Lkb1 is required for TGFβ-mediated myofibroblast differentiation

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Accepted 21 July 2008
Journal of Cell Science 121, 3531-3540 Published by The Company of Biologists 2008
doi:10.1242/jcs.032706

Summary

Inactivating mutations of the tumor-suppressor kinase gene *LKB1* underlie Peutz-Jeghers syndrome (PJS), which is characterized by gastrointestinal hamartomatous polyps with a prominent smooth-muscle and stromal component. Recently, it was noted that PJS-type polyps develop in mice in which *Lkb1* deletion is restricted to *SM22*-expressing mesenchymal cells. Here, we investigated the stromal functions of *Lkb1*, which possibly underlie tumor suppression. Ablation of *Lkb1* in primary mouse embryo fibroblasts (MEFs) leads to attenuated Smad activation and TGFβ-dependent transcription. Also, myofibroblast differentiation of $Lkb1^{-/-}$ MEFs is defective, resulting in a markedly decreased formation of α-smooth muscle actin (SMA)-positive stress fibers and reduced contractility. The myofibroblast differentiation defect was not associated with altered serum response factor (SRF) activity and was rescued by exogenous TGFβ, indicating that inactivation of *Lkb1* leads to defects in myofibroblast differentiation through attenuated TGFβ signaling. These results suggest that tumorigenesis by *Lkb1*-deficient *SM22*-positive cells involves defective myogenic differentiation.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/121/21/3531/DC1

Key words: Lkb1, Myofibroblast differentiation, TGFβ

Introduction

Peutz-Jeghers syndrome (PJS) is caused by inactivating mutations of the *LKB1* (*STK11*) serine/threonine kinase gene, and is characterized by gastrointestinal polyposis and an increased risk of cancer (Hemminki et al., 1998). PJS polyps are hamartomas, consisting of differentiated epithelia and a prominent stromal compartment with a characteristic smooth-muscle core. Recently, it was demonstrated that stromal mutations of *Lkb1* are sufficient for epithelial proliferation and polyposis in a PJS mouse model by conditional deletion of *Lkb1* in *SM22* (also known as *Tagln*)-expressing mesenchymal cells (Katajisto et al., 2008).

The identified tumor-suppressive function of *Lkb1* in the $Lkb1^{lox/+};Tagln^{Cre/+}$ mice focuses interest towards changes in *SM22*-expressing cells following *Lkb1* deletion, especially because these changes probably underlie polyposis also in PJS patients (Katajisto et al., 2008). Immunohistochemical analysis of mouse and human polyps revealed a prominent increase in stromal α-smooth muscle actin (SMA)-expressing cells, which did not display the smooth-muscle cell (SMC) marker desmin. This phenotype is characteristic of myofibroblasts that were originally identified in the granulation tissue of wounds as highly contractile fibroblastic cells with SMC features (Hinz et al., 2007; Tomasek et al., 2002). Importantly, characteristics include the expression of SM22 in several tissues (Chiavegato et al., 1999; Faggin et al., 1999). Thus, the additional myofibroblasts in the conditional *Lkb1* polyposis model could originate from the small number of myofibroblasts that are normally present, which proliferate following *Lkb1* deletion; a similar proliferation of pericryptal myofibroblasts has been suggested as a possible mechanism for the increased number of myofibroblasts in other polyposis models (Powell et al., 2005).

Shared markers, such as SMA and SM22, and the high plasticity of fibroblasts, myofibroblasts and SMCs in, for example, vascular injury have raised the concept that these cell types represent a continuous spectrum of differentiation stages (Hinz et al., 2007; Tomasek et al., 2002; Zalewski et al., 2002). Furthermore, dedifferentiation of SMCs to myofibroblasts has been noted, e.g. following vascular injury (Rajkumar et al., 2005) and in severe lesions of the coronary artery (Hao et al., 2006). Thus, an alternative model for the increased number of myofibroblasts in $Lkb1^{lox/+};Tagln^{Cre/+}$ polyps is dedifferentiation of the more abundant SMCs following *Lkb1* deletion. At present, *Lkb1* has not been directly implicated in myogenic differentiation despite conditional mutagenesis studies in skeletal (Sakamoto et al., 2005a) and cardiac (Sakamoto et al., 2005b) muscle. However, the importance of *Lkb1* in neuronal polarization, migration and differentiation (Asada et al., 2007; Barnes et al., 2007; Shelly et al., 2007) suggests a cell-type-specific requirement for Lkb1 in differentiation.

Deficient TGFβ signaling from *Lkb1*-deficient stroma to epithelia was identified as one possible mechanism for epithelial hyperproliferation and tumorigenesis (Katajisto et al., 2008), but it is yet unknown how deletion of *LKB1* can induce the formation of myofibroblasts in polyposis. Interestingly, TGFβ is a crucial factor in both myofibroblast (Desmouliere et al., 1993; Hinz et al., 2001b) and SMC (Grainger et al., 1998; Roelofs, 1998; Qiu et al., 2006) differentiation.

When fibroblasts that are isolated from tissues are grown on standard tissue-culture plates, focal-adhesion attachment to the rigid

non-deformable plastic leads to tension and subsequent differentiation of cells to myofibroblasts (Wang et al., 2003; Wipff et al., 2007). Here, we used this myofibroblast-differentiation model using primary mouse embryonic fibroblasts (MEFs) with a loxed allele of *Lkb1* to investigate the involvement of Lkb1 in TGFβ signaling and myofibroblast differentiation.

Results

Lkb1 regulates mRNA levels of TGFβ1

Supernatants from $Lkb1^{-/-}$ MEF culture were previously noted to contain lower levels of TGFβ (Katajisto et al., 2008), which could reflect defective production (Yue and Mulder, 2000), defective secretion (Miyazono et al., 1991) or defective extracellular processing (Annes et al., 2003) of TGFβ. To investigate possible mechanisms behind the decreased levels of TGFβ in culture supernatants, we used primary MEFs with a loxed allele of *Lkb1* (Bardeesy et al., 2002), enabling conditional deletion of *Lkb1* following the introduction of Cre recombinase using a recombinant adenovirus (AdCre) (Anton and Graham, 1995).

To determine possible changes in TGFβ production, mRNA levels of TGFβ1 were determined from $Lkb1^{lox/-}$ MEFs infected with recombinant adenovirus containing *lacZ* (AdLacZ) (referred herein as control; see Materials and Methods) and AdCre-infected $Lkb1^{lox/-}$ MEFs ($Lkb1^{-/-}$) grown in the presence of 10% fetal calf serum (FCS). Real-time PCR analysis of GAPDH-normalized mRNA levels (see Materials and Methods) demonstrated a 47% reduction of mRNA levels of TGFβ1 in $Lkb1^{-/-}$ MEFs, suggesting that the previously noted comparable changes in supernatants (Katajisto et al., 2008) were due to *Lkb1*-mediated intracellular changes that were reflected in altered mRNA levels.

Lkb1 modulates Smad-dependent transcription

Decreased mRNA levels of TGFβ1 in $Lkb1^{-/-}$ MEFs could reflect attenuated TGFβ signaling activity because regulation of mRNA transcription of TGFβ1 is partly mediated by autoinduction (Kim et al., 1990; Piek et al., 2001; Van Obberghen-Schilling et al., 1988; Yue and Mulder, 2000). Supernatants of normal primary MEFs in standard culture conditions contain a relatively high level of active TGFβ (228 pg/ml), suggesting that the TGFβ pathway is activated. In response to TGFβ-pathway activation, receptor-activated Smads (R-Smads; Smad1, Smad2, Smad3, Smad5 and Smad8), together with Smad4, activate or repress primary TGFβ target genes together with other transcription factors, co-activators and co-repressors (Itoh and ten Dijke, 2007). To address intracellular TGFβ signaling in $Lkb1^{-/-}$ MEFs, Smad3- and Smad2-dependent TGFβ signaling was assessed using a Smad3-dependent $(CAGA)_{12}$-luc reporter (Dennler et al., 1998) or a Smad2-dependent ARE-luc (activin response element) reporter together with a FAST-1 expression plasmid (Yakymovych et al., 2001), respectively. In AdCre-infected $Lkb1^{lox/lox}$ MEFs, a 70% decrease in $(CAGA)_{12}$-luc activity ($P=8.3\times10^{-14}$) and a 57% decrease in ARE-luc activity ($P=6.4\times10^{-7}$) was noted when compared with AdLacZ (control)-infected cells (Fig. 1A). A smaller, but still significant, decrease in $(CAGA)_{12}$-luc activity was also noted in AdCre-infected $Lkb1^{lox/+}$ MEFs (16%; $P=0.0014$) (Fig. 1A, lox/+ bars), demonstrating haploinsufficiency of *Lkb1* in this model and indicating similarity in this regard to the tumor-suppressor function of *Lkb1* (Hernan et al., 2004; Jishage et al., 2002; Miyoshi et al., 2002; Rossi et al., 2002).

To validate these results, a second genetic approach using siRNA-mediated knockdown of Lkb1 in wild-type MEFs was used. At 3 days after transfection of non-targeting (control) or *Lkb1*-targeting (siLkb1) siRNAs, $(CAGA)_{12}$-luc activity was significantly reduced in siLkb1 samples ($P=0.0012$) (Fig. 1A, right panel), in which Lkb1 protein levels were reduced by 75% (data not shown). These results demonstrate that Lkb1 is required for normal Smad2- and Smad3-dependent TGFβ signaling in MEFs.

To investigate the specificity of regulation by Lkb1, the activity of the Smad1-, Smad5- and Smad8-dependent BMP signaling pathway was assessed following *Lkb1* deletion using an ID1 BMP-response element reporter (BRE-luc) (Korchynskyi and ten Dijke, 2002). The results indicated unaltered BRE-luc activity in unstimulated $Lkb1^{-/-}$ MEFs (Fig. 1A), and this activity was similarly inducible with exogenous BMP4 (100 ng/ml, 19 hours) in control and $Lkb1^{-/-}$ MEFs (data not shown). Thus, the requirement of Lkb1 is restricted to Smad2- and Smad3-dependent signaling.

Attenuated activity of the Smad2 and Smad3 pathways in $Lkb1^{-/-}$ cells

To further investigate Smad2 activation in $Lkb1^{-/-}$ MEFs, levels of active Smad2 phosphorylated on serines 465 and 467 (Smad2-*P*) were analyzed by western blotting. Smad2-*P* levels in untreated samples were robust in control MEFs, but significantly lower in $Lkb1^{-/-}$ MEFs (Fig. 1B). The addition of high levels (1 ng/ml) of exogenous TGFβ increased Smad2-*P* levels both in control and $Lkb1^{-/-}$ MEFs (Fig. 1B). Attenuated Smad activation in $Lkb1^{-/-}$ MEFs was also suggested by immunofluorescence analysis. In $Lkb1^{-/-}$ MEFs, Smad2 and Smad3 were mostly detected in a cytoplasmic localization, which changed to a predominant nuclear staining following the addition of TGFβ (1 ng/ml). This staining was comparable to control MEFs even in the absence of exogenous TGFβ (Fig. 1C).

Although TGFβ signaling is attenuated at the R-Smad and transcriptional level in *Lkb1*-deficient MEFs, the results above did not allow determination of the level of regulation due to autoinduction by TGFβ1. To now differentiate whether defects following deletion of *Lkb1* were primarily at the level of TGFβ production or in intracellular TGFβ signaling, the inducibility of the Smad3 reporter $(CAGA)_{12}$-luc to exogenously added TGFβ was assessed. The addition of a saturating concentration (1 ng/ml; based on saturation curves) of active TGFβ to the culture medium resulted in the induction of $(CAGA)_{12}$-luc both in control and $Lkb1^{-/-}$ MEFs, but activity was still significantly lower in $Lkb1^{-/-}$ MEFs (37% of control) (Fig. 1D, vector + TGFβ). The result suggested a defect between the formation of active TGFβ and target-gene induction. To further dissect the level at which the defect occurs, $(CAGA)_{12}$-luc activity was compared in cells transfected with a constitutively active (ca) TGFβ type-I receptor (TGFβRI) (Nakao et al., 1997); again, $(CAGA)_{12}$-luc activity was significantly lower in $Lkb1^{-/-}$ MEFs (22% of controls) (Fig. 1D, caALK5). These results suggest that the defects noted in $Lkb1^{-/-}$ MEFs are primarily due to downregulation of intracellular TGFβ signaling resulting in reduced TGFβ levels in culture supernatants.

Decreased expression of the myofibroblast markers SMA and SM22 following Lkb1 ablation

The observed attenuated TGFβ signaling suggested that myofibroblast differentiation might be deregulated in $Lkb1^{-/-}$ MEFs, because several differentiation models implicate TGFβ as a crucial regulator of myofibroblast differentiation (Desmouliere et al., 1993; Ronnov-Jessen and Petersen, 1993). This was able to be addressed

in the MEF cultures because a significant fraction of MEFs spontaneously differentiate into myofibroblasts in a rigid environment (Chen et al., 2004; Greenberg et al., 2006; Sousa et al., 2007), represented, for example, by a normal-tissue-culture plate. Here, this spontaneous differentiation of MEFs was found to be dependent on the endogenously produced TGFβ, because addition of a TGFβRI inhibitor, SB431542 (Inman et al., 2002), led to significantly decreased expression of SMA, the expression of which was otherwise noted in 75% of the cells thereby defined as myofibroblasts (Fig. 2A).

This observation allowed us to investigate whether Lkb1 is required for myofibroblast differentiation. Interestingly, only 25% of *Lkb1*$^{-/-}$ MEFs demonstrated detectable SMA expression (Fig. 2B, *Lkb1*$^{-/-}$). Similar reduced staining was noted with the myofibroblast marker SM22 (Qiu et al., 2006) in *Lkb1*$^{-/-}$ MEFs (Fig. 2C), and both SMA and SM22 total levels were decreased, as analyzed by western blotting (data not shown).

Myofibroblast differentiation, as well as TGFβ signaling, has been associated with various effects on proliferation depending on the experimental conditions (Desmouliere et al., 1992; Grotendorst et al., 2004; Ng et al., 2005; Sorrentino and Bandyopadhyay, 1989; White et al., 2006) or concentration of TGFβ (Battegay et al., 1990). However, the noted differences in SMA and SM22 levels and in TGFβ signaling of *Lkb1*$^{-/-}$ MEFs were not associated with a significant change in proliferation rates, because comparable fractions of BrdU-positive cells following a 6-hour pulse of BrdU were observed (supplementary material Fig. S1), which is consistent with previous observations on early-passage *Lkb1*$^{-/-}$ MEFs (Bardeesy et al., 2002).

Lkb1 MEFs have decreased contractility

Another important feature of myofibroblasts is the development of contractile stress fibers that define the 'proto-myofibroblasts' that precede myofibroblasts expressing SMA (Hinz et al., 2007; Tomasek et al., 2002). To test whether *Lkb1*$^{-/-}$ MEFs also exhibit defective stress-fiber formation, F-actin was visualized with fluorescent phalloidin. In contrast to the prominent stress fibers observed in control MEFs, contractile bundles were lacking in *Lkb1*$^{-/-}$ MEFs, in which F-actin was restricted to the cell periphery (Fig. 2D, phalloidin). Concomitantly with the reduction of stress fibers and reorganization of F-actin in *Lkb1*$^{-/-}$ MEFs, focal

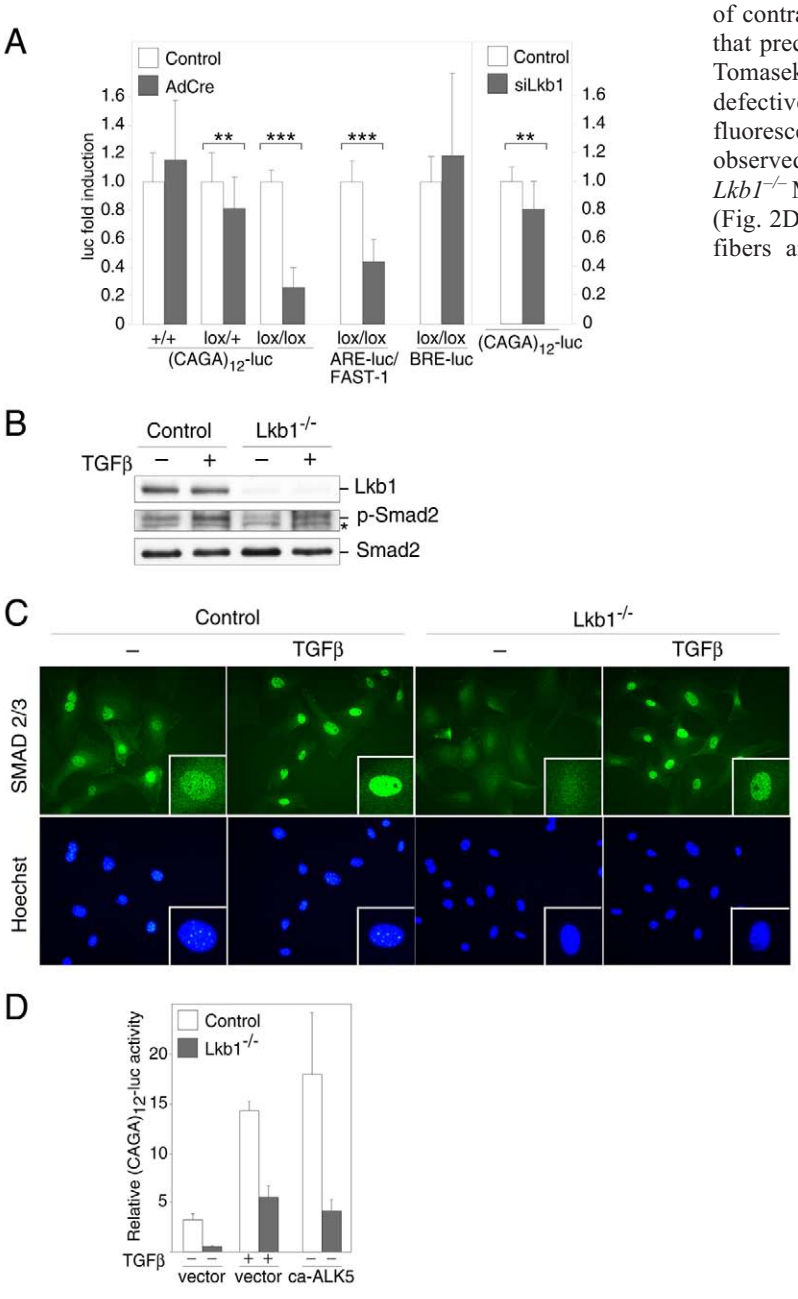

Fig. 1. Lkb1 modulates TGFβ signaling. (A) Fold induction of luciferase activity in MEFs with the indicated *Lkb1* genotypes following infection with AdCre or AdLacZ (control) recombinant adenoviruses and subsequently transfected with either $(CAGA)_{12}$-luciferase, ARE-luciferase or BRE-luciferase plasmids and RL-TK to correct for transfection efficiency. Fold induction is indicated relative to control samples and all bars represent averages of three experiments performed with independently isolated MEFs using triplicate samples, except for the $(CAGA)_{12}$-luc *Lkb1*$^{lox/+}$ and $(CAGA)_{12}$-luc *Lkb1*$^{lox/lox}$ bars, which represent averages from ten and five experiments, respectively. Right panel shows similar analysis with MEFs transfected with non-targeting (control) or *Lkb1*-targeting (siLkb1) siRNAs and $(CAGA)_{12}$-luc. Bars represent averages from two experiments using four to ten samples per experiment. Error bars indicate s.d.; $^{**}P<0.01$, $^{***}P<0.001$. (B) Western blotting analysis of Lkb1, activated Smad2 (Smad2-*P*, shown as p-Smad2) and total Smad2 from serum-starved (0.2% FCS) untreated (–) or TGFβ-treated (+; 1 ng/ml) control (AdLacZ-infected *Lkb1*$^{lox/-}$ MEFs) or *Lkb1*$^{-/-}$ (AdCre infected *Lkb1*$^{lox/-}$ MEFs) MEFs. A background band in the Smad2-*P* blot is indicated with an asterisk. (C) Immunofluorescence staining using an antibody recognizing Smad2 and Smad3 of serum-starved (0% FCS, 22 hours) and subsequently untreated (–) or TGFβ-treated (1 ng/ml) control and *Lkb1*$^{-/-}$ MEFs. The apparently lower total intensity of signal might be due to more diffuse localization of Smad2 and Smad3 in the cytoplasm of *Lkb1*$^{-/-}$ MEFs compared with controls because no difference in total levels were noted in western blotting (see Fig. 1B). Nuclei are visualized with Hoechst. A single-cell magnification is shown in the inset. (D) $(CAGA)_{12}$-luc activity in control and *Lkb1*$^{-/-}$ MEFs transfected with constitutively active ALK5 (ca-ALK5) or vector control and treated with added TGFβ as indicated. The value of relative $(CAGA)_{12}$-luc activity represents the ratio of $(CAGA)_{12}$-luc to *Renilla*-luciferase–thymidine-kinase (RL-TK), with the average from a representative experiment out of three using triplicate samples shown; error bars indicate s.d.

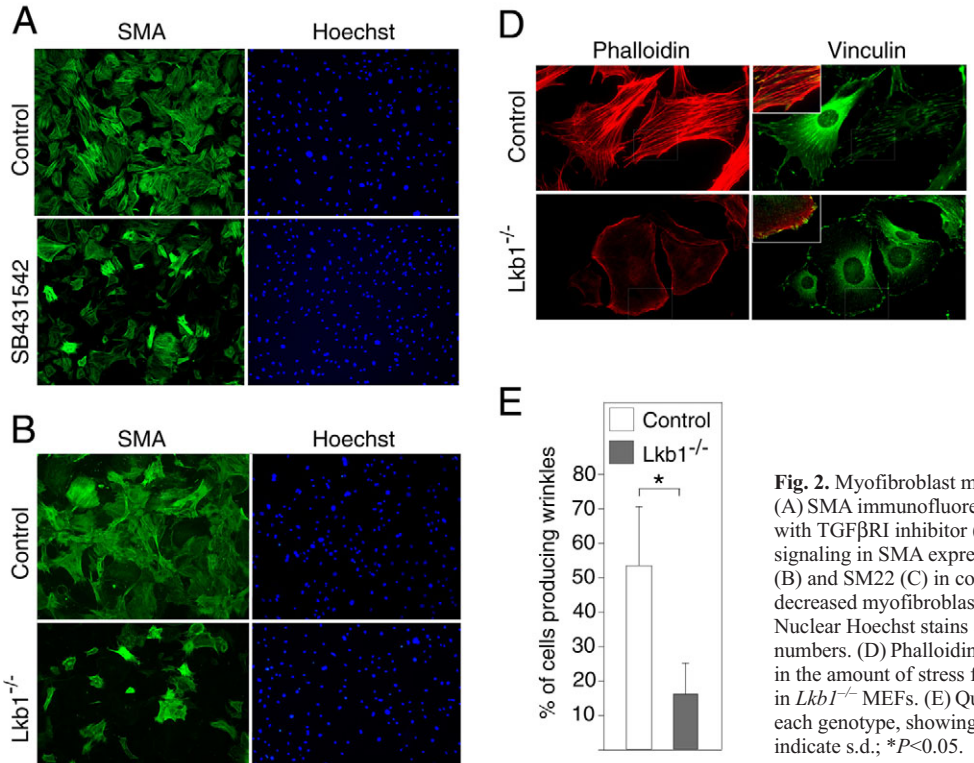

Fig. 2. Myofibroblast marker expression is dependent on *Lkb1*. (A) SMA immunofluorescence staining of MEFs treated for 72 hours with TGFβRI inhibitor (SB431542), indicating a requirement for TGFβ signaling in SMA expression by primary MEFs. (B,C) Staining of SMA (B) and SM22 (C) in control and *Lkb1*$^{-/-}$ MEFs, demonstrating decreased myofibroblast marker expression in the absence of *Lkb1*. Nuclear Hoechst stains are shown to demonstrate comparable cell numbers. (D) Phalloidin and vinculin staining, demonstrating a decrease in the amount of stress fibers and the mislocalization of focal adhesions in *Lkb1*$^{-/-}$ MEFs. (E) Quantitation of the percentage of wrinkling cells in each genotype, showing the average out of three experiments. Error bars indicate s.d.; **P*<0.05.

adhesions were reduced in size and number, as shown by staining for vinculin. Whereas control MEFs exhibited the typical large 'supermature' focal adhesions in myofibroblasts at stress-fiber termini (Dugina et al., 2001; Hinz, 2006), *Lkb1*$^{-/-}$ MEFs exhibited untypical localization of focal adhesions at cell edges (Fig. 2D, vinculin).

A functional hallmark of myofibroblasts is their high contractile activity, which is dependent on SMA expression (Hinz et al., 2001a; Hinz et al., 2002) and focal-adhesion maturation (Dugina et al., 2001; Goffin et al., 2006). To monitor contractile state, control and *Lkb1*$^{-/-}$ MEFs were cultured on deformable silicone substrates of a stiffness of 10 kPa. Upon 12 hours of culture, MEFs were able to generate surface distortions, perceived as 'wrinkles', as previously shown for highly contractile rat lung myofibroblasts (Hinz et al., 2001a; Hinz et al., 2002). Quantification of the percentage of wrinkling cells demonstrated that 53.2% of control MEFs exhibited high contraction, in contrast to only 15.9% of *Lkb1*$^{-/-}$ MEFs (Fig. 2E) (*P*=0.042). Together, these results demonstrate that deletion of *Lkb1* in MEFs leads to the loss of myofibroblast phenotype and of contractile function.

Unaltered focal-adhesion-kinase activity in *Lkb1*$^{-/-}$ MEFs

SMA expression and myofibroblast differentiation are triggered via interdependent signal-transduction pathways involving, for example, focal adhesion kinase (FAK) (Hinz, 2006), RhoA (Hildebrand et al., 1996; Mack et al., 2001; Zhai et al., 2003), serum response factor (SRF) (Du et al., 2003; Miralles et al., 2003; Schratt et al., 2002; Yoshida et al., 2003) and AMPK (Lee et al., 2007), a substrate of Lkb1. Furthermore, recent studies have suggested crosstalk between these pathways and TGFβ signaling (Chen et al., 2006; Edlund et al., 2002; Fan et al., 2007; Lee et al., 2007; Mishra et al., 2008; Qiu et al., 2003; Thannickal et al., 2003).

The dramatic effect of Lkb1 depletion on focal-adhesion morphology and distribution provoked the question: is FAK activation altered in *Lkb1*$^{-/-}$ MEFs? To assess FAK activation upon cell adhesion, control and mutant MEFs were seeded either on adhesive fibronectin-coated plates or on non-adhesive bovine serum albumin (BSA)-coated plates. Analysis of FAK activity 50 minutes after plating demonstrated comparable FAK activity specifically on the fibronectin-coated plates [Fig. 3A, Tyr-*P* (p-Tyr), FN]. Because fibronectin coating did not alter the level of SMA expression in control or *Lkb1*$^{-/-}$ MEFs (data not shown), the results suggest that initial cell adhesion and FAK activation are normal in the absence of Lkb1.

Lkb1$^{-/-}$ MEFs exhibit normal SRF activity

TGFβ signaling has been suggested to promote myofibroblast differentiation via CArG-box-mediated transcription through increasing SRF levels (Jeon et al., 2006; Qiu et al., 2003) and inducing nuclear localization of the SRF cofactor MRTF-A (also known as MKL1 and MAL) (Wang et al., 2002) in a RhoA-dependent manner (Fan et al., 2007; Miralles et al., 2003). To monitor MRTF-A and

SRF activity, a serum-response element (SRE)-luc reporter composed of tandem repeats of the CArG element (Cen et al., 2003; Murai and Treisman, 2002) was transfected into control and *Lkb1*$^{-/-}$ MEFs, followed by a serum-starvation/restimulation experiment. SRE-luc activity was comparable between control and *Lkb1*$^{-/-}$ MEFs under starved conditions (Fig. 3B, 0% FCS), in which both TGFβ signaling and myofibroblast markers are deregulated in *Lkb1*$^{-/-}$ MEFs. Following serum stimulation, induction of SRE-luc was maximal at 3 hours and decreased at 15 hours in both control and mutant MEFs, with the small and opposite differences observed between the controls and mutants at the two time points considered to not be likely to represent meaningful differences (Fig. 3B, 20% FCS). The addition of TGFβ (see Materials and Methods) to the starved sample had only a modest and comparable effect on SRE-luc activity in control and *Lkb1*$^{-/-}$ MEFs at 3 and 15 hours (Fig. 3B, TGFβ). Consistent with unaltered SRE-luc activity, immunofluorescence analysis of MRTF-A indicated comparable subcellular distribution in control and *Lkb1*$^{-/-}$ MEFs (data not shown). Unaltered SRF activity was also supported by the fact that PKB (AKT) activity (as assessed by Ser473 phosphorylation) was similar between control and *Lkb1*$^{-/-}$ MEFs [Fig. 3C, PKB-*P* (p-PKB)], because PKB has been suggested to regulate TGFβ-dependent SMA expression by inducing SRF binding to SREs (Lien et al., 2006). Also, independent evidence for unaltered RhoA activity was provided by the fact that there were comparable levels of moesin that was phosphorylated on Thr558 [Fig. 3C, moesin-*P* (p-Moesin)], a site that is phosphorylated by the RhoA-activated Rho kinase (Matsui et al., 1998; Oshiro et al., 1998). These results suggest that the changes in expression of SMA and SM22 in *Lkb1*$^{-/-}$ MEFs are not mediated through alterations of RhoA-SRF signaling.

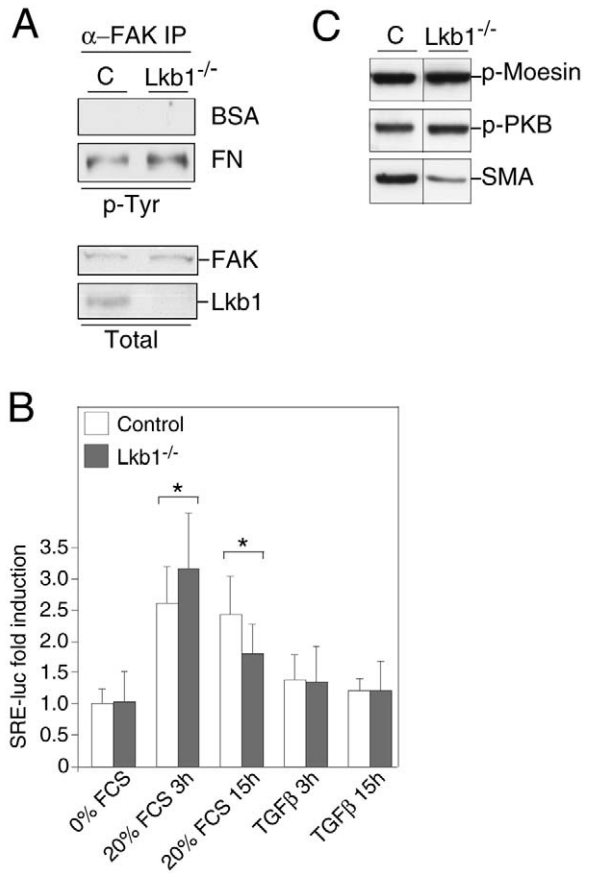

Fig. 3. Unaltered FAK and SRF activity in *Lkb1*$^{-/-}$ MEFs. (A) FAK activity measured by anti-phosphotyrosine (p-Tyr) immunoblot signals from anti-FAK immunoprecipitates (Owen et al., 1999) after 50 minutes of adhesion on adhesive fibronectin (FN) plates or non-adhesive BSA-coated plates. Below are total cell extracts used for immunoprecipitations to indicate similar FAK levels between control (indicated by 'C') and *Lkb*$^{-/-}$ immunoprecipitates. (B) SRE-luc activity in semiconfluent control and *Lkb1*$^{-/-}$ MEFs following a 24-hour serum starvation (0% FCS) or subsequent 3- or 15-hour treatment with serum (20% FCS) or TGFβ. Corrected luciferase values are normalized to the 0% FCS sample of control MEFs. The averages from five experiments using triplicate samples are shown, except for the 15-hour treatments, which represent averages from three experiments. Error bars indicate s.d.; **P*<0.05. (C) Western blotting analysis of control and *Lkb1*$^{-/-}$ MEFs was performed with anti-moesin-*P* (p-Moesin), anti-PKB-*P* (p-PKB) and anti-SMA antibodies.

Unaltered myofibroblast differentiation in *AMPKα1*$^{-/-}$*;AMPKα2*$^{-/-}$ MEFs

The kinase AMPK has been suggested to regulate the contractile machinery in parallel with RhoA via phosphorylation of the regulatory myosin light chain (MLC) (Lee et al., 2007), and AMPK requires Lkb1 phosphorylation for activation (Hawley et al., 2003). To study whether defects in myofibroblast differentiation were due to decreased activation of AMPK after depletion of Lkb1, we stained control and *AMPKα1*$^{-/-}$*;AMPKα2*$^{-/-}$ MEFs (see Materials and Methods) for SMA. *AMPKα1*$^{-/-}$*;AMPKα2*$^{-/-}$ MEFs exhibited no alterations in the levels of SMA or SM22 (supplementary material Fig. S2) in conditions in which *Lkb1*$^{-/-}$ MEFs demonstrated a significant decrease in SMA expression (Fig. 2A). Thus, whereas stimulation of AMPK by exogenously added AMP-mimic AICAR appears to interfere with myofibroblast differentiation (Mishra et al., 2008), lack of AMPK does not significantly alter myofibroblast differentiation in unstimulated conditions, and AMPK does not appear to be involved in the observed loss of differentiation in *Lkb1*$^{-/-}$ MEFs.

Exogenous TGFβ is sufficient to restore SMA and SM22 expression in *Lkb1*$^{-/-}$ MEFs

The ability of the ALK inhibitor SB431542 to downregulate SMA in primary MEFs (Fig. 2A) implied that attenuated TGFβ signaling was responsible for decreased myofibroblast marker expression in *Lkb1*$^{-/-}$ MEFs. To investigate this directly, *Lkb1*$^{-/-}$ MEFs were treated with 1 ng/ml exogenous TGFβ for 45-48 hours. Importantly, the added TGFβ significantly increased the number of SMA-positive *Lkb1*$^{-/-}$ MEFs (Fig. 4A, compare *Lkb1*$^{-/-}$ and *Lkb1*$^{-/-}$+ TGFβ), in addition to significantly increasing total levels of both SMA and SM22 (Fig. 4B) as shown by western blotting analysis (CDK7 levels are shown as a loading control).

TGFβ treatment was also able to rescue the contractile phenotype in *Lkb1*$^{-/-}$ MEFs by inducing the relocalization and maturation of focal adhesions from the cell edge towards the cell center and by increasing the number of central stress fibers (Fig. 4C). Furthermore, the addition of exogenous TGFβ increased the percentage of *Lkb1*$^{-/-}$ MEFs that were capable of deforming 10 kPa silicone substrates from 15.9% to control levels (50.7%; s.d.=15.6%, *P*=0.040). Thus, defective contractility of *Lkb1*-deficient MEFs is mediated by downregulated TGFβ signaling.

Decreased TGFβ-dependent promoter activity of myofibroblast marker genes in *Lkb1*$^{-/-}$ MEFs

TGFβ can regulate myofibroblast differentiation through a variety of different transcriptional and non-transcriptional mechanisms, among which Smad signaling appears to be the most prominent (Gu et al., 2007; Li et al., 2002). To investigate the mechanism in

our model, MEFs were transfected with a SMA-luciferase reporter construct (SMA-luc) (Yoshida et al., 2003) and were treated with the TGFβRI inhibitor SB431542. After a 20-hour inhibition, SMA-luc activity was reduced to 58% of control levels (Fig. 4D, SB431542, $P=4.45\times10^{-5}$). Also, transfection of MEFs with Smad7, an inhibitor of R-Smads, reduced SMA-luc reporter activity (72%, Fig. 4D, Smad7, $P=0.019$), indicating that endogenous Smad signaling contributes on the transcriptional level to SMA expression, and thus to myofibroblast differentiation, in primary MEF cultures.

To address whether the altered levels of SMA and SM22 in *Lkb1*$^{-/-}$ MEFs were associated with alterations in transcription, SMA-luc and SM22-luciferase (SM22-luc) (Yoshida et al., 2003) activities were investigated. *Lkb1*$^{-/-}$ MEFs expressing SMA-luc (Fig. 4D, *Lkb1*$^{-/-}$) or SM22-luc (data not shown) demonstrated a 59% ($P=3.0\times10^{-9}$) and a 45% ($P=3.7\times10^{-5}$) lowered normalized luciferase activity, respectively, compared with controls. A small but significant decrease in SMA-luc activity (by 16%; $P=0.0064$) was noted in heterozygote cells (Fig. 4D, *Lkb1*$^{+/-}$), which is consistent with haploinsufficiency of *Lkb1*. Importantly, TGFβ treatment of *Lkb1*$^{-/-}$ MEFs also increased SMA-luc and SM22-luc activities, which reached 102% and 94% of untreated control MEFs, respectively (Fig. 4E). These results strongly implicate deregulated TGFβ signaling in mediating the downregulation of *SMA* and *SM22* transcription in, and myofibroblast differentiation of, *Lkb1*$^{-/-}$ MEFs.

Discussion

Lkb1 modulates TGFβ signaling

The studies presented here demonstrate that Lkb1 is required for myofibroblast differentiation by modulating TGFβ signaling. This was noted through the observation of reduced Smad2- and Smad3-dependent reporter activities, reduced Smad2-*P* levels, and the inability of exogenous TGFβ or constitutively active ALK5 to fully activate TGFβ signaling in *Lkb1*$^{-/-}$ MEFs. Taken together, the results suggest a modulatory role for Lkb1 downstream of TGFβRI and upstream of TGFβ-dependent transcription, and provide a mechanism for the noted decreased secretion of active TGFβ by *Lkb1*$^{-/-}$ MEFs (Katajisto et al., 2008). Previously, Lkb1 has been reported to indirectly interact with Smad4 through LIP1, a protein that has been identified as a two-hybrid interaction partner of Lkb1 (Smith et al., 2001). However, our study provides genetic evidence for normal Smad4, Smad6 and Smad7 signaling in the absence of Lkb1 based on normal BMP signaling activity (Miyazawa et al., 2002). Thus, other reported interactions (Al-Hakim et al., 2005; Brajenovic et al., 2004; Lizcano et al., 2004) are considered as more probable candidates in mediating the activity of Lkb1 on the TGFβ signaling pathway but, at this point, indirect mechanisms cannot be ruled out.

The involvement of Lkb1 in regulating TGFβ signaling suggest a novel function for Lkb1, which is likely to be independent of the previously reported LKB1-AMPK signaling (Hawley et al., 2003). Considering the multiple functions of Lkb1, it is interesting to note that a small but significant part of the transcriptomic response following loss of *Lkb1* appears to be mediated via TGFβ signaling (K.V., Jianmin Wu and T.P.M., unpublished). Regulation of TGFβ signaling by Lkb1 is not restricted to fibroblasts, because deletion of *Lkb1* in endothelial cells results in decreased Smad activation (as measured by Smad2-*P* and mRNA levels of TGFβ1 and PAI1)

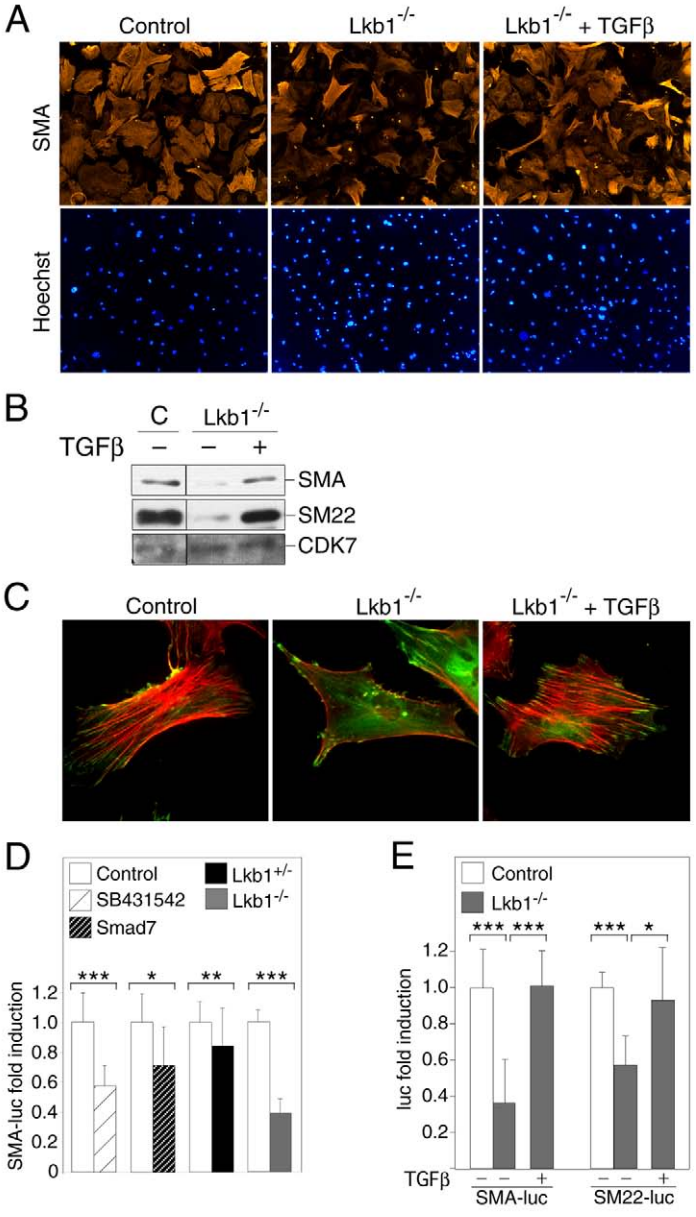

Fig. 4. Exogenous TGFβ rescues the defective myofibroblast differentiation of *Lkb1*$^{-/-}$ MEFs. (A) Immunofluorescence staining for SMA of semiconfluent control and *Lkb1*$^{-/-}$ MEFs following serum starvation (0.2% FCS), as well as subsequent treatment with TGFβ, demonstrating a rescue of SMA staining upon TGFβ treatment. (B) Lysates from non-treated and TGFβ-stimulated control (indicated by 'C') and *Lkb1*$^{-/-}$ MEFs were analyzed by western blotting with antibodies specific for SMA, SM22 or CDK7 (a loading control). Lanes have been reorganized, as indicated by the black bar. (C) Immunofluorescence staining for vinculin (green) and phalloidin (red) of control and *Lkb1*$^{-/-}$ MEFs, demonstrating recovery of mature focal adhesions and stress fibers in TGFβ-treated *Lkb1*$^{-/-}$ MEFs. (D) SMA-luc activity following 20 hours of TGFβRI inhibitor (SB431542) treatment, 48 hours after Smad7 transfection or upon deletion of one (*Lkb1*$^{+/-}$) or both (*Lkb1*$^{-/-}$) alleles of *Lkb1*. DMSO-treated (left), vector-transfected or control-infected samples are shown with white bars. Fold induction is indicated relative to these controls. Bars represent the average out of three experiments using triplicate samples, except for the *Lkb1*$^{+/-}$ and *Lkb1*$^{-/-}$ bars, which represent averages of nine and four experiments, respectively. Error bars indicate s.d.; *$P<0.05$, **$P<0.01$, ***$P<0.001$. (E) SMA-luc and SM22-luc activity in control or *Lkb1*$^{-/-}$ (AdCre) MEFs with added TGFβ as indicated. Fold induction of corrected luciferase values of non-treated and TGFβ-treated *Lkb1*$^{-/-}$ MEFs to non-treated control samples are shown. Bars represent averages from three experiments using triplicate samples. Error bars indicate s.d.; *$P<0.05$, ***$P<0.001$.

and defective embryonal angiogenesis in vivo, which are rescued by exogenous TGFβ ex vivo (Londesborough et al., 2008).

Role of LKB1 in myofibroblast and SMC differentiation

Here, *Lkb1*$^{-/-}$ MEFs lack the characteristics of both the first phase of proto-myofibroblast differentiation, characterized by stress-fiber formation, and further development into the differentiated myofibroblast, characterized by SMA expression and increased contractility. Platelet-derived growth factor (PDGF) has previously been suggested to stimulate early myofibroblast differentiation (Bostrom et al., 1996; Rubbia-Brandt et al., 1991). However, this pathway appears to be unaffected upon deletion of *Lkb1*, as was deduced from the observation of normal SRE activities in *Lkb1*$^{-/-}$ MEFs, and similar growth stimulatory effects of PDGFβ on control and *Lkb1*$^{-/-}$ MEFs (data not shown). In support of these findings, a previous study demonstrated unaltered ERK activity in *Lkb1*-deficient MEFs (Shaw et al., 2004). Instead, the essential role of R-Smads in SMA-luc reporter activity in primary MEFs, the defective Smad2 and Smad3 activation and TGFβ signaling in *Lkb1*$^{-/-}$ MEFs, and the ability of exogenous TGFβ to rescue, in *Lkb1*$^{-/-}$ MEFs, (1) stress-fiber formation, (2) SMA and SM22 expression, (3) reporter activities and (4) force production suggest TGFβ signaling and TGFβ control elements (TCE) (Adam et al., 2000; Hautmann et al., 1997; Liu et al., 2003), and/or Smad-binding elements (SBE) (Hu et al., 2003; Qiu et al., 2005), as the effectors of Lkb1-dependent regulation of myofibroblast differentiation. These results support the previous report arguing sufficiency of TGFβ in inducing both the early and later phases of myofibroblast differentiation (Vaughan, 2000) as opposed to a role for PDGF in the early phases. In this regard, it is interesting to note that *LKB1* overexpression was sufficient to increase expression of both PAI1 and SM22 in HeLa cells (Lin-Marq et al., 2005), indicating that LKB1 expression alone is sufficient to trigger expression of a TGFβ target gene (*PAI1*) and a myofibroblast marker gene (*SM22*) in epithelial cells directly.

Polyps in both *Lkb1*$^{+/-}$ mice (Bardeesy et al., 2002; Jishage et al., 2002; Miyoshi et al., 2002; Rossi et al., 2002) and mice in which *Lkb1* deletion is restricted to *SM22*-expressing cells (Katajisto et al., 2008) contain an increased ratio of myofibroblast-like cells to SMCs in comparison to normal stomach tissue (Katajisto et al., 2008). Enrichment of myofibroblasts might be caused by their increased proliferation or dedifferentiation of SMCs. Primary myofibroblasts, the differentiation of which is controlled by the same elements controlling SMC differentiation (Hautmann et al., 1999), provide a valuable tool for in vitro studies to dissect mechanisms leading to polyp development in vivo. Whereas loss of *Lkb1* and subsequent dedifferentiation of myofibroblasts did not noticeably affect proliferation of MEFs in culture, responses of various SMC-like lineages to dedifferentiation in vivo can include changes also in the proliferation of dedifferentiated cells (Han et al., 2008; Kocher et al., 1991). Interestingly, the observation of haploinsufficiency of *Lkb1* in myofibroblast differentiation as measured by SMA reporter activity (Fig. 4D) resembles the situation in vivo in heterozygote PJS patients and mice, in which haploinsufficiency of *Lkb1* leads to polyposis (Hernan et al., 2004; Jishage et al., 2002; Miyoshi et al., 2002; Rossi et al., 2002). Thus, the role of *Lkb1* in the differentiation of SM22-positive cells that was investigated here suggests that aberrant differentiation of the SM22-positive cell lineage in mice underlies polyposis.

Previously, Lkb1 has been shown to be required for differentiation of neurites in vitro and in vivo (Asada et al., 2007; Barnes et al., 2007; Shelly et al., 2007), and deletion of *Lkb1* has been suggested to influence lineage choice of a common lung-cancer progenitor cell (Ji et al., 2007). By contrast, ablation of *Lkb1* in vivo in keratinocytes (Gurumurthy et al., 2008), liver (Shaw et al., 2005), skeletal muscles (Sakamoto et al., 2005a) or heart (Sakamoto et al., 2005b) did not provide evidence for dedifferentiation, which is in correlation with our observation of normal myoblast differentiation of *Lkb1*$^{-/-}$ MEFs upon adenoviral-mediated MyoD (also known as MyoD1) expression (M.T. and T.P.M., unpublished). Thus, the requirement of Lkb1 for myofibroblast differentiation demonstrates that a highly cell-type-specific function is involved in tumor suppression by Lkb1.

Materials and Methods

Cell culture, adenovirus infections and treatments

Targeted inactivation and genotyping of the murine *Lkb1* gene have been described previously (Bardeesy et al., 2002; Rossi et al., 2002). MEFs were isolated from embryonic day 12.5 (E12.5) embryos and cultured according to the 3T3 protocol (Nilausen and Green, 1965) in high glucose (4.5 g/ml) DMEM (Gibco) supplemented with 10% FCS, glutamine and antibiotics at 37°C, in the presence of 5% CO_2. *Lkb1*$^{+/+}$ (Fig. 1A and Fig. 4D), *Lkb1*$^{lox/+}$ (Fig. 1A and Fig. 4D), *Lkb1*$^{lox/-}$ (Fig. 1B,C; Fig. 2B,C; Fig. 3A,C; Fig. 4B), *Lkb1*$^{lox/lox}$ (Fig. 1A,D; Fig. 2A,D,E; Fig. 3B; Fig. 4A,C-E; supplementary material Fig. S1), *AMPKα1*$^{+/-}$;*α2*$^{lox/+}$ (supplementary material Fig. S2) and *AMPKα1*$^{-/-}$;*α2*$^{lox/-}$ (supplementary material Fig. S2) primary MEFs were infected for 2 hours at 37°C, 5% CO_2 at multiplicity of infection (MOI) of 1500 with adenovirus encoding Cre recombinase (AdCre) (Anton and Graham, 1995) or *lacZ* (AdLacZ) (Badie et al., 1995) at passage 2 (p2), and experiments were done at p4. Cell-culture plates were either uncoated or coated with human fibronectin (BD Biosciences) or BSA (Sigma) as indicated. To stimulate TGFβ signaling, cells were treated with TGFβ1 (human platelet derived, R&D Systems) at a final concentration of 5 ng/ml for 3 hours (Fig. 3B), or 1 ng/ml for 15 (Fig. 3B), 24 (Fig. 1B-D; Fig. 4E), 45-48 (Fig. 4A,B), 60 (Fig. 2E) or 72 (Fig. 4C) hours in the presence of 10% FCS (Fig. 1C,D; Fig. 4B,C,E) or subsequent to serum starvation for 22-26 hours with 0.2% FCS and 0.1% BSA (Fig. 1B; Fig. 4A), or with 0% FCS and 0.1% BSA (Fig. 3B). To inhibit TGFβRI, SB431542 (Sigma) was used for 24 (Fig. 4D) or 72 (Fig. 2A) hours at a concentration of 5 μM. For analysis of S-phase entry, semiconfluent control and *Lkb1*$^{-/-}$ MEFs were labeled for 6 hours with 20 μM BrdU (Sigma).

Quantitative real-time PCR

RNAs of duplicate samples were isolated using the RNeasy isolation kit (Qiagen) according to the manufacturer's protocol. Total RNA was converted using Taqman reverse-transcription reagents (Applied Biosystems). The ABI Prism 7500 was used with Power SYBR Green PCR Master Mix (Applied Biosystems). Relative mRNA amounts were assayed by using 7500 Fast Real-Time PCR System software. Values were first compared with those obtained with GAPDH and then normalized to mRNAs isolated from control cells. The primers used were: TGFβ1 forward, 5′-ACTTTAGGAAGGACCTGGGTTG-3′; TGFβ1 reverse, 5′-GTAGTAGACGA-TGGGCAGTGG-3′; GAPDH forward, 5′-AAGGTCGGAGTCAACGGATT-3′; GAPDH reverse, 5′-TTGATGACAAGCTTCCCGTT-3′.

Western blotting

For western blotting analysis, cells were lyzed with ELB buffer (150 mM NaCl, 50 mM HEPES pH 7.4, 5 mM EDTA, 0.1% NP-40) (Fig. 1B and Fig. 3C), SDS boiling buffer (SB; 2.5% SDS, 0.25 M Trizma base) (Fig. 4A; supplementary material Fig. S2A) or Triton buffer (TB; 150 mM NaCl, 20 mM Trizma base, 1% Triton X-100, 0.05% Tween 20) (Fig. 3A) including 50 mM NaF, 10 mM β-glycerophosphate, 0.5 mM DTT, 0.5 mM PMSF, 2.5 μg/ml Approtinin and 1 μg/ml Leupeptin. Cell concentrations were measured using Bio-Rad protein assay or Bio-Rad DC protein assay (for SB samples) kits (Bio-Rad Laboratories). 10 μg or 40 μg of protein were used per sample and run in SDS-PAGE gel and blotted using the semi-dry transfer method. Filters were blocked with TBS including 0.05% Tween 20 and 5% BSA for phospho-specific antibodies or 5% milk for other antibodies. Filters were probed with the following antibodies: anti-Lkb1 (Abcam), anti-SMA (Sigma), anti-FAK (BD Transduction Laboratories), anti-phospho-tyrosine (4G10, Upstate), anti-actin (Sigma) anti-SM22 (Abcam), anti-phospho-SMAD2 (Ser465/467) (Chemicon International), anti-Smad2/3 (BD Transduction Laboratories), anti-MRTF-A (Santa Cruz), anti-phospho-ERM (Santa Cruz), anti-phospho-Akt (Ser473, New England BioLabs) and anti-AMPK (Cell Signaling). Anti-rabbit-HRP, anti-mouse-HRP and anti-goat-HRP (Chemicon International) were used as secondary antibodies.

Immunoprecipitation

50 minutes after plating on fibronectin- or BSA-coated plates, cells were lyzed with Triton buffer, and 400 μg of the lysates were incubated with anti-FAK (BD Transduction Laboratories) for 1 hour and, subsequently, rabbit anti-mouse (AP160,

Chemicon) was added. After 30 minutes, protein A sepharose (Sigma) was added for 1 hour. Immunoprecipitates were washed three times with the lysis buffer and were subsequently incubated for 10 minutes at 95°C in 1× Laemmli sample buffer (0.2M Trizma base, 1.5% SDS, 10% glycerol, 1 mM EDTA, 0.004% bromophenol blue, 1% β-mercaptoethanol).

Transfection and reporter-gene assay

Transcription of luciferase in the $(CAGA)_{12}$-luc reporter construct is controlled by 12 repeats of the consensus binding site (CAGA) of a complex consisting of Smad3 and Smad4 (Dennler et al., 1998), in ARE-luc by two repeats of ARE that is specific for Smad2- and Fast-1-dependent TGFβ-induced transcription (Yakymovych et al., 2001), in BRE-luc by SBEs from the *ID1* gene that are specific for Smad1-, Smad5- and Smad8-dependent BMP signaling (Korchynskyi and ten Dijke, 2002), in SMA-luc by the promoter fragment (–2560 to +2784) of the *SMA* gene (Yoshida et al., 2003), in SM22-luc by the promoter fragment (–450 to +88) of the *SM22* gene (Yoshida et al., 2003) and in SRE-luc by tandem repeats of SRE (Clontech's Mercury Pathway Profiling System).

Semiconfluent MEFs were transiently transfected on 12-well plates with 1 μg of DNA using Superfect transfection reagent (Qiagen) according to the manufacturer's instructions. $(CAGA)_{12}$-luc, ARE-luc, BRE-luc, SRE-luc, SMA-luc or SM22-luc and *Renilla*-luciferase–thymidine-kinase (RL-TK) plasmid constructs were co-transfected in the ratio of 10:1. When caALK5, Smad7 or vector control was co-transfected with $(CAGA)_{12}$-luc or SMA-luc and RL-TK DNA, a ratio of 10:4:1 was used. For measurements of $(CAGA)_{12}$-luc activity subsequent to RNAi-mediated silencing of Lkb1, siRNA oligos J-044342-5, J-044342-7 and J-044342-8 (Dharmacon) were used with a pooled concentration of 20 nM (siLkb1). The non-targeting pool D-001206-13 was used as control. Cells were plated on 12-well plates at p4, and transfected with siRNAs 1 and 2 days post plating followed by transfection of 1 μg CAGA–firefly-luciferase reporter and 0.6 μg of TK–*Renilla*-luciferase reporter 2 days post plating. Samples were collected 4 days post plating.

Luciferase activity was measured at 24-48 hours after reporter-construct transfection with the Dual luciferase reporter assay system (Promega) according to the manufacturer's recommendations. The activity of firefly luciferase was normalized to that of *Renilla* luciferase from the co-transfected RL-TK plasmid, and fold induction was calculated with respect to the DMSO-treated (Fig. 4D) or control infected/transfected and non-treated samples.

Immunofluorescence and microscopy

Cells were fixed with 3.5% PFA for 20 minutes, permeabilized (0.25% Triton X-100 in PBS) for 10 minutes and blocked (5% normal goat serum in PBS) for 30 minutes. Immunolabeling was performed with the following antibodies: anti-SMAD2/3 (BD Transduction Laboratories), anti-SMA (Sigma), anti-SM22 (Abcam), anti-vinculin (BD Transduction Laboratories) or anti-MRTF-A (Santa Cruz). Anti-mouse-Alexa-Fluor-488 (Molecular Probes), anti-rabbit-Alexa-Fluor-594 (Molecular Probes), anti-rabbit-Alexa-Fluor-488 (Molecular Probes), anti-rabbit-TRITC (Molecular Probes) and anti-goat-TRITC (Molecular Probes) were used as secondary antibodies. Filamentous actin was stained with Alexa-Fluor-594–Phalloidin (Molecular Probes). After washing with PBS, nuclei were stained with Hoechst (0.1 μg/ml in PBS) for 5 minutes, and coverslips were mounted with Mowiol (Calbiochem) or Immu-mount (Thermo Scientific). The stained coverslips were analyzed and photographed using Zeiss Axioplan 2 upright epifluorescence microscope and Axiovision software.

Contractility assay

Untreated and TGFβ-treated (1 ng/ml, 48 hours) MEFs were plated on wrinkling silicone elastomer substrates with a stiffness of 10 kPa (provided by B.H.). At this stiffness, formation of wrinkles in the substrate surface is restricted to highly contractile myofibroblasts (Hinz et al., 2001a; Hinz et al., 2002). Following 12 hours of culture, cells were fixed with 3% PFA and 300 cells/culture were scored for the ability to form wrinkles.

Statistical analysis

Statistical analyses for significance of results were performed by two-tailed Student's *t*-test assuming unequal variance.

We are grateful to Peter ten Dijke for Smad2-*P* antibody, $(CAGA)_{12}$-luc, ARE-luc, BRE-luc and Fast-1 expression vectors; to Aristidis Moustakas for caALK5 and Smad7 expression vectors; to Gary Owens for SMA-luc and SM22-luc reporters; to Yuan Zhu and Luis Parada for AdCre and AdLacZ constructs; to Daniel Rifkin for MLEC cells; to Nabeel Bardeesy and Ronald De Pinho for $Lkb1^{lox/lox}$ mice; and to Benoit Viollet for the $AMPK\alpha1^{-/-}$ and $AMPK\alpha2^{lox/lox}$ mice. Olli Carpen, Jorma Keski-Oja, Katri Koli, Kaisa Lehtinen, Ana-Maria Osiceanu, Minna Pulkki and Olli Ritvos are acknowledged for useful advice, and Katja Helenius, Anou Londesborough and Lina Udd for critical comments on the manuscript. We thank Jenny Bärlund, Outi Kokkonen, Saana Laine and Sari Räsänen for technical assistance and Susanna Räsänen for animal husbandry. Biomedicum Helsinki Molecular Imaging Unit and Biomedicum Virus Core are acknowledged for services. This work was supported by ENFIN (EC FP6 contract number LSHG-CT-2005-518254), Academy of Finland, Biocentrum Helsinki, Finnish Cancer Organization and Sigrid Juselius Foundation. The work of P.-J.W. and B.H. was supported by the Swiss National Science Foundation, grant #3100A0-113733/1. K.V. is a graduate student in Helsinki Graduate School in Biotechnology and Molecular Biology.

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