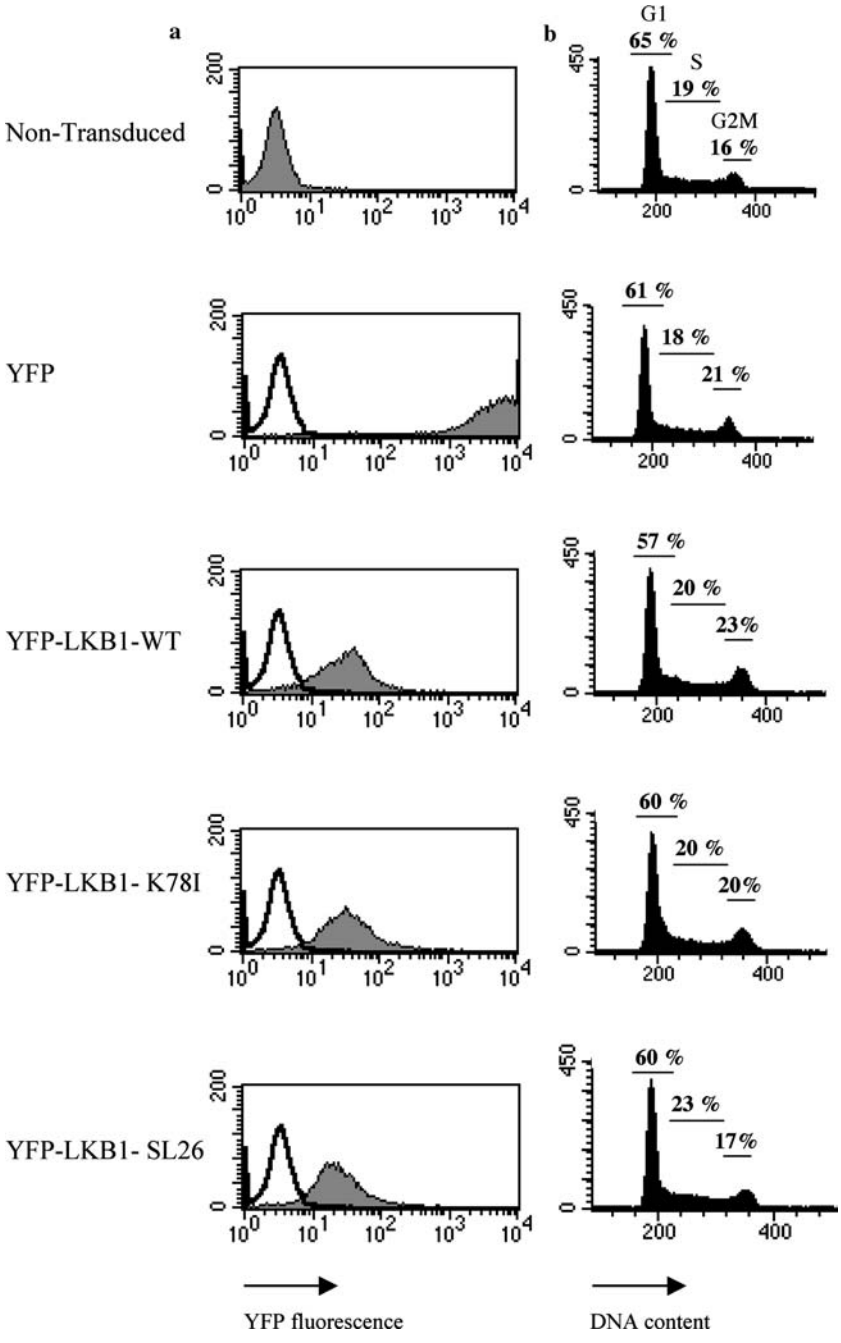

Fig. 1 FACS analysis showing viability of HeLa cells transduced with lentiviral vectors expressing wild type or mutant LKB1. Parental HeLa cells (*HeLa*) or cells transduced with lentiviral vectors expressing YFP, YFP-LKB1-WT, YFP-LKB1-K78I or YFP-LKB1-SL26 were analyzed by FACS 5 days post-infection, and RNA samples for microarray analysis were prepared. **a** YFP fluorescence analysis shows that 100% of cells express the indicated proteins, and that all fusion proteins are expressed at similar levels. **b** Cell cycle analysis of the cells described in a were performed as described in Materials and methods, and the percentages of cells in G1, S or G2/M phases are indicated

nase activity but is nevertheless still pathogenic probably because the mutant protein accumulates in the nucleus (Nezu et al. 1999). All constructs were tagged with YFP or the HA (haemagglutinin) epitope, and immunofluorescence experiments were performed to monitor the cellular localization of the fusion proteins in transduced cells. As previously described, YFP or HA-tagged LKB1-K78I displays the same cellular localization as wild-type LKB1 (i.e. it is found both in the nucleus and the cytoplasm), whereas LKB1-SL26 accumulates predominantly in the nucleus (Nezu et al. 1999; Karuman et al. 2001; data not shown). FACS analysis showed that all YFP-LKB1 fusion proteins were expressed at similar levels (Fig. 1a).

No difference in cell viability was observed when the cell cycle of HeLa cells expressing WT or mutant LKB1 was analyzed 5 days post-infection, indicating that low LKB1 expression levels do not affect HeLa cell viability (Fig. 1b). Five days was the minimum time required for cells to recover from virus infection and divide sufficiently often to provide enough RNA and protein for further analyses. One major advantage of this expression system is that, in contrast to previous studies, it does not induce growth inhibition upon ectopic expression of WT LKB1; thus transcriptome comparisons between cells expressing either WT or mutant LKB1 proteins (K78I or SL26) could be performed. Since the analyses were done 5 days post-infection, the data reflect the consequences of prolonged expression of WT or mutant LKB1 proteins rather than identifying direct targets of LKB1.

Comparative analysis of transcriptomes reveals that the K78I and SL26 mutations affect LKB1 functions differentially

In order to investigate how mutations found in PJS patients might affect LKB1 functions, we compared gene expression profiles between cells expressing wild type or mutant LKB1 proteins. Microarray analysis was performed using total RNAs from uninfected HeLa cells and from cells transduced with lentiviral vectors expressing YFP, YFP-LKB1-WT, YFP-LKB1-K78I or YFP-LKB1-SL26. cRNAs prepared from all samples (each analyzed in duplicate) were hybridized to Human Genome 133A arrays (HG-U133A; Affymetrix) containing ~22,500 transcripts. Transcripts were defined as up-regulated or down-regulated if: (1) variation showed the same trend in the four comparison sets, (2) the ratio of the signal intensities was >2 or <0.5 for induced or repressed genes respectively, or (3) a t test for significance yielded a P value of <0.05.

First, we compared uninfected with YFP-transduced HeLa cells (two arrays each). On the basis of the criteria listed above, YFP expression has only minor effects on the transcription profile when compared to uninfected cells (data not shown). Cells transduced with YFP were then used as the control for comparison with the cells transduced with YFP-LKB1-WT. In this analysis we detected 64 up-regulated and 65 down-regulated transcripts in cells expressing the wild-type LKB1 fusion compared to YFP alone (see Tables S1 and S2, respectively, in the Electronic Supplementary Material). Among the up-regulated set were transcripts encoding PAI1, thrombospondin 1, type IV collagen α1, transgelin (SM22α) and calponin 3—derived from genes that have been shown to be transcriptionally activated by the SMAD4/TGF-β pathway (Dennler et al. 1998; Schwarte-Waldhoff et al. 2000). These results indicate that the cellular system used here is appropriate for functional analysis of LKB1 since the protein has already been suggested to play a role in TGF-β signaling (Smith et al. 2001).

We were then interested in identifying genes whose LKB1-dependent expression is affected by the LKB1 mutations. Further comparisons were therefore performed between cells expressing LKB1-WT and LKB1-K78I or LKB1-SL26, and differentially regulated transcripts are depicted in Fig. 2.

We first noted that expression of the two mutants affected global gene expression differentially. Indeed when compared to cells expressing wild type LKB1, fewer transcripts were misregulated in K78I- (22 transcripts up- and 20 down-regulated; Fig. 2 and Supplementary Table S3) than in SL26 (90 transcripts up- and 60 transcripts down-regulated; Fig. 2 and Supplementary Table S4)-expressing cells. We found very few transcripts that were specifically affected by expression of the K78I variant (two up- and three down-regulated; shown in yellow in Table S3, and see Fig. 2a, b). In contrast, upon expression of SL26, 40 up- and 18 down-regulated transcripts were found to respond specifically in this mutant (Fig. 2a, b; shown in yellow in Table S4). Taken together, these results show that the two mutations are not functionally equivalent, and suggest that global gene expression is affected more by a mutation that alters the cellular

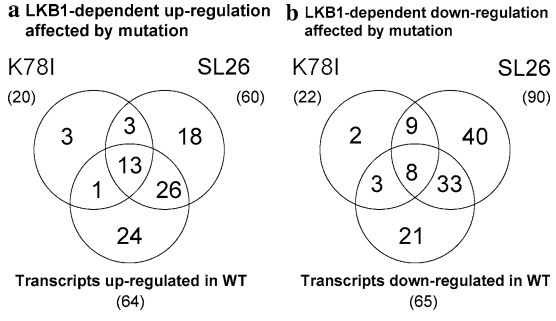

Fig. 2 Microarray analysis. Transcripts are grouped as up- (**a**) or down (**b**)-regulated in cells expressing wild type LKB1 and the numbers of these that are misregulated by a given LKB1 mutation are indicated. Only transcripts whose levels changed by more than twofold (ratios >2 or <0.5) are considered, and the following comparisons were done: K78I versus WT, SL26 versus WT and WT versus YFP. Each number represents a specific Net Affyx ID and is associated with a $P<0.05$. Numbers in *brackets* indicate the total number of transcripts with up- or down-regulated expression in the comparisons listed above

localization of LKB1 than one that reduces its kinase function. Finally, the SL26 mutant exhibits novel functions, since it induces a specific cellular response (transcripts marked in yellow in Supplementary Table S4).

A common subset of transcripts responds to both mutant forms of LKB1

The transcriptome comparisons described in the previous section between HeLa cells expressing normal and mutant LKB1 revealed that a subset of LKB1-dependent genes is affected by both the K78I and SL26 mutations. For instance, 43 (26 + 13 + 1) transcripts that are up-regulated by LKB1-WT (Fig. 2a) and 44 (33 + 8 + 3) transcripts that are down-regulated were affected by K78I or SL26 mutations (Fig. 2b). A detailed analysis identified eight transcripts that are down-regulated by LKB1-WT but not by either of the LKB1 mutants (Fig. 2a and Table 1; transcripts highlighted in blue in Supplementary Tables S3 and S4). Similarly, LKB1-dependent up-regulation of 13 transcripts is affected by K78I and SL26 mutations (Fig. 2b and Table 1; transcripts highlighted in blue in Tables S3 and S4). These results suggest that, although the SL26 mutant retains its kinase activity in vitro, it also fails to regulate some genes that do not respond to the kinase-dead K78I mutant.

Among these commonly regulated transcripts we identified several components of the Wnt signaling pathway. Wnt proteins are secreted molecules that transduce signals by binding to two distinct families of cell surface receptors, namely the Frizzled (Fzd) family

Table 1 Genes differentially regulated in HeLa cells expressing wild-type versus mutant LKB1 kinase

Net affyx ID	Accession no.	Gene	Relative change[a]		
			WT vs. YFP	K78I vs. WT	SL26 vs. WT
Genes up-regulated by LKB1-WT and down-regulated by LKB1 mutants					
203570_at	NM_005576	Lysyl oxidase-like 1 (LOXL1)	16.5	0.27	0.1
202628_s_at	NM_000602.1	Plasminogen activator inhibitor type 1 (PA1)	8.1	0.2	0.1
204298_s_at	NM_002317.1	Lysyl oxidase (LOX)	6.7	0.3	0.2
221029_s_at	NM_030775.1	WNT5B	6.5	0.4	0.3
211719_x_at	BC005858.1	Unknown (protein for MGC:3255)			
215446_s_at	L16895	Lysyl oxidase (LOX) gene, exon 7	5.6	0.4	0.2
212143_s_at	NM_000598.1	Insulin-like growth factor binding protein 3 (IGFBP3)	5.3	0.2	0.16
210495_x_at	AF130095.1	Fibronectin 1	4.76	0.42	0.19
216442_x_at	AK026737.1	Fibronectin 1	4.59	0.43	0.21
214247_s_at	AU148057	Dickkopf 3 (DKK3)	4	0.5	0.4
213909_at	AU147799	*Homo sapiens* cDNA FLJ12280 fis, clone MAMMA1001744	4	0.3	0.3
201578_at	NM_005397.1	Podocalyxin-like (PODXL)	3.36	0.49	0.35
201952_at	L38608.1	Activated leucocyte cell adhesion molecule	3.2	0.5	0.3
Potentially interesting					
205547_s_at	NM_003186.2	Transgelin (TAGLN)	20.7	**0.3**	0.1
210220_at	NM_001466	Frizzled homolog 2 (FZD2)	3	**0.52**	0.4
Genes down-regulated by LKB1-WT and up-regulated by LKB1 mutants					
204920_s_at	W80357	Carbamoyl-phosphate synthetase 1, mitochondrial (CPS1)	0.05	5.2	21
201926_s_at	NM_000574.1	Decay accelerating factor for complement (CD55)	0.1	4.5	8.1
211919_s_at	AF348491.1	Chemokine receptor CXCR4	0.12	3.8	5.9
202946_s_at	NM_014962.1	*Homo sapiens* KIAA0952 protein	0.19	2.98	6.61
217388_s_at	D55639.1	Kynureninase (L-kynurenine hydrolase)	0.28	2.73	3.36
209869_at	NM_000681.1	Alpha-2A adrenergic receptor mRNA	0.30	2.88	3.8
202388_at	NM_002923.1	Regulator of G-protein signaling 2, 24 kD (RGS2)	0.3	2.2	3
202887_s_at	NM_019058.1	Hypothetical protein (FLJ20500)	0.36	2.59	3.03
Potentially interesting					
202890_at	NM_003980	Microtubule-associated protein 7 (MAP7)	**0.04**	**11.3**	28
204748_at	NM_000963	COX-2	0.15	**3.8**	2.12
205935_at	NM_001451.1	Forkhead box F1 (FOXF1)	**0.4**	1.9[b]	2.4
204198_s_at	NM_004350.1	Runt-related transcription factor 3 (RUNX3)	0.4	1.6[b]	2.2

[a]Expression levels of transcripts found up- or down–regulated upon YFP-LKB1-WT were compared with those found in YFP-LKB1-K78I and YFP-LKB1-SL26 mutant expressing cells. Transcripts whose expression levels are affected by both mutations are listed. Only transcripts with expression (signal intensity) ratios of >2 or <0.5 for induced or repressed transcripts, respectively, and a P value of <0.05 are considered. Relative changes shown in *bold* are associated with P values of >0.05

[b]Changes of less than twofold

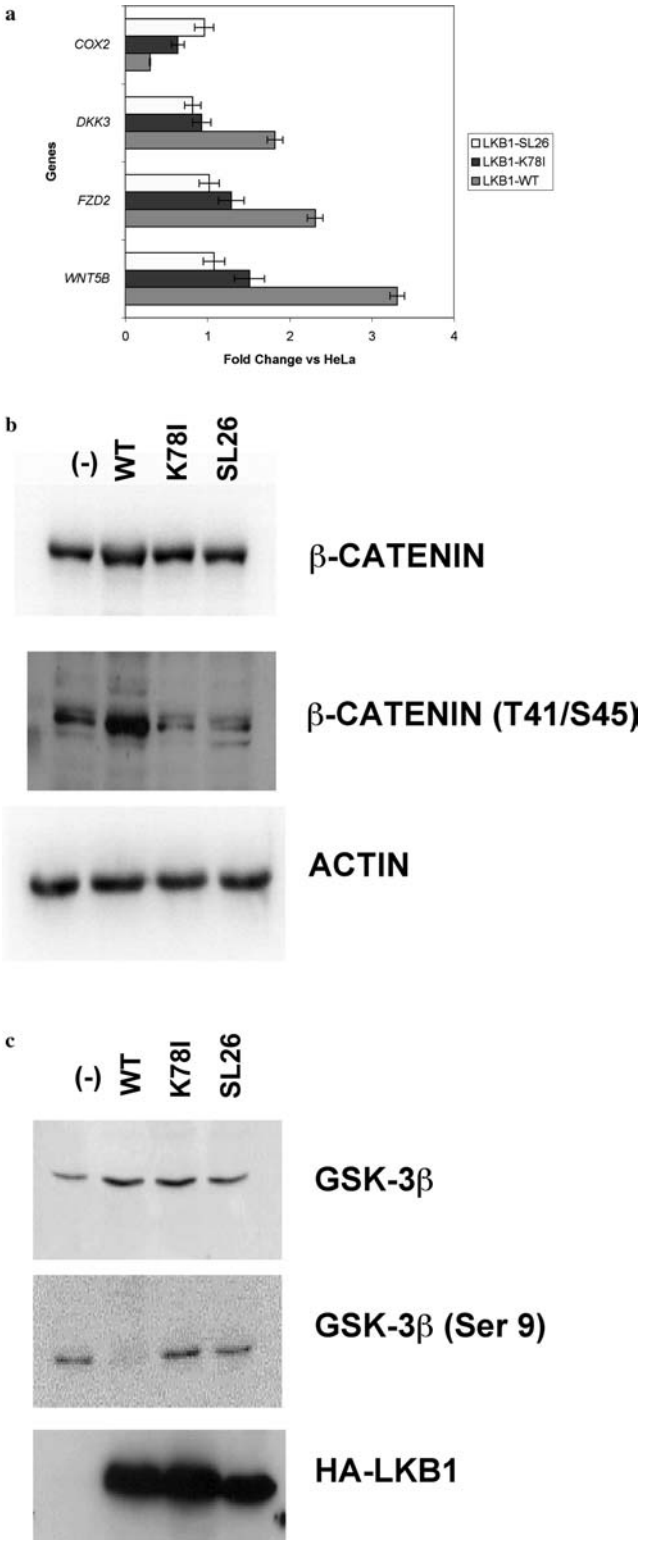

Fig. 3 Expression of WT but not mutant LKB1 proteins in HeLa cells dys-regulates transcription of several genes involved in the Wnt signaling and induces β-catenin phosphorylation by activation of GSK-3β **a** qRT-PCR was performed on RNA extracts from parental HeLa cells and from cells expressing WT or mutant (K78I and SL26) LKB1 proteins. Data are means (± SD) from three independent experiments analyzed in triplicate. Data are normalized with reference to the housekeeping genes mentioned in Materials and methods. Data obtained with RNA from HeLa cells served as reference and the relative changes correspond to the following ratios: LKB1-WT/HeLa, LKB1-K78I/HeLa, LKB1-SL26/HeLa. Genes for components of the Wnt signaling pathway (*DKK3, FZD2* and *WNT5B*) and the Wnt target gene *COX2* were assayed. **b, c** Lysates of parental HeLa cells (-) or cells tranduced with HA-LKB1-WT, HA-LKB1-K78I or HA-LKB1-SL26 were prepared 5 days post-infection and fractionated by SDS-PAGE, and selected endogenous proteins were detected by immunoblotting with the following antibodies: anti-β-catenin and T41/S45 phospho-specific anti-β-catenin antibodies (**b**), or anti-GSK-3β, S9 phospho-specific anti-GSK-3β and anti-HA antibodies (**c**). Anti-actin antibodies were used to check that equal amounts of total proteins (50 µg) had been loaded

and the LDL-receptor-related-protein (LRP) family. In contrast, members of the Dickkopf (Dkk) protein family inhibit Wnt signal transduction. In particular, we found that transcription of *WNT5B* and *DKK3* (Dickkopf-3) is induced—6.5-fold and fourfold, respectively—upon stable expression of YFP-LKB1-WT (Table 1), and these differences were confirmed by qRT-PCR (Fig. 3a). The *Frizzled-2* gene (*FZD2*) was tested by qRT-PCR because it was found to be up-regulated in LKB1-WT-expressing cells and down-regulated in cells that express K78I or SL26 (albeit with a $P > 0.05$ for the K78I vs. WT comparison); qRT-PCR confirmed the microarray data. Similarly, *COX2*, which has been identified as a target of Wnt (Araki et al. 2003) is up-regulated twofold to fourfold in mutant-expressing cells (Table 1). qRT-PCR also confirmed the microarray data for *COX2* (Fig. 3a). As *DKK3* expression is induced and *COX2* expression is repressed in YFP-LKB1-WT but not in mutant-expressing cells, these results suggest that wild-type LKB1 inhibits the Wnt signal transduction pathway and that this function is compromised by mutations in LKB1.

GSK-3β activation is impaired by the K78I and SL26 mutations in LKB1

Canonical Wnt signaling is initiated by the formation of a Wnt-Fzd-LRP complex, resulting in nuclear accumulation of β-catenin and subsequent activation of target genes. The secreted protein Dickkopf 1 (Dkk1) specifically inhibits complex formation by binding to the LRP, thus inhibiting Wnt signaling by preventing accumulation of β-catenin in the nucleus (Bafico et al. 2001). Unphosphorylated β-catenin accumulates in the nucleus and activates transcription of Wnt target genes containing DNA binding sites for the TCF/LEF family of transcription factors (TCF-LEF dependent), whereas phosphorylated β-catenin is degraded by the proteasome (Miller 2002). Based on the sequence homology between, and the functional equivalence of, Dkk1 and Dkk3 in vitro (Caricasole et al. 2003), we further tested the hypothesis that up-regulation of *DKK3* in LKB1-

WT-expressing cells could inhibit canonical Wnt signal transduction by inducing phosphorylation of β-catenin. We used phospho-specific antibodies to analyze by Western blotting the phosphorylation state of residues T41 and S45 of β-catenin in protein extracts from cells transduced with the empty YFP vector, or the LKB1-WT, K78I or SL26 construct. We found that β-catenin is phosphorylated only in LKB1-WT-expressing cells (Fig. 3b), which is consistent with the idea that *DKK3* overexpression results in the inhibition of canonical Wnt signaling. In contrast, LKB1 proteins with mutations affecting either its kinase activity (K78I) or its subcellular localization (SL26) fail to induce β-catenin phosphorylation and therefore could potentially activate canonical Wnt signaling. However, although β-catenin is phosphorylated in LKB1-expressing cells, it is not degraded as expected: amounts of β-catenin in all extracts are similar.

Regulation of the Wnt/β-catenin pathway is mediated by the glycogen synthase kinase 3-beta (GSK-3β; reviewed in Doble and Woodgett 2003)). De-phosphorylation of GSK-3β at residue S9 activates its catalytic activity, allowing it to phosphorylate its substrate β-catenin (Harwood 2001). We therefore assayed GSK-3β activity by Western analysis of protein extracts from cells transduced with LKB1 WT or mutant LKB1. We found that GSK-3β is phosphorylated at S9 in uninfected HeLa cells, and in cells expressing mutant (K78I and SL26) but not WT LKB1 proteins (Fig. 3c). These data demonstrate that LKB1 expression in HeLa cells is sufficient to allow GSK-3β to become active, either by inhibiting a kinase or activating a phosphatase specific for GSK-3β. Furthermore, we show that, in LKB1-WT-expressing cells, GSK-3β activity correlates with the presence of phosphorylated β-catenin. These data indicate that LKB1 negatively regulates transcription of Wnt target genes by inducing-dephosphorylation of GSK-3β. Therefore, we hypothesize that LKB1 mutants that are defective in the ability to regulate the phosphorylation state of GSK-3β are likely to activate the canonical Wnt pathway.

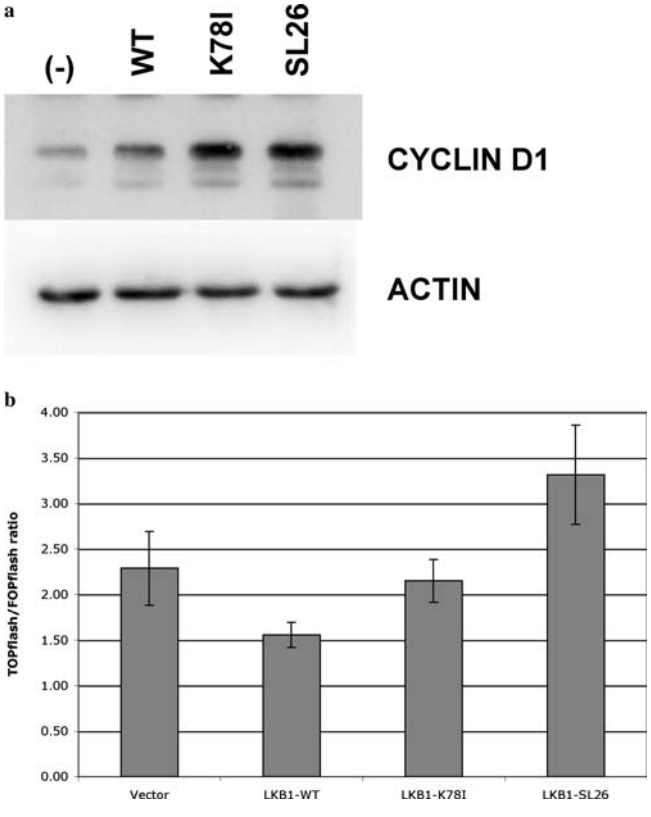

Fig. 4 Wild-type LKB1 inhibits canonical Wnt signaling in A549 lung epithelial cells. **a** Lysates of parental HeLa cells (-) or cells tranduced with HA-LKB1-WT, HA-LKB1-K78I or HA-LKB1-SL26 were prepared 5 days post-infection and fractionated by SDS-PAGE, and selected endogenous proteins were detected by immunoblotting with anti-cyclin D1 antibodies. **b** Constructs expressing WT or mutant K78I or SL26 LKB1, as well as active and mutated TCF-LEF luciferase reporter plasmids (TOPFLASH and FOPFLASH), were transfected into A549 cells. Transcription of the reporter gene was assayed by measuring luciferase activity according the manufacturer's instructions. Values are means (± SD) of triplicate samples of three independent experiments

Wild-type LKB1 inhibits the canonical Wnt signaling pathway

Microarray and biochemical data from HeLa cells thus suggest that LKB1 may inhibit Wnt signaling. Therefore, loss of functional LKB1 could lead to activation of the canonical Wnt pathway. We proceeded to test the hypothesis that LKB1 is a negative regulator of Wnt-responsive genes and that LKB1 mutants found in PJS patients are defective for this inhibitory activity on the Wnt/β-catenin pathway. To explore Wnt signaling, transcription of a TCF-LEF dependent luciferase reporter gene was analyzed following co-transfection of the reporter gene together with expression plasmids for WT or mutant LKB1. Two reporter genes under the control of TCF promoters were used to assay the activity of the canonical Wnt pathway. The TOPFLASH plasmid contains multimeric TCF-binding sites upstream of the luciferase reporter gene, whereas the FOPFLASH version has mutations in the TCF-binding sites. Activation of the canonical Wnt pathway is usually measured by the ratio between the levels of luciferase activity in cells transfected with the TOPFLASH and FOPFLASH plasmids. In HeLa cells, this ratio is 1, indicating that Wnt signaling is not constitutively active. Therefore in the absence of any positive stimulus we were unable to draw any conclusions regarding the inhibitory effect of LKB1 expression on Wnt signaling in HeLa cells using this reporter system (data not shown).

We then tested the TCF-LEF reporter luciferase in A549 adenocarcinoma lung epithelial cells which, -like Hela cells, do not express the endogenous *LKB1* gene. We found weak Wnt signaling in cells that had not been transfected with any of the LKB1 constructs, since the TOPFLASH/FOPFLASH ratio was around 2. We found 30% inhibition of Wnt signaling upon expression

of WT LKB1, whereas the K78I mutant had no effect and SL26 actually activated signaling by up to 40% (Fig. 4b). We also found that the level of cyclin D1 protein is higher in cells expressing mutant LKB1 than in non-transduced HeLa cells, which is consistent with increased transcription of at least some endogenous Wnt target genes in LKB1 mutant-expressing cells (Fig. 4a). This result suggests that LKB1 mutants are indeed able to activate canonical Wnt signaling on their own, and is consistent with the presence of less phosphorylated β-catenin in cells expressing mutant LKB1 than in non-transduced cells (Fig. 3b). However, in contrast to SL26, the K78I mutant has no effect on Wnt signal transduction in A549 cells, suggesting that the ability of LKB1 mutants to activate Wnt signaling could be dependent on the cell type considered.

Taken together, our data suggest that LKB1 negatively regulates the Wnt/β-catenin pathway and that activation of this pathway could be a common feature of PJS mutants. As activation of Wnt signaling has been implicated in the development of many cancers, including colorectal and ovarian cancers (Giles et al. 2003), this finding is compatible with the cancer predisposition of PJS patients.

Other notable transcriptome differences

Among the other transcriptome differences listed in Table 1, several interesting transcripts that appear to be functionally relevant for the involvement of LKB1 in tumor suppression, embryogenesis or vasculogenesis were identified. One of these encodes transgelin (*TAGLN* alias SM22α), which is a marker for differentiated smooth muscle cells and is important in blood vessel formation (Zhang et al. 2001); its expression is highly increased in LKB1 expressing cells. Because the *P* value for *TAGLN* was >0.05 in K78I-expressing cells, *TAGLN* expression was determined at the mRNA and protein levels using quantitative qRT-PCR and Western analysis, respectively. Our data show that TAGLN is only expressed in LKB1-tranduced cells; the ability to induce TAGLN expression is abolished by both K78I and SL26 mutations (Fig. 5a, c).

We also confirmed by qRT-PCR that LKB1 mutations affect the capacity of LKB1 to down-regulate genes

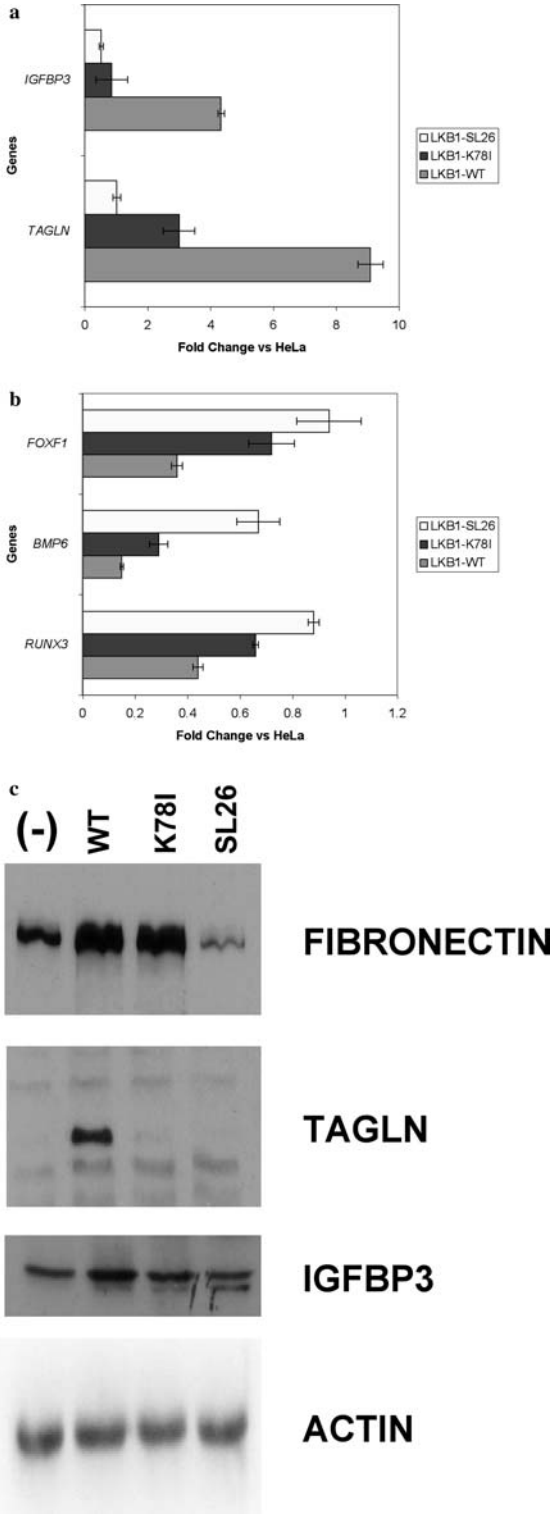

Fig. 5 Expression of exogenous LKB1 in HeLa cells is associated with mis-regulation of other genes mainly involved in vasculogenesis. qRT-PCR was performed on RNA extracts from parental HeLa cells and from cells expressing WT or mutant (K78I and SL26) LKB1 proteins. Data are means (± SD) from three independent experiments analyzed in triplicate. Data are normalized with respect to the housekeeping genes mentioned in Materials and methods. Data obtained with RNA from HeLa cells served as reference and the relative changes correspond to the following ratios: LKB1-WT/HeLa, LKB1-K78I/HeLa, LKB1-SL26/HeLa. Genes up-regulated by LKB1-WT (such as *IGFBP3* and *TAGLN*) were tested in **a** and genes down-regulated by LKB1-WT (such as *FOXF1*, *BMP6* and *RUNX3*) were tested in **b, c** Lysates from parental HeLa cells (-) or cells tranduced with HA-LKB1-WT, HA-LKB1-K78I or HA-LKB1-SL26 were prepared 5 days post-infection and fractionated by SDS-PAGE, and selected endogenous proteins were detected by immunoblotting with the following antibodies: anti-fibronectin, anti-IGFBP3, anti-transgelin antibodies. Anti-actin antibodies were used to check that equal amounts of total protein (50 μg) had been loaded

such *FOXF1* (which encodes a transcription factor important for cell migration; Kalinichenko et al. 2002), *BMP6* (which codes for a signaling molecule important during development for cell communication; Perr et al. 1999) and *RUNX3* (a tumor suppressor gene for gastric cancer; Li et al. 2002) (Fig. 5b).

We also found that the expression of genes involved in vasculogenesis, such as *LOXL1*, *PODXL* and *IGFBP3*, was similarly affected in cells expressing either LKB1 mutant. *LOXL1* encodes lysyl oxidase-like 1 protein, which catalyzes the oxidative deamination of lysine residues in elastin. Interestingly, vascular abnormalities occur in mice lacking *LOXL1* (Liu et al. 2004). *PODXL* (Kershaw et al. 1997) and *IGFBP3* (Lee et al. 1999) are highly expressed in vascular endothelium cells and might regulate vascularization. We confirmed by qRT-PCR and Western analysis that *IGFBP3* transcripts are induced upon expression of wild type LKB1 but not mutant proteins (Fig. 5a, c).

The amount of fibronectin in protein extracts from cells expressing wild type or mutant LKB1 was also analyzed, since fibronectin levels are reduced in transformed cells (Spicer and Branton 1980). While fibronectin RNA levels are decreased in all transductants expressing mutant LKB1 (Table 1), the protein level was found to be reduced only in extracts obtained from SL26-expressing cells (Fig. 5c). This result supports the notion that *LKB1* mutations have distinct effects and suggests that the SL26 mutant might be the more potent promoter of tumorigenesis. Moreover, these data identify new candidate proteins for involvement in metabolic pathways regulated by LKB1.

Discussion

This study was designed to investigate the molecular pathogenesis of PJS by comparing transcription profiles of cells expressing exogenous WT or mutant LKB1. The use of both K78I and SL26 mutants in our transcriptome analysis makes the present work complementary to previous array studies performed in lung epithelial A549 (Jimenez et al. 2003) or LKB1 null MEF cells (Bardeesy et al. 2002). We took advantage of the ability of lentiviral vectors to drive the expression of low levels of LKB1 protein in a homogeneous cell population. In contrast to previous studies, this allowed us to perform microarray analysis in growing HeLa cells, simply because LKB1 is not overexpressed. HeLa cells have been extensively used to study LKB1 functions because they lack endogenous LKB1 expression (Smith et al. 2001; Sapkota et al. 2002) and therefore are useful for functional analysis of wild-type or mutant LKB1 proteins. Our microarray data analysis confirmed the suitability of this cell line as a model system for the study of LKB1 functions since it is consistent with the activation of SMAD4/TGF-β signaling upon expression of LKB1, and with *COX2* up-regulation in association with loss of LKB1 function, two features previously linked to LKB1 function (Smith et al. 2001; Jishage et al. 2002; Rossi et al. 2002; Wei et al. 2003).

In $Lkb1^{+/-}$ mice, haploinsufficiency for *LKB1* is responsible for polyp formation (Jishage et al. 2002). In humans, despite the fact that loss of heterozygosity (LOH) affecting the WT allele is found in 70% of tumors isolated from PJS patients (Hemminki et al. 1997), there is no evidence that polyposis and cancer predisposition arise from LOH only. Although further investigations are necessary to establish that *LKB1* mutant alleles are expressed in PJS patients, comparisons of transcriptomes from cells expressing wild-type versus mutant LKB1 proteins highlights signaling pathways that are potentially affected by *LKB1* mutations found in PJS patients.

Transcriptome comparisons of cells expressing mutant versus wild type LKB1 show that: (1) the LKB1-dependent transcription of some genes is affected by both K78I and SL26 mutations, and (2) these LKB1 mutations also have different effects on overall gene expression. We determined that almost all genes affected by the LKB1-K78I mutant (which lacks kinase activity) are also misregulated in cells that express the SL26 mutant (which accumulates in the nucleus). This result is surprising, because SL26 retains its kinase activity, as indicated by in vitro tests (data not shown), but confirms previous evidence that both the kinase activity and the cytoplasmic localization of LKB1 are necessary for this protein to perform its normal cellular function (Nezu et al. 1999). This implies that at least some of the targets of LKB1 are likely to be cytoplasmic.

In addition, de novo expression of YFP-LKB-SL26 affects specific genes, whose expression is not modified by either YFP-LKB-WT or YFP-LKB-K78I. This suggests that nuclear accumulation of SL26 induces a specific cellular response and that this particular mutant might have acquired new functions, probably phosphorylating novel nuclear substrates.

Many amino acid substitutions found in PJS patients result in loss of kinase activity. How the SL26 mutation leads to the PJS phenotype has remained an open question. Our microarray experiment partially answers this question by pinpointing several genes that are commonly affected in both K78I- and SL26-expressing cells. In agreement with the proposed function of LKB1, genes identified by our microarray data as being differently regulated between cells expressing WT or mutant LKB1 proteins are mostly involved in cellular differentiation, and in cell adhesion and migration. Several of these genes play a role in vasculogenesis (*LOX, IGFBP3, TAGLN*, and *PODXL*). This finding is of interest, since $Lkb1^{-/-}$ mice exhibit vascular abnormalities, and show a complete absence of vascular smooth muscle cells in the dorsal aorta and somites (Ylikorkala et al. 2001).

Interestingly, we found several of the genes that are misregulated in mutant-expressing cells are involved in Wnt signaling, supporting recent observations that LKB1 regulates developmental processes through this pathway (Ossipova et al. 2003; Spicer et al. 2003). We identified *DKK3*, *WNT5B* and *FZD2* as potential

candidates for this regulation. Several studies provide evidence that WNT5A (a close homolog of WNT5B) is able to antagonize the Wnt/β-catenin pathway (Miller et al. 1999). WNT5A may act as a tumor suppressor since it is able to suppress transformation by WNT1. Furthermore, expression of *Xenopus* Wnt5A leads to an increase in Ca^{2+} fluxes and to activation of protein kinase C (Miller et al. 1999). In contrast, very little is known about functions of WNT5B, and further investigations are necessary to establish whether its functions are similar to those of WNT5A. FZD2, WNT5A and PLC activate the Wnt/Ca^{2+} pathway, while proteins belonging to the Dickkopf family inhibit the Wnt/β-catenin pathway (Miller 2002). Based on the sequence homologies between WNT5A and WNT5B, as well as those between DKK1 and DKK3, our microarray data suggest that LKB1 could be a molecular switch for Wnt signaling, activating the Wnt/Ca^{2+} pathway and inhibiting Wnt/β-catenin signaling. In contrast, LKB1 mutants might be impaired in this switch regulation. As the canonical Wnt pathway is essential for the integrity of intestinal crypt cells and for epithelium homeostasis (Pinto et al. 2003), failure to properly regulate this pathway could explain the propensity of PJS patients to develop polyposis.

LKB1 has recently been reported to be a regulator of GSK-3β activity (Ossipova et al. 2003). Experimental data presented in that report suggested that GSK-3β might be a substrate for LKB1, since reduction of *LKB1* expression resulted in diminished phosphorylation of GSK-3β at residue S9. In contrast, we found that de novo LKB1 expression results in dephosphorylation of GSK-3β at the same S9 residue. Hence, our data support the idea that GSK-3β is not a direct substrate for LKB1. Furthermore, our data are consistent with the failure of immunoprecipitated *Xenopus* LKB1 (XEEK1) to directly phosphorylate the GSK-3β protein (Ossipova et al. 2003). Our finding that LKB1 protein activates GSK-3β is particularly instructive since both proteins have previously been implicated in regulating cell polarity (Watts et al. 2000; Etienne-Manneville and Hall 2003; Martin and St Johnston 2003; Baas et al. 2004a). The inability of mutant forms of LKB1 to activate GSK-3β might therefore be responsible for the lethality of mutations in LKB1 orthologs in *C. elegans* and *D. melanogaster*, as well as in $Lkb1^{-/-}$ knockout mice.

Finally, we show that expression of LKB1 inhibits Wnt signaling in A549 cells, another cell line that lacks endogenous LKB1 (Carretero et al. 2004). However, in transduced HeLa cells, LKB1-mediated phosphorylation of β-catenin did not seem to lead to degradation of the latter by the proteosome. In order to explain this apparently conflicting result, β-catenin localization was investigated by performing immunostaining for total and phosphorylated β-catenin in HeLa cells expressing WT or mutant LKB1 proteins. Upon expression of mutant LKB1, the number of cells with nuclear β-catenin staining is increased, whereas β-catenin localizes preferentially at membranes in non-transduced cells and in the cytoplasm in cells expressing WT LKB1 (data not shown). One possible explanation for the accumulation of β-catenin in WT LKB1 expressing cells is that, upon LKB1 expression, phosphorylated β-catenin is not ubiquitinated and therefore not targeted for degradation. Although we cannot explain how LKB1 blocks Wnt signal without promoting β-catenin proteolysis, this result is consistent with the effect of LKB1 expression in HCT116 cells (in which one β-catenin allele is mutated at Ser45 and cannot be targeted for degradation to the proteosome). Although TCF/β-catenin dependent transcription is constitutively activated in this particular HCT116 cell line, exogenous expression of LKB1 decreases Wnt signaling by 50% (data not shown). This observation supports the notion that degradation of β-catenin might be unrelated to the ability of LKB1 to inhibit Wnt signaling.

In A549 cells, the LKB1 kinase activity is necessary for this inhibition. In contrast, SL26 stimulates transcription of Wnt target genes, as revealed using a reporter gene and by the response of the endogenous target gene encoding cyclin D1. This probably occurs because SL26 is impaired in its ability to regulate GSK-3β activity, resulting in the presence of unphosphorylated β-catenin in SL26-expressing cells. This finding is of interest, as somatic mutations at codons 41 and 37 in the β-catenin gene (Miyaki et al. 2000), as well as nuclear accumulation of β-catenin (Back et al. 1999), have been reported in gastrointestinal polyps from PJS patients. Notably, mutations in the *APC* gene that are associated with familial adenomatous polyposis (FAP) result in the development of polyposis and colon carcinomas through activation of the Wnt/β-catenin pathway (Giles et al. 2003). This suggests that mutations in genes that regulate β-catenin phosphorylation are generally involved in the development of polyposis and cancer predisposition. The ability of the SL26 mutant to activate the Wnt/β-catenin pathway is consistent with this hypothesis. The question whether activation of this pathway, either by the positive effect of a gain-of-function LKB1 mutant (such as SL26) or by loss of the inhibitory LKB1 function, is a common feature of PJS mutants must remain open. As activation of the Wnt pathway has been implicated in the development of many cancers, including colorectal and ovarian cancers (Giles et al. 2003), this finding is compatible with the cancer predisposition of PJS patients. Moreover, our study has identified novel candidates for a second potential PJS locus.

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