LKB1 Deficiency Sensitizes Mice to Carcinogen-Induced Tumorigenesis

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Abstract

Lkb1 is a central regulator of cell polarity and energy metabolism through its capacity to activate the AMP-activated protein kinase (AMPK)–related family of protein kinases. Germ line–inactivating mutation of Lkb1 leads to Peutz-Jeghers syndrome, which is characterized by benign hamartomas and a susceptibility to malignant epithelial tumors. Mutations in Lkb1 are also found in sporadic carcinomas, most frequently in lung cancers associated with tobacco carcinogen exposure. The basis for Lkb1-dependent tumor suppression is not defined. Here, we uncover a marked sensitivity of Lkb1 mutant mice to the chemical carcinogen 7,12-dimethylbenz(*a*)anthracene (DMBA). Lkb1$^{+/-}$ mice are highly prone to DMBA-induced squamous cell carcinoma (SCC) of the skin and lung. Confirming a cell autonomous tumor suppressor role of Lkb1, mice with epidermal-specific Lkb1 deletion are also susceptible to DMBA-induced SCC and develop spontaneous SCC with long latency. Restoration of wild-type Lkb1 causes senescence in tumor-derived cell lines, a process that can be partially bypassed by inactivation of the Rb pathway, but not by inactivation of p53 or AMPK. Our data indicate that Lkb1 is a potent suppressor of carcinogen-induced skin and lung cancers and that downstream targets beyond the AMPK-mTOR pathway are likely mediators of Lkb1-dependent tumor suppression. [Cancer Res 2008;68(1):55–63]

Introduction

The Lkb1 tumor suppressor encodes a serine-threonine kinase that is mutated in individuals with the Peutz-Jeghers polyposis and cancer syndrome (1, 2). Peutz-Jeghers syndrome patients develop benign polyps (hamartomas) during adolescence and have a high incidence of gastrointestinal and lung carcinomas as adults (3, 4). Carcinomas in Peutz-Jeghers syndrome patients arise independently of the hamartomas; hence, the mechanisms by which Lkb1 controls benign polyposis and malignancy may be distinct (5). Lkb1 is also mutated in sporadic cancers whose spectrum of tumor types suggests cooperation with exposure to environmental carcinogens. Lkb1 alterations are most prevalent in lung cancers with mutations detected in ~30% of specimens (6–8). Significantly, Lkb1 mutations in lung cancer are highly correlated with a history of tobacco smoking and show a preferential occurrence of GC:TA substitutions, suggesting a mutational effect of polycyclic aromatic hydrocarbon adducts from tobacco carcinogens (8). Other carcinomas exhibiting Lkb1 mutations include head and neck squamous cell carcinoma (SCC) and pancreatic cancer (9), which are also associated with tobacco smoking.

Lkb1 regulates cellular energy metabolism and cell polarity through its capacity to phosphorylate and activate AMP-activated protein kinase (AMPK), as well as other members of the AMPK subfamily (10–14). Activated AMPK restores ATP levels by promoting ATP-producing catabolic processes (e.g., glycolysis) and blocking ATP-consuming biosynthetic processes (including mTOR-directed protein synthesis; ref. 15). Lkb1-mediated regulation of AMPK has tissue-specific effects on glucose metabolism. In skeletal muscle, Lkb1 deletion results in loss of AMPK function and improved glucose uptake (16). Lkb1 knockout in the liver causes inactivation of both AMPK and of the AMPK family member SIK2 and produces metabolic defects, including deregulated gluconeogenesis and lipogenesis (17). LKB1 is also an essential modulator of cellular structure and polarity through activation of AMPK, as well as the microtubule affinity–regulating kinases (MARK1–MARK4) and SAD/Brsk kinases (SAD-A and SAD-B; refs. 18–24). Collectively, these data indicate that energy sensing and cell polarity may be broadly integrated under the control of LKB1-AMPK family signaling.

The specific signaling pathways, by which Lkb1 suppresses both malignant and benign tumorigenesis, and the relative contributions of the AMPK-related family members to these processes are unknown. In this regard, it is provocative that PTEN, TSC, and LKB1, tumor suppressor genes associated with hamartoma and cancer syndromes, are all negative regulators of mTOR activity (25). Hence, the AMPK-mTOR pathway is a plausible mediator of Lkb1 in restraining tumor development. In support of this notion, both Lkb1 mutant hamartomas and lung cancer cell lines show deregulation of mTOR signaling (11, 26), although the biological significance of mTOR activation has yet to be defined in these tumors. At the cellular level, Lkb1 has been implicated in the direct control of cell growth *in vitro* through several mechanisms. Lkb1 overexpression in melanoma and lung cancer cell lines blocks colony formation associated with the induction of the p53-p21 and PTEN pathways, respectively (27, 28). Mouse embryonic fibroblasts (MEF) lacking Lkb1 escape culture-induced senescence, suggesting a potential role in cellular response to oxidative stress (29). Despite their immortal growth, Lkb1$^{-/-}$ MEFs are resistant to transformation by activated H-ras, a property that distinguishes Lkb1-deficient MEFs from those with classic immortalizing lesions in the Rb and p53 pathways.

Genetic models in which Lkb1 inactivation promotes carcinoma formation enable the study of Lkb1 tumor suppressor pathways *in vivo*. Given the association of Lkb1 mutation with carcinogen-associated malignancies and the need for Lkb1 mutant cancer models, we assessed the effect of 7,12-dimethylbenz(*a*)anthracene (DMBA) exposure on Lkb1-deficient mice.

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Materials and Methods

Mouse strains and DMBA treatment. Lkb1$^{+/-}$ and Lkb1$^{L/L}$ strains have been previously described (29). To generate K14-Cre Lkb1$^{L/L}$ and littermate control, K14-Cre/Lkb1$^{L/+}$ males were crossed with Lkb1$^{L/L}$ females. The Lkb1$^{+/-}$ mice and littermates were on an inbred FVB/n genetic background. The K14-Cre Lkb1$^{L/L}$ mice and control animals were ~87.5% FVB/n. For carcinogenesis studies, 5-day-old to 7-day-old mice were treated with a single dose of 50 μL of DMBA (Sigma) in acetone (0.5% w/v) applied directly onto mouse's back.

Antibodies. Keratin-14, Involucrin, keratin-1 (Covance), p63 (Sigma), phosphorylated and total AMPK, phosphorylated acetyl-CoA carboxylase (ACC), phosphorylated and total p70 S6 kinase (S6K), phosphorylated and total S6, phosphorylated and total Erk, phosphorylated and total Akt, phosphorylated Jnk, phosphorylated mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase (MEK), phosphorylated p38, cyclin D1, phosphorylated and total Rb (Cell Signaling), phosphorylated epidermal growth factor receptor (EGFR; Abcam), p16^{Ink4a} (M-156), p21 (C-19; Santa Cruz Biotechnology), p19^{Arf} (Ab-80, Abcam), BrdUrd (BD Transduction Laboratories). Rabbit Lkb1 1K antibody was described previously (29). Secondary antibodies used were Alexa Fluor 488 (Molecular Probes), anti-rabbit and anti-mouse biotin and anti–horseradish peroxidase antibodies (Vector Labs), MOM kit, and ABC Vector kit (Vector Labs).

Immunostaining. Dorsal skin samples were fixed for overnight in 4% paraformaldehyde, then embedded in paraffin or frozen in optimum cutting temperature (OCT). Before freezing in OCT, tissue was shaken in PBS, 10%, 20%, and 30% sucrose for 1 h each. Ten-micron sections were used for all experiments. After immunohistochemical staining, images were taken using Leica DM100 microscope and Leica DC500 camera. After immunofluorescent staining, images were taken with a Leica confocal microscope under a 40× oil immersion objective using the same laser intensity and Z-settings and analyzed with LCS Advanced software. For each marker, at least three independent samples were evaluated for each genotype.

H-ras mutational analysis. H-ras codon 61 status was ascertained by allele-specific PCR analysis. Oligonucleotides H-rasF-wt 5′-GACATCTTAGACACAGCAGGTCA and H-rasR 5′-TGGTGTTGTTGATGGCAAATACT were used to detect the wild-type allele. H-rasF-mut 5′-GACATCTTAGACACAGCAGGTCT and H-rasR were used to detect the CAA→CTA mutation. Sequence analysis of H-ras codons 12, 13, and 61 was performed after amplification of genomic DNA sequences flanking these regions.

SCC cell lines. Cell lines were derived from SCC tumor tissue as described in ref. 30. Briefly, tumor tissue was rinsed in PBS, minced finely with sterile razor blades, incubated in 0.25% trypsin-EDTA at 37°C for 20 min with intermittent tituration, and plated in fibronectin/vitrogen-coated plates in keratinocyte media (Ca^{+}-free EMEM, 8% chelexed serum, 50 ng/mL cholera toxin, 1 μg/mL hydrocortisone, 2 mmol/L glutamine, 0.1–0.3 mmol/L Ca). The AB-B9 cell line, a murine DMBA–12-*O*-tetradecanoylphorbol-13-acetate (TPA)–induced SCC cell line with wild-type Lkb1, was used as a control (31).

Molecular and cellular analysis. RNA and DNA isolation was performed as described using standard procedures. SCC or papilloma tissue was homogenized in lysis buffer [50 mmol/L HEPES, 250 mmol/L NaCl, 2 mmol/L EDTA, 25% glycerol, 1% NP40, 0.1% SDS, protease inhibitors (Roche), and phosphatase inhibitors (Calbiochem)] on ice. SCC cell lines were infected with retroviruses expressing vector, GFP, Lkb1, or Lkb1-KD, selected with puromycin, harvested in lysis buffer at indicated time points, subjected to SDS-PAGE, and immunoblotted with indicated antibody. For cell proliferation assays, the cells were plated in 96-well plate 24 h postinfection and assayed using the WST-1 cell proliferation assay reagent (Roche).

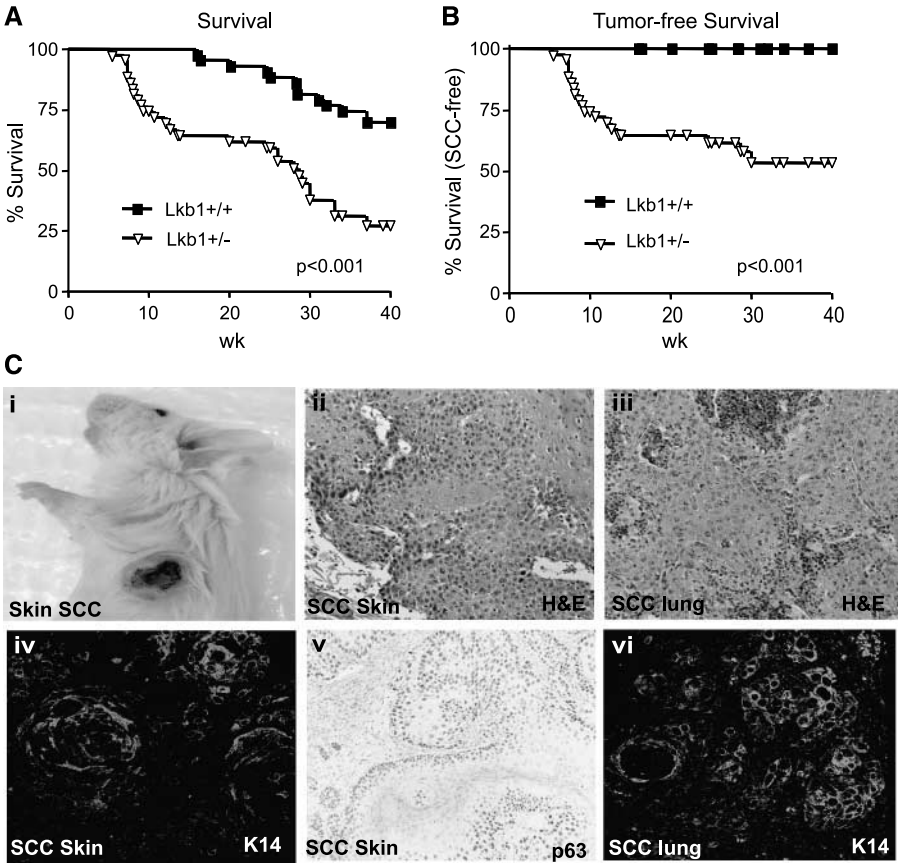
Results

Lkb1$^{+/-}$ mice are highly sensitive to DMBA-induced SCC. We examined the function of Lkb1 in suppression of carcinogen-induced tumors by treating 5-day-old to 7-day-old Lkb1$^{+/+}$ and Lkb1$^{+/-}$ mice (29) with a single topical dose of DMBA. DMBA acts systemically under this protocol, promoting the gradual development of lymphomas, sarcomas, and lung adenomas in wild-type mice (32). The mouse cohorts were monitored for tumor incidence and spectrum up to 40 weeks of age. Lkb1$^{+/-}$ mice showed a significantly reduced cancer-free survival (28.6 weeks versus >40 weeks) and altered tumor spectrum relative to Lkb1$^{+/+}$ animals (Fig. 1*A* and *D*; Lkb1$^{+/-}$ mice developing benign hamartomas were censored in the survival analysis). The increased mortality in the Lkb1$^{+/-}$ cohort was due to the development of invasive skin and lung cancers (present in 18 of 42 Lkb1$^{+/-}$ mice), tumors types that were not observed in wild-type animals (Fig. 1*B, C,i,* and *D*). Histologic analysis and staining for both cytokeratin-14 and p63 revealed that these lung and skin tumors were all malignant SCC (Fig. 1*C,ii–vi*). Lymphomas were observed in both wild-type and mutant cohorts with similar incidence and latency. DMBA exposure did not affect the incidence or histopathology of hamartomas in Lkb1$^{+/-}$ animals (not shown). Hence, Lkb1 heterozygosity specifically sensitizes mice to DMBA-induced SCC.

Lkb1 mutant SCC do not evolve from the classic DMBA-induced papilloma-to-SCC sequence. The molecular progression of DMBA-induced SCC in wild-type mice is well described (33). Specifically, A-T transversions at H-ras codon 61 (CAA→CTA) are a hallmark of DMBA-induced skin carcinogenesis, serving to initiate the development of benign papillomas that undergo gradual multistage progression to malignant SCC (33, 34). Mutations of various tumor suppressor genes can increase papilloma number or accelerate papilloma-to-SCC progression. Notably, papillomas were not observed before SCC development in serially monitored DMBA-treated Lkb1$^{+/-}$ mice. Furthermore, we did not detect papillomatous changes adjacent to carcinoma in our histologic analysis. Finally, the incidence of papillomas was comparable in the wild-type and mutant cohorts (4 of 42 Lkb1$^{+/+}$ mice and 3 of 42 Lkb1$^{+/-}$ mice developed papillomas).

We used allele-specific PCR and direct sequencing to test the mutational status of H-ras in the papillomas and SCC arising in our study. All papillomas had activating H-ras mutations, regardless of Lkb1 genotype (four of four papillomas from Lkb1$^{+/+}$ mice and three of three from Lkb1$^{+/-}$ mice; Fig. 2*C* and data not shown). On the other hand, none of the SCC arising in the Lkb1$^{+/-}$ mice (0 of 17 SCC tested) exhibited H-ras mutations (Fig. 2*A* and data not shown), a finding consistent with the lack of an observed papilloma-SCC sequence in these mice. Together, these results suggest that SCC associated with Lkb1 loss may involve pathways distinct from those in H-ras–induced SCC. Alternatively, Lkb1 loss could lead to activation of H-ras or its effectors obviating the need for concurrent H-ras mutations (see below).

The wild-type Lkb1 allele is inactivated in SCC from Lkb1$^{+/-}$ mice. The benign hamartomas arising in Lkb1$^{+/-}$ mice and Peutz-Jeghers syndrome patients seem to be driven primarily by Lkb1 haploinsufficiency since loss of wild-type Lkb1 expression is not an obligate event in these tumors (29, 35–37). We sought to assess the status of the wild-type Lkb1 allele in the malignant SCC arising in Lkb1$^{+/-}$ mice. Southern blot analysis of tumor DNA revealed loss of wild-type Lkb1 in 8 of 20 specimens (Fig. 2*B* and data not shown). Furthermore, Western and Northern blot analyses showed that all SCC and SCC-derived cell lines lacked Lkb1 expression regardless of the status of the wild-type allele (Fig. 2*C* and *D*), suggesting that the Lkb1 wild-type allele is inactivated by multiple mechanisms in SCC, including deletion and possibly point mutation or promoter hypermethylation. In comparison, robust Lkb1 expression was



D

Tumors in DMBA-treated mice.

Genotype	total mice	SCC-S	SCC-L	Lym	Lung Ad	Sarc	Pap	Ham
$Lkb1^{+/+}$	43	0	0	9	3	1	4	0
$Lkb1^{+/-}$	43	18	3	6	2	1	3	10

SCC-S, squamous cell carcinoma of the skin; SCC-L, squamous cell carcinoma of the lung; Lym, lymphoma; Lung Ad, lung adenoma; Sarc, soft tissue sarcoma; Pap, papilloma; Ham, hamartoma.

Figure 1. $Lkb1^{+/-}$ mice are highly prone to DMBA-induced SCCs. *A,* survival analysis of $Lkb1^{+/-}$ and $Lkb1^{+/+}$ mice treated with DMBA at ages 5 to 7 d. $Lkb1^{+/-}$ mice developing hamartomas were censored from the survival analysis. *B,* survival analysis of the DMBA-treated cohorts documenting mortality due to SCC. *C, i,* gross image of SCC in an $Lkb1^{+/-}$ mouse 15 wk post-DMBA application; *ii,* histology of cutaneous SCC; and *iii,* lung SCC. Immunostaining of SCC for keratin-14 (*iv*) and p63 (*v*). *vi,* immunofluorescence staining for keratin-14 in lung SCC.

detected in papillomas from both $Lkb1^{+/-}$ and $Lkb1^{+/+}$ mice, in SCC cell lines generated from DMBA-TPA–treated wild-type mice (31), and in epidermal keratinocytes, the normal cellular counterparts to SCC (Figs. 2*C* and 3*B*). Hence, inactivation of the wild-type Lkb1 allele is specifically associated with SCC pathogenesis in $Lkb1^{+/-}$ mice.

Selective inactivation of Lkb1 in the epidermis sensitizes mice to carcinogen-induced and spontaneous SCC. Cutaneous SCC arises from the transformation of epidermal progenitors (38). We sought to examine Lkb1 function in the epidermis and to determine whether homozygous inactivation in this compartment is sufficient to promote SCC development. To this end, we generated mice with selective epidermal deletion of Lkb1 by crossing the $Lkb1^{L/L}$ and keratin-14–Cre strains (29, 39). Keratin-14–Cre $Lkb1^{L/L}$ mice (hereafter, designated K14:$Lkb1^{-}$) were born at the expected frequency, but were smaller than $Lkb1^{lox/lox}$ and K14-Cre $Lkb1^{+/+}$ controls. The K14:$Lkb1^{-}$ animals exhibited delays in hair growth and had wavy and less dense hair as adults (Fig. 3*A*). Northern blot analysis of primary keratinocytes confirmed Lkb1 was specifically inactivated in the epidermis of these mice (Fig. 3*A, bottom*). Cutaneous histology revealed a diminution in hair shaft diameter, increased erythema of the skin (reddening associated with congestion of the capillaries), and mild follicular plugging (filling of follicular openings with keratinous debris; Fig. 3*B,i*). In addition, these animals had corneal opacity associated with hyperkeratinization of the corneal epithelium (data not shown). Immunofluorescence analysis of K14, K1, and involucrin revealed comparable staining in the epidermis of K14:$Lkb1^{-}$ and control mice, indicating that epidermal differentiation is not compromised in the absence of Lkb1 (Fig. 3*B,ii–iv*). *BrdUrd* analysis of the epidermal compartment showed similar rates of proliferation between 3-week-old control and K14:$Lkb1^{-}$ animals (the frequency of BrdUrd + nuclei/field was 8 $\pm$ 1.7 and 7.5 $\pm$ 1.7, respectively), indicating that Lkb1 does not influence keratinocyte

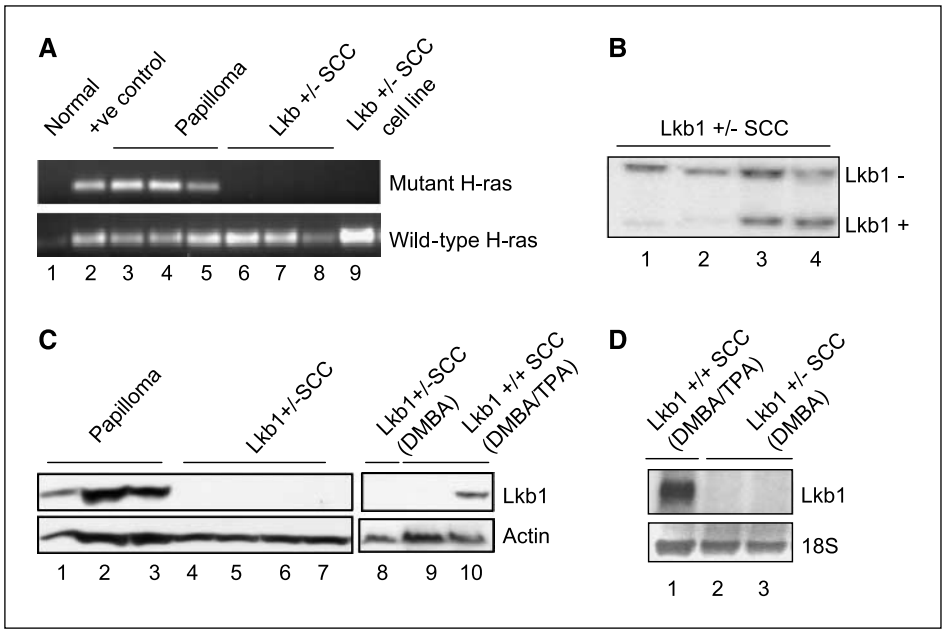


Figure 2. Genetic analysis of SCC in Lkb1$^{+/-}$ mice. *A,* allele-specific PCR to detect H-ras codon 61 wild-type (*bottom*) and CAA→CTA mutant alleles (*top*). Lkb1-deficient SCC have only wild-type H-ras (*lanes 6–9*), whereas papillomas (*lanes 3–5*) and SCC from mice with wild-type Lkb1 (*lane 2*) harbor the mutant H-ras allele. *B,* Southern blot analysis showing loss of wild-type Lkb1 in a subset of SCC from Lkb1$^{+/-}$ mice (note loss of the Lkb1+ band in lanes 1 and 2). *C,* Western blot showing absence of Lkb1 expression in SCC from Lkb1$^{+/-}$ mice (*lanes 4–7*) and cell lines derived from SCC from Lkb1$^{+/-}$ (*lanes 8* and *9*). Lkb1 expression is readily detected in papillomas (*lanes 1–3*) and in an SCC cell line derived from a DMBA/TPA-treated wild-type mouse (*lane 8*). *D,* Northern blot analysis showing Lkb1 mRNA expression in an SCC cell line from a wild-type mouse (*lane 1*) and absence of Lkb1 expression in SCC from Lkb1$^{+/-}$ mice (*lanes 2 and 3*; the tumor in lane 3 showed retention of the wild-type Lkb1 allele at the DNA level). 18S rRNA is shown as a loading control.

proliferation in untreated mice (Fig. 3*B,v*). Similarly, histologic analysis failed to reveal significant alterations in epidermal cell death or defects in epidermal polarity in the K14:Lkb1^{-} animals. Despite their grossly normal epidermal development and homeostasis, a subset of K14:Lkb1^{-} mice developed spontaneous SCC by the age of 40 weeks (3 of 20 mice). The relatively long latency and absence of an early hyperproliferative phenotype in these mice suggest that Lkb1 functions as a tumor suppressor in the epidermis but that the development of SCC requires additional oncogenic changes.

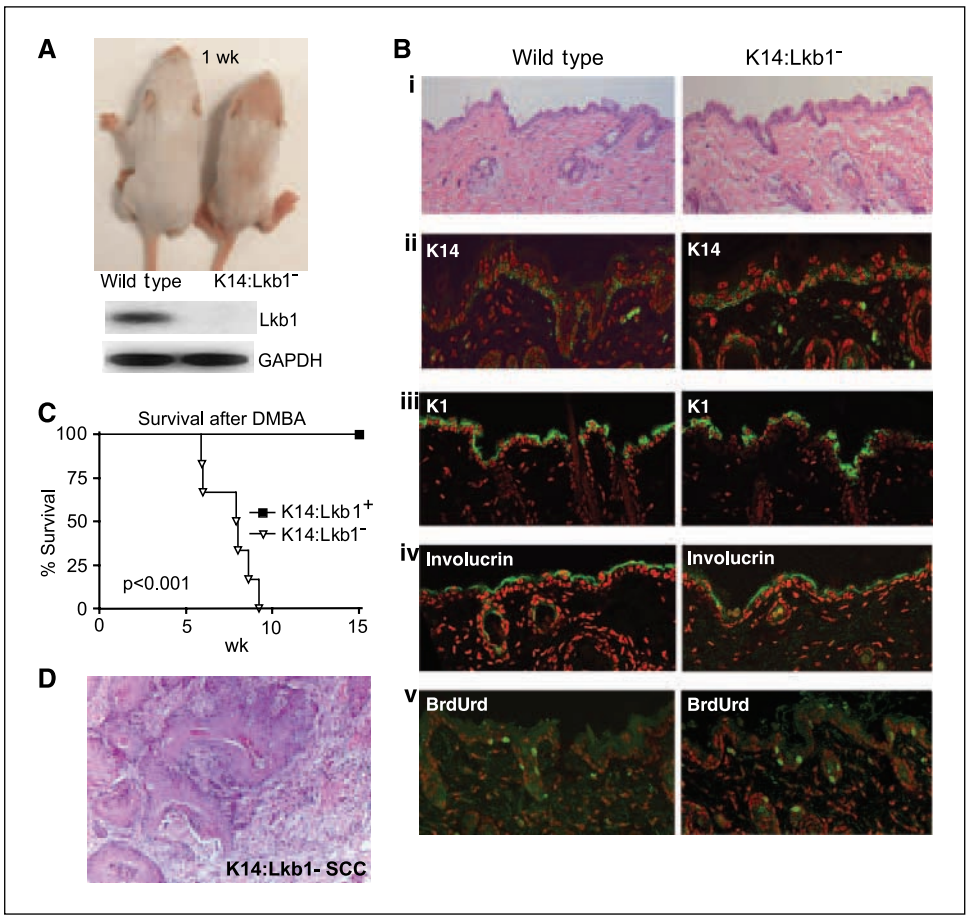


Figure 3. Analysis of mice with epidermal specific deletion of Lkb1. *A,* gross image of K14:Lkb1^{-} and litter mate control mice at 1 wk (*top*) and Northern blot showing Lkb1 expression for keratinocytes from K14:Lkb1^{-} and wild-type mice (*bottom*); *B,* histologic analysis of K14:Lkb1^{-} and litter mate control dorsal skin (*i*). Epidermises from K14:Lkb1^{-} and wild-type mice show comparable staining for the differentiation markers: *ii,* keratin-14; *iii,* keratin-1; *iv,* involucrin; *v,* comparable BrdUrd staining. *Green,* immunofluorescence; *red,* 4′,6-diamidino-2-phenylindole. *C,* survival analysis of DMBA-treated cohorts documenting mortality due to SCC in K14:Lkb1^{-} mice and littermate control mice; *D,* histology of SCC from a DMBA-treated K14:Lkb1^{-} mouse.

In contrast to the long latency in spontaneous tumor development, the K14:Lkb1^{-} mice were highly tumor-prone after DMBA administration. Invasive skin cancers were observed in six of six DMBA-treated K14:Lkb1^{-} mice (average latency, 8 weeks) whereas 0 of 10 DMBA-treated control animals developed tumors by 15 weeks (Fig. 3*C*). Histologic analysis confirmed that these tumors were SCC resembling those observed in the DMBA-treated Lkb1$^{+/-}$ mice (Fig. 3*D*). Hence, homozygous deletion of Lkb1 in the epidermis renders mice highly sensitive to SCC initiated by a chemical carcinogen.

Signaling pathways in Lkb1 mutant SCC. Having shown a critical role for Lkb1 in suppression of DMBA-induced SCC, we wished to define molecular alterations associated with tumorigenesis in Lkb1 mutant mice. RAF pull-down assays showed elevated levels of activated Ras-GTP in Lkb1 mutant SCC relative to papillomas and normal skin despite the absence of H-ras mutations in these tumors (Fig. 4*A*). Previous genetic studies have shown that RAF-MEK-ERK–cyclin D1 and EGFR–phosphoinositide 3-kinase (PI3K)–AKT pathways are critical effectors of Ras-directed skin carcinogenesis and that the activity of these pathways increases gradually during papilloma SCC progression (40–43). Western blot analysis showed that five of six Lkb1 mutant SCC tested expressed p-ERK, whereas p-ERK was absent in all six papillomas tested (Fig. 4*B* and data not shown). Cyclin D1 and p-c-Jun levels were elevated in all SCC relative to papillomas. Finally, p-EGFR was detectable in all SCC, and robust p-AKT levels were noted in four of six of these tumors (Fig. 4*B* and data not shown). Together, these results indicate that although no activating H-Ras mutations are present in the Lkb1 mutant SCC, Ras signaling pathways are deregulated in these tumors.

The AMPK-TSC-mTOR pathway is a candidate mediator of Lkb1-dependent tumor suppression. Western blot analysis showed that Lkb1 mutant SCC (n = 6 SCC) had diminished ACC Ser^{79} phosphorylation relative to papillomas (n = 6 papillomas), consistent with loss of AMPK activity in these tumors (Fig. 4*C* and data not shown). Correspondingly, p-S6K Thr^{421}/Ser^{424} levels were increased in the Lkb1 mutant SCCs, whereas there was heterogeneous expression of p-S6 Ser$^{435/436}$. Hence, AMPK activity is compromised in Lkb1 mutant SCC, although the level of mTOR signaling was not markedly increased in these tumors relative to papillomas.

Restoration of Lkb1 in SCC cell lines results in growth arrest that cannot be rescued by disruption of AMPK signaling. The Lkb1 mutant SCC cell lines that we established from DMBA-treated mice provided a system to address the mechanisms of Lkb1-dependent tumor suppression. SCC cell lines generated from DMBA/TPA-treated wild-type mice served as controls for these studies (31). Human cancer genetics studies suggest that Lkb1 kinase activity is critical for tumor suppression because most cancer-associated Lkb1 mutations result in impaired catalytic activity (9). Correspondingly, introduction of retroviruses expressing wild-type Lkb1, but not a kinase-dead mutant, resulted in rapid induction of growth arrest in these cell lines (Fig. 5*A, top*). This phenotype was specific because Lkb1 overexpression did not affect the growth characteristics of SCC cell lines harboring wild-type Lkb1 (Fig. 5*A, bottom*).

Next, we assessed the role of the AMPK pathway in Lkb1-directed growth arrest. Wild-type Lkb1 specifically activated AMPK in SCC cells, as reflected by increased phosphorylation of AMPKα Thr^{172} and of the AMPK target ACC Ser^{79} (Fig. 5*B*). Consistent with AMPK activation, mTOR activity was repressed by wild-type Lkb1

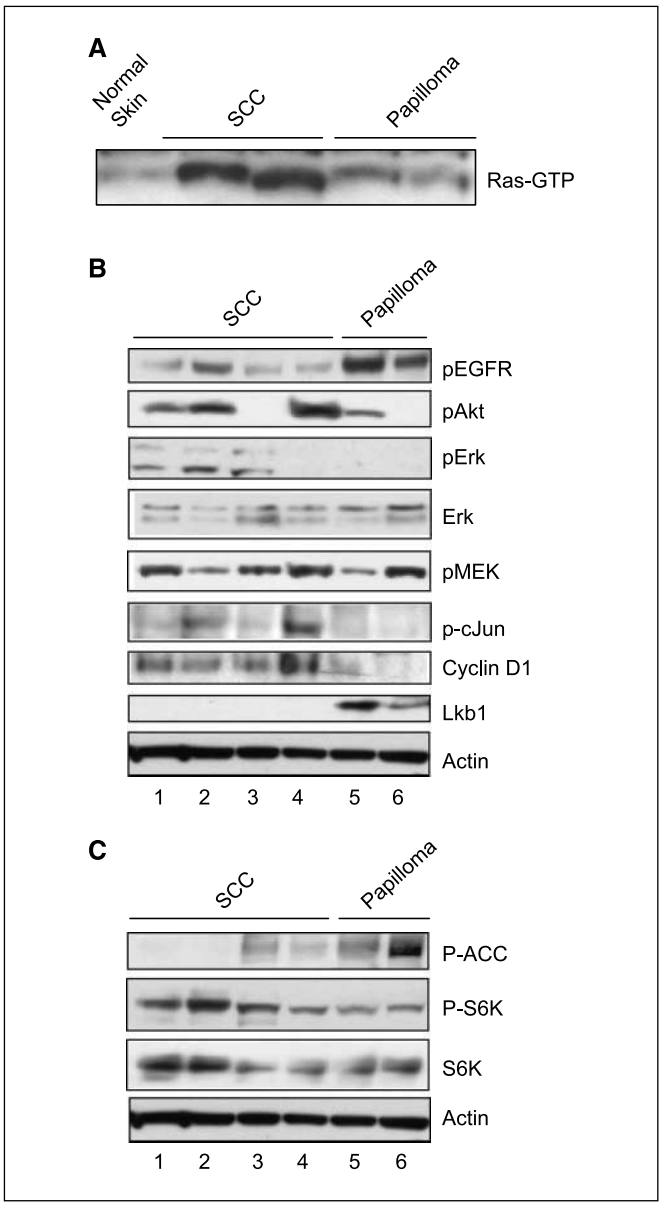


Figure 4. Signaling pathways in Lkb1 mutant SCC. *A,* RAF pull-down assay showing levels of activated Ras-GTP in normal skin (*lane 1*), SCC (*lanes 2 and 3*), and papillomas (*lanes 4 and 5*). *B,* Western blot analysis of ERK and PI3K pathway components in papillomas (*lanes 5 and 6*) and Lkb1-mutant SCC (*lanes 1–4*). *C,* Western blot analysis of the AMPK/mTOR signaling pathway in papillomas (*lanes 5 and 6*) and SCC (*lanes 1–4*). All SCCs were derived from Lkb1$^{+/-}$ mice.

as shown by a reduction in levels of p-S6K. P-S6 levels were also reduced 24 to 48 h after Lkb1 restoration; however, this effect was transient, and elevations in p-S6 were noted at later time points (>96 h).

To test the requirement of AMPK in growth arrest, we introduced adenoviruses expressing dominant-negative AMPK into the SCC cells and assessed whether Lkb1 arrest was abrogated. DN-AMPK effectively inactivated AMPK signaling because it blocked Lkb1-induced phosphorylation of AMPK and of the AMPK target ACC. Correspondingly, DN-AMPK rescued the Lkb1-mediated down-regulation of p-S6K and p-S6 seen at early time points. On the other hand, despite this disruption in AMPK signaling,

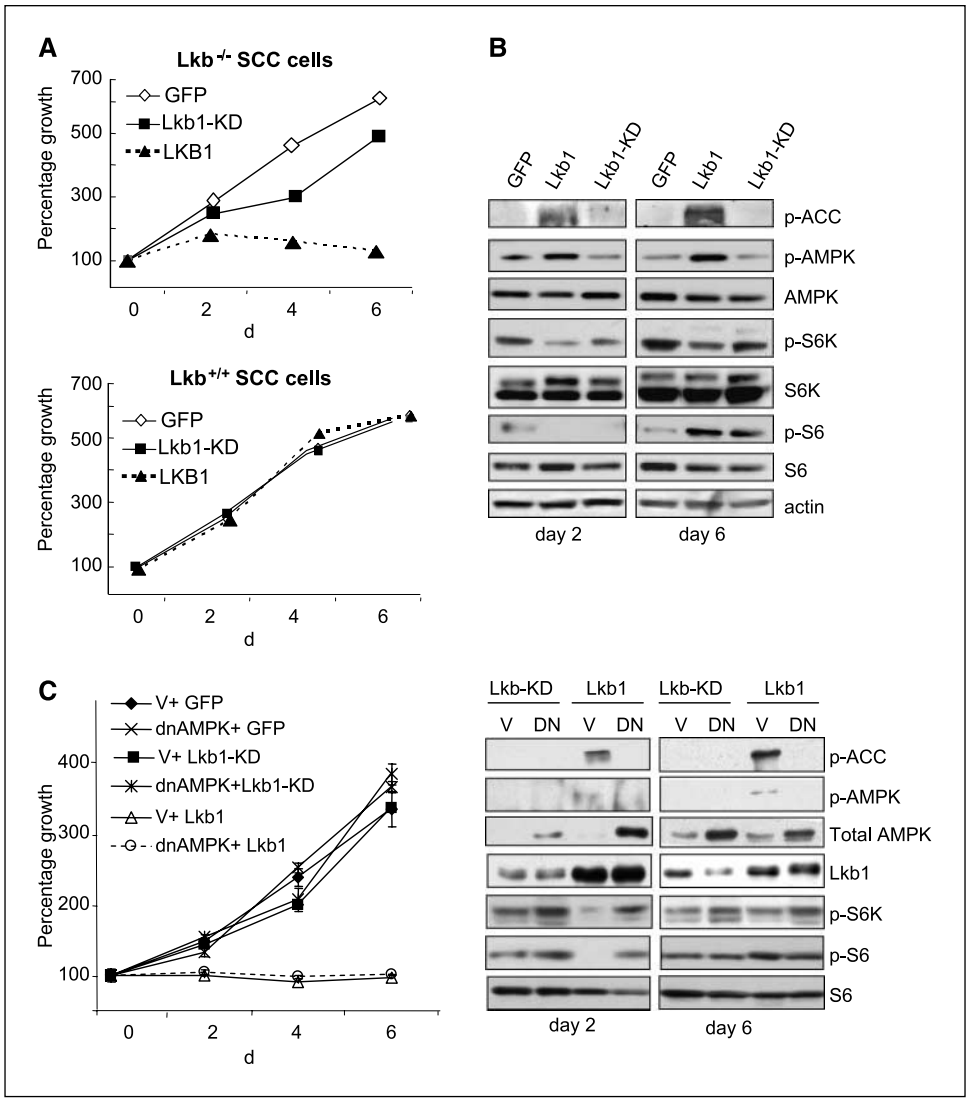


Figure 5. Lkb1 restoration provokes AMPK-independent growth arrest in Lkb1-mutant SCC cells. *A,* growth curves of Lkb1$^{-/-}$ (*top*) and Lkb1$^{+/+}$ (*bottom*) SCC cells after transduction with retroviruses expressing GFP, Lkb1, or a kinase-dead Lkb1 mutant. *B,* Western blot analysis of AMPK-mTOR pathway at 2 d (*left*) and 6 d (*right*) after retroviral transduction. Note that Lkb1 provokes sustained p-AMPK and p-ACC expression and that p-S6K levels remain repressed whereas p-S6 reduction is only transiently observed. *C, left,* growth curves of SCC cells infected with adenoviruses expressing dominant-negative AMPK or GFP and subsequently transduced with retroviruses encoding wild-type Lkb1 or Lkb-KD mutant. *Right,* Western blot analysis showing that DN-AMPK blocks Lkb1-mediated activation of AMPK, as measured by p-AMPK and p-ACC, and repression of mTOR (p-S6K) at 2 and 6 d postretroviral transduction.

DN-AMPK did not rescue the growth arrest phenotype (Fig. 5*C, left and right*). Along these lines, the pharmacologic AMPK inhibitor compound C was unable to rescue Lkb1-mediated growth arrest (data not shown). These results indicate that AMPK-mTOR signaling is not required for Lkb1-induced growth inhibition of SCC cell lines and, therefore, may not be critical for tumor suppression downstream of Lkb1.

Lkb1-mediated growth arrest shows features of oncogene-induced senescence. Oncogene-induced senescence refers to a specific type of growth arrest that occurs in primary cells in response to strong oncogenic signals and, therefore, serves as a barrier to tumor progression (44). We noted that Lkb1-arrested SCC cells became enlarged, took on a flattened appearance, were frequently binucleated, and stained for senescence-associated β-galactosidase, indicating that wild-type Lkb1 restored a senescence response in these cancer cells (Fig. 6*A*).

Oncogene-induced senescence is associated with feedback signals that inactivate the RAF–MAP kinase (MAPK) and PI3K-AKT pathways, with the induction of p38-mediated oxidative stress responses (45, 46). Western blot analysis showed that Lkb1-mediated growth arrest did not require inactivation of PI3K signaling because p-AKT levels were elevated after Lkb1 expression (Fig. 6*B*), a finding consistent with reduced mTOR/S6K signaling and a resulting loss of feedback inhibition insulin-like growth factor/PI3K signaling (47). Lkb1 expression led to an acute and sustained decrease in p-MEK, whereas p-ERK levels were only transiently decreased, suggesting that Lkb1 represses the RAF-MAPK pathway at a level of upstream of p-ERK (Fig. 6*B*). Finally, we found that Lkb1 restoration resulted in the pronounced activation of p38, indicating that Lkb1 restoration provokes an acute stress response in these cells.

Role of the Rb and p53 pathways in Lkb1-induced growth arrest. Intact Rb and p53 pathway function are broadly required for senescence responses. Lkb1 restoration did not lead to increased expression of p53 or p21, a p53 target gene indicating that arrest provoked by Lkb1 was likely to be p53-independent (Fig. 6*C, top*) and data not shown. In contrast, the wild-type Lkb1-expressing SCC cells showed increased levels of the hypophosphorylated, activated form of Rb and decreases in hyperphosphorylated, inactivated Rb (Fig. 6*C, bottom*). Rb phosphorylation is controlled by cyclin/cyclin-dependent kinase (CDK) complexes. Both wild-type and kinase-dead Lkb1 induced expression of the

CDK4/CDK6 inhibitor $p16^{Ink4a}$, whereas wild-type Lkb1-expressing cells showed a specific reduction in cyclin D1 levels at both early and late time points (Fig. 6*C* and data not shown). These data indicate that Lkb1-induced growth arrest is associated with activation of Rb and a corresponding altered balance of negative and positive regulators of the Rb checkpoint.

We sought to directly test the requirement for the Rb and p53 pathways in Lkb1-mediated growth arrest of SCC cells by expression of viral oncogenes that inactivate these pathways. Before introduction of Lkb1 retroviruses, the SCC cells were transduced with retroviruses encoding the human papilloma virus proteins E6 (to inactivate p53), E7 (to inactivate Rb), or both E6 and E7. The expression of E6 had no effect on Lkb1-induced arrest (Fig. 6*E, top*), whereas expression of either E7 alone or E6 and E7 led to a partial rescue of this growth arrest phenotype (Fig. 6*E, bottom* and data not shown). These results suggest that Lkb1 suppression of SCC proliferation involves the Rb function, but not p53 function, and that additional pathways are required to mediate Lkb1 activity.

Discussion

In this study, we describe the development of genetic models that show an important role of Lkb1 in suppression of carcinogen-induced tumorigenesis. Mice with germ line heterozygous mutations of Lkb1, or with selective deletion of Lkb1 in the epidermis, were highly prone to the development of DMBA-induced SCC. Restoration of wild-type Lkb1 in tumor-derived SCC cell lines resulted in a senescence-like growth arrest that involved Rb function but was independent of the p53 and AMPK pathways. This genetic model and associated cell lines provide a framework to elucidate the mechanisms of Lkb1-dependent suppression of

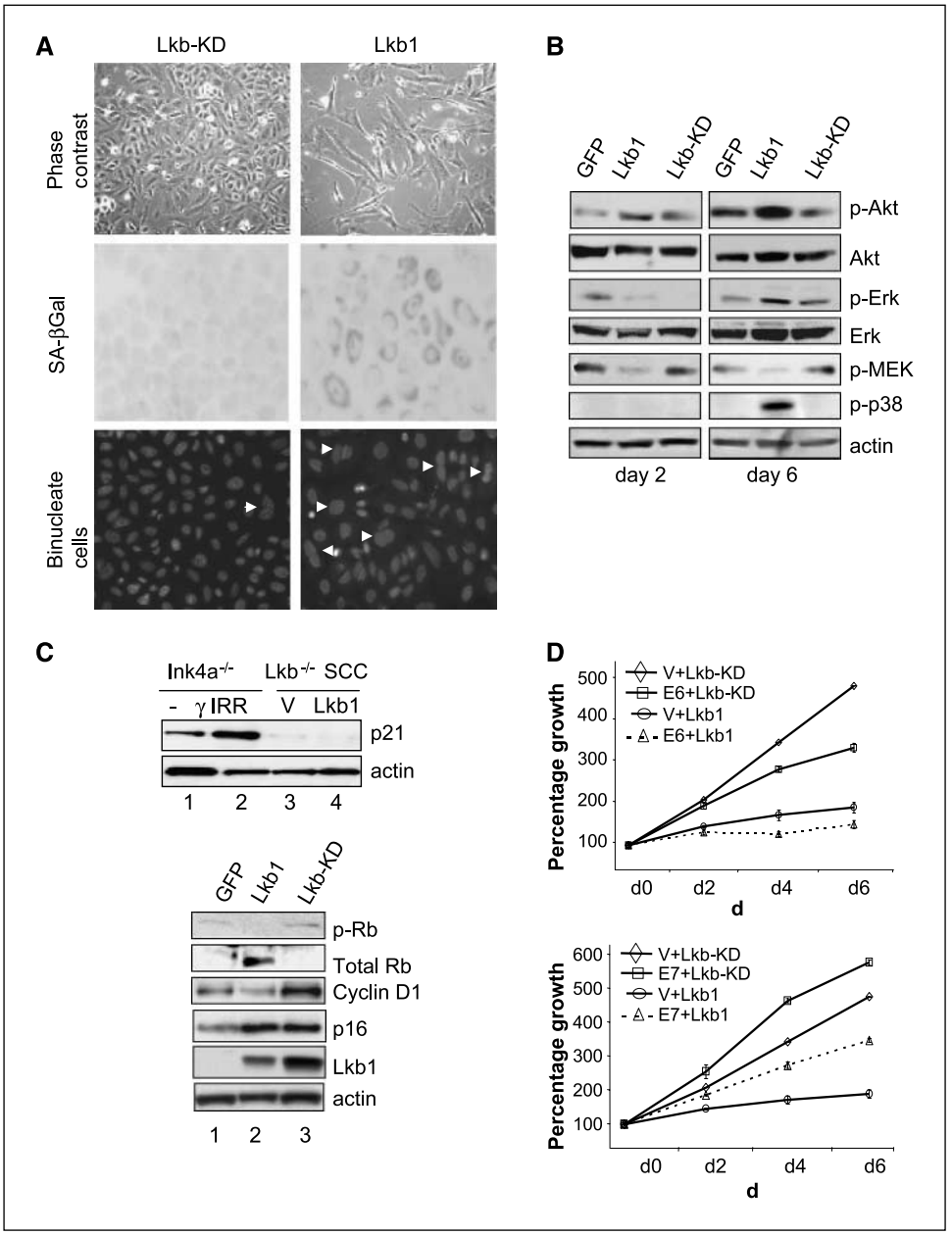


Figure 6. Analysis of Lkb1-dependent growth arrest in SCC cells. *A,* wild-type Lkb1 induces senescence in Lkb1 mutant SCC cells, as reflected by the enlarged, flattened cells (*top*), senescence-associated β-gal staining (*middle*), and binucleated cells (*bottom*). *B,* Western blot analysis of SCC cells at days 2 and 6 postinfection with the indicated retroviruses. Lkb1 expression is associated with decreased p-MEK and increased p-Akt, p-Erk, and p-p38. *C, top,* Western blot at the day 6 time point showing that Lkb1 does not induce p21 in SCC cells compared with empty vector (*lanes 3 and 4*). Untreated and γ-irradiated fibroblasts (*lanes 1 and 2*) were used as controls for p21 induction. *Bottom,* the Rb pathway is activated by Lkb1 (*lane 2*) as reflected by loss of p-Rb-S795, an increase in hypophosphorylated Rb, and a decrease in cyclin D1 relative to GFP or Lkb1-KD (*lanes 1 and 3*). *D,* growth curves showing that expression of E6 does not effect Lkb1-mediated growth inhibition (*top*), whereas E7 partially rescued this phenotype (*bottom*). Error bars are depicted but may be too small to see.

epithelial cancers and may uncover specific roles for Lkb1 in response to environmental carcinogens.

We observed that the wild-type Lkb1 allele was inactivated in all DMBA-induced SCC from $Lkb1^{+/-}$ mice and that absence of Lkb1 in the epidermis led to greatly accelerated SCC progression (SCC latency was 15 weeks in $Lkb1^{+/-}$ mice and 7 weeks in K14:$Lkb1^{-}$ mice). Regardless of allelic loss, all SCC lacked expression of Lkb1. Hence, in contrast to the benign gastrointestinal polyposis associated with Lkb1 deficiency, malignant SCC pathogenesis seems to require biallelic inactivation of Lkb1.

The AMPK-TSC-mTOR pathway has been a prime candidate for mediating tumor suppression downstream of the Lkb1 kinase (48). Consistent with this, deregulation of this pathway was observed in Lkb1 mutant SCCs *in vivo* and in derivative cell lines. However, the inactivation of AMPK using pharmacologic inhibitors or expression of dominant-negative AMPK mutants had no effect on Lkb1-induced growth arrest in SCC cell lines. The results indicate that in Lkb1-deficient cancers, pathways other than AMPK-TSC-mTOR are likely to be the critical downstream effectors of Lkb1. It remains possible that deregulation of this pathway may be important for the initiating stages of Lkb1 mutant tumors and dispensable in the later stages of cancer progression.

Our demonstration that Lkb1 restoration causes senescence in SCC cell lines is notable in light of our previous observation that Lkb1 forms a barrier to passage-induced senescence in primary MEFs. Senescence in primary cells arises due to the generation of reactive oxygen species (ROS) that are genotoxic and provoke Rb and p53-dependent checkpoint responses (44, 45). Genetic alterations that reduce ROS production or that bypass these checkpoints can result in escape from senescence. We noted that Lkb1 restoration in SCC cell lines was associated with induction of p38 suggesting that Lkb1 provokes an acute stress response. We speculate that the tumor suppressor role of Lkb1 may involve induction of senescence in cells receiving aberrant growth signals.

Based on the requirement of Lkb1 in squamous tumor suppression, it was surprising that mice with epidermal specific deletion of Lkb1 showed largely normal skin development. The overall architecture of the skin was not impaired in these mice, and the normal expression of keratin-1, keratin-14, and involucrin indicated that Lkb1 mutant keratinocytes underwent normal differentiation. The prominent increase in SCC development in the context of DMBA exposure may indicate that Lkb1 plays a particular role in restraining proliferation in response to chemical carcinogens or more broadly to stresses that result in increased cell turnover. Such a role could account for the strong association between Lkb1 mutations and smoking-associated cancers.

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Correction: Carcinogen-Induced Tumors in Lkb1 Mutant Mice

In the article on carcinogen-induced tumors in Lkb1 mutant mice in the January 1, 2008 issue of *Cancer Research* (1), Dr. Ergun Sahin should have been included as the third author.

1. Gurumurthy S, Hezel AF, Sahin E, Berger JH, Bosenberg MW, Bardeesy N. LKB1 deficiency sensitizes mice to carcinogen-induced tumorigenesis. Cancer Res 2008;68: 55–63.

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LKB1 Deficiency Sensitizes Mice to Carcinogen-Induced Tumorigenesis

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