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Liver Kinase B1 (LKB1) in the Pathogenesis of UVB-induced Murine Basal Cell Carcinoma

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Abstract

LKB1, a known tumor suppressor, is mutated in Peutz-Jeghers Syndrome (PJS). It is responsible for the enhanced cancer risk in patients with PJS. Dysregulation of LKB1-dependent signaling also occurs in various epithelial cancers. UVB alters the expression of LKB1, though its role in the pathogenesis of skin cancer is unknown. Here we describe upregulation of LKB1 expression in UVB-induced murine basal cell carcinoma (BCC) and in human skin tumor keratinocytes. AMP-kinase and acetyl Co-A carboxylase, the downstream LKB1 targets, are also enhanced in this neoplasm. In addition, p-Akt, a kinase which inactivates GSK3 β by its phosphorylation, is enhanced in BCCs. Consistently, an accumulation of p-GSK3 β and an increase in activated nuclear β -catenin are found. mTOR signaling, which is also inhibited by LKB1, remains upregulated in BCCs. However, a marked decrease in the expression of sestrins, which function as potent negative regulators of mTOR is observed. Metformin, a known chemical inducer of this pathway, was found effective in immortalized HaCaT keratinocytes, but failed to activate the LKB1-dependent signaling in human carcinoma A431 cells. Thus, our data show that the LKB1/AMPK axis fails to regulate mTOR pathway, and a complex regulatory mechanism exists for the persistent mTOR activation in murine BCCs.

Introduction

Basal cell carcinoma (BCC) is the most common cancer in Caucasian populations, affecting at least 800,000 Americans annually and exhibiting a worldwide increase in incidence [1]. While multiple factors play a role in the pathogenesis of BCCs, genetic predisposition and exposure to ultraviolet (UV) radiation are considered the most significant. [2]. BCC incidence strongly correlates with geographic latitude and degree of sun exposure, with the highest incidence reported in Australia and South Africa, where 1–2 out of 100 people develop BCCs annually [3]. UV exposure is an important initiating factor in BCC development that likely results in dysregulation of multiple biochemical pathways involved in cellular metabolism, growth, and replication. The precise mechanism underlying the pathogenesis of BCCs is currently under investigation. Dysregulation of sonic hedgehog (SHH) signaling is critical to the development of BCCs [4]. Inactivating mutations in the

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Ptch1 gene, which encodes a receptor for diffusible morphogen Hedgehog, are found in both sporadic BCCs as well as in a hereditary nevoid basal cell carcinoma syndrome (NBCCS) [4]. Patients afflicted with NBCCS are predisposed to multiple developmental defects and various neoplasms. Significantly, these patients develop tens to hundreds of BCCs. Ptch1 is a negative regulator of SHH signaling via inhibition of the transmembrane protein Smoothened (Smo). Thus, loss of the Ptch1 protein results in persistent activation of SHH signaling, which is thought to lead to tumorigenesis.

Recently engineered heterozygous Ptch1 ($Ptch1^{+/-}$) mice served as the first murine model of UVB-induced cutaneous carcinogenesis. Although the skin of these mice appears grossly intact, chronic UVB-radiation results in the development of multiple BCCs that histologically closely resemble those occurring in humans. While Ptch1 is believed to be the 'gatekeeper' for BCC development, recent data suggest that a combined effect of multiple disrupted pathways is likely to play an important role in BCC carcinogenesis [6]. The availability of a murine $Ptch1^{+/-}$ model of UVB-induced skin cancer now allows for *in vivo* investigations of these pathways.

The tumor suppressor LKB1 is a serine/threonine kinase that modulates cellular growth, proliferation, polarity, and survival in response to energy stress. LKB1 is a master kinase that directly phosphorylates at least 14 downstream targets, including AMP-activated protein kinase (AMPK). Intracellular ATP depletion, as occurs following nutrient deprivation or hypoxia, leads to LKB1 directed phosphorylation of AMPK [7]. LKB1-AMPK-dependent signaling activation is important in the suppression of protein synthesis, lipogenesis, and carbohydrate metabolism, leading to an overall arrest in cell growth and proliferation. LKB1 germline mutations are seen in Puetz-Jeghers Syndrome, a known cancer susceptibility syndrome. [8,22]. In addition, sporadic inactivating mutations of LKB1 have also been implicated in the pathogenesis of some lung and cervical carcinomas as well as a number of other epithelial cancers [8,9]. Loss of LKB1 in fibroblasts has recently been reported to result in unabated cell growth and failure to undergo senescence in culture [10]. Also, selective deletion of LKB1 in murine epidermis leads to the enhanced development of carcinogen-induced as well as spontaneous squamous cell carcinomas (SCCs) [11].

There is emerging evidence that the LKB1-AMPK-dependent pathway may play a role in UVB-induced skin cell damage and carcinogenesis. However, its role in BCC development is not defined. In this study, we assessed LKB1 expression and its downstream signaling pathways in UVB-induced BCCs derived from $Ptch1^{+/-}$ mice. We demonstrated increased expression of both LKB1 and its downstream targets AMPK and ACC in UVB-induced murine tumors. To further probe the possible effect of increased LKB1 in BCC development, we investigated mTOR, Wnt/ β -catenin, and MAPK signaling pathways. We observed that both mTOR and MAPK signaling were independent of LKB1/AMPK expression, and that LKB1 may potentiate Wnt/ β -catenin signaling via suppression of GSK3 β , which acts as a potent inhibitor of this pathway. Our data suggest that LKB1 expression and -dependent downstream signaling are dysregulated in UVB-induced murine BCCs.

Materials and Methods

Reagents and antibodies

Phospho-Ser-428 LKB1, LKB1, phospho-Thr-172 AMPK, AMPK, phospho-Ser-79-Acetyl-CoA Carboxylase, Acetyl-CoA Carboxylase, phospho-Ser-235/236-S6 Ribosomal protein, S6-ribosomal protein, phospho-Thr-389-p70 S6 kinase, p70 S6 kinase, phospho-Ser-65-4E-BP1, 4E-BP1, p44/42 MAPK (Erk1/2), TSC1, p-TSC1 (S1389), and TSC2 antibodies were purchased from Cell Signaling Technology (Danvers, MA). Phospho-Ser-9-GSK3 β , GSK3 β ,

phospho-Thr-308-Akt1/2/3, Akt1, phospho p38, p38, p-TSC1 and phospho-p44/42 MAPK (Erk1/2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Sestrin1 and sestrin2 antibodies were purchased from Abcam (Cambridge, MA). Anti- β -actin antibody was purchased from Sigma-Aldrich (St. Louis, MO). β -catenin antibody was purchased from BD Transduction Laboratories (San Jose, CA). Secondary antibodies were purchased from Thermo Fisher Scientific (Rockford, IL). The use of antibodies is summarized in Table 1. Metformin hydrochloride (98.8% purity) was purchased from LKT laboratories (St. Paul, MA).

Cell culture and cell lysate preparation

Immortalized keratinocytes (HaCaT), and epidermoid carcinoma A431 cells were obtained from the American Type Culture Collection (Manassas, VA). HaCat and A431 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum. Neonatal human epidermal keratinocytes (NHEK) were derived from neonatal foreskin in our SDRC Skin Cell Culture Core. NHEK were grown in keratinocyte basal medium KBM-2 (Lonza, Walkersville, MD). For western blotting, cells were grown to approximately 70% confluency and harvested using a lysis buffer containing 1M Tris (pH 7.5), 5M NaCl, 10% Triton X-100, 10% sodium deoxycholate, 10% SDS, 0.1M PMSF, and protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN).

Metformin treatment

A431 and HaCaT cells were grown in serum-supplemented medium to approximately 70% confluency and exposed to serum-free medium containing 0mM, 0.15mM, 1mM, and 5mM metformin for 24 hours at 37°C. Cells were harvested and cell lysates were prepared for western blotting as described above.

Animals

UVB-induced BCCs that developed in *Ptch1*^{+/-} mice were used in this study as described previously [17].

Western blotting

Tumor and skin tissue lysates were prepared as previously described [16]. 50 μ g of total protein was separated on a 10% SDS-PAGE gel and blotted onto nitrocellulose membrane. Following transfer, non-specific sites were blocked using 5% (w/v) nonfat-dry milk in Tween-Tris base-saline (TBST) buffer (0.1% Tween-20, 20mM Tris base, 137 mM NaCl, 3.8 mM HCl, pH 7.5–7.6) for 2 hours at room temperature followed by incubation with primary antibody in nonfat-dry milk in TBST overnight at 4°C (dilutions were chosen per manufacturer's recommendations). After washing the blots in TBST with 5 buffer changes 10 min each, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:3000 dilution). Blots were washed four times for 15 min each and developed with ECL according to the manufacturer's protocol (Thermo Fisher Scientific, Rockford, IL). For sequential antibody reprobing, blots were stripped using Restore Plus Western Blot Stripping Buffer (Pierce Biotechnology, Rockford, IL) according to manufacturer's instructions. Nuclear and cytoplasmic protein fractions were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents ((Pierce Biotechnology, Rockford, IL). Band densities were measured using NIH Image J software and results were normalized to corresponding actin band densities.

RNA isolation and RT-PCR

Total RNA from tumor and skin samples was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Script cDNA synthesis kit was used to

generate corresponding cDNA (Bio-Rad, Hercules, CA). The following primers were used to amplify a significant portion of the coding region of LKB1: forward primer, 5'-CCCGAGCCGTTGGGCCTTTT-3'; reverse primer, 5'-CCTGGCTTGTCCGGCAGGTG-3'. PCR was carried out in a volume of 25 µl containing 2µl of cDNA, 21 µl of 1X PCR SuperMix (Invitrogen, Carlsbad, CA), and 1µl of each primer. Cycling conditions were performed as described previously [17].

Statistical analysis

Statistical analyses were performed using GraphPad Instat® V.3 software. The significance between the two test groups was determined using Student's 't' test. *P* values less than 0.05 were considered significant.

Results

Different groups have reported conflicting results regarding the role of LKB1 in skin carcinogenesis. For example, Cao *et al* reported that UVB radiation dose correlates with the increased protein expression of LKB1 and AMPK in an EGFR-dependent manner [12], while Zhang and Bowden found that UVB decreases LKB1 and AMPK protein expression [13]. More recently, two independent studies implicated LKB1 in the pathogenesis of melanoma [14,15]. Melanoma cells harboring the BRAFV600E mutation manifest inactivated LKB1 via Erk-and Rsk-dependent phosphorylation leading to reduced AMPK signaling. This mechanism also potentiated mTOR signaling, which is considered a key regulator of melanoma cell growth, proliferation, and tumorigenesis. To understand whether there is a similar role of LKB1 in BCCs, we conducted the following experiments.

A431 epidermoid carcinoma cells express higher levels of LKB1 and p-AMPK compared to normal and immortalized human keratinocytes

To evaluate the status of LKB1 expression in an *in vitro* model of cutaneous carcinogenesis, we compared its expression in NHEK, immortalized human keratinocytes (HaCaT), and A431 epidermoid carcinoma cell lines. We observed that LKB1 expression in A431 epidermoid carcinoma cells was markedly increased when compared to normal and immortalized human keratinocytes (Fig. 1). Concurrently, phospho-AMPK levels were also elevated in A431 cells indicating that the LKB1/AMPK axis is functional in these cells.

Metformin activates LKB1/AMPK signaling in immortalized keratinocytes but not in A431 epidermoid carcinoma cells

There is emerging evidence that metformin, a widely prescribed oral hypoglycemic agent, exhibits a direct anti-cancer effect through inhibition of the AMPK/mTOR/S6K pathways both in various human cancer cell lines as well as in murine models [20,21]. We employed metformin to assess the responsiveness of the LKB1/AMPK pathway on the growth of immortalized (HaCaT) and SCC keratinocytes. Metformin treatment enhanced expression of both LKB1 and p-AMPK in HaCaT cells in a dose-dependent manner but not in A431 keratinocytes (Fig. 2). These findings suggest that in immortalized human keratinocytes, tumor suppressor functions of LKB1 remain intact, whereas LKB1 tumor suppressor functions are overcome in SCC cells, presumably by the activation of oncogenic signaling.

LKB1 expression and -dependent AMPK signaling are increased in UVB-induced non-melanoma skin cancers (NMSCs) in *Ptch1*^{+/-} mice

To show the importance of LKB1 and AMPK proteins in skin carcinogenesis, we first assessed their expression levels in UVB-induced SCCs and BCCs harvested from *Ptch1*^{+/-} mice. Expression levels of both LKB1 and AMPK were increased in tumor samples as

compared to adjacent histologically normal skin (Fig. 3). Moreover, LKB1/AMPK activation was more pronounced in BCCs than in SCCs. We also observed an increase in phospho-LKB1 expression in BCC tumors compared to adjacent tumor-free skin and non-irradiated age-matched control skin (Fig. 4, A and B), confirming increased activation of LKB1/AMPK signaling in BCCs. Similar to enhanced protein expression of LKB1, BCCs exhibited increased transcription of LKB1 mRNA (Fig. 4D). As described earlier, under conditions of nutrient deprivation LKB1 directly phosphorylates AMPK on Thr-172 which leads to AMPK activation and subsequent inhibition of anabolic reactions and activation of catabolic reactions [23]. AMPK directly phosphorylates Ser-29 of ACC, resulting in ACC's inactivation, which stimulates fatty acid oxidation (Fig. 4C). Mirroring the pattern of LKB1 activation, AMPK expression was increased in murine BCCs accompanied by the enhanced phosphorylation of ACC (Fig. 4A).

Upregulation of mTOR signaling in UVB-induced BCCs in *Ptch1*^{+/-} mice occurs independent of LKB1/AMPK activation

LKB1/AMPK axis activation is known to downregulate mTOR signaling under conditions of nutrient deprivation [23,24]. In this regard, LKB1-mediated AMPK activation leads to accumulation of phosphorylated TSC2 [20], which inhibits mTOR signaling by blocking phosphorylation of its two key translational regulators, p70 ribosomal S6 kinase 1 (S6K) and eukaryote initiation factor 4E binding protein 1 (4E-BP1) (Fig. 5C) [25,26]. Therefore, we investigated the mTOR signaling pathway in these tumors by examining the expression of total and phosphorylated TSC1/2, 4E-BP1, S6K, and its direct downstream target S6 protein. Enhanced mTOR signaling in murine BCCs occurs as evidenced by augmented levels of p-4E-BP1, p-S6K, and p-S6-proteins (Fig. 5, A and B). These results suggest that activated LKB1-AMPK pathway in BCCs fails to downregulate mTOR signaling. In addition, we did not observe significant changes in the expression of TSC2 (Fig. 5C and D), which is known to downregulate mTOR signaling [20]. This finding further strengthens the conclusion that LKB1 and-dependent signaling fails to regulate mTOR pathway during the pathogenesis of UVB- induced BCCs in experimental animals.

Expression of sestrins is reduced in UVB-induced BCCs in *Ptch1*^{+/-} mice

Recently, sestrins, a group of highly conserved proteins, were found to function as novel negative regulators of mTOR signaling [27]. Sestrins are known downstream targets of tumor suppressor p53, which induces their expression in response to stress signals. Sestrins shut down protein synthesis and cell growth by inhibiting mTOR (Fig. 6C). Since UVB-induced BCCs frequently harbor mutations in p53, we hypothesized that sestrin expression may be altered in these tumors, and may contribute to dysregulation of mTOR signaling. To test this hypothesis, we examined the expression profile of sestrins in murine UVB-induced BCCs. We observed a consistent decrease in both sestrin1 and sestrin2 expression in these tumors (Fig. 6A and B). This suggests that a decrease in negative feedback inhibition via sestrins is likely to contribute to persistent mTOR activation in murine BCCs.

LKB1 may potentiate Wnt/ β -signaling in UVB- induced BCCs in *Ptch1*^{+/-} mice

Activation of Wnt signaling with subsequent nuclear accumulation of β -catenin has been shown to be associated with increased proliferative and invasive potential in human BCCs [28,29]. Recent evidence suggests that LKB1 functions as an upstream activator of canonical Wnt signaling by regulating glycogen synthase kinase (GSK) 3 β [30]. It is also known that GSK3 β can be deactivated by AKT, a serine-threonine kinase that is mutated and overexpressed in many cancers [31,32]. Thus, inhibitory phosphorylation of GSK3 β by LKB1 and/or AKT may be a cardinal event leading to constitutive activation of Wnt/ β -catenin signaling (Fig. 7C). We, therefore, investigated the expression of GSK3 β , AKT, and β -catenin in UVB-induced murine BCCs. We observed that expression of p-AKT was

markedly increased in BCCs with a concomitant inactivation of GSK3 β and increased accumulation of nuclear β -catenin (Fig. 7, A and B). Recently, it has also been shown that GSK3 β may be inactivated via direct phosphorylation at its C-terminus by p38 mitogen activated protein kinase (MAPK) in a tissue specific manner [33]. Interestingly, ERK1/2 and p38 signaling pathways were shown to be upregulated in response to UVB irradiation in cultured human keratinocytes [34]. Therefore, we characterized the protein expression of ERK1/2 and p38 in murine BCCs. We observed that ERK1/2 and p38 are not activated in BCCs (Fig. 8), suggesting that p38 does not contribute to GSK3 β inactivation. Thus, LKB1/AMPK and Akt contribute to GSK3 β inactivation and play an important role in Wnt signaling upregulation in UVB-induced BCCs.

Discussion

In the present study, we investigated the role of LKB1 and LKB1-dependent signaling in UVB-induced cutaneous BCCs. Although LKB1 is a well-characterized tumor suppressor, our results suggest that LKB1 activation in NMSCs may not be related to its tumor suppressor function and may contribute to the pathogenesis of skin cancer through indirect mechanisms. Our observation that the LKB1/AMPK pathway is upregulated in human SCC cells as well as in murine NMSCs, suggests that LKB1 and -dependent AMPK/ACC signaling is not effective in blocking the growth of UVB-induced tumors. This is also consistent with our observation that LKB1/AMPK upregulation failed to block mTOR signaling. Instead mTOR seems to be regulated through disrupted sestrin signaling. However, we demonstrate that LKB1 activation may have potentiated Wnt/ β -catenin signaling via inactivation of GSK3 β . This may ultimately contribute to BCC growth in coordination with activated Shh signaling.

The loss of LKB1 was found to be important in the development of non-small cell lung [35], breast [36,37], prostate [38], cervical [39], and other epithelial cancers [8]. Consistently, murine tissue-specific LKB1 knock-out models have demonstrated an increased predisposition for multiple epithelial malignancies [11]. Unexpectedly, in this study, we found that LKB1 expression is enhanced in murine cutaneous BCCs, suggesting that LKB1 signaling may be pro-carcinogenic in specific contexts. While AMPK/ACC signaling remained intact in our studies, other downstream pathways, including mTOR signaling were dysregulated. It is likely that inability of LKB1 to effectively downregulate mTOR may be related to its failure to block tumorigenesis. It has been recently reported that aberrant Akt activation can lead to augmented mTOR signaling [20,40]. In our studies, we observed elevated AKT expression in BCCs. Therefore, activated Akt may have overridden the inhibitory effects of LKB1 on mTOR, thus partly explaining the mTOR upregulation in UVB-induced BCCs. Although this hypothesis needs further strengthening, our results support a novel concept that LKB1 regulation of mTOR signaling may become ineffective in the setting of activation of other pro-oncogenic signaling such as Akt.

Recently, LKB1 has been shown to activate Wnt/ β -catenin signaling during vertebrate development via inhibitory phosphorylation of GSK3 β , a known negative regulator of this pathway [30]. In this regard, it was shown that LKB1 physically associates with PKC- ζ , which is known to inhibit GSK3 β kinase activity by promoting its phosphorylation. This inactivation of GSK3 β results in constitutive activation of Wnt/ β -catenin signaling. A similar pattern of activation of LKB1 and inhibition of GSK3 β was observed in our studies, suggesting that enhanced LKB1 activity may potentiate Wnt/ β -catenin signaling-mediated pathogenesis of murine BCCs. This is consistent with the reported upregulation of Wnt/ β -catenin signaling in human BCCs [29]. Interestingly, recent studies in prostate cancer have shown that loss, rather than increased expression of Lkb1, results in similar upregulation of Wnt/ β -catenin signaling [38].

Although LKB1 is historically known as a tumor suppressor, our study provides evidence that it may drive proliferation in UVB-induced BCCs. Interestingly, two recent reports have also demonstrated previously undefined pro-oncogenic roles for LKB1. Zhong *et al* showed that in the setting of aberrantly activated Akt, LKB1 is required for FoxO3a phosphorylation and subsequent suppression of apoptosis in lung and colon cancer cells [41]. Elevated LKB1 expression has also been observed in cells isolated from hepatocellular carcinoma [42]. These studies implicate LKB1's complex role in the pathogenesis of various malignancies which seems to be highly context-dependent. In summary, we show that LKB1 activation along with the activation of multiple signaling pathways, including AMPK/ACC, mTOR/S6K/4E-BP1, and AKT/GSK3 β / β -catenin, occur during the pathogenesis of BCCs. While some of these pathways appear to be directly regulated by LKB1, others are overridden by oncogenic stimuli that contribute to the tumor pathogenesis.

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Fig. 1. Expression of LKB1 and p-AMPK is increased in A431 epidermoid carcinoma cells compared with normal and immortalized human keratinocytes

A. Western blot analysis of cell lysates prepared from NHEK, HaCaT, and A431 cell lines. Cells were grown to 70%–90% confluency and harvested. Cell lysates containing 50µg of total protein were analyzed on a 10% SDS-polyacrylamide gel, electroblotted onto nitrocellulose membrane, and probed with indicated antibodies.

B. Quantification of LKB1, AMPK, and p-AMPK expression. Relative band intensities were measured using Image J software, normalized to the respective actin bands to account for sample loading variation, and shown as a bar graph. Each bars represent mean ± S.E. of three experiments. Significance between the expression of proteins in different cell lines was calculated using Student's t-test: ¹ $p=0.009$, ² $p=0.51$, ³ $p=0.16$, ⁴ $p=0.99$, ⁵ $p=0.006$, and ⁶ $p=0.39$. $P<0.01$ were described as highly significant (**) whereas $p<0.05$ were described as significant (*).

Fig. 2. Metformin enhances LKB1/AMPK signaling in immortalized human skin keratinocytes but not in A431 epidermoid carcinoma cells

A. Western blot analysis of metformin-treated A431 and HaCaT cells. Cells were grown to 70% confluency and exposed to metformin-containing serum-free medium for 24 hours at 37°C. When cells reached near-confluency, they were harvested and analyzed by Western blotting as described in Fig. 1.

B. Quantification of LKB1, p-LKB1, AMPK, and p-AMPK expression. Relative band intensities were measured using Image J software, normalized to the respective actin band intensities to account for sample loading variation, and shown as a line graph. Each marker represents mean $\pm$ S.E. of three experiments. Significance between the expression of proteins in 5mM treatment group compared to no treatment group was calculated using Student's t-test: ¹ $p=0.0003$, ² $p=0.71$, ³ $p=0.0072$, and ⁴ $p=0.63$. $P<0.01$ were described as highly significant (**).

Fig. 3. LKB1 and p-AMPK expression is increased in UVB-induced murine squamous cell and basal cell carcinoma

A. Western blot analysis of tumor lysates prepared from UVB-induced murine BCCs SCCs. Adjacent normal skin from corresponding animals was used as control. Tissue lysated containing 50μg of total protein were analyzed by Western blotting as described in Fig. 1 and sequentially probed with anti-LKB1, anti-p-AMPK, and anti-β-actin antibodies.

B. Quantification of LKB1 and p-AMPK expression in UVB-induced SCCs and BCCs. Relative band intensities were measured using Image J software and normalized to the respective actin band intensities to account for sample loading variation. Each bar represents mean ± S.E. of three tumors harvested from three different animals. Error bars indicate S.E. Significance between the expression of proteins in tumors and tumor-adjacent normal skin was calculated using Student's t-test: ¹ $p=0.01$, ² $p=0.003$, ³ $p=0.23$, and ⁴ $p=0.02$. $P<0.01$ were described as highly significant (**) whereas $p<0.05$ were described as significant (*).

Fig. 4. LKB1 expression and LKB1-dependent signaling is increased in UVB- induced murine BCCs

A. Western blot analysis of tumor sample lysates prepared from four different UVB-induced murine BCCs (T1-T4). Adjacent normal skin from corresponding animals (S1-S4) as well as non-UVB exposed skin from age-matched mice (C) were used as controls. Tissue lysates containing 50μg of total protein were analyzed by Western blotting as described in Fig. 1 and sequentially probed with anti-LKB1, anti-p-LKB1, anti-AMPK, anti-p-AMPK, and anti-β-actin antibodies.

B. Quantification of LKB1 and p-AMPK expression in UVB-induced BCCs and control skin. Relative band intensities were measured using Image J software and normalized to the respective actin band intensities to account for sample loading variation. Each bar represents mean ± S.E. of four tumors harvested from four different mice. Error bars indicate S.E. Significance between the expression of proteins in tumors and tumor-adjacent normal skin was calculated using Student's t-test: ¹ $p=0.004$, ² $p=0.01$, ³ $p=0.05$, ⁴ $p=0.007$, ⁵ $p=0.83$, and ⁶ $p=0.34$. $P<0.01$ were described as highly significant (**) whereas $p<0.05$ were described as significant (*).

C. Schematic representation of LKB1/AMPK/ACC axis. Under conditions of cellular stress and nutrient deprivation, LKB1 directly phosphorylates AMPK and activates its kinase activity. p-AMPK in turn phosphorylates ACC resulting in its inactivation and increase in fatty acid oxidation

D. Semiquantitative RT-PCR of mRNA isolated from three different murine BCCs (T1-T3) and adjacent normal skin (S1-S3). Total RNA from tumor and skin samples was extracted and amplified using the following primers: forward primer, 5'-CCCGAGCCGTTGGGCCTTTT-3'; reverse primer, 5'-CCTGGCTTGTCCGGCAGGTG-3'. A 625kb band was detected on gel-electrophoresis blot corresponding to the expected size of product for LKB1 using this set of primers.

Fig. 5. mTOR signaling is upregulated in UVB-induced murine BCCs despite LKB1/AMPK activation

A. Western blot analysis of four different tumors (T1-T4), adjacent normal skin (S1-S4), and skin from age-matched non-UVB exposed control mice (C) was performed as described in Fig. 1. Blots were sequentially probed with anti-p-p70 S6K, anti-p70 S6K, anti-p-S6-protein, anti-S6-protein, anti-p-4E-BP1, anti-4E-BP1, and anti-β-actin antibodies.

B. Quantification of mTOR signaling proteins in UVB-induced BCCs and controls was performed as described in Fig. 4. Each bar represents mean ± S.E. of four tumors harvested from four different mice. Error bars indicate S.E. Significance between the expression of proteins in tumors and tumor-adjacent normal skin was calculated using Student's t-test: ¹ $p=0.02$, ² $p=0.009$, ³ $p=0.08$, ⁴ $p=0.02$, ⁵ $p=0.05$, ⁶ $p=0.22$. $P<0.01$ were described as highly significant (**) whereas $p<0.05$ were described as significant (*).

C. Schematic representation of LKB1/AMPK/mTOR signaling cascade. LKB1-mediated activation of AMPK results in the phosphorylation of TSC2 and subsequent inhibition of mTOR signaling. TSC2 inhibits phosphorylation of the two key translational regulators 4E-BP1 and S6 kinase with its target ribosomal protein S6. These events result in inhibition of protein synthesis and cell growth.

D. Western blot analysis of four different tumors (T1-T4), adjacent normal skin (S1-S4), and skin from age-matched non-UVB exposed control mice (C) was performed as described in Fig. 1. Blots were sequentially probed with anti-TSC1, anti-p-TSC1, anti-TSC2, and anti-p-TSC2 antibodies.

E. Quantification of TSC1/2 proteins in UVB-induced BCCs and controls was performed as described in Fig. 4. Each bar represents mean ± S.E. of four tumors harvested from four different mice. Error bars indicate S.E. Significance between the expression of proteins in tumors and tumor-adjacent normal skin was calculated using Student's t-test: ¹ $p=0.019$, ² $p=0.14$, ³ $p=0.15$ and ⁴ $p=0.2$. $P<0.05$ were described as significant (*)

Fig. 6. Sestrin1 expression is reduced in UVB-induced murine BCCs

A. Western blot analysis of four different tumors (T1–T4), adjacent normal skin (S1–S4), and skin from age-matched non UVB-exposed control mice (C) was performed as described in Fig. 1. Blots were sequentially probed with anti-sestrin1, anti-sestrin2, and anti-β-actin antibodies.

B. Quantification of sestrins expression in UVB-induced BCCs and control skin was performed as described in Fig. 4. Each bar represents mean $\pm$ S.E. four tumors harvested from four different mice. Error bars indicate S.E. Significance between the expression of proteins in tumors and tumor-adjacent normal skin was calculated using Student's t-test: $^1p=0.0003$ and $^2p=0.06$. $P < 0.001$ was described as extremely significant (***).

C. Schematic representation of sestrin-mediated inhibition of mTOR signaling. Activation of p53 by metabolic stress induces the expression of sestrin proteins. Sestrin1 and 2 preferentially activate AMPK towards inhibition of mTOR signaling.

Fig. 7. LKB1 potentiates Wnt signaling in UVB-induced BCCs

A. Western blot analysis of four different tumors (T1–T4), adjacent normal skin (S1–S4), and skin from age-matched non-UVB exposed control mice (C) was performed as described in Fig. 1. Blots were sequentially probed with anti-p-Akt, anti-Akt, anti-p-GSK3β, anti-GSK3β and anti-β-actin (not shown) antibodies. Nuclear fraction of BCCs and control skin was extracted and probed with anti-β-catenin antibody.

B. Quantification of Wnt signaling proteins in UVB-induced BCCs and controls was performed as described in Fig. 4. Each bar represents mean $\pm$ S.E. four tumors harvested from four different mice. Error bars indicate S.E. Significance between the expression of proteins in tumors and tumor-adjacent normal skin was calculated using Student's *t*-test: ¹*p*=0.01, ²*p*=0.03, ³*p*=0.009, ⁴*p*=0.0005, ⁵*p*=0.04, ⁶*p*=0.23. *P* values less than 0.01 were considered highly significant (**). *P* < 0.05 were described as significant (*).

C. Schematic representation of Wnt/β-catenin signaling regulation. Activation of LKB1, Akt, and p38 kinases results in inhibitory phosphorylation of GSK3β, which results in translocation of β-catenin into the nucleus and transcriptional activation of multiple targets contributing to carcinogenesis.

Fig. 8. MAPK signaling is upregulated both in UVB-induced BCCs and in normal skin adjacent to the tumors

A. Western blot analysis of four different tumors (T1–T4), adjacent normal skin (S1–S4), and skin from age-matched non-UVB exposed control mice (C) was performed as described in Fig. 1. Blots were sequentially probed with anti-p-ERK1/2, anti-ERK1/2, anti-p-p38, anti-p38, and anti-β-actin antibodies.

B. Quantification of MAPK signaling proteins in UVB-induced BCCs and controls was performed as described in Fig. 4. Each bar represents mean ± S.E. four tumors harvested from four different mice. Error bars indicate S.E. Significance between the expression of proteins in tumors and tumor-adjacent normal skin was calculated using Student's t-test: ¹ $p=0.83$, ² $p=0.85$, ³ $p=0.71$, ⁴ $p=0.12$