Identification of TGFβ signaling, p53, and actin stress fibers as targets of LKB1 tumor suppressor activity

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Academic dissertation

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HELSEINKI 2011
"Tieteelliset tutkimukset ovat fikiota siinä kuin romaanikin, erona se että ne ovat huonosti kirjoitettuja ––“

Pentti Saarikoski, Euroopan reuna
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ABBREVIATIONS

aa  amino acid
ACC  acetyl co-enzyme A
AMP  adenosine 3', 5'-cyclic monophosphate
AMPK  AMP-activated kinase
APC  adenomatous polyposis coli
ARE  activin response element
aPKC  atypical protein kinase C
ATM  ataxia telengiectasia mutated
BMP  bone morphogenic protein
BMPR1  BMP receptor 1
BRSK1-2  brain-specific kinase 1-2
CamKKβ  calcium/calmodulin-dependent protein kinase kinase β
CDK  cyclin-dependent kinase
c-MYC  v-myc myelocytomatosis viral oncogene homolog
CPI-17  protein phosphatase 1 regulatory subunit 14A
CREB  cAMP response-element binding protein
CRM1  chromosome region maintenance 1
CRTC2  CREB-regulated transcription coactivator 2
C-terminus  carboxy-terminus
DDR1  discoidin domain receptor 1
ECM  extra cellular matrix
EMT  epithelial-mesenchymal transition
EGF  epidermal growth factor
EGFp  enhanced green fluorescent protein
EGFR  epidermal growth factor receptor
FAK  focal adhesion kinase
FoxO  forkhead box class O
FSP1  fibroblast-specific protein 1
GAP  GTPase-activating protein
GEF  guanine nucleotide-exchange factor
GF  growth factor
GFR  growth factor receptor
GI  gastrointestinal
GSK3  glycogen synthase kinase-3
GST  glutathione S-transferase
GTP  guanosine-5'-triphosphate
GTPase  GTP- hydrolase
H2B  histone 2B
HMGR  3-hydroxy-3-methylglutaryl-CoA reductase
IF  immunofluorescence
IHC  immunohistochemistry
IP  immunoprecipitation
kDa  kilodalton
KRAS  Kirsten rat sarcoma viral oncogene homolog
LAPs  latency-associated proteins
<table>
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<tr>
<th>Abbreviation</th>
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<td>LIP1</td>
<td>LKB1-interacting protein</td>
</tr>
<tr>
<td>LOH</td>
<td>loss-of-heterozygosity</td>
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<tr>
<td>LTBP</td>
<td>latent-TGFβ-binding protein</td>
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<tr>
<td>MAL</td>
<td>megakaryoblastic leukemia 1</td>
</tr>
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<td>microtubule-associated protein</td>
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<tr>
<td>MARKK</td>
<td>MARK activating kinase</td>
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<tr>
<td>MDM2</td>
<td>murine double minute 2</td>
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<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
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<tr>
<td>MELK</td>
<td>maternal embryonic leucine zipper kinase</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MLC</td>
<td>myosin light chain</td>
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<td>myosin light chain phosphatase</td>
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<tr>
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<td>matrix metalloproteinase</td>
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<td>MO25</td>
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<td>nuclear export signal</td>
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<td>NLS</td>
<td>nuclear localization signal</td>
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<tr>
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<td>NSCLC</td>
<td>non-small cell lung cancer</td>
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<td>p90RSK</td>
<td>p90 ribosomal S6 kinase</td>
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<td>plasminogen activator inhibitor type 1</td>
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<td>PAI-1 luciferase assay</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>PI3K</td>
<td>phosphatidylinositol-3 kinase</td>
</tr>
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<td>PJS</td>
<td>Peutz-Jegher’s syndrome</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
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<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PP1</td>
<td>protein phosphatase 1</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
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<tr>
<td>pRB</td>
<td>retinoblastoma susceptibility protein</td>
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<tr>
<td>RAS</td>
<td>rat sarcoma viral oncogene homolog family (KRAS, HRAS and NRAS)</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<td>ROCK</td>
<td>Rho-associated protein kinase</td>
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<td>salt inducible kinase 1-3</td>
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<td>short interfering RNA</td>
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<td>α-smooth muscle actin</td>
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<td>SMA and MAD related</td>
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<td>smooth muscle cell</td>
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<td>SRF</td>
<td>serum response factor</td>
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<td>STRAD</td>
<td>Ste20 related adaptor</td>
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<tr>
<td>TF</td>
<td>transcription factor</td>
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<td>TGFβ</td>
<td>transforming growth factor β</td>
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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 are presented in this
text.

I Tiainen M, Vahtomeri K, Ylikorkala A, Mäkelä TP. “Growth arrest by the LKB1
tumor suppressor: induction of p21 (WAF1/CIP1)”. *Human Molecular Genetics*. Jun

II Katajisto P, Vahtomeri K, Ekman N, Ventelä E, Ristimäki A, Bardeesy N, Feil R,
DePinho RA, Mäkelä TP. “LKB1 signaling in mesenchymal cells required for

Tiainen M, Mäkelä TP. “Lkb1 is required for TGFβ-mediated myofibroblast

IV Vallenius T*, Vahtomeri K*, Kovac B, Osiceanu AM, Viljanen M, and Mäkelä TP.
“An association between NUAK2 and MRIP reveals a novel mechanism for regulation of

* =equal contribution

Publication II was also used in the thesis of Ph.D. Pekka Katajisto.
ABSTRACT

Tumorigenesis is a consequence of inactivating mutations of tumor suppressor genes and activating mutations of proto-oncogenes. Most of the mutations compromise cell autonomous and non-autonomous restraints on cell proliferation by modulating kinase signal transduction pathways. \textit{LKB1} is a tumor suppressor kinase whose sporadic mutations are frequently found in non-small cell lung cancer and cervical cancer. Germ-line mutations in the \textit{LKB1} gene lead to Peutz-Jeghers syndrome with an increased risk of cancer and development of benign gastrointestinal hamartomatous polyps consisting of hyperproliferative epithelia and prominent stromal stalk composed of smooth muscle cell lineage cells. The tumor suppressive function of LKB1 is possibly mediated by 14 identified LKB1 substrate kinases, whose activation is dependent on the LKB1 kinase complex. The aim of my thesis was to identify cell signaling pathways crucial for tumor suppression by LKB1.

Re-introduction of \textit{LKB1} expression in the melanoma cell line G361 induces cell cycle arrest. Here we demonstrated that restoring the cytoplasmic LKB1 was sufficient to induce the cell cycle arrest in a tumor suppressor p53 dependent manner. To address the role of LKB1 in gastrointestinal tumor suppression, \textit{Lkb1} was deleted specifically in SMC lineage \textit{in vivo}, which was sufficient to cause Peutz-Jeghers syndrome type polyposis. Studies on primary myofibroblasts lacking \textit{Lkb1} suggest that the regulation of TGF\(\beta\) signaling, actin stress fibers and smooth muscle cell lineage differentiation are candidate mechanisms for tumor suppression by LKB1 in the gastrointestinal stroma. Further studies with LKB1 substrate kinase NUAK2 in HeLa cells indicate that NUAK2 is part of a positive feedback loop by which NUAK2 expression promotes actin stress fiber formation and, reciprocally the induction of actin stress fibers promote NUAK2 expression. Findings in this thesis suggest that p53 and TGF\(\beta\) signaling pathways are potential mediators of tumor suppression by LKB1. An indication of NUAK2 in the promotion of actin stress fibers suggests that NUAK2 is one possible mediator of LKB1 dependent TGF\(\beta\) signaling and smooth muscle cell lineage differentiation.
INTRODUCTION

Tumorigenesis is a condition of uncontrolled cell proliferation. Recently, it has become evident that tumors should not be considered as independent entities composed entirely of tumor cells but instead as complex tissues. For example, epithelial tumors are comprised of hyperproliferative epithelial cells that reciprocally interact with altered stroma consisting of fibroblasts, immune cells and vasculature. Tumor cells can be relieved from the restraints set by cell extrinsic and intrinsic tumor suppression. However, for this to occur, multiple loss-of-function mutations of tumor suppressor genes and dominant activating mutations of proto-oncogenes (called oncogenes subsequent to activating mutation) are required. Hereditary cancer syndromes have provided a valuable tool for the identification of several tumor suppressor genes that include adenomatous polyposis coli (APC) tumor suppressor whose hereditary mutations are causative of familial adenomatous polyposis coli. The LKB1 tumor suppressor kinase was cloned in 1998 in the search for a cause of Peutz-Jeghers syndrome (PJS), a syndrome that is characterized by an increased risk of cancers and gastrointestinal (GI) polyposis. Subsequently, LKB1 was identified as being frequently mutated in non-small cell lung cancer (NSCLC) and especially in lung adenocarcinomas. In my thesis work I have studied the signaling networks and cellular functions that are essential for tumor suppression by LKB1. In the literature review I will first discuss the role of tumor suppressors and oncogenes in regulating the cell division cycle and tumor stroma interactions. Accordingly, special focus is laid on p53 and transforming growth factor β (TGFβ) tumor suppressor signaling, which mediate cell autonomous and non-autonomous tumor suppression, respectively. Both p53 and TGFβ are also central to this thesis work. Finally, genetic evidence for tumor suppression by LKB1, LKB1 signaling network, and suggested tumor suppressive functions of LKB1 are presented. The most recent publications that complement or contrast the results presented in this thesis are discussed in the results and discussion section.
REVIEW OF LITERATURE

1. Tumor suppressors inhibit kinase signal transduction pathways and cell proliferation

1.1 Kinases are central mediators of cell growth

Cell growth is triggered by external mitogenic cues, which induce multiple cell signal transduction pathways that relay the signals from the cell surface to exert their effects on, inter alia, cell division and protein translation machinery in the nucleus and in the cytoplasm, respectively. Cell signaling takes place in protein complexes in which the signal is delivered by various means to modify activity and/or localization of enzymes and transcription factors (TF). These changes are achieved by the regulated production and release of second messengers, such as phospholipids and calcium, and/or by covalent modifications of proteins by phosphorylation among other things.

Signaling pathways that are essential in cell growth and tumorigenesis are characterized by kinase cascades. The human genome encodes 518 kinases that comprise 90 tyrosine kinases and 428 serine/threonine kinases, which together constitute about 2% of human genes. Almost half of the kinases, such as epidermal growth factor receptor (EGFR) and protein kinase B (PKB/AKT) are located in genetic loci linked to cancer or other diseases, which highlights the immense importance of this class of proteins. Indeed, kinase domain encoding genes are the most prevalent group of genes indicated in tumorigenesis. Kinases phosphorylate tyrosine (Tyr), serine (S), and threonine (T) residues, which may regulate allosteric conformation of proteins or protein-protein interactions and thus have an effect on the activity of the target protein. There are also approximately 50 pseudokinases encoded by the human genome, which are inactive due to substitution of at least one conserved residue in the kinase domain of these proteins. Pseudokinases have been indicated in the regulation of conformation and activity of proteins. Accordingly, multiple kinases with intact kinase domains, such as RAF-1, B-RAF, and discoidin domain receptor 1 (DDR1), have also been reported to possess kinase activity independent functions.
1.2 Growth factor signaling is inhibited by a variety of tumor suppressors

Many of the proto-oncogenes mediate growth factor (GF) signaling, whereas the role of the tumor suppressors involved is to ensure that these signals are transient (Figure 1). Tumor suppressor signaling dominates in conditions in which the cell is not capable of exerting a cell division without a risk of severe genetic perturbations \(^{10}\). Prototypical GF signaling, such as EGFR signaling will be depicted below as an example of interactions between proto-oncogenes and tumor suppressors (Figure 1). Most of the growth promoting GF receptors (GFRs) are tyrosine kinases. In the event of oncogenic mutations or increased expression of GFs by the tumors themselves, the tyrosine kinases are constitutively activated and thus become independent of GF signaling from adjacent normal tissue \(^{20}\). Upon GFR activity proto-oncogene RAS, which is frequently mutated in cancers, is recruited in the vicinity of the GFR \(^{21}\). RAS is a small Guanosine-5'-triphosphate hydrolase (GTPase), which in its GTP bound form activates its effector proteins that include the proto-oncogene phosphatidylinositol-3 kinase (PI3K) \(^{22}\) and also the RAF kinases \(^{23-26}\). RAS GTPase activating proteins (RAS-GAP), such as tumor suppressor neurofibromatosis 1 (NF1), induce the intrinsic GTPase hydrolyze activity of RAS, which leads to the inactivation of the RAS \(^{27-31}\). RAS effector PI3K \(^{22}\) phosphorylates phosphatidylinositol lipids and is counteracted by tumor suppressor phosphatase tensin homologue (PTEN) \(^{32-35}\). Under conditions of high PI3K and low PTEN activity proto-oncogene protein kinase B is activated (PKB1-3/AKT1-3) \(^{36, 37}\). Active PKB phosphorylates and thus inactivates the pro-apoptotic proteins \(^{38, 39}\), tumor suppressors of forkhead box class O (FoxO) family of transcription factors (TF) \(^{40, 41}\), glycogen synthase kinase-3 (GSK3) \(^{42}\), tumor suppressor p21\(^{C}p1\)/WAF1 \(^{43, 44}\), tumor suppressor p27\(^{K}p1\) \(^{45, 46}\) and tumor suppressor tuberous sclerosis complex 2 (TSC2) \(^{47}\). These phosphorylation events promote cell survival, cell proliferation and protein translation. The downstream signaling pathways of EGFR and RAS with their multiple points of negative regulation are fine examples of complex networks of proto-oncogenes and tumor suppressors. The identities of mutated genes in single tumors vary especially between tumor types, which could reflect tissue specific differences in expression and activity of tumor suppressors and proto-oncogenes.
Figure 1: Signaling network of proto-oncogenes and tumor suppressors downstream of epidermal growth factor (EGF).

EGF signaling is an example of a signaling network that promotes: cell proliferation, protein translation, and cell survival. The network includes multiple proteins that have been indicated in cancer. Several less-characterized connections between depicted downstream pathways of EGF have also been reported but omitted from figure 1 for clarity. However, an intriguing suggestion for the activation of TSC2 by LKB1 signaling has been included and marked with dim colors (see 5.1). *= tumor suppressors and proto-oncogenes whose genetic alterations have been indicated in tumorigenesis (http://www.sanger.ac.uk/genetics/CGP/Census) are marked with red and green, respectively. Proteins indicated in tumorigenesis but whose genetic alterations have not been identified in tumors are in blue. See text for references.

1.3 Tumor suppression by p53
Whereas multiple tumor suppressors such as PTEN suppress GF signaling, tumor suppressor p53 induces cell cycle arrest, senescence, autophagy or apoptosis in response to cell intrinsic stress, such as DNA damage. Most of the p53 responses are credited to
its ability to function as a transcription factor \(^{48}\). The induction of transcription of p21\(^{Cip/waf}\) cyclin-dependent kinase (CDK) inhibitor has consistently been suggested to be critical for p53 induced cell cycle arrest \(^{49,50}\) (Figure 2). Moreover, p53 mutated cells are not arrested upon DNA damage and thus accumulate mutations and genome rearrangements at an increasing rate, which provides material for selection of tumorigenic properties \(^{51-53}\). Indeed, hereditary p53 mutations are causative for Li-Fraumeni syndrome, which is characterized by an increased risk of cancer in several organs \(^{54,55}\). A large fraction of sporadic cancers also carry mutations of p53 or p53 signaling pathway components highlighting the importance of intact p53 responses (http://www-p53.iarc.fr/) \(^{56,57}\). Consistent with the critical role of p53 in tumor suppression, re-activation of p53 in mouse models of various cancers has been shown to lead to apoptosis, cell senescence or cell cycle arrest in a context dependent manner \(^{58-61}\).

Although p53 resides constantly on its target promoters \(^{62,63}\), only the disruption of the interaction of p53 with its negative regulator murine double minute 2 (MDM2) causes p53 to be activated and thus the expression of target genes such as p21\(^{Cip/WAF}\) are induced \(^{64-66}\). As an example, p53–MDM2 interaction is disrupted by the phosphorylation on p53-S15 by tumor suppressor kinase ataxia telangiectasia mutated (ATM) when DNA-damage occurs \(^{67,68}\). Studies on p53 non-modifiable knock-in and upstream regulator mutant mice have suggested both redundancy and context specificity in p53 activation by post-translational modifications \(^9\).

1.4 Tumor suppressors in controlling cell cycle

The p53 and TGFβ signaling pathways in part suppress tumorigenesis by preventing the progression of the cell division cycle under conditions of oncogenic stress \(^{69}\) (Figure 2). The cell division cycle is composed of four phases: the G1-phase, during which cell achieves its normal size subsequent to cell division, the S-phase during which the DNA is replicated, the G2-phase, and the M-phase during which the DNA and organelles are equally divided between the daughter cells during mitosis \(^{69}\). The cell cycle check points exist throughout the cell cycle. However, it is only during the G1-phase before the restriction point (R-point) that the cell is sensitive to both cell extrinsic and intrinsic stress signals and the cell either continues to grow or exits the cell cycle \(^{70}\). Subsequent to
passing the R-point cell division occurs with a similar speed regardless of whether the cells are normal or tumor cells. The difference being that the tumor cells pass the restriction point under conditions in which the normal cells would not \(^{70}\). Decisive factors for passing the R-point are the increased activities of G1- and S-phase CDKs (CDK4, CDK6, CDK2) that are the ultimate targets of positive and negative cell division signals. In the event of a prolonged repression of CDK activity cells will not pass the R-point and will exit the cell cycle (to the G0 phase) by entering quiescence, senescence or terminal differentiation \(^{71}\).

CDK4/6 and CDK2 are partly activated by increased levels of cyclins, whereas CDK inhibitors, such as p15\(^\text{ink4b}\), p16\(^\text{ink4a}\), p21\(^\text{Cip1/WAF1}\), p27\(^\text{Kip1}\) of p57\(^\text{Kip2}\), inhibit CDK activity \(^{69}\). Mitogenic signals activate CDK4/6 and CDK2 by increasing cyclin D and cyclin A/E levels, respectively. They also activate CDK4/6 and CDK2 by downregulating CDK inhibitors partly via the RAS activated proto-oncogenes v-myc myelocytomatosis viral oncogene homolog (c-MYC) and PKB \(^{43-46, 72-82}\) (Figure 2). The increase in CDK 4/6 activity leads to the phosphorylation of the transcriptional repressor tumor suppressor retinoblastoma protein (pRB) \(^{83}\). Phosphorylation of pRB releases the repression of E2F1-3 TFs and their transcriptional targets including cyclin E and E2Fs \(^{83}\). This event subsequently leads to enhanced CDK2 activity and also to the hyperphosphorylation of pRB, which thus creates a positive feedback loop \(^{83}\). The CDK inhibitors p15\(^\text{ink4b}\) and p21\(^\text{Cip1/WAF1}\) are partly induced by external cues that are mediated by TGFβ tumor suppressive signaling pathway \(^{84-88}\), whereas the internal stress sensing pathways that include ATM- p53 DNA damage signaling induces p21\(^\text{Cip1/WAF1}\) expression \(^{68, 89}\) (Figure 2). Increased CDK inhibitor expression reduces CDK activity and pRB phosphorylation, which prevents the cell cycle from passing the R-point \(^{69, 70}\). Mutations of CDK inhibitors and other tumor suppressors essential for G1/S check points are frequent in tumors (http://www.sanger.ac.uk/genetics/CGP/Census/) and lead to premature cell divisions, which cause genomic aberrations. These, in turn, lead to further activation of oncogenes and inactivation of tumor suppressors \(^{69}\).
Figure 2: Central role of p21 as an integration hub of signals from proto-oncogenes and tumor suppressors.

CDK2 inhibitor p21\textsuperscript{Cip1/WAF1} expression is positively regulated at the transcriptional level by TGFβ signaling and by p53. However, p21\textsuperscript{Cip1/WAF1} expression is negatively regulated by growth factor induced inactivation of FoxO by PKB. In addition, PKB directly phosphorylates p21\textsuperscript{Cip1/WAF1} which thus affects the stability and localization of p21\textsuperscript{Cip1/WAF1} and prevents p21\textsuperscript{Cip1/WAF1} binding to CDK2/cyclin complex. Active CDKs phosphorylate pRB, which leads to increased CDK activity via a positive feedback loop and eventually passing of the R-point of the cell cycle. See text for references and details on other components of the cell cycle machinery.
2. Alterations in TGFβ signaling and actin cytoskeleton dynamics are frequent in epithelial tumor cells and stromal cells of tumor microenvironments

2.1 Tumor suppression by the stroma

Although cancer causing mutations mostly occur in tumor cells, tumor growth is largely regulated by the surrounding stroma. The stroma is composed of, *inter alia*, immune cells and SMC lineage cells (fibroblasts, myofibroblasts, SMC), which together with the epithelial cells secrete collagens, elastins, laminins, fibronectins, heparan-sulphate proteoglycans and nidogens to form a meshwork of the extra cellular matrix (ECM) and basal lamina \(^1,90\) (Figure 3). Attachment of epithelial cells to the basal lamina by integrins induces polarization of epithelial cells \(^91\). Proper polarization, in turn, has been suggested to prevent induction of cell proliferation in response to oncogenic activity \(^92,93\).

Supporting the notion of a crucial role played by stromal cells in the production of the basal lamina, the tumor suppressive activity of myoepithelial cells in cell culture has been associated with the expression of laminin-111, a constituent of the basal lamina \(^94\). The GFs secreted by the epithelial and stromal cells can be stored within the ECM from which they can be liberated \(^95\). For example, secreted TGFβ is anchored to ECM by its interaction with latency-associated proteins (LAPs), which in turn interact with latent-TGFβ-binding protein (LTBP) \(^96\). TGFβ is liberated from the complex by proteolytic processing of LTBP and/or LAP by matrix metalloproteinases (MMP) or plasmin \(^97-99\). In addition, LAP-TGFβ interaction may be disrupted due to a conformational change in LAP molecule in response to mechanical traction that is conveyed from contracting cell to LAP by integrins \(^96,100,101\). Liberated TGFβ and other GFs affect the proliferation of adjacent epithelial cells \(^1,95\) (Figure 3).

Recently, the contribution of the stromal cells as an active participant in epithelial tumorigenesis has been highlighted by the identification of stromal mutations. Initially loss-of-heterozygocity (LOH) of multiple genetic loci was indicated in the stroma of breast ductal carcinomas in situ \(^102\). Subsequently, *p53* and *PTEN* mutations have also been identified in breast cancer tumor stroma \(^103,104\). Furthermore, it has been suggested that the inactivation of the TGFβ signaling component, Smad4, in the stroma drives GI polyposis of juvenile polyposis syndrome patients \(^105\). However, this suggestion is
controversial and counter arguments have been put forward. Accordingly, the lack of TGFβ type II receptor (Tgfβrii) in the fibroblast-specific protein 1 (Fsp1) expressing cells led to the increased proliferation of fibroblasts in abnormally developed ductal units in murine breast tissue, highly aggressive squamous cell carcinomas in the GI-tract and intraepithelial neoplasia in the prostate.

2.2 Epithelial TGFβ signaling mediates non-cell-autonomous tumor suppression

The TGFβ that is produced and activated by the stroma and the epithelia inhibits the proliferation of normal epithelial cells via the induction of cell cycle inhibitors p15Ink4b and p21Cip1/WAF1. Accordingly, the inactivating epithelial mutations of TGFβ receptors have been found in hereditary and sporadic colon cancers. Moreover, alterations in TGFβ downstream pathways have been reported to occur frequently in invasive colorectal cancers.

TGFβ binding to TGFβ receptors (TGFβRI and TGFβRII) activates several downstream pathways, of which Smad (SMA and MAD related protein) signaling pathway has been especially indicated in growth suppression (Figure 3). Activated TGFβRI phosphorylates and activates receptor Smads (R-Smad: Smad2 and Smad3), which consequently bind Smad4 (co-Smad) and accumulate within the nucleus possibly because of inhibited nuclear export. Nuclear Smad complex binds DNA with low affinity and thus interactions with other transcription factors are needed. Co-factors of Smads, including FoxO, induce Smad mediated activation of target gene transcription, which includes the cell cycle inhibitors p15Ink4b and p21Cip1/WAF1. The crucial role of TGFβ signaling in growth control is highlighted by the existence of complex feedback loops. Positive feedback loops include the auto induction of the TGFβ gene, whereas the negative feedback loops include the induction of the inhibitory Smad, denoted Smad7, which attenuates the TGFβ signaling at several levels (Figure 3).
2.3 Altered actomyosin fiber dynamics in stromal and epithelial cells of tumors are regulated by RhoA signaling

In addition to regulating CDK inhibitor expression TGFβ affects tumorigenesis by inducing actin cytoskeleton contractility in epithelial and stromal cells \(^1,125\). TGFβ induced contractility may induce adverse effects in tumorigenic epithelial cells by contributing to the epithelial-mesenchymal transition (EMT). This is characterized by the acquisition of mesenchymal characteristics: disruption of epithelial cell-cell junctions, increase in ECM component production and increase in cell motility \(^125-127\). On the other hand, TGFβ induced cell contraction in stromal cells leads to the modification of ECM and subsequent GF activation \(^1\). Thus contraction of stromal cells, in turn, affect the proliferation of adjacent epithelial cells (see 2.1) \(^1\).
TGFβ induces mesenchymal characteristics and contractility by driving the expression of actomyosin fibers, such as actin stress fibers. Actin stress fiber formation is initiated from within focal adhesions, which is followed by the incorporation of myosin units. Stress fibers from the opposing poles of the cell converge and form contractile ventral stress fiber bundles. These bundles consist of several actin filaments of alternating polarity, intercalating myosin units, alpha actinins that cross link the stress fibers and tropomyosins. Myosin units use ATP as an energy source to induce the contraction of the ventral stress fiber bundles. The resulting contraction is mediated by the sliding over of antiparallel actin filaments. Contraction of the stress fiber bundle is causative for cell contraction, force transmission to ECM and thus tension between these two due to the tethering of stress fibers to focal adhesions.

Cell contractility and TGFβ are essential for SMC lineage differentiation. Thick contractile actin stress fiber bundles define proto-myofibroblasts that are found in the granulation tissue of healing wounds. Increased tension upon contractility together with TGFβ induces myofibroblast differentiation that is characterized by a-smooth muscle actin (α-SMA) expression and its subsequent incorporation into the stress fibers. Cells with α-SMA positive stress fibers have increased force production that leads to increased integrin mediated activation of TGFβ (see 2.1). Thus there is a positive feedback loop between contractility and TGFβ activation. Concomitant with the stress fiber strengthening, focal adhesions mature and enlarge. In addition to regulating actin stress fibers TGFβ has also been indicated in the formation of non-contractile actin filament structures such as lamellopodias.

RhoA GTPase is a key regulator of actin stress fiber contractility and thus it is not surprising that the regulation of RhoA in response to TGFβ has been suggested to be essential for TGFβ induced EMT. RhoA is predominantly active in the tail ends of migrating cells. In this location, RhoA possibly induces the contraction and dissociation of posterior focal adhesions. Accordingly, ventral actin stress fibers have been found to locate mostly in the rear of motile cells. Recently, it was proposed that motile tumor cells could switch between adhesion dependent to adhesion independent (amoeboid) types of motility, similar to that observed in leukocytes. Moreover, ameboid-type motility requires RhoA signaling for the induction of cortical
actomyosin contractility. The activation of RhoA signaling has been associated with tumorigenesis that is consistent with the central role of RhoA in modulating contractility in epithelial and stromal cells.

RhoA regulates stress fiber contraction via a complex network of protein interactions. The key role downstream of RhoA is played by phosphorylation status of the myosin light chain (MLC), which induces ATPase activity of myosin 2 and leads to contraction. In non-muscle cells RhoA signaling induces MLC phosphorylation mostly via the activation of Rho kinase (ROCK). Negative regulation of phosphorylated MLC is exerted by myosin light chain phosphatase (MLCP), which is composed of catalytic protein phosphatase 1 (PP1), myosin phosphatase targeting 1 (MYPT1) and small 20kDa subunit. Negative regulation by MLCP can be blocked by RhoA via the induction of MYPT phosphorylation at several sites by ROCK or other kinases, which leads to the inhibition and/or sequestration of the phosphatase complex. Alternatively, ROCK could inhibit MLCP via the phosphorylation of protein phosphatase 1 regulatory subunit 14A (CPI-17).

3. LKB1 as a tumor suppressor

3.1 Inherited mutations of LKB1 are causative for gastrointestinal polyposis

The search for the causative mutations of Peutz-Jeghers syndrome (PJS) identified serine threonine kinase LKB1 (also known as STK11) in 1998. The most severe manifestation of PJS is gastrointestinal (GI) polyposis. Its onset occurs during the second or third decade of life and is diagnosed by abdominal pain, bleeding or endoscopy/colonoscopy. Benign polyps arise throughout the GI-tract, are pedunculated in shape and classified as hamartomas. Polyps contain most of the differentiated cell types found in the adjacent normal tissue. Juvenile polyposis syndrome, instead, is caused by mutations in SMAD4 or in bone morphogenic protein receptor 1 (BMPRI) and Cowden syndrome is caused by mutations in PTEN. Both conditions are characterized by hamartomatous polyps in the GI-tract. When polyps of these different hamartoma syndromes are compared PJS polyps are found to have the most predominant stromal stalk. Consistently, the PJS polyps also have the highest occupancy of SMC-lineage cells.
Recent reports indicate \textit{LKB1} mutations in up to 94\% of PJS patients\textsuperscript{164-168}, which suggests that all the PJS patients are \textit{LKB1} deficient. Causality between the \textit{LKB1} mutations and PJS is indicated by the analysis of \textit{Lkb1} deficient mice. \textit{Lkb1} heterozygose mice survive and develop remarkably similar polyps to those found in PJS patients. The polyps predominately locate within the pyloric region and to a lesser extent elsewhere in the stomach. They can also occur in the intestine though this is comparatively rare. Polyps begin to appear at the age of 5 months and cause the death of the mice at the age of 8 months and onwards\textsuperscript{169-172}. In contrast, analysis of nullizygous mice demonstrated embryonic lethality between days E8.5-E9.5\textsuperscript{173}. This was possibly because of vascular defects and indicates an essential role for \textit{LKB1} during embryo development\textsuperscript{173}.

In addition to polyposis, epidemiological studies suggest that PJS patients have an increased risk of cancers of the GI-tract, gynecological cancers, and also breast cancer\textsuperscript{174, 175}. PJS patients have between a 41 to 60\% risk of developing a first cancer before the age of 60, compared to only 8.5\%, for the general population, which indicates a 5-7 fold increase in cancer risk at 60\textsuperscript{174, 175}.

\subsection*{3.2 \textit{LKB1} mutations in sporadic cancers}

The status of \textit{LKB1} as a tumor suppressor has been highlighted by recent reports that demonstrate frequent \textit{LKB1} mutations in sporadic tumors. In contrast to tumor spectrum of PJS patients, sporadic mutations of \textit{LKB1} were identified in up to 20\% of cervical cancers\textsuperscript{176, 177} and up to 34\% of non-small cell lung carcinomas (NSCLC)\textsuperscript{6, 7, 176, 178-180}. In NSCLC, \textit{LKB1} mutations are the most frequent in adenocarcinomas but are also found in squamous cell carcinomas and in large cell carcinomas\textsuperscript{6, 179, 181, 182}. Kinome and genome wide mutation analyses of diverse tumor samples have consolidated the notion of tissue specificity in tumor suppression by \textit{LKB1}. Screenings confirmed \textit{LKB1} mutations in NSCLC\textsuperscript{51, 181, 183, 184} but did not find significant numbers of \textit{LKB1} mutations in other types of tumors\textsuperscript{181, 184-189}. The tissue specificity of \textit{LKB1} in tumor suppression is in contrast to its ubiquitous expression pattern\textsuperscript{190} and could reflect differential expression of \textit{LKB1} substrates and/or differential function of \textit{LKB1} in various tissues. Consistent with tissue specific tumorigenesis upon \textit{LKB1} mutations, accumulation of mutations in tumor
type specific signaling pathways have been reported \(^{191}\). For example, mutations of the BMP pathway are frequent in tumors of GI-tract but more rare in other tumor types \(^{191}\).

Analysis of those mutations that co-segregate or are mutually exclusive with the \textit{LKB1} mutations could reveal signaling pathways essential for tumor suppression by LKB1. Interestingly, studies on lung adenocarcinomas indicate mutual exclusiveness between \textit{EGFR} activating and \textit{LKB1} inactivating mutations \(^{51,179}\). In contrast, activating mutations of \textit{KRAS}, a downstream target of EGFR, co-segregate with \textit{LKB1} inactivating mutations \(^{179}\). These observations suggest that one of the following occurs: 1) LKB1 is involved in EGFR signaling but is part of an EGFR downstream pathway other than KRAS, 2) \textit{LKB1} mutations sensitize cells to oncogenic stress by EGFR, or 3) \textit{LKB1} and \textit{EGFR} mutated lung adenocarcinomas represent separate sub-types that can not be distinguished from each other with current methods.

3.3 \textit{LKB1} mutations promote both initiation and progression in tumorigenesis

Studies on tumors associated with \textit{LKB1} mutations have implicated that a loss of \textit{LKB1} promotes both tumor initiation and aggressiveness. The former is supported by the benign nature of PJS polyps together with an increased number of tumors upon \textit{Lkb1} inactivation in the lung adenocarcinomas of mice expressing oncogenic \textit{Kras} \(^{6}\). Moreover, \textit{LKB1} mutation frequency did not correlate with the clinical stage of NSCLC patients, which indicated that \textit{LKB1} mutations occur early on in tumor development \(^{179}\). However, \textit{LKB1} mutations were associated with the shorter survival of lung adenocarcinoma patients, although the sample size was not enough to ensure statistical significance \(^{179}\). Consistently, \textit{Lkb1} inactivation together with \textit{Kras} activation induced lung adenocarcinoma metastasis, whereas \textit{Kras} mutation alone was not sufficient for induction of metastasis or local invasions \(^{6}\). Furthermore, cervical cancer \textit{LKB1} mutations were reported to associate with poor prognosis \(^{177}\). The mechanisms of the underlying increased tumor aggressiveness enhanced by \textit{LKB1} mutations are not known but obvious candidates include the induction of EMT and/or changes in cell cytoskeleton upon the loss of \textit{LKB1}.
4. Regulation of LKB1 kinase activity and downstream signaling

4.1 LKB1 kinase complex

*LKB1* gene encodes a serine/threonine kinase of 433 amino acids (aa) \(^4, 5, 192\). The integrity of the LKB1 kinase domain and kinase activity are crucial for the tumor suppressive function of LKB1 as almost all missense, nonsense or frameshift mutations of *LKB1* in tumors have been demonstrated or predicted to interfere with the kinase function \(^51, 192, 193\). LKB1 forms a kinase complex with pseudokinase Ste20 related adaptor (STRAD\(\alpha\) and STRAD\(\beta\)) \(^{194, 195}\) and the scaffolding protein MO25 (MO25\(\alpha\) and MO25\(\beta\); also known as calcium-binding protein 39) \(^{196}\). Upon interaction between STRAD and MO25, STRAD binds to ATP and shifts to the closed “active” conformation \(^{197}\), which promotes the complex formation with LKB1 and a conformational change of the LKB1 activation loop \(^{198}\). Kinase complex formation induces LKB1 kinase activity \textit{in vitro} and also in cell culture and it has been suggested to be essential for LKB1 signaling \(^{194, 196, 199}\).

4.2 Regulation of LKB1 activity

How LKB1 activity is regulated is still largely uncharacterized to date. Currently, the focus of investigations is on the post-translational modifications of LKB1. LKB1 is not regulated by T-loop phosphorylation \(^{193}\), which is the classic mechanism of regulating kinase activity \(^{200}\). Moreover, there are no reports about regulation of expression levels of LKB1 or its kinase complex partners. However, LKB1 has been reported to be covalently modified by phosphorylation of several residues in cells \(^{201-206}\) in addition to farnesylation \(^{203, 207}\) and acetylation \(^{208}\). Phosphorylation of LKB1-T363 is exerted by the ATM tumor suppressor kinase in response to DNA damage \(^{202}\) and has been implicated in LKB1 mediated cell cycle arrest in B-cells that are undergoing immunoglobulin gene remodeling \(^{209}\). LKB1-S428, in turn, has been suggested to be a direct target of protein kinase A (PKA) \(^{203, 207}\), p90 ribosomal S6 kinase (p90RSK) \(^{203}\) and atypical protein kinase C (aPKC) \(^{210}\). Its phosphorylation has been suggested to contribute to the regulation of epithelial \(^{211}\) and neuronal polarity by LKB1 \(^{212-214}\). In contrast to these studies, phosphorylation of LKB1-S428 by p90RSK was reported to inhibit LKB1 signaling activity in SK-MEL-28 cells when coinciding with phosphorylation of LKB1-S325 by extracellular signal-regulated kinase (ERK, see 5.1) \(^{215}\). Regulation of LKB1 function by
phosphorylation could take place at the level of LKB1-substrate complex formation as in vitro kinase activity was not affected upon inhibition of upstream kinases or mutagenesis of phosphorylated residues \(^{201-203}\).

As to whether the phosphorylation of LKB1 at T363 or at S428 is critical for LKB1 mediated tumor suppression is still an open question. Mutations of PKA, aPKC or p90\(^{RSK}\) have not been identified in lung adenocarcinomas \(^{51, 181, 184}\) or in PJS, which could be due to a possible redundancy in phosphorylation of LKB1-S428. Interestingly, mutations of ATM can be found in lung adenocarcinomas although this is rarer than for LKB1 mutations \(^{51}\). Co-segregation studies of ATM and LKB1 in lung adenocarcinomas or the creation of LKB1-T363A knock–in mice could provide an answer as to the relevance of a direct link between these two tumor suppressors.

Alternatively, the LKB1 signaling activity could be regulated spatially via changes in LKB1 localization in a similar manner to the tumor suppressors p53 and Smad4 \(^{216-218}\). Various patterns of LKB1 localization have been observed: Caenorhabditis elegans homologue of LKB1 (PAR-4) localized at cell cortex and in the cytoplasm \(^{219}\), in Drosophila melanogaster LKB1 localized at the cell membrane and in the cytoplasm \(^{211}\), Xenopus laevis homologue Xeek1 was reported to localize exclusively in the cytoplasm \(^{220}\), mouse Lkb1 was predominantly nuclear although modest amounts were also found in the cytoplasm and membranes \(^{203, 221}\) and, finally, human LKB1 has been reported to localize into both the nucleus and the cytoplasm \(^{222}\). The context dependent LKB1 localization and the ability of co-expressed Lkb1 interacting protein 1 (Lip1) to induce cytoplasmic re-localization of Lkb1 \(^{223}\) support the idea of the dynamic regulation of LKB1 localization and encourage further studies on regulation of LKB1 localization and its significance on LKB1 function.

### 4.3 LKB1 activates 14 substrate kinases indicated in the regulation of metabolism and polarity

LKB1 on its own or in the context of the kinase complex has been implicated in the phosphorylation of p53 \(^{203}\), AGS3 \(^{224}\), STRADA \(^{194}\), p21-activated kinase-1 (PAK1) \(^{225}\), GSK3\(\beta\) \(^{226}\), and PTEN \(^{227, 228}\). Interestingly, PTEN, whose hereditary mutations cause
hamartoma syndromes similar to those associated with LKB1 \(^{162}\), was reported to be activated upon phosphorylation on several residues by LKB1 \(^{228}\). A downstream target of PTEN, denoted PKB (Figure 1), was consistently inhibited upon LKB1 dependent activation of PTEN in endothelial cells. In contrast to these results, PKB has been found to be inhibited in several LKB1 deficient cell lines and primary mouse embryonic fibroblasts (MEF) \(^{229,230}\). Furthermore, synergistic effects of \(LKB1\) and \(PTEN\) deletion on bladder tumorigenesis in mice \(^{231}\) indicates that in some tissues and cell types these tumor suppressors are part of distinct signaling pathways.

Identification of AMP-activated kinase (AMPK\(\alpha_1\) and AMPK\(\alpha_2\)) as LKB1 substrate \(^{232,233}\) proved to be extremely significant for LKB1 research as it paved the road for a subsequent study that indicated LKB1 in the phosphorylation of 12 kinases closely related to AMPK \(^{199}\) (figure 4). Phosphorylation of the T-loop threonine of AMPK\(\alpha_1\)-2, brain-specific kinase 1-2 (BRSK1-2, also known as SAD-B and SAD-A), microtubule-affinity regulating kinase 1-4 (MARK1-4), NUAK 1-2 (also known as ARK5 and SNARK), salt inducible kinase 1-3 (SIK1-3, SIK2 is also known as QIK and SIK3 as QSK) and SNF1-related kinase (SNRK) by LKB1 induced the kinase activities in vitro \(^{199,232,234}\). In contrast to other reported LKB1 substrates (see previous paragraph), the requirement for LKB1 in the activation of most of these kinases has been indicated by studies in MEFs and HeLa cells that lack \(LKB1\) \(^{199,232,234}\) in addition to tumors and tissues deficient in their \(Lkb1\) expression \(^{6,213,235-238}\). As an exception, AMPK\(\alpha_1\) activity was not lost in the heart and skeletal muscle in the absence of \(Lkb1\) \(^{239,240}\). This was probably due to the homologous role played by calcium/calmodulin-dependent protein kinase kinase \(\beta\) (CamKK\(\beta\)) in respect of the T-loop kinase of AMPK \(^{241,242}\). Accordingly, CamKK\(\beta\) has been shown to activate only AMPK\(\alpha_1\) and AMPK\(\alpha_2\) of all 14 AMPK-related kinases in vitro and in cell culture \(^{243}\). The ability of MARK activating kinase (MARKK) to interact with MARK1-4 in cells and to activate MARK1-4 in vitro \(^{244}\) suggests that in some specific conditions kinases other than LKB1 or CamKK\(\beta\) might be involved in the activation of LKB1 substrate kinases.

Identification of the LKB1 substrate kinases has linked LKB1 kinase activity to several cellular functions (Figure 4). The best characterized LKB1 substrate kinase is AMPK, whose activation is a two-step process in which both the phosphorylation of the T-loop
by LKB1 and the AMP binding by AMPK γ-subunit are crucial for robust kinase activity. AMPK was initially identified as an upstream kinase and inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) and acetyl co-enzyme A (ACC), which are essential respective enzymes in cholesterol and lipid synthesis pathways. Based on the sequence around these phosphorylation sites and in vitro peptide library screening the AMPK consensus target sequence was proposed. The consensus sequence contains the following positions in relation to the target serine: a basic residue on the aa -3 or -4, a hydrophobic bulky aa at position -5 and +4 and preference for polar aa at +3, all of which make AMPK a highly selective kinase. The establishment of AMPK consensus sequence has facilitated identification of several AMPK targets one of which was tumor suppressor TSC2. Most of the AMPK substrates are linked to the regulation of metabolism similar to that of AMPK homologue sucrose non-fermenting 1 (SNF1) in Saccharomyces cerevisiae. In addition to AMPK, SIK1 and SIK2 have been indicated in metabolic regulation. SIK1 and SIK2 inhibit the expression of genes involved in gluconeogenesis via phosphorylation and subsequent sequestration of cAMP response element-binding protein (CREB)-regulated transcription coactivator 2 (CRTC2). SIK1-2 phosphorylates CRTC2 in response to hormonal signals, whereas AMPK has been indicated in the phosphorylation of the same residues of CRTC2 in response to energetic stress. The regulation of metabolism and thus cell growth by AMPK and SIK1-2 brings them forth as candidate mediators of LKB1 tumor suppression as tumor cells are forced to reprogram their metabolism to survive under conditions of low oxygen and constant need of building blocks. Instead of regulation of metabolism, MARK1-4 and BRSK1-2 have been indicated in the phosphorylation of microtubule-associated proteins (MAPs), which normally bind to and stabilize microtubules (MT) but upon phosphorylation by MARK1-4 or BRSK1-2 detach, which leads to MT destabilization. NUAK1-2, SIK3, and SNRK are poorly characterized. Induction of NUAK2 activity in response to energetic stress and ultraviolet radiation associates NUAK2 with molecular mechanisms involved in tumorigenesis.
Paucity of knowledge on LKB1 substrate kinases together with their ability to phosphorylate common targets \textit{in vitro} \textsuperscript{199} and ability of AMPK and SIKs to induce CRTG2 phosphorylation \textit{in vivo} \textsuperscript{255} (Figure 4) has raised the hypothesis of there being some redundancy of LKB1 downstream signaling pathways \textsuperscript{263}. However, the differential preferences on \textit{in vitro} substrates \textsuperscript{199} and the inducibility of SIK1 \textsuperscript{264}, AMPK\(\alpha_2\) \textsuperscript{265}, NUAK1 \textsuperscript{266} and NUAK2 \textsuperscript{260} expression levels in response to conditions specific for each of the kinases imply that LKB1 substrate kinases are not redundant \textit{in vivo}. Indeed, embryonic lethality of BRSK1\(^{-/-}\);BRSK2\(^{-/-}\) \textsuperscript{258}, AMPK\(\alpha_1\)\(^{-/-}\);AMPK\(\alpha_2\)\(^{-/-}\) \textsuperscript{267}, and MARK2\(^{-/-}\);MARK3\(^{-/-}\) \textsuperscript{268} double knock out mice in addition to increased plasma glucose levels in AMPK\(\alpha_2\)\(^{+/-}\) mice \textsuperscript{269} and other phenotypes of mice harboring deletion of single LKB1 substrate kinase gene \textsuperscript{269-276}, support the hypothesis of unique functions of LKB1 substrate kinases. These indications of non-redundant functions allow genetic and cell biological
studies in addressing, which of the 14 substrate kinases mediate the LKB1 tumor suppression in different situations.

5. Suggested tumor suppressive functions of LKB1

Cell culture and in vivo studies have demonstrated associations between LKB1 and several cellular functions. Involvement of LKB1 in regulation of cell proliferation, metabolism, polarity, and cytoskeleton are potentially relevant for tumor suppression by LKB1 as these cellular processes are commonly deregulated in tumors.

5.1 LKB1 is indicated in the suppression of cell proliferation

Cell culture experiments by several laboratories have provided evidence for the involvement of LKB1 in the suppression of cell proliferation. In primary MEFs Lkb1 deletion was suggested to abrogate culture-induced senescence and led to the immortalization of the cells whereas the overexpression of LKB1 has been demonstrated to restrict the growth of several cancer cell lines. The re-introduction of LKB1 expression in the G361 melanoma cell line, A549 lung adenocarcinoma cell line and MDA-MB-435 breast cancer cell line suppressed cell proliferation, which was possibly due to arrest of the cell cycle at the G1 stage. Whether LKB1 affects cell proliferation directly by regulating CDK activity, or indirectly through the regulation of metabolism or polarity merits further investigation. Nevertheless, cell cycle phenotype in response to the manipulation of LKB1 levels indicate LKB1 as being a gatekeeper of cell division and thus provides a platform for more detailed studies of LKB1 downstream signaling in the suppression of cell proliferation.

Phosphorylation of LKB1-S325 and LKB1-S428 by ERK and p90RSK, respectively, were suggested to prevent the suppression of proliferation of SK-MEL-28 melanoma cells by LKB1. In this context ERK and p90RSK activities were induced by B-RAF-V600E oncogenic mutation, which are frequent in melanomas. The suggested link between B-RAF and LKB1 places LKB1 downstream of EGFR signaling and could thus provide an explanation for the mutual exclusiveness of LKB1 inactivating and EGFR activating mutations and co-segregation of LKB1 inactivating and KRAS activating mutations in lung adenocarcinomas (see 3.2). In tumors B-RAF has been suggested to be
activated by EGFR downstream effector RAP1 \(^{282, 283}\) whereas RAF-1 is activated by RAS \(^{23-26}\). Thus, mutations of \(LKB1\) and \(KRAS\) in lung adenocarcinomas would activate or partly activate both RAF1 and B-RAF signaling pathways downstream of EGFR. However, as the re-introduction of \(LKB1\) induces cell cycle arrest in G361 melanoma cells \(^{280}\), which harbor the \(B-RAF-V600E\) mutation (Cosmic database, www.sanger.ac.uk/genetics/CGP/cosmic/), it is plausible that the inhibition of LKB1 by B-RAF occurs only in certain contexts or involves LKB1 downstream pathways that are not responsible for LKB1 induced cell cycle arrest in G361 cells.

AMPK activity has also been implicated in the suppression of cell proliferation \(^{284, 285}\), which indicates the possibility of AMPK being a potential mediator of tumor suppression by LKB1. Interestingly, mutations of the AMPK substrate \(TSC2\) (Figures 1 and 4) \(^{229, 252}\) are causative for tuberous sclerosis, which is a condition that is characterized by hamartomatous growth of the skin, kidney, heart, and brain \(^{286}\) whereas hereditary mutations of \(TSC1/2\) activator \(PTEN\) lead to multiple syndromes similar to PJS, which are characterized by hamartomatous polyps in the GI-tract \(^{162}\). Tumorigenesis upon \(TSC1/2\) mutations has been suggested to be caused by the activation of the mammalian target of rapamycin complex 1 (mTORC1) as a treatment of tuberous sclerosis patients and model rats with mTORC1 inhibitor rapamycin led to the regression of tumors \(^{287, 288}\). Intriguingly, elevated phospho-S6 levels, an indicator of mTORC1 activity (Figure 1), have been observed in the epithelia of PJS polyps, which is suggestive of AMPK attenuation \(^{229}\). Moreover, several epidemiological studies on type 2 diabetes patients have revealed a significant decrease in the incidence of cancer and mortality among patients treated with metformin \(^{289-291}\) an AMPK activator \(^{292, 293}\), which is evidence that supports the tumor suppressive role of AMPK. However, the presence of \(LKB1\) mutations and lack of \(AMPK\) mutations in lung adenocarcinomas \(^{51}\) or PJS \(^{262}\) (see 3.1 and 3.2) imply that LKB1 substrate kinase other than AMPK might be relevant for tumor suppression by LKB1 and that multiple LKB1 substrates are involved.

5.2 Regulation of cell polarity and cytoskeleton by LKB1

Initially, a role for LKB1 in cell polarity was demonstrated by studies on \(Caenorhabditis elegans\) in which LKB1 homologue PAR-4 was required for the asymmetry of an early
embryo. Subsequently, LKB1 was shown to be required for the polarity of an oocyte and epithelial cells in *Drosophila melanogaster*. In the eye rhabdomere of *Drosophila melanogaster* and the developing mouse pancreas, Lkb1 deletion was associated with a deficiency of tight junctions (TJ) and adherence junctions (AJ). These findings could be indicative of a mechanism for the noted polarity defects of *Drosophila melanogaster* follicle epithelia as integrity of the cell-cell junctions is a prerequisite for proper epithelial polarity. Consistently, the downregulation of LKB1 in a three-dimensional cell culture model of MCF10A mammary epithelial cells led to the inability to form hollow acinar structures and was associated with deficient cell-cell junctions. Thus it is intriguing that the inactivation of the mouse mammary tissue Lkb1 led to tumorigenesis. Furthermore, PJS patients have an increased risk of breast cancer. Together with the association of LKB1 inactivation with EMT and increased invasiveness of Lkb1 deficient lung adenocarcinomas, these results suggest that the regulation of epithelial polarity contributes to tumor suppression by LKB1. More specifically, it has been postulated that deficient epithelial polarity drives PJS polyposis.

Epithelial cell-cell junction formation and maintenance are interdependent on junctional complex formation and carefully controlled microtubule and actomyosin fiber dynamics. To date studies have not yielded any evidence for the direct modification of cell junction components by LKB1. Therefore attention has been redirected to the regulation of cell cytoskeleton by LKB1. Consistently, it has been suggested that LKB1 activity regulates actin polarization in single colorectal cancer cells in culture and microtubules in various cell types, which indicates that regulation of cell cytoskeleton by LKB1 is not secondary to the regulation of cell-cell contacts.
Figure 5. LKB1 signaling in control of epithelial cell polarity

In polarized epithelial cells TJs and AJs, whose formation and maintenance are dependent on the actomyosin ring, separate basolateral (bottom) and apical (top) domains. In intestinal epithelial cells integrins and the nucleus are located in the basolateral domain, whereas the actin brush border and microvilli are located in the apical domain. Microtubules originate in the centrosome and traffic vesicles from the golgi to the apical or to the basolateral domain. LKB1 substrate kinase AMPK has been implicated in the regulation of actin cytoskeleton and microtubules whereas MARKs have been shown to regulate microtubule stability, cell-cell junctions and apico-basal polarity. Potentially relevant for epithelial polarity are also BRSK1 and SIK2, which regulate centrosome duplication and separation, respectively. However, these have only been studied in non-polarized cell types. See text for references.

LKB1 substrate kinases have been indicated in the direct phosphorylation of cytoskeletal components, which could provide a mechanism for the cytoskeleton and cell-cell junction regulation by LKB1. It has been suggested that MARK1-4 and BRSK1-2 destabilize microtubules by phosphorylating the microtubule associated proteins (MAP) whereas AMPK has been reported to stabilize microtubules by phosphorylating...
cytoplasmic linker protein 170 (CLIP-170)\textsuperscript{309}. Moreover, AMPK has been suggested to directly modulate actomyosin fiber dynamics by phosphorylating MLC\textsuperscript{310}. The ability of MLC mutant, which mimics the phosphorylated state, to rescue the epithelial polarity defects of AMPK mutant \textit{Drosophila} and to mimic the activation of LKB1 on the polarization of actin cytoskeleton in LS174T-W4 colorectal cancer cells\textsuperscript{310} suggests a mediator role for AMPK in the cell cytoskeletal regulation by LKB1. Consistently, constitutively active AMPK mutant was able to rescue the epithelial polarity defects of LKB1 deleted \textit{Drosophila}. Another LKB1 substrate implicated in the regulation of actin dynamics is NUAK2. The overexpression of NUAK2 in HEPG2 induced cell detachment and disruption of actin fibers in glucose starved cells\textsuperscript{261}. Therefore, it appears likely that LKB1 also regulates actin cytoskeleton in mammalian cells as based on the studies presented above. Nonetheless, further studies are required to establish detailed molecular mechanisms of the regulation of the actin cytoskeleton by mammalian LKB1 and their possible relevance for tumor suppression by LKB1.
AIMS OF THE STUDY

Prior to writing this thesis the first cell culture model of LKB1 induced cell cycle arrest had been established\(^{280}\) and polyposis prone \(Lkb1^{+/}\) mice were created in the laboratory. Analysis of \(Lkb1^{+/}\) mice revealed development of remarkably similar polyps to those in PJS patients characterized by prominent stalk composed of SMC lineage cells\(^{172}\). Based on these tools the aims were set as follows:

1. Identification of the molecular mechanism that contributes to LKB1 tumor suppressive function by studying LKB1 induced cell cycle arrest in G361 melanoma cells and the regulation of LKB1 localization in other cell lines (publication I).

2. Characterization of potential tumor suppressive LKB1 functions and LKB1 signaling pathways in SMC lineage cells by deleting \(Lkb1\) in the SM22 positive cell \textit{in vivo} and in cell culture (publication II and III).

3. The studies on the 2\textsuperscript{nd} aim identified Lkb1 as a regulator of actin stress fibers (publication III). The third aim was to investigate whether the interaction between the LKB1 substrate kinase NUAK2 and an actin binding protein MRIP, which was found in the yeast two hybrid screen carried out in the laboratory, would play a role in the regulation of actin stress fibers (publication IV).
MATERIALS AND METHODS

DNA constructs (unpublished data)
Amino-terminally (N-terminally) tagged GST-STRADα was constructed by recombining the STRADα insert from pDONR221-STRADα with the pDEST27 vector via a LR-recombination reaction according to manufacturers protocol (Invitrogen). Enhanced green fluorescent protein (EGFP) tagged wild type LKB1, EGFP-LKB1-G163D, EGFP-LKB1-KD and EGFP-LKB1-D176N were subcloned by EcoRI-SalI digestion of pAHC-LKB1\textsuperscript{192}, pAHC -LKB1-G163D\textsuperscript{192}, pAHC-LKB1-KD (publication I) and pAHC-LKB1-D176N (publication I) vectors and subsequent ligation of extracted LKB1 inserts to EcoRI-SalI cut pEGFP vector.

GST-pulldown (unpublished data)
293 embryonic kidney cells were transfected with pDEST27-STRADα (GST-STRADα), pAHC-MO25α (HA-MO25α) and pEGFP-LKB1, pEGFP-LKB1-G163D, pEGFP-LKB1-KD, pEGFP-LKB1-D176N, pAHC-LKB1, pAHC-LKB1-SL26 or pAHC-LKB1-SL8 using Fugene (Roche) according to the manufacturer’s instructions. Subsequently, at 48 hours after transfection the cells were washed once with room temperature phosphate buffered (pH 7.4) isotonic saline (PBS), placed on ice, and washed once with ice cold PBS followed by lysis in buffer C containing 50mM Tris pH7.5, 1mM EGTA, 1mM EDTA, 1% triton, 0.27 M sucrose and cocktail of protease and phosphatase inhibitors. Subsequent to centrifugation at 20 800 g for 10 minutes 500 ug of protein lysate was first incubated with gluthatione-sepharose beads (Amersham) under rotation at +4 celsius degrees for 1 hour and then washed 10 times with ice cold buffer C. Western blotting analysis was carried out as described in publication I of this thesis with the exception that the antibodies used were specific for LKB1 (Upstate Biotechnologies), HA (12CA5, Babco), and GST (Genescript).
Methods, antibodies, cells, siRNA oligos, plasmids and mouse strains used in publications (I-IV)

The materials and methods used in publications I-IV of this study are tabulated below, and described in detail in the original publications, which are referred to here by their roman numerals.

Table 1. Methods used in this study

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| α-SM22 | goat polyclonal ab, WB | Abcam | III |
| α-a-SMA, a-5691 | mouse monoclonal ab, WB/IF/IHC | Sigma | II, III |
| α-Smad2/3, 18/Smad2/3 | mouse monoclonal ab, IF | BD Transduction laboratories | III |
| α-vinculin, hVIN1 | mouse monoclonal ab, IF | BD Transduction laboratories | III |

Table 3. Cells used in this study

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Table 4. short interfering (si) ribonucleic acid (RNA) oligos used in this study

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<td>siMRIP</td>
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Table 5. Expression plasmids used in this study

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<td>pRLTK-luc</td>
<td>Renilla fire fly</td>
<td>Promega</td>
<td>III</td>
</tr>
<tr>
<td>pTAL-SRE-Luc</td>
<td>SRE-response element drives the luciferase expression</td>
<td>Clontech</td>
<td>III</td>
</tr>
<tr>
<td>RC-cycD1-HA</td>
<td>Cyclin D1, C-terminal HA-tag</td>
<td></td>
<td>III</td>
</tr>
</tbody>
</table>

Table 6. Mouse strains used in this study

<table>
<thead>
<tr>
<th>allele</th>
<th>description</th>
<th>source</th>
<th>used in study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lkb1&lt;sup&gt;Lox&lt;/sup&gt;</td>
<td>Conditional Lkb1 allele, exons 3-6 are flanked by Lox sites and are deleted upon Cre-recombinase expression.</td>
<td>Provided by Dr. DePinho and Dr. Bardeesy</td>
<td>II, III</td>
</tr>
<tr>
<td>Lkb1&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Lkb1 &quot;null&quot; allele, genomic sequences encompassing exons were deleted</td>
<td>produced in the lab of Tomi Mäkelä</td>
<td>II, III</td>
</tr>
<tr>
<td>SM22&lt;sup&gt;+&lt;/sup&gt;/Cre</td>
<td>SM-Cre-ERT2 was created by targeted integration of Cre-ERT2 transgene to SM22 locus</td>
<td>Provided by Dr. Feil</td>
<td>II</td>
</tr>
<tr>
<td>AMPK&lt;sup&gt;a&lt;/sup&gt;</td>
<td>AMPK&lt;sup&gt;a&lt;/sup&gt; &quot;null&quot; allele, genomic sequences corresponding to aa’s 97–157 of catalytic domain were deleted.</td>
<td>Provided by Dr. Viollet</td>
<td>III</td>
</tr>
<tr>
<td>AMPK&lt;sup&gt;a&lt;/sup&gt;2&lt;sup&gt;Lox&lt;/sup&gt;</td>
<td>Conditional AMPK&lt;sup&gt;a&lt;/sup&gt;2 allele, exon C is flanked by LoxP sites</td>
<td>Provided by Dr. Viollet</td>
<td>III</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

6. Cytoplasmic LKB1 induces p53 dependent growth arrest (I)

6.1 LKB1 localizes and is active in both the cytoplasm and the nucleus

The various patterns of LKB1 localization in mammalian cells and model organisms reviewed above (section 4.2) and reported elsewhere \(^{203, 211, 219-223}\) prompted us to study the regulation of LKB1 localization. Consistent with previous reports, LKB1 localization in all cell lines studied (U2OS, COS-7, C2C12) was classified into three categories: (i) predominantly nuclear, (ii) predominantly cytoplasmic, and (iii) nuclear and cytoplasmic (publication I, Figures 1A-B). Surprisingly, most of the LKB1 kinase dead mutants (LKB1-KD, LKB1-G163D, LKB1-SL26, LKB1-SL8, and LKB1-SL31, see materials and methods for details on mutations) localized exclusively in the nucleus. In contrast, kinase dead mutant LKB1-D176N localized in a similar manner to that of wild type LKB1 (I Figures 1A, C) as noted in a previous study \(^{222}\). The nuclear localization of many LKB1 kinase inactive mutants identified here prompted us to study whether there are differences in LKB1 kinase activity in the nucleus and the cytoplasm. Consistent with the immunofluorescence (IF) analysis, overexpressed LKB1 was found in comparable amounts in the nuclear and cytoplasmic fractions of U2OS and COS-7 cells. Surprisingly, nuclear and cytoplasmic LKB1 displayed similar specific autophosphorylation activities in vitro (I Figure 1D). Most importantly, endogenous nuclear and cytoplasmic LKB1 fractions had comparable kinase activities (I Figure 1E). Collectively, these results indicate that mammalian LKB1 localizes into both the nucleus and the cytoplasm unlike the LKB1 homologues in Caenorhabditis elegans and in Drosophila melanogaster, which have been reported to reside exclusively in the cytoplasm and the plasma membrane \(^{211, 219, 320}\). This suggests the conservation of cytoplasmic LKB1 functions across the phyla, but not those of the nuclear LKB1. Although the IF studies did not provide evidence on plasma membrane localization of LKB1, minute amounts of LKB1 were found in the membrane fractions of U2OS and COS-7 cells (data not shown). This finding is consistent with other studies that used mammalian cells \(^{203, 321}\).
6.2 Cytoplasmic localization of LKB1 correlates with the ability to bind STRAD (I and unpublished results)

Due to the reported re-localization of LKB1 into the cytoplasm in response to interaction with STRAD and MO25 we wanted to study whether nuclear LKB1 mutants were able to bind to STRADα. Glutathione S-transferase (GST)-pulldown assays of cell lysates that overexpressed either the wild type or mutant LKB1 demonstrated strong interaction of GST-STRADα and HA-MO25α with the wild type LKB1 or LKB1-D176N. However, there were no such interactions with LKB1-KD, LKB1-G136D, LKB1-SL26, or LKB1-SL8, which demonstrated an inverse correlation between exclusively nuclear localization of LKB1 and the ability to bind to STRADα (Figure 6), which is consistent with other reports. In addition, LKB1 binding to STRADα correlated with the ability of MO25α also to bind to STRADα. The stabilizing effect of LKB1 on STRADα–MO25α interaction was probably caused by reported interaction sites between LKB1 and STRADα and also, between LKB1 and MO25α. Our results suggest that cytoplasmic localization of LKB1 is dependent on STRADα binding but independent of LKB1 kinase activity. It is plausible that additional mechanisms including LKB1 phosphorylation by tyrosine kinase FYN, the lack of phosphorylation by aPKCζ, and the lack of interaction with LKB1 interacting protein (LIP1) contribute to partial nuclear localization of wild type LKB1 and exclusively nuclear localization of some of the LKB1 kinase inactive mutants reported here (Figure 6).

In LKB1 heterozygote (LKB1+/mut) cells the ability of some kinase inactive LKB1 mutants to bind to STRAD could result in the limitation of available STRAD and possibly LKB1 substrates in regard to the wild type LKB1. Consequently, PJS patients or lung adenocarcinoma patients that carry such mutations could have more severe tumorigenesis than those patients who have the LKB1 kinase inactivating non-STRAD binding mutation. Although some initial attempts have been made to correlate LKB1 mutations with tumorigenic phenotype of PJS STRAD binding was not included as one of the parameters in these analyses.
Results and discussion

Figure 6: LKB1 mutants that localize in the nucleus do not form a kinase complex with STRADα and MO25α.

Western blotting analyses of total lysates and GST-pulldown precipitates from 293 cell expressing GST-STRADα and HA-MO25α together with EGFP- or HA- epitope tagged wild type LKB1 or various kinase inactive mutants as denoted in the figure. LKB1 mutations that are causative for nuclear localization (EGFP-LKB1-KD, EGFP-LKB1-G136D, HA-LKB1-SL26 and HA-LKB1-SL8) do not interact with GST-STRADα and do not stabilize the interaction between STRADα and MO25α. Lower expression levels of EGFP-LKB1-KD and EGFP-LKB1-G136D mutants in comparison to wild type and D176N mutant LKB1 are probably a consequence of lack of stabilizing effect of LKB1-STRADα complex formation. White lines indicate the removal of intervening lanes.

Localization of LKB1 into both the cytoplasm and the nucleus could be a consequence of LKB1 shuttling between these compartments. Indeed, Lkb1 has been demonstrated to have a functional nuclear localizing signal (NLS, I Figures 2A and 221), which has been suggested to be masked by STRAD binding (Figure 7). Alternatively, cytoplasmic localization of LKB1 could be induced by cytoplasmic retention of the LKB1 or by nuclear export. Nuclear export could be mediated by a putative nuclear export signal (NES) on LKB1 (Figure 7) identified here or by NES sequences on STRADα that have
been suggested to be essential for chromosome region maintenance 1 (CRM1) and exportin7 dependent nuclear export of LKB1 kinase complex. Possible redundancy between the two alternative export mediators of LKB1 kinase complex could explain the unresponsiveness (Figure 1F) and the modest nuclear accumulation of LKB1 in response to Leptomycin b, an inhibitor of CRM1 dependent nuclear export.

With regard to the model by which LKB1 kinase complex formation relocalizes LKB1 into the cytoplasm and concomitantly induces LKB1 kinase activity, it is surprising that our results demonstrate similar LKB1 kinase activities in both compartments. This similarity also suggests the existence of LKB1-STRAD-MO25 kinase complex in the nucleus. Indeed, a recent study reported the localization of LKB1 and STRADα on the promoter regions in the nucleus (Figure 7). Thus it is possible that LKB1 kinase complex is not automatically exported from the nucleus but is subjected to an additional level of regulation. Moreover, nuclear and active LKB1 could be a consequence of a complex formation with STRADα isoforms that lack either the N- and/or the C-terminal ends or with STRADβ. These STRADα isoforms and STRADβ lack the characterized NES sequences of full length STRADα and thus are expected to be unable to fully mediate the LKB1 export out of the nucleus. It is also plausible that in some contexts LKB1 kinase activity is not dependent on STRAD as has been shown in vivo in Caenorhabditis elegans. These results and the lack of STRADα mutations in LKB1 associated tumorigenesis, leaves it open as to whether STRAD is essential for tumor suppression by LKB1. This conundrum calls for detailed comparative studies on cytoplasmic and nuclear LKB1 complexes and their activities in the model system that should reflect in vivo tumor suppressive functions of LKB1.

6.3 Cytoplasmic LKB1 is sufficient for growth arrest in G361 melanoma cells

As LKB1 was found to be active in both the cytoplasm and the nucleus we wanted to address the question as to which compartment was it that LKB1 activity suppresses cell proliferation. To this end exclusively cytoplasmic LKB1ΔNLS mutant was expressed in the G361 melanoma cell line in which the expression of the wild type LKB1 but not the kinase dead LKB1 caused G1 arrest. LKB1-ΔNLS mutant induced comparable cell cycle arrest to the wild type LKB1 in a kinase dependent manner (Figure 2C), which
suggests that in certain conditions the proposed nuclear functions of LKB1 are dispensable for LKB1 induced inhibition of cell proliferation. Later on LKB1 kinase complex partner STRAD was shown to be essential for G1 growth arrest in G361 cells, which is in line with the role of STRAD in inducing cytoplasmic localization and kinase activity of LKB1. These results do not exclude the possibility that plasma membrane localization would be essential for LKB1 induced growth arrest as suggested for the regulation of epithelial polarization by the D. melanogaster homologue of LKB1. However, immunofluorescence staining of the overexpressed LKB1 in G361 cells did not find any evidence of membrane localization. Moreover, the LKB1 truncation mutant (LKB1 1-416) that lacks the Caax-box, whose farnesylation has been implicated in plasma membrane localization, induced a comparable increase in G1 cells in response to the wild type LKB1 (data not shown).

6.4 LKB1 induced growth arrest in G361 melanoma cells is p53 dependent
The levels of p21Cip1/WAF1 and p27Kip1 were studied to address the question as to whether LKB1 induced growth arrest would be mediated by changes in expression of CDK inhibitor. Western blotting analysis revealed an increase in p21Cip1/WAF1 levels specifically in LKB1 arrested G361 cells (Figures 4A-C). The causality of p21Cip1/WAF1 induction and LKB1 induced cell cycle arrest was supported by the ability of cyclinD1 and CyclinE1 to override LKB1 induced arrest (Figure 3). This was possibly achieved by sequestering surplus p21Cip1/WAF1 and inducing CDK2 activity, respectively. Whether LKB1 induced p21Cip1/WAF1 expression and G1 arrest are transient or lead to quiescence, melanocyte differentiation, or senescence requires further investigations.

Increased activity of p21P-luciferase reporter construct in LKB1 expressing G361 cells (Figure 5A, B) suggested that LKB1 induced p21Cip1/WAF1 promoter activity. Moreover, LKB1 re-introduction induced p21Cip1/WAF1 messenger RNA (mRNA) expression consistently in the A549 lung cancer cell line and in MEFs. These findings and the lack of any known evidence on p21Cip1/WAF1 post-translational stabilization by LKB1 suggest that the induction of p21Cip1/WAF1 by LKB1 takes place at the transcriptional level. p21Cip1/WAF1 is a known transcriptional target of the tumor suppressor p53. Consistently, transfected dominant negative p53 abolished the LKB1 induced increase in
p21P-luciferase activity (Figure 5C) and prevented the LKB1 induced G1 arrest in G361 cells (Figure 5D). These results indicate that p53 is essential for LKB1 induced growth arrest in G361 cells and that this possibly occurs via p21\textsuperscript{Cip1/WAF1} induction. Our results do not support a major role for post-translational stabilization of p27\textsuperscript{Kip1} (Figure 4A) though it is plausible that a LKB1 mediated transcriptional repression of cyclinD1 contributes to LKB1 induced cell cycle arrest in G361 cells (Figure 3).

**Figure 7: Regulation of LKB1 localization and models of LKB1 induced p21\textsuperscript{Cip1/WAF1} expression.** A Schematic figure of LKB1 with NLS, putative NES, Caax-box and phosphorylation sites thought to be involved in the regulation of LKB1 localization. It has been suggested that elements in red enhance nuclear localization, whereas elements in green or yellow promote cytoplasmic or plasma membrane association of LKB1, respectively. STRAD\textalpha with two NES sequences is not depicted. B In G361 cells cytoplasmic and most likely STRAD and MO25 bound...
LKB1 induces the expression of p21\textsuperscript{Cip1/WAF1} possibly by activating one of the LKB1 substrate kinases, which could either (1) re-localize into the nucleus and phosphorylate p53 on p21\textsuperscript{Cip1/WAF1} promoter directly or (2) phosphorylate p53 in the cytoplasm from which the p53 would, in turn, re-localize to the nucleus. In other contexts (3) nuclear LKB1 has been suggested to bind to p53 on p21\textsuperscript{Cip1/WAF1} promoter where it is needed for AMPK or possibly SIK1 mediated phosphorylation of p53 and histone 2B (H2B) and subsequent p21\textsuperscript{Cip1/WAF1} expression. The shuttling of LKB1 between nucleus and cytoplasm is depicted with black dashed arrows. See text for references.

LKB1 and its downstream substrates AMPK and SIK1 have been implicated in the phosphorylation of the p53 S15 and S392 \cite{323, 331-333} that provide a possible mechanism for LKB1 induced p53 dependent cell cycle arrest. Furthermore, both the p53 phosphorylation and phosphorylation of histone 2B have been implicated in LKB1 initiated and AMPK-p53 mediated p21\textsuperscript{Cip1/WAF1} induction \cite{323, 328, 333}. In these same reports LKB1 was suggested to interact with p53 and AMPK on the p21\textsuperscript{Cip1/WAF1} promoter region in the nucleus in MEFs, U2OS and HCT116 cells \cite{323, 328, 333}. As cytoplasmic LKB1 was sufficient to cause p21\textsuperscript{Cip1/WAF1} induction in G361 cells (I, Figure 2C) it is plausible that LKB1 could phosphorylate one of the LKB1 substrate kinases in the cytoplasm from which activated substrate kinase would re-localize to the nucleus and phosphorylate p53 on the p21\textsuperscript{Cip1/WAF1} promoter (Figure 7). Accordingly, AMPK and SIK1 have been demonstrated to localize in both the nucleus and the cytoplasm \cite{334, 335}. Alternatively, LKB1 or its substrate kinases could phosphorylate p53 in the cytoplasm from which p53 would re-localize to the p21\textsuperscript{Cip1/WAF1} promoter within the nucleus (Figure 7). Interestingly in this regard, LKB1 was reported to induce apoptosis by interaction with p53 in the mitochondria of HT1080 cells \cite{336}.

When the possible importance of p53 S15 phosphorylation for tumor suppression by LKB1 in particular is considered, an interesting parallel is provided by the phosphorylation of p53 S15 by the tumor suppressor kinase ATM. Mutations of p53 and of ATM are mutually exclusive in lung adenocarcinomas and mutations of either of these promote increased mutation rates \cite{51}, which suggests that p53 is essential for ATM tumor suppression. In contrast, LKB1 and p53 mutations co-segregate in lung adenocarcinomas \cite{6, 7, 51} and, apparently, LKB1 mutations do not cause increased mutation rates \cite{51}. Similarly, in cervical cancers LKB1 mutations promote tumorigenesis against a
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background in which p53 has already been inactivated by the E6 oncogene encoded by human papilloma virus (HPV)\textsuperscript{177}. Furthermore, the synergistic effects of \textit{LKB1} and p53 deletion on gastrointestinal tumorigenesis of mouse models\textsuperscript{337, 338} and the lack of hamartomatous polyps upon \textit{p53} mutations\textsuperscript{339-342} suggest that p53 is not the sole mediator of tumor suppression by LKB1. However, the correlation of \textit{p21Cip1/WAF1} with LKB1 levels in \textit{p53} wild type but not in mutant pancreatic tumors\textsuperscript{343} suggests a model by which the relative importance of \textit{p53} in LKB1 mediated tumor suppression may vary between the tumor types and tumor grade. Moreover, the genetic loss of \textit{p53} in \textit{LKB1} deleted tumors may confer additional resistance for, or promote, genetic instability that benefits tumor growth.

7. \textbf{LKB1 is essential for TGFβ signaling, production and TGFβ dependent smooth muscle cell lineage differentiation (II, III)}

Inherited heterozygous mutations of \textit{LKB1} cause growth of the hamartomatous gastrointestinal polyps with hyperproliferative epithelia. The existence of a prominent stromal stalk that is composed of SMC lineage cells and the lack of solid evidence on biallelic \textit{LKB1} inactivation in epithelia motivated us to address the two following questions (i) is the decreased \textit{Lkb1} activity in the stromal compartment sufficient for polyp formation \textit{in vivo}? (ii) What are the cellular functions of Lkb1 in SMC lineage cells?

7.1 \textbf{Lkb1 gene deletion in smooth muscle cell lineage causes gastrointestinal polyposis \textit{in vivo}}

To address the role of stromal LKB1 in gastrointestinal tumor suppression the conditional \textit{Lkb1} allele was deleted in the SM22 expressing SMC lineage cells\textsuperscript{318}. Development of gastrointestinal polyps in \textit{Lkb1\textsuperscript{+/Lox,SM22\textsuperscript{+/Cre}}} mice was evident from 11 months of age onwards leading to death of 12 out of 25 mice by the age of 18 months (II Figures 1A, B). Polyps caused by stromal inactivation of \textit{Lkb1} were indistinguishable from the polyps observed in the PJS mouse model (\textit{Lkb1\textsuperscript{+/+}}) and also in PJS patients (II Figure 1C and 172), suggesting that \textit{Lkb1} activity in SMC-lineage cells is tumor suppressive and stromal deletion of the \textit{Lkb1 gene} is sufficient to drive GI polyposis.
We attempted to characterize the possible changes in stromal cells upon Lkb1 deficiency by quantifying the relative amounts of SMC and myofibroblast. This revealed the enrichment of myofibroblasts in polyps (II Figure 2A). Similarly, myofibroblasts were enriched in the polyp stroma in Lkb1+/− mice and, importantly, in the polyps of PJS patients (II Figure 4A). The enrichment of the myofibroblasts could have been caused by the proliferation of the existing myofibroblasts subsequent to Lkb1 deletion or by de-differentiation of SMCs to myofibroblasts as noted in other diseases such as vascular injury and severe lesions of coronary artery.

7.2 Lkb1 is required for myofibroblast differentiation

It has been suggested that SMCs partially develop via the fibroblast-myofibroblast-SMC sequence. Consequently, the primary MEFs are appropriate cell culture model of SMC-lineage differentiation as they have been shown to differentiate spontaneously into myofibroblasts in response to the tension caused by cell contraction against focal adhesions on a rigid cell culture plate. Here control and Lkb1 deleted primary MEFs were created by adenoviral expression of LacZ or Cre-recombinase in cell culture. The control MEFs displayed robust expression of myofibroblast markers α-SMA and SM22, whereas their expression was lost in the majority of Lkb1−/− MEFs (III Figure 2B, C). These findings were consistent with a deficient SMC lineage differentiation upon Lkb1 inactivation.

Actin stress fibers in Lkb1−/− MEFs were studied in order to map which stage of the myofibroblast differentiation process was deficient upon Lkb1 deletion. The formation of actin stress fiber bundles precedes α-SMA expression during myofibroblast differentiation and defines a differentiation stage named the proto-myofibroblast (see 2.3). Actin stress fibers were lost in most of Lkb1−/− MEFs (Figure 8) and this loss was accompanied by deficient focal adhesion maturation (III Figure 2D). Moreover, contractility of Lkb1−/− MEFs was dramatically decreased as measured by their ability to deform silicon substrate in cell culture (III Figures 2D, E). Comparable focal adhesion kinase (FAK) activation in response to cell attachment in the control and Lkb1−/− MEFs (III Figure 3A) suggest that initial cell adhesion and focal complex formation were not
affected by \textit{Lkb1} deletion. Consequently, defects in factors that promote cell contractility and actin stress fibers were more likely to contribute for the deficient myofibroblast differentiation of \textit{Lkb1}^+/− MEFs.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure8}
\caption{Attenuated actin stress fibers in \textit{Lkb1}^+/− MEFs. Acute deletion of LKB1 in primary MEFs led to the loss of actin stress fibers as seen by the decreased staining of actin fibers by phalloidin (green). Nuclei are stained with hoechst (blue).}
\end{figure}

\section*{7.3 \textit{Lkb1} potentiates Smad dependent TGFβ signaling in primary fibroblasts}

TGFβ signaling drives the differentiation of myofibroblast and SMCs in cell culture and \textit{in vivo} \cite{353,354}. Thereby, TGFβ signaling status was tested both in our \textit{in vivo} and cell culture models in which the \textit{Lkb1} gene deletion was associated with deficient SMC-lineage differentiation. Interestingly, phosphorylation of Smad2 was compromised in \textit{Lkb1} deleted MEFs (III Figure 1B). Consistently, Smad2/3 were mostly cytoplasmic (III Figure 1C) and Smad3 and Smad2 dependent transcription were attenuated \textit{Lkb1}^+/− MEFs as monitored by (CAGA)$_{12}$-Luc and activin response element (Are)-Luc reporters (III Figure 1A), respectively. Importantly, (CAGA)$_{12}$-Luc activity was significantly decreased in \textit{Lkb1}^+/− MEFs (III Figure 1A), which are similar to mesenchymal cells in PJS polyps in regard to the \textit{LKB1} genotype \cite{262}. In support of these results, p-Smad2 levels were decreased in \textit{Lkb1} deficient but not in adjacent \textit{Lkb1} wild type stromal cells in the polyps of \textit{Lkb1}^{+/Lox},\textit{SM22}^{+/Cre} mice \textit{in vivo} (II Figure 2G-I and 3D). These results are further supported by the demonstration of decreased levels of plasminogen activator inhibitor
type 1 (PAI-1), a TGFβ target gene, by three independent Lkb1\(^{-/-}\) MEF studies (our unpublished observation and \(^{169,355}\)) and also a study in which LKB1 was downregulated in an immortalized HUVEC cell line \(^{356}\).

TGFβ signaling activity of control and Lkb1\(^{-/-}\) MEFs were measured in response to exogenous TGFβ1 (1ng/ml, 24h) or constitutive activation of TGFβRI ALK5 to map the point of interaction between LKB1 and TGFβ signaling pathways in Lkb1\(^{-/-}\). Although (CAGA)\(_{12}\)-Luc reporter activity was induced in Lkb1\(^{-/-}\) MEFs by exogenous TGFβ and constitutively active ALK5, it did not reach the levels observed in treated control MEFs (III Figure 1D). This result suggests that Lkb1 potentiates but is not essential for the TGFβ pathway activity and that Lkb1 interacts with the TGFβ signaling pathway downstream of receptor activation. In consideration of LKB1 substrates mediating modulation of TGFβ signaling activity by LKB1, it is interesting that LKB1 substrate NUAK2 has been reported to interact with TGFβRI, Smad2 and Smad4 \(^{357}\) and may thus promote full activation of Smad signaling. Keeping in mind reported interactions between LKB1 and p53 (see 6.4), it is intriguing that p53 has been thought to be essential for full Smad dependent transcriptional activation of p21 expression \(^{358}\). Thus p53 activation via phosphorylation of p53-S15 and p53-S392 by LKB1 or its substrate kinases could contribute to the regulation of Smad dependent promoter activation. On the other hand, activation of TGFβ signaling upon LKB1 expression could contribute to an increased p21 expression in conditions of high LKB1 levels in cell culture (I Figures 4A-C) and \textit{in vivo} \(^{278,343}\).

In contrasting the role of LKB1 as an activator of TGFβ signaling in primary MEFs (III Figure 1) and HUVEC cells \(^{356}\), decreased LKB1 activity was found to be associated with enhanced TGFβ signaling in multiple epithelial cell lines, HepG2 cell line of hepatocyte origin, and C2C12 cell line of mesenchymal origin \(^{355,359}\). This association might possibly provide an explanation for an induction of EMT and increased cell motility in cell culture \(^{299,355,359}\) and in lung adenocarcinomas \(^{6,359}\) upon Lkb1 deficiency. Suppression of TGFβ signaling by LKB1 in epithelial cells might be mediated by the interactions between LKB1, Smad4 binding protein LIP1 and Smad4 \(^{355}\) or by LKB1 substrate kinase SIK1 \(^{264}\). SIK1 was reported to be induced in response to the activation
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of TGFβ signaling and to mediate downregulation of TGFβRI/ALK5, which would create a negative feedback loop in TGFβ signaling \(^{264}\).

### 7.4 TGFβ mediates Lkb1 function in regulation of myofibroblast differentiation

*Lkb1*^-/- MEFs were treated with exogenous TGFβ to directly address whether attenuation of TGFβ signaling was causative for myofibroblast dedifferentiation of *Lkb1*^-/- MEFs. Indeed, TGFβ induced the α-SMA and SM22 expression in *Lkb1*^-/- MEFs to levels comparable with non-treated control MEFs (III Figures 4A, B). Concomitantly, actin stress fibers, maturation of focal adhesions (III Figure 4C), and contractility of *Lkb1*^-/- MEFs were rescued. SMC-lineage components including α-SMA and SM22 are targets of Smad dependent transcription \(^{360-365}\). Decreased α-SMA-luciferase and SM22-luciferase reporter activities in *Lkb1*^-/- MEFs in comparison to control and TGFβ treated *Lkb1*^-/- MEFs (III Figure 4D, E) suggest that deficient TGFβ dependent transcription contribute to attenuated myofibroblast differentiation subsequent to Lkb1 inactivation. The ability of TGFβ to rescue both proto-myofibroblast characteristics as determined by actin stress fiber expression and myofibroblast characteristics measured by α-SMA and SM22 expression (III Figure 4A-E) supports the idea that LKB1-TGFβ signaling promotes myofibroblast differentiation at several levels. Such levels possibly include post-translational modification of contractile machinery via activation of RhoA \(^{137,139,366}\).

The *in vivo* study, for which *Lkb1* deletion in SM22 positive cells resulted in increased ratio of myofibroblasts to SMC (II Figure 2A), and also the cell culture study, in which exogenous TGFβ rescued the loss of myofibroblast marker expression in *Lkb1*^-/- MEFs (III Figure 4A-E), suggest the dedifferentiation of SMC lineage cells is a possible cause of PJS polyposis. Similarly, it has been suggested that in breast cancer vascular SMCs contribute to myofibroblasts \(^{367}\). In malignant tumors myofibroblasts also known as cancer associated fibroblasts have been thought to partly originate from fibroblasts in response to increased PDGF secretion by tumorigenic epithelia and by the subsequent increase in TGFβ secretion emanating from infiltrating myeloid cells \(^{368,369}\). In addition, other sources of myofibroblasts/cancer associated fibroblast, such as epithelial cells undergoing EMT \(^{370,371}\), have been suggested. Nevertheless, our model on SMC dedifferentiation as causative for enrichment of myofibroblasts in the PJS polyps is
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supported by following results: 1) α-SMA negative stroma, which most likely represents fibroblasts and which was adjacent to Lkb1 deleted myofibroblasts, lacked deleted Lkb1 allele (II Figures 2G-I) and 2) Lkb1 deficient GI epithelial cells have normal polarity in vivo. Unlike in our cell culture model in which Lkb1 deletion in early passage MEFs did not cause alterations in cell proliferation (III Figure S1), the dedifferentiation in vivo could be associated with an increased proliferation of SMC lineage cells.

Recently, Lkb1 has been implicated as a factor in the differentiation of neurites, hematopoietic cells and gastrointestinal epithelial cells. In addition it has a possible role in the lineage choice of lung cancer progenitor cells in a mouse model. In contrast, Lkb1 inactivation in keratinocytes, striated muscle, heart and liver have provided no evidence on defective differentiation. Collectively, these studies support the concept of LKB1 being a context specific gatekeeper of differentiation and suggest differentiation defects as a possible cause for LKB1 associated tumorigenesis also in other tissues than the GI-tract.

7.5 Decreased secretion of TGFβ by Lkb1 deficient stroma as a possible mechanism of epithelial hyperproliferation

Our model of the dedifferentiation of SMCs under conditions of LKB1 deficiency could explain the development of the prominent stromal stalk in PJS polyps but do not provide a direct mechanism for the hyperproliferation of adjacent polyp epithelia. Interestingly, there is a positive feedback loop in TGFβ production and, consistently, TGFβ1 mRNA levels were decreased both in Lkb1−/− MEFs and in polyps of Lkb1+/Lox;SM22+/Cre mice (II Figure 3M). Consequently, TGFβ secretion, as measured by the PAI-1 luciferase (PAIL) assay, was attenuated in Lkb1−/− MEF cultures (II Figure 3I). These findings imply that decreased TGFβ secretion by Lkb1 deficient stromal cells could induce epithelial proliferation observed in vivo in Lkb1+/Lox;SM22+/Cre mice (II Figure 3G-I) and PJS polyps. Consistently, p-Smad staining in epithelial cells adjacent to Lkb1 deleted stroma was decreased and coincided with the increased expression of Ki-67, which is a marker of cell proliferation (II Figure 3D-I). Additionally, the possible attenuation of Lkb1 deficient stromal cell contractility in vivo could reduce amounts of activated TGFβ available for the epithelial cells (see 2.1). Whether attenuated TGFβ signaling...
in the epithelia could contribute to epithelial differentiation defects noted in \textit{Lkb1}^{+/Lox;SM22+/-Cre} and \textit{Lkb1}^{+/-} mice\textsuperscript{373} in addition to PJS patients, requires further investigation.

Our studies suggest a non-autonomous role for LKB1 in the suppression of epithelial hyperproliferation in the GI-tract. Moreover, they are in contradiction to the hypothesis that proposes polarity defects of epithelial cells and subsequent relapse as causative of PJS polyposis\textsuperscript{300, 301}. In support of our model, \textit{Lkb1} deletion in the gastrointestinal epithelia has not been reported to lead to tumorigenesis\textsuperscript{372} and thus \textit{LKB1} could be categorized as a landscaper tumor suppressor similar to what has been suggested for \textit{Smad4}\textsuperscript{105}. Our results together with the induction of GI tumorigenesis upon \textit{Smad4} deletion in the T cells\textsuperscript{382} and the deletion of \textit{Tgfb}rII in fibroblasts\textsuperscript{108} provide novel examples of the active role of stroma in the suppression of tumorigenesis. In contrast to its landscaper function, LKB1 has been shown to suppress tumorigenesis in a cell autonomous fashion in several tissues: sporadic mutations of LKB1 have been found in tumor cells themselves in cervical cancer\textsuperscript{177}, non small cell lung adenocarcinoma\textsuperscript{6, 7} and mouse model of induced skin tumorigenesis\textsuperscript{237}. This in combination with cell type specific regulation of TGFβ signaling by LKB1 reported in this study and in other studies\textsuperscript{355, 356, 359} underlines the context specificity of LKB1 tumor suppression.

7.6 \textbf{Is AMPK involved in LKB1 tumor suppression in the gastrointestinal tract?}

Although decreased AMPK activity is evident in several \textit{LKB1} mutated tumors, mouse models, and tumor cells\textsuperscript{6, 235-238}, it remains unclear whether the deregulation of AMPK is causative of tumorigenesis. Increased levels of phosphorylated ribosome subunit S6 in the PJS polyp epithelia have been used to suggest that attenuated AMPK activity as a cause for PJS\textsuperscript{229, 383} (Figures 1 and 4). However, increased levels of phosphorylated S6 are most likely secondary to that of polyp growth or caused by unidentified additional mutations other than those of \textit{Lkb1} as similar increase in epithelial p-S6 levels was observed in mice in which \textit{Lkb1} was inactivated only in the stromal cells (II Figures S2E-F). Thus the decreased polyposis of \textit{Lkb1} heterozygote mice upon mTOR inhibitor rapamycin\textsuperscript{383, 384} does not provide evidence for a causal association between the decreased AMPK activity and polyposis. Of course this does not rule out the possibility
that AMPK would contribute to epithelial hyperproliferation or induction of stromal stalk noted in PJS polyps. Intriguingly, mTOR activation has been implicated in the dedifferentiation of vascular smooth muscle cells \(^{385}\). Furthermore, TSC1/2 complex, a target of AMPK, is essential for actin stress fiber regulation in MEFs \(^{386}\). However, our results showed normal actin stress fibers (data not shown) and myofibroblast differentiation in AMPK deficient primary MEFs (III Figure S2), which implies that AMPK does not regulate actin stress fibers and differentiation via phosphorylation of MLC \(^{310}\) or other targets in SMC-lineage cells. Thus deficient actin stress fibers in TSC1/2 attenuated MEFs might reflect deficient PTEN signaling as PTEN deleted MEFs were reported to have enhanced myofibroblast differentiation \(^{387}\). Moreover, the lack of AMPK\(^{\alpha1}\) and \(\alpha2\) mutations in PJS patients \(^{262}\) suggests that either AMPK\(^{\alpha1}\) or \(\alpha2\) are not major mediators of tumor suppression by LKB1 in these tissues or alternatively that AMPK\(^{\alpha1}\) and \(\alpha2\) are redundant. The potential tumor suppressive role of AMPK in the GI tract could be addressed by studies on AMPK\(^{\alpha1\,-}\) or \(\alpha2\,-\) mice or by stroma-specific deletion of both alleles.

8. LKB1 substrate NUAK2 promotes actin stress fibers (IV)

8.1 NUAK2 interacts with actin binding myosin phosphatase Rho-interacting protein (MRIP) and localizes to the actin stress fibers

The possible involvement of LKB1 in promoting actin stress fibers and contractility (see results 7.2 and 7.4) suggests that LKB1 might either positively regulate RhoA signaling, which promotes contractility, or negatively regulate MLCP, which counteracts the RhoA pathway by dephosphorylation of MLC \(^{388}\) (see 2.3).

In regard to the involvement of the LKB1 substrate kinases in actin stress fiber regulation, it was of great interest that NUAK2 interacted with the actin binding protein MRIP (also known as p116\(^{\text{rip}}\)) in two independent yeast two hybrid screens carried out in our laboratory (IV Figure 1A). A total of 26 out of 28 clones that interacted with NUAK2 encoded for various C-terminal fragments of MRIP. MRIP is a large protein that binds to actin with its N-terminus and to MYPT, a subunit of MLCP, with its C-terminus. Thus it functions as an adapter between the actin cytoskeleton and MLCP. MRIP has been
suggested to be crucial for MLCP activity towards MLC as the overexpression of MRIP attenuated and downregulation induced actin stress fibers\textsuperscript{389-391}.

The established role of MRIP with regard to actin fibers prompted us to study whether NUAK2 could localize onto the actin stress fibers. The interaction of NUAK2 with MRIP was studied in the HeLa cell line in which the earlier characterization of MRIP had been carried out\textsuperscript{389, 392}. The co-localization of epitope tagged NUAK2 and MRIP on the stress fibers (IV Figure 1D) strongly supported the interaction observed in the yeast two hybrid assay. Furthermore, GST-pulldown assays demonstrated NUAK2 and MRIP interaction in the soluble pool of the cell lysates (IV Figures 1B, C). Localization of NUAK2 on actin stress fibers was MRIP dependent as demonstrated by the loss of stress fiber localization of NUAK2 upon MRIP downregulation (IV Figures 3A, B and S3A, B). Despite extensive studies on LKB1 localization using several cell types there are no reports of LKB1 localizing on actin stress fibers, which suggests that either LKB1 localization on actin stress fibers is transient or that NUAK2 is activated by LKB1 in the soluble pool of cytoplasm from which activated NUAK2 would re-localize to the stress fibers. Consistent with possible dynamic NUAK2 localization mechanism, overexpressed NUAK2 was observed both in the cytoplasm and the nucleus in addition to the actin stress fibers (IV Figures S2, S3A).

**8.2 NUAK2 promotes actin stress fibers in kinase independent and dependent manner by inhibiting myosin light chain phosphatase (MLCP)**

In order to study whether NUAK2 contributed to the regulation of actin stress fibers, phalloidin intensities of NUAK2 overexpressing HeLa cells were quantified. Interestingly, wild type NUAK2 increased actin stress fibers significantly. Apparently this increase was mediated in a kinase activity independent manner as HeLa cells do not express LKB1. Moreover, kinase deficient T-loop mutant NUAK2-T208A\textsuperscript{199} promoted stress fibers in a comparable manner to wild type NUAK2 (IV Figures 1D, E and S1). Expression of constitutively active NUAK2-T208E mutant\textsuperscript{199} caused a further increase in phalloidin intensity in comparison to the wild type and T208A mutant NUAK2 (IV Figures 3D, E and S1). This suggested partially kinase independent and kinase dependent functions for NUAK2 in the regulation of actin stress fibers in HeLa cells. Consistent
with the stress fiber induction by NUAK2 overexpression, NUAK2 downregulation led to the attenuation of stress fibers that was concomitant with the decrease in phosphorylated MLC (IV Figures 4A-E). This finding together with the NUAK2 interaction with MLCP adaptor MRIP suggests that NUAK2 might inhibit MLCP activity.

We investigated whether NUAK2 might inhibit MLCP. Therefore, we acutely inhibited the MLC kinase ROCK by using the specific inhibitor Y27632 \(^{393, 394}\). ROCK inhibition in the control cells led to the rapid loss of stress fibers. This was most likely due to the lack of ongoing phosphorylation of MLC and thus domination of MLC dephosphorylation by MLCP \(^{392}\). Thus changes in MLCP activity should be seen as changes in the kinetics of loss of stress fibers. Expression of NUAK2 caused the resistance of central stress fibers to Y27632 treatment (IV Figures 4A, B) but not to cytochalasin D treatment, which prevents fiber formation in an MLCP independent manner by binding to actin monomers \(^{395}\). Consistent with a role of NUAK2 in the inhibition of MLCP via MRIP interaction, actin stress fiber disruption and decreased levels of phosphorylated MLC upon NUAK2 downregulation were dependent both on MRIP and MYPT1 (IV Figures 7A-D). These studies identified MRIP as a hub at which both negative and positive MLC regulation are integrated.

Other possible LKB1 downstream pathways involved in actin stress fiber regulation include RhoA activation by RhoA GTP exchange factor (RhoA-GEF) DBL (also known as MCF2). Overexpressed LKB1 was suggested to interact with DBL in HeLa cells and induce actin stress fiber formation in an LKB1 kinase activity independent manner (Figure 9) \(^{396}\). In addition, the LKB1 substrate AMPK has been implicated in the direct phosphorylation of MLC in *Drosophila melanogaster* \(^{310}\) (Figure 9, see 5.2). It is plausible that one or multiples of these LKB1 regulated signaling pathways could contribute to the loss of stress fibers in *Lkb1* deleted MEFs (III Figure 2D). However, normal phosphorylation levels of ROCK target moesin in *Lkb1*\(^{−/−}\) MEFs \(^{397, 398}\) (III Figure 3C) in addition to normal actin stress fibers (data not shown) and myofibroblast differentiation in AMPK deleted MEFs (III Figure S2) suggest that in MEFs it is NUAK2, instead of DBL or AMPK that should be considered as a candidate mediator of LKB1 activity in actin stress fiber regulation.
Our observation of the TGF\(\beta\) signaling mediated induction of actin stress fibers by Lkb1 in MEFs and the involvement of LKB1 substrate kinase NUAK2 in the positive regulation of actin stress fibers in HeLa cells support the idea that NUAK2 could mediate LKB1 dependent regulation of TGF\(\beta\) signaling. Interestingly, TGF\(\beta\) induced RhoA-ROCK activity in differentiating SMCs was suggested to be essential for the nuclear localization of Smads and Smad dependent promoter activation. Therefore it is plausible that the phosphorylation of MLC is a prerequisite for the proper activation of Smads in differentiating SMCs, though this hypothesis has not been tested yet. Thus positive regulation of MLC phosphorylation by kinase active NUAK2 could provide the mechanism for the potentiation of TGF\(\beta\) signaling by LKB1 (Figure 9). Alternatively, NUAK2 could affect TGF\(\beta\) signaling via interactions with the TGF\(\beta\) signaling pathway components.

### 8.3 Identification of a positive feedback loop between actin stress fibers and NUAK2 expression

The partially kinase independent function of NUAK2 in promoting actin stress fibers suggested that NUAK2 expression levels can act as a potential means of regulation of NUAK2 function. Indeed, NUAK2 mRNA and protein levels were low in serum starved cells in which the stress fibers were attenuated but high in the presence of prominent actin stress fibers induced by one hour of treatment with 10% serum (IV Figures 5A-E). Direct evidence on the role for actin in regulating NUAK2 levels was provided by the following: decreased NUAK2 mRNA levels in response to one-hour of treatment with ROCK kinase inhibitor Y27632, myosin 2 inhibitor blebbistatin, or actin binding drug cytochalasin D, all of which caused a rapid loss of actin stress fibers (IV Figures 5A-E). The rapid regulation of NUAK2 mRNA in response to altered actin fiber dynamics suggests the existence of actin responsive elements on the NUAK2 promoter. Potential transcription factors involved in mediating the effects of altered actin dynamics on gene expression include: a complex between serum response factor (SRF) and megakaryoblastic leukemia 1 (MAL), which is inhibited by monomeric actin and thus activated in response to actin polymerization, and also the transcriptional repressor yin yang 1 (YY1), which is inhibited upon actin fiber formation. Our current studies are aimed to resolve whether
these TFs are involved in the regulation of NUAK2 expression levels. Increased expression of NUAK2 in response to prominent actin stress fibers with the induction of actin stress fibers in response to NUAK2 expression suggests the existence of a positive feedback loop, which promotes the maintenance of actin stress fibers. Previously, similar positive feedback loops had been suggested to involve structural components of actin cytoskeleton, such as actin and tropomyosin. However, to the best of the author’s knowledge NUAK2 is the first actin stress fiber regulating kinase whose expression levels rapidly respond to dynamic changes in the actin cytoskeleton.

Recently, NUAK1 was shown to bind to PP1 and phosphorylate MYPT1 on three sites in the soluble fraction of cells which led to the sequestration of MLCP by 14-3-3. The findings of their study combined with our results suggest a model by which NUAK2 inhibits MRIP bound MLCP in a partially kinase independent manner in the presence of abundant actin stress fibers whereas activation of NUAK1 by LKB1 might allow local MLCP inhibition in conditions of low actin fiber content (Figure 9). Obviously these mechanisms between NUAK1 and NUAK2 might be partially redundant (see 4.3) as suggested by our observation of further increase in stress fibers by constitutive activation of NUAK2 (NUAK2-T208E) in comparison to that found for the wild type NUAK2 (IV Figures 1E and S1).

UNC-82 a homologue of NUAK2 and NUAK1 in Caenorhabditis elegans is essential for muscle integrity in vivo, which suggests a general role for NUAK2 in the regulation of actomyosin fibers. Interestingly, the expression of MRIP and NUAK2 were induced by the 18-hour TGFβ treatment in epithelial MTLn3E cells. Similar to that found for RhoA/C MRIP was required for TGFβ dependent disruption of epithelial cell-cell junctions and the retraction of the tail end of motile cells. These results together with the finding in this study of an identified NUAK2–MRIP interaction suggest that NUAK2 is a potential inducer of EMT, at least in MTLn3E cells (Figure 10). Consistently, meta-analysis of the EST-database revealed a high NUAK2 expression in ovarian cancer and peritoneal adenocarcinoma samples that represent highly invasive cancers (publication III, www.genesapiens.org, and). In contrast to these, LKB1 mutations have been associated with increased invasiveness of tumors, which suggests that one or several
LKB1 substrate kinases normally inhibit EMT\(^6, 177, 359\) (see literature review 3.3, Figure 10). The discrepancy between reported inhibition of tumor invasiveness by LKB1 and potential promotion by NUAK2 might be explained by a cell type specific regulation of TGF\(\beta\) signaling by LKB1\(^{223, 355, 359}\) (see results & discussion 7.3, Figure 10). Accordingly, LKB1-NUAK2-MRIP could promote the invasiveness of cells in which LKB1 potentiates TGF\(\beta\) signaling and inhibit the invasiveness of cells in which LKB1 suppresses TGF\(\beta\) signaling. Alternatively, MRIP dependent induction of EMT could represent NUAK2 kinase independent function (Figure 10).

**Figure 9:** Actin stress fibers and TGF\(\beta\) pathway activity are possible targets of LKB1-NUAK2 signaling.

1. LKB1 substrate kinase NUAK2 is able to inhibit PP1 (MLCP) activity in a kinase independent manner and possibly in kinase dependent manner via the phosphorylation of MYPT on three
amino acid residues, which leads to the sequestration of MLCP by 14-3-3. Alternative mechanisms of actin fiber regulation by LKB1 include (2) the direct phosphorylation of MLC by activated AMPK and (3) LKB1 binding to RhoA-GEF DBL and thus the activation of RhoA-ROCK signaling, which leads to phosphorylation of MLC. (4) Co-regulation of TGFβ signaling and actin fibers by LKB1 suggest it is plausible that LKB1-NUAK2 induces TGFβ signaling by affecting actin cytoskeletal dynamics, which would be consistent with the reported observation of RhoA induced Smad signaling.
PERSPECTIVES

Studies presented here revealed possible links between LKB1 and two major tumor suppressive pathways namely, the p53 and the TGFβ signaling pathways. First, the observation on LKB1 induced p53 dependent and p21 associated G1 arrest in G361 cells have been followed by several publications that suggest p53-S15 phosphorylation as a link between LKB1 and p53. Due to the discrepancies in the in vivo evidence on the importance of p53 as a downstream target of LKB1 (see 6.4) further studies on the possible LKB1–p53 link in Lkb1 associated tumor models are required. The effect of p53-S15A knock in (S18A in mice) in model mice of KRAS driven lung cancer and studies on possible synergistic effects of p53-S15A knock-in and Lkb1 deletion in polyposis of GI tract (and this study), tumorigenesis of lung and tumorigenesis of pancreas could reveal whether p53-S15 phosphorylation is downstream of LKB1 tumor suppression. Second, the potentiation of TGFβ signaling in primary mesenchymal cells by Lkb1 and the data of recent studies suggesting TGFβ suppression by LKB1 in other model systems links these two tumor suppressive pathways. It is intriguing that in mesenchymal cells LKB1 may enhance TGFβ signaling and thus suppress epithelial proliferation via increased secretion/activation of TGFβ. On the other hand, LKB1 could suppress the adverse effect of TGFβ signaling (EMT) in epithelia as suggested by the increased TGFβ signaling and metastatic capability of Lkb1 deficient lung tumors (Figure 10). Indications of LKB1 in the regulation of TGFβ signaling calls for further studies on the contribution of LKB1 in tumorigenesis of colon and pancreas in which frequent alterations of TGFβ signaling have been reported.

Third, these studies are the first to demonstrate the dependency of actin stress fibers on Lkb1 and its substrate kinase NUAK2. The critical role of stress fibers in cell contractility associates LKB1 with the remodeling of ECM and cell motility. These studies could also provide insight into the mechanisms to explain earlier observations in which regulation of polarity by LKB1 has been implied to be partially dependent on the actin cytoskeleton. The intriguing connection of NUAK2 with both TGFβ signaling and actin cytoskeleton warrants further studies on the possible role of NUAK2 as a mediator of Lkb1 and TGFβ dependent regulation of the actin cytoskeleton.
and mesenchymal cell differentiation (Figures 9 and 10). Furthermore, the finding of increased adenoma and aberrant crypt foci formation in the large intestine of azoxymethane treated NUAK2 heterozygote mice in comparison to wild type mice supports a tumor suppressive role for NUAK2 in the initiation of tumorigenesis \(^{276}\). The combination of NUAK2 overexpression or deficiency with Lkb1 associated tumor prone mouse models (\(^{236-238, 412}\) and this study) are essential to clarify whether NUAK2 contributes to tumor suppression by LKB1.

Future studies will expand our knowledge on LKB1 signaling network. It is possible that all LKB1 substrate kinases have already been identified as LKB1 does not phosphorylate maternal embryonic leucine zipper kinase (MELK), NIM1 and testis-specific serine/threonine kinases (TSSK1-4) \(^{199, 234}\), all of which are most closely related to the known 14 LKB1 substrate kinases. Instead, studies on context dependent activation of known LKB1 signaling pathways could shed light on the mechanism of LKB1 tumor suppression. There is a possibility that LKB1 could function as a hub in which extracellular and intracellular cell growth regulating cues are integrated (Figure 10). It has been suggested that growth factor responsive ERK inhibits LKB1 activity by phosphorylation of LKB1-S325 \(^{215}\). In contrast, phosphorylation of LKB1-S428 by lipid and hormone sensitive PKA and other kinases was essential for LKB1 activity \(^{203, 211}\). On the other hand, LKB1 signaling is responsive to cell intrinsic stress as an increase in AMP levels potentiates the activation of AMPK by LKB1 \(^{246}\). Moreover, various cellular stresses cause the upregulation of LKB1 substrate kinase NUAK2 \(^{260}\). Technologies such as fluorescence resonance energy transfer and the availability of phospho specific antibodies enable spatiotemporal studies on the activation of particular LKB1 signaling pathways in response to specific stimuli involved in tumorigenesis.
The studies in this thesis and the studies reported elsewhere suggest the following: 1) NUAK2 should be considered as a possible LKB1 substrate kinase that mediates cell contraction, TGFβ signaling, and SMC lineage differentiation that are induced by LKB1 in mesenchymal cells of the stroma. 2) LKB1 increases amounts of active TGFβ and thus also induces TGFβ signaling in adjacent epithelial/tumor cells via the enhanced contractility and TGFβ auto-loop. This event may lead to the inhibition of cell proliferation or induction of EMT in a context dependent manner. 3) LKB1 in epithelial cells may concomitantly inhibit tumor cell proliferation by activating p53 possibly through AMPK or SIK1. 4) LKB1 may inhibit EMT by suppressing TGFβ signaling through NUAK2, SIK1 or one of the other substrates. In contrast to suppression of EMT by LKB1, it is plausible that in some conditions NUAK2 should be considered as a candidate inducer of EMT. It is possible that NUAK2 does this in an LKB1 independent manner (see 8.3). Signaling activity of LKB1 can be regulated by both extra- and intracellular cues. 5) Extracellular cues may include...
mitogenic growth factors, lipids, and hormones whereas 6) intracellular stress signals contribute to activation of LKB1 signaling via increased AMP (AMPK) or LKB1 substrate kinase levels (NUAK2).
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