LKB1 Is Necessary for Akt-Mediated Phosphorylation of Proapoptotic Proteins

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Abstract

LKB1 plays the role of tumor suppressor, opposite to Akt, by negatively regulating mammalian target of rapamycin through the activation of AMP-activated protein kinase and TSC signaling. We have discovered a novel, potentially oncogenic role for LKB1 as a supporter of Akt-mediated phosphorylation of proapoptotic proteins. We found that Akt activation led to increased phosphorylation of FoxO3a at Thr22 in LKB1 wild-type cells but not in LKB1-null cells. Depletion of LKB1 in the cells with wild-type LKB1 resulted in attenuation of that phosphorylation of FoxO3a by activated Akt, whereas the restoration of LKB1 function in LKB1-null cells reestablished Akt-mediated FoxO3a phosphorylation. On expanding our analysis to other Akt targets, using isogenic LKB1 knockout cell line pairs and a phospho-specific antibody microarray, we observed that there was a requirement for LKB1 in the phosphorylation of other Akt downstream targets, including Ask1 (Ser83), Bad (Ser136), FoxO1 (Ser319), FoxO4 (Ser197), and glycogen synthase kinase 3β (GSK3β; Ser9). Because the phosphorylation of these sites by Akt suppresses apoptosis, the requirement of LKB1 suggests that LKB1 may have an antiapoptotic role in tumor cells with constitutively active Akt. Indeed, we found that the suppression of LKB1 expression led to apoptosis in three cell lines in which Akt is constitutively active but not in two cell lines without Akt activation. This observation may explain the lack of LKB1 somatic mutations in brain, breast, and colon cancers, where Akt is frequently activated due to mutations in phosphatidylinositol 3-kinase, PTEN, or Akt itself. [Cancer Res 2008;68(18):7270–7]

Introduction

LKB1 is a serine/threonine kinase gene located on chromosome 19p13.3 (1). Inherited mutations in LKB1 give rise to Peutz-Jeghers syndrome, a disorder characterized by benign hamartomas of the gastrointestinal tract and a predisposition to certain cancers (2). In addition, somatic mutation analyses indicate that biallelic inactivation of LKB1 is present in ~30% of non–small cell lung cancer (NSCLC) primary tumors and cell lines (3–6). Recent progress on the function of LKB1 places this protein at the apex of a novel signaling pathway that ultimately serves to inhibit the activity of mammalian target of rapamycin (mTOR) kinase, a key mediator of phosphatidylinositol 3-kinase (PI3K)/Akt-driven survival signals (7). LKB1 is linked to mTOR regulation through the sequential activation of AMP-activated protein kinase (AMPK) and the tumor suppressor TSC2, a GTPase-activating protein that negatively regulates mTOR through the small G protein Rheb (8). Together, LKB1, AMPK, and TSC2 constitute a cell stress pathway that counteracts PI3K/Akt signaling, thus suppressing mTOR-related translation under energy stress.

Although extensive genetic and functional evidence suggest that LKB1 is a tumor suppressor (2), the biological properties associated with LKB1 deficiency are complex. Some studies in model organisms suggest that inactivation of LKB1 may not always be compatible with the activation of certain oncogenic signaling. For example, in mouse embryonic fibroblast (MEF) cells, a LKB1 deficiency leads to resistance to Ha-Ras transformation via a p19arf/p53-independent growth-inhibitory pathway, suggesting that the early loss of LKB1 function may render cells resistant to subsequent oncogene-induced transformation (9). In Xenopus and mouse MEF cells, LKB1 depletion leads to decreases in glycogen synthase kinase 3β (GSK3β) phosphorylation at Ser9 and subsequent down-regulation of WNT signaling (10). These data are of particular interest because the Ser9 of GSK3β is a target of aberrantly activated Akt in many human cancers, which suggests that LKB1 may be required for the phosphorylation of Akt downstream targets. This would constitute a previously unknown pro-oncogenic role for LKB1. In this study, we directly tested the role of LKB1 in Akt signaling. Our results showed that the presence of LKB1 is required for Akt-mediated phosphorylation of the proapoptotic proteins.

Materials and Methods

Materials

2-Deoxyglucose (2-DG) was purchased from Sigma. Mouse monoclonal antibody against LKB1 was purchased from Abcam. Antibodies against AMPK, phospho-AMPKα (Thr172) Akt, phospho-Akt (Ser473), phospho-Bad (Ser136), phospho-GSK3β (Ser9), p21, p27, cyclin D1, and caspase-3 were purchased from Cell Signaling Technology, Inc. Antibodies against FoxO3a and phospho-FoxO3a (Thr24) were purchased from Upstate. Rabbit polyclonal antibody against Bim-1 was purchased from Affinity BioReagents. Rabbit polyclonal anti-actin antibody was purchased from Sigma Chemical Co. The NSCLC cell lines H1792, A549, H23, H157, H460, H1299, H1650, H1705, HCC827, and H520 and colorectal cancer cell line HCT116 were purchased from the American Type Culture Collection (ATCC) and propagated according to the conditions recommended by ATCC.

Immunoblot Analysis

The procedures for the preparation of whole-cell protein lysates and for immunoblot were as described previously (5). Whole-cell protein lysates
(20 μg/lane) were processed for immunoblot analysis using antibodies against specified proteins. The same blots were used in probing for phospho-specific antibodies and antibodies against total protein. Actin served as a loading control.

**Selection of Cells**

**Transient small interfering RNA treatment.** LKB1 small interfering RNA (siRNA) duplexes were purchased from Dharmacon. To control for any nonspecific off-target effects of the siRNA transfection, the company’s lamin A/C siControl was also used. The LKB1 siRNA sequence was 5’-GGACUGACGUAGAACAATT-3’. Gene silencing was achieved by transfecting cells with siRNAs delivered by Oligofectamine reagent (Invitrogen), according to the manufacturer’s recommendation. Briefly, cells were grown to approximately 60% to 70% confluence. Oligofectamine reagent was incubated with Opti-MEM1 reduced serum medium for 10 min and then a mixture of siRNA was added. After incubating for 15 min at room temperature, the siRNA mixture was diluted with medium and added to each well of cells. We used 200 pmol of siRNA per 2 mL of medium. To improve gene silencing, we transfected the same cells 48 h after the first transfection. Twenty-four hours after their second transfection, cells were washed and then resuspended in new culture medium in the presence or absence of 2-DG for a given period. Total cell lysates were used in the immunoblot blot analysis described above.

**Adenovirus infection.** NSCLC cell lines were infected with adenovirus as described previously (5). Briefly, AdEasy-GFP-LKB1 plasmid was first transfected into 293 cells for the generation of adenovirus containing GFP-LKB1. Adeno-GFP virus was a gift from Dr. Lily Yang (Emory University). H157 was infected with either GFP-LKB1 or GFP-only adenovirus at 20 multiplicities of infection for 24 h. The infection rate of the cells was ~ 90% as determined by GFP expression.

**LKB1 Stable Knockdown Using Lentiviral Short Hairpin RNA**

Five premade lentiviral LKB1 short hairpin RNA (shRNA) constructs and a negative control construct that was created in the same vector system (pLKO.1) were purchased from Open Biosystems. Lentiviral helper plasmids (pCMV-dR8.2 dvpr and pCMV-VSV-G) were obtained from Addgene. Transient lentivirus stocks were prepared following the manufacturer’s protocol. Briefly, 1.5 × 10⁷ 293T cells were plated in 10-cm dishes. Cells were cotransfected with siRNA constructs (3 μg) together with 3 μg pCMV-dR8.2 dvpr and 0.3 μg pCMV-VSV-G helper constructs. Two days later, viral stocks were harvested from the culture medium, which was filtered to remove non-adherent 293T cells. To select for the NSCLC cells that were stably expressing shRNA constructs, cells were plated at subconfluent densities and infected with a cocktail of 1 mL of virus-containing medium, 3 mL of regular medium, and 8 μg/mL polybrene. Selection with 0.5 to 2 μg/mL of puromycin was started 48 h after lentivirus infection. After several weeks of selection (2 wk for H1299 and H1703 and 4 wk for H1650, HCC827, and H520), monolayers of stably infected pooled clones were harvested for use and cryopreserved.

**Cell Proliferation Assay**

LKB1 shRNA knockdown stable cells (H1650/LKB1-shRNA, H1299/LKB1-shRNA, and H1703/LKB1-shRNA) and their corresponding controls (H1650/pLKO.1, H1299/pLKO.1, and H1703/pLKO.1) were seeded in 96-well cell culture plates at a density of 2,000 per well. The attached cells were seeded in six-well plate culture plates at a density of 2 × 10⁶ per well in six-well plates overnight. The following day, cells were transfected in triplicate with Lipofectamine 2000 using either LKB1-shRNA or the negative control pLKO.1 plasmid. For H1299 cells, 2 μg plasmid and 6 μL Lipofectamine were used for each well. Cells were selected with 2 μg/mL puromycin 72 h after transfection for 2 wk. For H1650 cells, cells were transfected with 0.5 μg plasmid and 1.5 μL Lipofectamine. Cells were selected with 0.5 μg/mL puromycin 72 h after transfection for 4 wk. Medium was changed every 4 d. Finally, the cells were fixed with 10% TCA solution and stained with 0.5% crystal violet. Colony numbers were assessed visually and colonies containing >50 normal-appearing cells were counted. Statistical differences in colony numbers between LKB1-shRNA and pLKO.1 plasmid-transfected cells were calculated using the two-sided Student’s t test.

**Phospho-Specific Protein Microarray Analysis**

Phospho-specific protein microarray was obtained from Full Moon Biosystems, Inc. Protein microarray analysis was carried out using the protocol provided. Briefly, 100 μg of cell lysate in 50 μL of reaction mixture were labeled with 1.43 μL biontin in 10 μg/mL N,N-dimethylformamide. The resulting biotin-labeled proteins were diluted 1:20 in coupling solution before applying to the array for conjugation. To prepare the antibody microarray, it was first blocked with blocking solution for 30 min at room temperature, rinsed with Milli-Q grade water for 3 min, and then dried with compressed nitrogen; finally, the array was incubated with the biotin-labeled cell lysates at 4°C overnight. After the array slide was washed thrice with 60 μL of 1× wash solution for 10 min each, the conjugated-labeled protein was detected using Cy3-streptavidin.

**Cell Cycle Analyses**

H1650/pLKO.1 or H1650/LKB1-shRNA cells (2 × 10⁶) were seeded in six-well cell culture plates. Only the live cells were harvested 4 d after cell seeding. Cells were washed with PBS and fixed in 70% ethanol overnight. Cells were then washed twice with PBS and stained using a Propidium Iodide/RNase Staining kit (BD PharMingen) for 30 min at room temperature in the dark. Cell cycle analysis was carried out using a FACSCan (Becton Dickinson) and Flowjo software version 7.2. A total of 10,000 cells were collected for each sample for analysis.

**Apoptosis Analyses**

Apoptosis was measured using the Annexin V-PE Apoptosis Detection kit (BD PharMingen) followed by flow cytometry. H1650/pLKO.1 and H1650/LKB1-shRNA cells (2 × 10⁶) were seeded in six-well cell culture plates. Both floating and attached cells were collected 4 d after cell seeding, washed twice with cold PBS, and suspended in 1× binding buffer. A 100 μL aliquot of cell suspension (representing 5 × 10⁵ cells) was transferred to a culture tube, to which 5 μL of Annexin V-PE and 5 μL of 7-amino-actinomycin D (7-AAD) were added, and the mix was incubated for 15 min at room temperature in the dark. Apoptosis analysis was carried out using a FACSCan and Flowjo software version 7.2. A total of 10,000 cells were collected for each sample for analysis.

**Results**

Phosphorylation of FoxO3a at Thr32 by Akt requires LKB1. When 2-DG inhibits glycolysis, it eventually leads to a decrease of intracellular ATP and an increase of intracellular AMP. In the presence of excess AMP, AMPK will undergo a conformational change on binding AMP, which allows LKB1 to access and phosphorylate the Thr32 of AMPK, the activation site of AMPK kinase activity (12, 13). Hence, the functional status of LKB1 in NSCLC cells could be monitored by examining the phosphorylation status of AMPK Thr32 after 2-DG treatment (Fig. 1A). As expected, 2-DG treatment augmented the phosphorylation of AMPK Thr32 only in LKB1 wild-type cells (H1299 and H1792) but not in LKB1-null cells (A549, H460, H157, and H23).

We recently discovered that 2-DG also activates Akt function via a mechanism that is PI3K dependent but LKB1 independent (14). A hyperphosphorylation of Akt can be detected as early as 15 min after 2-DG treatment. In fact, robust increases in Akt phosphorylation were observed in most NSCLC cell lines at 4 and 8 h after 2-DG treatment (Fig. 1A; ref. 14). The proapoptotic transcription factor FoxO3a is a downstream target of Akt kinase activity (15). Active FoxO3a promotes apoptosis through the transcription of...
Bim-1, a proapoptotic gene. Akt can suppress FoxO3a function via direct phosphorylation of three target sites on the molecule, including the Thr32 of FoxO3a, which has a consensus Akt target sequence (RPR5SpT; refs. 16, 17). Consistent with this, 2-DG treatment led to a subsequent increase in FoxO3a phosphorylation in the LKB1 wild-type H1299 and H1792 cells. Although a mild increase in FoxO3a phosphorylation at Thr32 was detected 4 h after 2-DG treatment, significant increases were not observed until 8 h after 2-DG treatment (Fig. 1A; ref. 14). Interestingly, Akt-mediated phosphorylation of FoxO3a (Thr25) was not detected in LKB1 mutants (A549, H460, H23, and H157). These data suggest that the induction of Thr32 phosphorylation of FoxO3a by Akt requires a functional LKB1.

To directly address the role of LKB1 in Akt-mediated phosphorylation of FoxO3a, we determined whether the transient depletion of LKB1 in LKB1 wild-type H1299 or HCT116 cells would attenuate FoxO3a phosphorylation in response to Akt activation. We used an LKB1 siRNA that was previously designed to transiently suppress the expression of LKB1 (14). Treatment with this LKB1 siRNA resulted in an 80% to 90% reduction in LKB1 protein, whereas, as expected, no reduction of LKB1 was detected with control siRNA (Fig. 1B, comparing lanes 3 and 4 with lanes 1 and 2 for H1299 and lanes 7 and 8 with lanes 5 and 6 for HCT116). The transient depletion of LKB1 did not alter the 2-DG–induced phosphorylation of Akt, consistent with our previous observation that 2-DG–induced Akt activation does not require LKB1 (14). The levels of 2-DG–induced FoxO3a phosphorylation 8 h after the addition of 25 mmol/L 2-DG, however, were significantly reduced in both H1299 and HCT116 cells when LKB1 expression was downregulated. These data showed that LKB1 is required for Thr32 phosphorylation of FoxO3a by Akt.

We also determined whether the restoration of LKB1 function in LKB1-null cells could reestablish Akt phosphorylation of the Thr32 of FoxO3a. We chose an adenovirus-based system to express wild-type GFP-LKB1 in LKB1-null H157 cells. Previously, we showed that this construct is capable of restoring the phosphorylation of AMPK (Thr172) under energetic stress condition (5). In our LKB1 restoration experiment, the GFP-LKB1 protein was detected only when our H157 cells were infected with adeno-LKB1 virus but not with a control adeno-GFP virus (Fig. 1C). It is known that adenovirus infection process activates Akt (18); therefore, we found that the total Akt protein levels remained the same and that infection with both the adeno-LKB1 and adeno-GFP viruses resulted in significant elevation of Akt phosphorylation at Ser473 (Fig. 1C, lanes 3–6). In contrast, significant elevation of FoxO3a phosphorylation at Thr32 was only observed in those cells infected with adeno-LKB1 virus (Fig. 1C, lanes 3 and 4) but not with adeno-GFP virus (Fig. 1C, lanes 5 and 6). Our data indicated that whereas adenoviral infection does induce Akt activation, the subsequent induction of FoxO3a phosphorylation at Thr32 required the presence of LKB1.

Figure 1. Phosphorylation of FoxO3a at Thr32 requires LKB1. A, an increase of FoxO3a, but not Akt phosphorylation by 2-DG treatment, is associated with the LKB1 gene status in human NSCLC cells. The indicated cell lines were treated with 25 mmol/L 2-DG for the given times, and then whole-cell protein lysates were prepared. The indicated proteins were detected by immunoblot analysis. In LKB1–null H1299 and HCT116 cells were transfected with control or LKB1 siRNA. Transfected cells were treated with 2-DG (25 mmol/L) for 8 h, and then whole-cell protein lysates were prepared and run by immunoblot analysis. C, activation of LKB1 in LKB1-null H157 cells. H157 cells were infected with adeno-GFP-LKB1 or adeno-GFP virus for 24 h, after which whole-cell protein lysates were prepared. The indicated proteins were detected by immunoblot analysis.

Depletion of LKB1 protein in H1650 cells leads to decreased cell proliferation. Considering our findings that LKB1 affected Akt-mediated phosphorylation of Thr32 of FoxO3a, we sought to determine the consequences of LKB1 knockdown in the context of Akt activation. H1650 has a constitutively high level of Akt phosphorylation due to the mutational activation of its epidermal growth factor receptor (EGFR), and its Akt phosphorylation cannot be further induced by 2-DG treatment (14). In contrast, 2-DG induces significant elevation of Akt phosphorylation in H1299 and H1703 cells, suggesting that these two cell lines do not have aberrantly activated Akt (Fig. 1A; data not shown). All three cell lines have wild-type LKB1, and we used LKB1shRNA lentivirus and puromycin to select for stable pools of cells with LKB1 depletion. The selection of stable H1650/LKB1shRNA cells took over a month, and we observed that the H1650/LKB1shRNA cells grew slowly and experienced extensive cell death. By comparison, puromycin

Cancer Res 2008; 68: (18). September 15, 2008 7272 www.aacrjournals.org
we carried out colony formation assays in H1650 and H1299 cells
contrast, transfection with LKB1shRNA plasmid in H1650 cells
includes 137 highly specific and well-characterized phospho-
status of both direct and indirect Akt targets. This antibody array
signaling pathway was used to compare the phosphorylation
targets, a phospho-specific antibody microarray for the Akt
whether LKB1 is required for the phosphorylation of other Akt
target sites that are involved in apoptosis. Our phospho-specific antibody microarray
context of Akt activation.

LKB1 depletion decreases phosphorylation of other direct
Akt target sites that are involved in apoptosis. To determine
whether LKB1 is required for the phosphorylation of other Akt
targets, a phospho-specific antibody microarray for the Akt
signaling pathway was used to compare the phosphorylation
status of both direct and indirect Akt targets. This antibody array
includes 137 highly specific and well-characterized phospho-
specific antibodies for proteins in the Akt pathway, each with six
replicates (raw data included in Supplementary Table S1). The
paired antibodies for the same (but unphosphorylated) target sites
are also included in the array to allow determination of the relative
level of phosphorylation. Because Akt is constitutively activated
in H1650 cells, a comparison between Akt target proteins from
H1650/LKB1shRNA and H1650/pLKO.1 cells enabled us to calculate
a ratio for any phosphorylation changes that are due to LKB1
depletion. Using a cutoff ratio of 0.8, we identified 21 sites that were
hypophosphorylated in H1650/LKB1shRNA cells compared with
H1650/pLKO.1 cells. Seven of the 21 sites contained the consensus
sequence for Akt phosphorylation and are direct targets of
Akt phosphorylation (Table 1). Therefore, the depletion of LKB1 in
H1650 cells not only resulted in a decrease in FoxO3a Thr32
phosphorylation but also attenuated the phosphorylation of other
direct Akt targets, such as Ask1 (Ser83), Bad (Ser136), FoxO1 (Ser199),
FoxO4 (Ser127), and GSK3β (Ser9). To validate the antibody array
results, we also directly tested the phosphorylation status of Bad
(Ser136) and GSK3β (Ser9) in the H1650/LKB1shRNA and H1650/
pLKO.1 cells. As with the array, hypophosphorylation of Bad
(Ser136) and GSK3β (Ser9) was observed in H1650/LKB1shRNA cells
(Fig. 3A). Therefore, we confirmed that depletion of LKB1 results
in decreases in the phosphorylation of multiple Akt targets.

Depletion of LKB1 in H1650 cells also resulted in hypophosph-
ylation of 12 serine/threonine sites and 2 tyrosine sites, all of
which were not known to be direct Akt targets. Of these, Ask1
(Ser866), Bad (Ser132 and Ser135), and Bcl-2 (Ser70 and Thr56) are
involved in apoptosis. Our phospho-specific antibody microarray
also evaluated eight phosphorylation sites on p53, but a change in
phosphorylation was only detected from residues 6 to 18, and no
alterations in phosphorylation were detected beyond Ser33. Ataxia-
telangiectasia mutated (ATM) can phosphorylate p53 at Ser15,
and emerging evidence indicates that LKB1 does physically interact
with ATM (19). Furthermore, LKB1 has been shown to directly
phosphorylate p53 at Ser15 in vitro (20). Therefore, these data

Figure 2. Depletion of LKB1 in H1650 cells attenuates
cell proliferation. A, LKB1 was depleted in H1650, H1299,
and H1703 cells, and stable pools of LKB1-depleted
cells were monitored for cell growth by SRB assay
using either parental cells or cells treated with pLKO.1
lentivirus as controls. Statistically significantly different
points were calculated using a two-sided Student’s t test
and marked by an asterisk. B, immunoblot analyses of
the LKB1 protein and phospho-Akt levels in H1650,
and H1703 cells was completed within 2 weeks. When stable cell pools were
analyzed for LKB1 protein expression by immunoblot, we found
that LKB1 protein expression was significantly suppressed in all
cell lines selected with LKB1shRNA lentivirus but not with the
control pLKO.1 virus (Fig. 2B). A SRB assay was used to evaluate
the effect of LKB1 on the rates of cell proliferation in all LKB1
knockdown cells (Fig. 2A). Whereas LKB1 knockdown had no effect
on H1299 or H1703 cell proliferation, the depletion of LKB1 in
H1650 cells significantly impeded cell growth (Fig. 2A). In addition,
we carried out colony formation assays in H1650 and H1299 cells
transfected with either the LKB1shRNA plasmid or a control pLKO.1
plasmid. Transfection with either plasmid in H1299 cells did not
result in significant differences in colony numbers (Fig. 2C). In contrast, transfection with LKB1shRNA plasmid in H1650 cells
significantly inhibited colony-forming ability, relative to the
transfection with the pLKO.1 plasmid. Because H1650 cells are
sensitive to Lipofectamine 2000 transfection, we also verify our
findings using LKB1shRNA or pLKO.1 lentivirus in colony formation
assays. Similarly, infection of H1650 cells with LKB1shRNA lentivirus
significantly attenuated cell proliferation compared with infection
with pLKO.1 lentivirus (Supplementary Fig. S1). Therefore,
although down-regulation of LKB1 expression did not
alter cell proliferation in H1299 and H1703 cells, it did result in a decrease
in cell proliferation, specifically in the H1650 cells. In summary, we
found that LKB1 depletion decreased cell proliferation only in the
context of Akt activation.
indicatethatLKB1maybeinvolvedinthephosphorylationofp53 at the NH2 terminus in vivo as well as phosphorylation of apoptosis-related genes. Depletion of LKB1 also resulted in hyperphosphorylation of several proteins. Using a cutoff ratio of >1.2, we identified 12 sites with increased phosphorylation. Loss of LKB1 resulted in hyperphosphorylation of several direct and indirect targets of mTOR, including 4E-BP1 (Thr45), IRS-1 (Ser639), S6 kinase (Ser424), and S6 (Ser235). In addition, eIF4E is also involved in the mTOR-related translation process (Table 1). Because Akt and LKB1 play opposite roles in regulating mTOR kinase activity, it was expected that the depletion of LKB1 in Akt-activated H1650 cells would lead to increases in the phosphorylation of these mTOR targets. These observations of variability in phosphorylation levels induced by changes in LKB1 provided independent internal control for the validity of our antibody microarray data set.

LKB1 depletion is associated with an increase in G1 cell cycle arrest and apoptosis in H1650 cells. Because the depletion of LKB1 in H1650 cell suppresses cell proliferation and LKB1 is required for Akt-mediated phosphorylation of apoptotic proteins, we sought to further characterize the observed cell growth phenotype in H1650 cells. We first carried out a cell cycle analysis in H1650/LKB1shRNA cells. We analyzed only the live cells that remained attached to tissue culture plates 4 days after seeding the cells. Compared with control H1650/pLKO.1 cells, the H1650/LKB1shRNA cells displayed an increase in the percentage of cells in G1 phase, indicating that LKB1 may be involved in the phosphorylation of p53 at the NH2 terminus in vivo as well as phosphorylation of apoptosis-related genes.

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### Table 1. Alteration of protein phosphorylation in Akt signaling pathway due to LKB1 depletion in H1650 cells

<table>
<thead>
<tr>
<th>Phosphorylation site</th>
<th>Ratio</th>
<th>Kinase involved</th>
<th>Biological effects</th>
<th>Supplementary References*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFX/FoxO4 (phospho-Ser297)</td>
<td>0.77</td>
<td>RRRAApSMDSS</td>
<td>Akt</td>
<td>Cell survival/apoptosis (1)</td>
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<td>ASK1 (phospho-Ser80)</td>
<td>0.71</td>
<td>RGRGSpSVDGG</td>
<td>Akt</td>
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<td>YLRLSpSLPV</td>
<td>Unknown</td>
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<td>Bad (phospho-Ser112)</td>
<td>0.58</td>
<td>RSRHSpSYPAG</td>
<td>p90RSK, PKA</td>
<td>Cell survival/apoptosis (4)</td>
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<td>Bad (phospho-Ser286)</td>
<td>0.67</td>
<td>RGRSpSAPPN</td>
<td>Akt</td>
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<td>Bad (phospho-Ser289)</td>
<td>0.64</td>
<td>ELRRMpsDEFV</td>
<td>PKA</td>
<td>Cell survival/apoptosis (6, 7)</td>
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<tr>
<td>Bcl-2 (phospho-Ser37)</td>
<td>0.61</td>
<td>PIVAPSpPLQT</td>
<td>II-3, JNK</td>
<td>Cell survival/apoptosis (8)</td>
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<tr>
<td>Bcl-2 (phospho-Thr45)</td>
<td>0.66</td>
<td>SQPGHspTPHHA</td>
<td>Unknown</td>
<td>Cell survival/apoptosis (9)</td>
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<tr>
<td>Bcl-3 (phospho-Ser37)</td>
<td>0.68</td>
<td>WLLDspSPAVN</td>
<td>JNK</td>
<td>Cell survival/apoptosis (10)</td>
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<tr>
<td>c-Jun (phospho-Ser286)</td>
<td>0.68</td>
<td>SDLTspSVPDV</td>
<td>Unknown</td>
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<tr>
<td>FAK (phospho-Tyr961)</td>
<td>0.78</td>
<td>NPQHpSVPVG</td>
<td>v-Src</td>
<td>FAK signaling (12)</td>
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<tr>
<td>FKHR/FoxO1 (phospho-Ser179)</td>
<td>0.78</td>
<td>BPRTSpSNAST</td>
<td>Akt</td>
<td>Cell survival/apoptosis (13)</td>
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<tr>
<td>FKHRL1/FoxO3a (phospho-Ser253)</td>
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<td>RRRAApSMDNS</td>
<td>Akt</td>
<td>Cell survival/apoptosis (14)</td>
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<tr>
<td>GSK3β (phospho-Ser9)</td>
<td>0.77</td>
<td>RpRTSpSFAES</td>
<td>Akt</td>
<td>Cell survival/apoptosis (15)</td>
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<td>IKKα (phospho-Thr21)</td>
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<td>RERLSpTFGFG</td>
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<td>NF-κB signaling (16)</td>
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<td>p53 (phospho-Ser15)</td>
<td>0.78</td>
<td>VEPQspsUTEF</td>
<td>ATM, ATR, DNA-PK</td>
<td>DNA damage (19, 20)</td>
</tr>
<tr>
<td>p53 (phospho-Thr18)</td>
<td>0.66</td>
<td>PLLQspFTSDL</td>
<td>Casein kinase 1</td>
<td>DNA damage (20)</td>
</tr>
<tr>
<td>Paxillin (phospho-Tyr118)</td>
<td>0.78</td>
<td>EEHVspYSFPN</td>
<td>Focal adhesion kinase</td>
<td>FAK signaling (21)</td>
</tr>
<tr>
<td>4E-BP1 (phospho-Thr45)</td>
<td>1.3</td>
<td>TLFSstpTPGGT</td>
<td>mTOR</td>
<td>mTOR-related translation (22)</td>
</tr>
<tr>
<td>c-Jun (phospho-Ser7)</td>
<td>2.2</td>
<td>LLKLApSPELE</td>
<td>Stress kinases</td>
<td>Cell survival/apoptosis (11)</td>
</tr>
<tr>
<td>c-Ki (phospho-Tyr213)</td>
<td>1.3</td>
<td>STNepYMDDM</td>
<td>Unknown</td>
<td>Activating PI3K (23)</td>
</tr>
<tr>
<td>elf4E (phospho-Ser221)</td>
<td>1.3</td>
<td>AKTSspSTTKN</td>
<td>Mnk1</td>
<td>mTOR-related translation (24, 25)</td>
</tr>
<tr>
<td>Gab1 (phospho-Tyr422)</td>
<td>1.7</td>
<td>KQVEpYLDLD</td>
<td>Unknown</td>
<td>C-Met signaling (26)</td>
</tr>
<tr>
<td>IKKα (phospho-Thr45)</td>
<td>1.5</td>
<td>KDELpYEQMV</td>
<td>Unknown</td>
<td>NF-κB signaling (27, 28)</td>
</tr>
<tr>
<td>IRS-1 (phospho-Ser286)</td>
<td>1.4</td>
<td>RSREspSTAT</td>
<td>JNK, IKK</td>
<td>Insulin signaling (29)</td>
</tr>
<tr>
<td>IRS-1 (phospho-Ser629)</td>
<td>7.0</td>
<td>PMSKpsVSAP</td>
<td>mTOR</td>
<td>Insulin signaling (29)</td>
</tr>
<tr>
<td>Met (phospho-Tyr130)</td>
<td>1.5</td>
<td>IGEHpYVHVN</td>
<td>Unknown</td>
<td>C-Met signaling (30)</td>
</tr>
<tr>
<td>p70S6 kinase (phospho-Ser244)</td>
<td>1.2</td>
<td>PRTVrsPSVKF</td>
<td>mTOR</td>
<td>mTOR-related translation (31, 32)</td>
</tr>
<tr>
<td>PDK1 (phospho-Ser411)</td>
<td>2.1</td>
<td>ARANspSVFGT</td>
<td>Autophosphorylation</td>
<td>Activating PI3K (33)</td>
</tr>
<tr>
<td>S6 (phospho-Ser235)</td>
<td>1.3</td>
<td>RRLpSSLRA</td>
<td>S6K</td>
<td>mTOR-related translation (34, 35)</td>
</tr>
</tbody>
</table>

NOTE: Ser256 of FKHR was excluded from the analysis because the replicates of FKHR (Ab-256) have a large SD. Abbreviations: PKA, protein kinase A; II-3, interleukin-3; JNK, c-Jun NH2-terminal kinase; ATR, ATM and Rad3-related; DNA-PK, DNA-dependent protein kinase; NF-κB, nuclear factor-κB.

*References are included in Supplementary Data.
G1 phase (from 47% to 61%) but a decrease of those in S phase (from 20% to 11%) and G2-M phase (from 21% to 14%; Fig. 3A). The change in cell cycle profile did not seem to be related to p21, as similar levels of p21 protein were observed in both the control and LKB1 knockdown cells (Fig. 3A). On the other hand, we did observe an increase in p27 and a decrease in cyclin D1 in the H1650/LKB1 shRNA cells, which suggested that the increase in G1 cells may have been related to p27-mediated cell cycle arrest.

For apoptosis assays, both the floating and attached cells were collected and analyzed 4 days after cell seeding. Approximately 17% of H1650/LKB1 shRNA cells underwent apoptosis. In stark contrast, only 0.7% of H1650/pLKO.1 cells were apoptotic (Fig. 3C). The appearance of apoptotic cells also correlated with a significant increase in caspase-3 cleavage 4 days after cell seeding (Fig. 3A). Consistent with LKB1 transient depletion analysis in Fig. 1, the stable down-regulation of LKB1 protein expression in H1650 cells was correlated with a decrease in the phosphorylation of FoxO3a at Thr32 (Fig. 3A). Unphosphorylated FoxO3a translocates to the nucleus, acting as a transcription factor to increase Bim-1 transcription (21). Indeed, we did observe an increase in total Bim-1 protein in the H1650/LKB1 shRNA cells. The activation of caspase-3 cleavage, however, was not detectable early on, only 4 or 5 days after cell seeding (data not shown).

LKB1 depletion in other NSCLC cells with aberrant Akt activation also results in increases in caspase-3 cleavage. Our data suggest that LKB1 depletion induces caspase-3 cleavage and apoptosis but only in the context of aberrant Akt activation. To determine whether this was true in other cell lines, we also depleted LKB1 in HCC827 and H520 cells. The HCC827 NSCLC cell line harbors an exon 19 deletion (DelE746A750) of EGFR, which results in the constitutive activation of Akt (22). We generated LKB1 stable knockdown pools in this cell line. Consistent with results found for H1650 cells, we detected significant caspase-3 cleavage 5 days after seeding the cells (Fig. 3D). Colony formation assay with LKB1 shRNA plasmid in HCC827 cells also had fewer colonies than the control pLKO.1 plasmid (Supplementary Fig. S2). H520 cells have a somatic amplification of the PIK3CA gene (23), and we previously showed that this amplification results in elevated Akt phosphorylation (14). LKB1 stable knockdown pools were generated for H520; LKB1 depletion also led to significant caspase-3 cleavage 5 days after seeding (Fig. 3D). In addition, SRB analysis indicated that the depletion of LKB1 in H520 cells significantly impeded cell growth compared with either the parental cells or H520/pLKO.1 cells (Supplementary Fig. S3). In contrast, caspase-3 cleavage was never detected in the H1299/LKB1 shRNA and H1703/LKB1 shRNA cells (data not shown). These
combined data suggested that the depletion of LKB1 in NSCLC cell lines that have aberrantly activated Akt promoted apoptosis, indicating that LKB1 is required for Akt-mediated phosphorylation of proapoptotic proteins and that the depletion of LKB1 induces apoptosis in the context of aberrant Akt activation.

**Discussion**

To date, **LKB1** has been considered a tumor suppressor because the hereditary and somatic loss of function mutations of this gene are associated with an increased risk of cancer development. This idea was further supported by functional data, as it was found that the restoration of LKB1 function in LKB1-null cells leads to either apoptosis or cell cycle arrest (24, 25). Current analysis of LKB1 function has focused on its regulation of AMPK and mTOR signaling. Because LKB1/AMPK signaling inhibits mTOR, but activated Akt stimulates mTOR activity, LKB1 and Akt are thought to play opposing roles with regard to mTOR regulation (7, 8). Here, we show that LKB1 is necessary for cancer cell survival in the context of aberrant Akt activation.

The aberrant activation of Akt occurs frequently in human cancers; thus, activated Akt is thought to contribute to tumor formation partly by preventing apoptosis. Activated Akt blocks apoptosis through the phosphorylation and inactivation of the FoxO transcription factors Ask1, Bad, and GSK3β (15, 26). For example, FoxO3α is a transcription factor that can promote apoptosis by activating the transcription of *Bim*-1, a proapoptotic gene. Akt inhibits FoxO3α function by phosphorylating FoxO3α on Thr32, Ser253, and Ser315, which results in cytoplasmic retention of FoxO3α (27). We carried out a detailed analysis on Thr32 phosphorylation of FoxO3α and showed that up-regulation of FoxO3α phosphorylation at Thr32 by Akt required the function of wild-type LKB1. The necessity of LKB1 in this phosphorylation process was supported by four lines of evidence. First, we had previously shown that 2-DG can activate Akt via PI3K and that 2-DG–induced Akt activation leads to subsequent FoxO3α phosphorylation at Thr32 in LKB1 wild-type cells (14). Concordant increases in Akt and FoxO3α phosphorylation levels, however, were not observed in four LKB1-null NSCLC cell lines (Fig. 1A). In addition, we showed that transient depletion of LKB1 by RNA interference in LKB1 wild-type H1299 or HCT116 cells did not affect 2-DG–induced Akt phosphorylation; it led instead to a decrease in 2-DG–induced phosphorylation of FoxO3α (Fig. 1B). Third, adenovirus infection, which induces Akt phosphorylation, only augmented FoxO3α phosphorylation in the LKB1-null cells that ectopically expressed LKB1 (Fig. 1C). Fourth, the stable depletion of LKB1 in H1650 cells with aberrantly activated Akt also resulted in the down-regulation of FoxO3α phosphorylation (Fig. 3A). Therefore, LKB1 was found to be necessary for Akt-mediated FoxO3α phosphorylation at Thr32. This is a novel finding because it provides the first evidence that LKB1 and Akt play cooperative roles with regard to the phosphorylation of FoxO3α on Thr32.

Our antibody microarray analysis indicated that the requirement for LKB1 in the phosphorylation of Akt targets was not limited to a single Akt target. On the contrary, the depletion of LKB1 in H1650 cells resulted in decreases in the phosphorylation of Ask1 (Ser85), Bad (Ser136), FoxO1 (Ser253), FoxO4 (Ser197), and GSK3β (Ser9). The transduction of these substrates, whose biological functions have been poorly studied to date (28), is expected to be important to determine in the future whether LKB1 or its downstream target(s) directly participates in the phosphorylation of Akt substrates.

Because Akt mediates its antiapoptotic activity through the phosphorylation of these apoptosis-promoting molecules, the depletion of LKB1 should promote apoptosis in cancer cells with aberrantly activated Akt function. Indeed, the depletion of LKB1 enhanced caspase-3 cleavage in three NSCLC cell lines (H1650, HCC827, and H520) with activated Akt functions (Fig. 3). However, LKB1 depletion did not alter the growth characteristics of two other NSCLC cell lines (H1299 and H1703) that were without Akt activation (Fig. 2). Therefore, LKB1 depletion only enhanced apoptosis in those cancer cells with aberrant Akt activation. Interestingly, the depletion of LKB1 in Akt-activating cells did not result in caspase-3 cleavage immediately after cell seeding; it took several days.

If active LKB1 is required for the phosphorylation of Akt targets that are involved in apoptosis, the inactivation of LKB1 will not provide a growth advantage to cancer cells with preexisting Akt activation. Consequently, a *LKB1* mutation should not be naturally selected for in cancer cells with aberrantly active Akt function. Oncogenic activation of Akt occurs through multiple mechanisms, including mutational activation of PI3K, mutational inactivation of PTEN phosphatase, or gene amplification of Akt itself (26), and is frequently observed in most solid tumor types except NSCLCs (29–33). In contrast, although somatic *LKB1* mutations frequently occur in NSCLC, they are rarely observed in the other major tumor types (34–38). We suspect that the cooperation between Akt and LKB1 in the phosphorylation of these proapoptotic genes may be a reason that somatic *LKB1* mutations are rarely observed in brain, breast, and colorectal tumors. These tumors are likely to have a high frequency of PI3K activations, PTEN deletions, or Akt amplifications. Because most of our studies were carried out in NSCLC cancer cell lines, our observation will require further evaluation in other cancer types.

In summary, we discovered that LKB1 is required for the phosphorylation of proapoptotic proteins by Akt. This is the first evidence that LKB1 plays a potentially oncogenic role in cells having activated Akt. Our data suggest that the mutational inactivation of LKB1 may not facilitate oncogenic transformation mediated by aberrant Akt activation.

**Disclosure of Potential Conflicts of Interest**

The authors declare no conflicts of interest.

**Acknowledgments**

Received 4/21/2008; revised 7/28/2008; accepted 8/5/2008.

Grant support: National Cancer Institute grants P01 CA116676-03 (W. Zhou, P.M. Vertino), P01 CA116676-01A1 (F.R. Khuri), and RO1 CA118470-01 (S-Y. Sun). W. Zhou, F.R. Khuri, P.M. Vertino, and S-Y. Sun are Georgia Cancer Coalition Distinguished Cancer Scholars. W. Zhou is an American Cancer Society Research Scholar.

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We thank Dr. Yaping Zong (Full Moon BioSystems, Inc.) for his help with phospho-specific protein microarray analysis.
References

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