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Tumor Suppressor LKB1 Inhibits Activation of Signal Transducer and Activator of Transcription 3 (STAT3) by Thyroid Oncogenic Tyrosine Kinase Rearranged in Transformation (RET)/Papillary Thyroid Carcinoma (PTC)

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The tumor suppressor LKB1 (STK11) is a cytoplasmic/nuclear serine/threonine kinase, defects in which cause Peutz-Jeghers syndrome (PJS) in humans and animals. Recent studies showed that loss of function of LKB1 is associated with sporadic forms of lung, pancreatic, and ovarian cancer. In cancer cells, LKB1 is inactivated by two mechanisms: mutations in its central kinase domain or complete loss of LKB1 expression. Inactivation of LKB1 is associated with progression of PJS and transformation of benign polyps into malignant tumors. This study examines the effect of LKB1 on regulation of STAT3 and expression of transcriptional targets of STAT3. The results show that LKB1 inhibits rearranged in transformation (RET)/papillary thyroid carcinoma (PTC)-dependent activation of signal transducer and activator of transcription 3 (STAT3), which is mediated by phosphorylation of STAT3 tyrosine 705 by RET/PTC. Suppression of STAT3 transactivation by LKB1 requires the kinase domain but not the kinase activity of LKB1. The centrally located kinase domain of LKB1 is an approximately 260-amino-acid region that binds to the linker domain of STAT3. Chromatin immunoprecipitation studies indicate that expression of LKB1 reduces the binding of STAT3 to its target promoters and suppresses STAT3-mediated expression of Cyclin D1, VEGF, and Bcl-xL. Knockdown of LKB1 by specific small interfering RNA led to an increase in STAT3 transactivation activity and promoted cell proliferation in the presence of RET/PTC. Thus, this study suggests that LKB1 suppresses tumor growth by inhibiting RET/PTC-dependent activation of oncogenic STAT3. (*Molecular Endocrinology* 21: 3039–3049, 2007)

THE SERINE/THREONINE KINASE LKB1 is a tumor suppressor, defects in which cause Peutz-Jeghers syndrome (PJS), a rare autosomal dominant human disease characterized by intestinal polyps and a strong predisposition to malignant tumors of the stomach, intestine, and colon (1, 2). Recent studies demonstrated that defects in LKB1 are also associated with sporadic lung, pancreatic, and ovarian cancer (1, 3, 4).

Wild-type but not kinase-inactive LKB1 inhibits cell cycle progression at G1/S by inducing $p21^{WAF1/CIP1}$ or via association with p53 (5–7), suggesting that the kinase activity of LKB1 is required for its tumor suppressor function (6–8). However, both wild-type and kinase-inactive LKB1 regulate Brg1, an ATPase that plays a role in chromatin remodeling and cell growth (8), suggesting that some functional roles of LKB1 may not require its kinase activity. LKB1-null mice exhibit lethal vascular abnormalities (9), but it is unclear at present whether these abnormalities are caused solely by loss of the LKB1 kinase activity or due to loss of another function of LKB1.

Gene expression studies in LKB1-null fibroblasts show multiple changes, including increased expres-

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Abbreviations: ChIP, Chromatin immunoprecipitation; DTT, dithiothreitol; GST, glutathione *S*-transferase; IP, immunoprecipitation; MEF, mouse embryo fibroblasts; OSM, oncostatin M; PJS, Peutz-Jeghers syndrome; PTC, papillary thyroid carcinoma; RET, rearranged in transformation; STAT3, signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor.

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sion of matrix metalloproteinase 2 (*MMP2*), matrix metalloproteinase 9 (*MMP9*), vascular endothelial growth factor (*VEGF*), IGF-binding protein 5 (*IGFBP5*), and prostaglandin-endoperoxide synthase 2 (*COX-2*) (10, 11). Interestingly, these genes are all regulated by signal transducer and activator of transcription 3 (STAT3) and play roles in cell growth, tumorigenesis, and angiogenesis (12–16). STAT3 is a latent cytoplasmic transcription factor that performs a variety of functions in regulating cell growth, inflammation (17), and early embryonic development (18). STAT3 is frequently constitutively activated in cancer cells (19, 20), and it is classified as an oncogene. Expression of a constitutively active dimer of STAT3 transforms cultured cells and promotes tumor formation in nude mice (21). Several nonreceptor tyrosine kinases that activate STAT3 stimulate malignant transformation of cultured cells (22–24). In addition, the constitutively active rearranged in transformation (RET)/papillary thyroid carcinoma (PTC) tyrosine kinase found in PTC cells phosphorylates tyrosine 705 of STAT3 and promotes dimerization and translocation and stimulates transac-

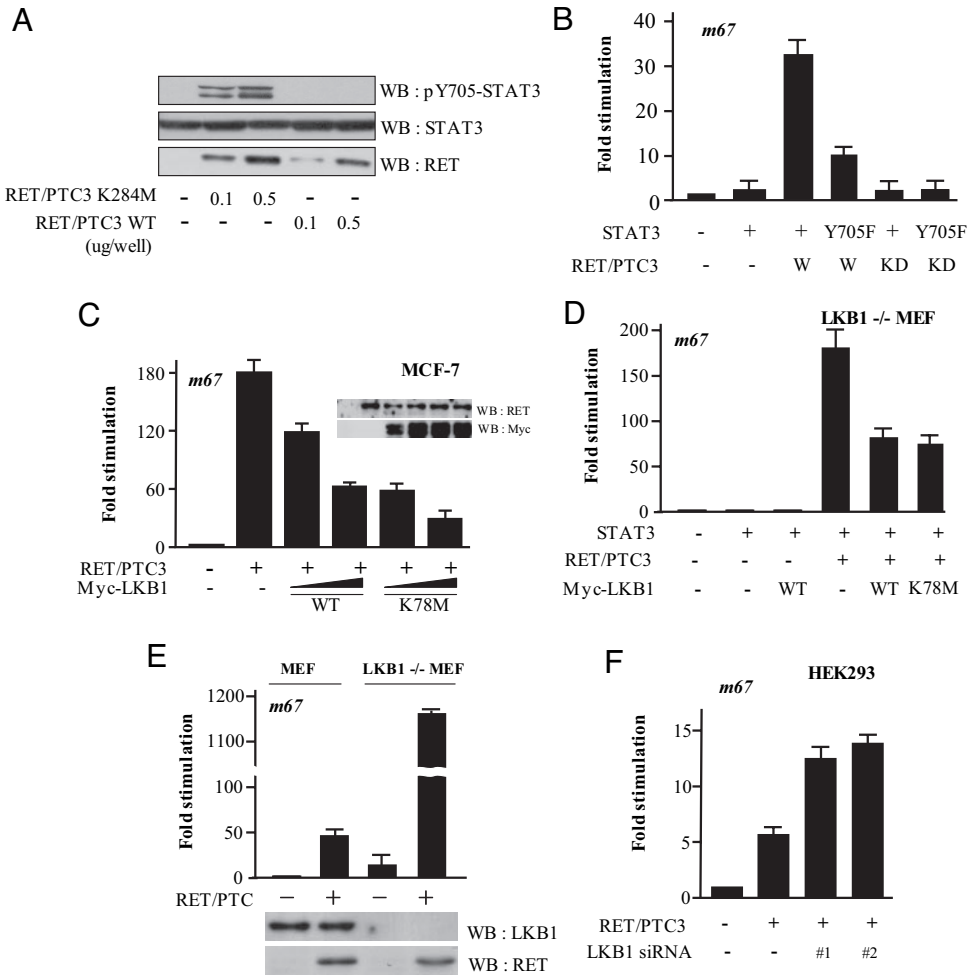

Fig. 1. RET/PTC3 Phosphorylates and Enhances the Transcriptional Activity of STAT3

A, RET/PTC3 phosphorylates STAT3 Y705. ARO cells were transiently transfected with 0.1 or 0.5 μg plasmids expressing RET/PTC3 (wild-type) or RET/PTC3-K284M (kinase-deficient). Cells were lysed 24 h after transfection and extracts immunoblotted with a phospho-specific STAT3 antibody (anti-pY705-STAT3). Total STAT3 and RET/PTC3 protein levels were determined using the antibodies indicated. WB, Western blot. B, RET/PTC3 phosphorylates the STAT3 Y705 residue and induces transcriptional activity. NIH3T3 cells were transiently cotransfected with 0.1 μg of the indicated plasmids. Luciferase activity was measured as described in *Materials and Methods* as an indicator of STAT3 activity on the m67 promoter. KD, Kinase deficient; W, wild type. C, RET/PTC3-mediated STAT3 transcriptional activity is decreased by wild-type (WT) and kinase-deficient LKB1. MCF-7 cells were transiently cotransfected with 0.1 μg of the following plasmids, as indicated: *m*67-Luc alone or *m*67-Luc and plasmids expressing RET/PTC3 and Myc-LKB1 or Myc-LKB1-K78M (0.2 or 0.5 μg each). D, Exogenous expression of LKB1 suppresses activation of STAT3 in LKB1$^{-/-}$ MEF. LKB1$^{-/-}$ MEF were transiently cotransfected with 0.1 μg of the following plasmids, as indicated: *m*67-Luc and plasmids expressing STAT3, RET/PTC3, and Myc-LKB1 or Myc-LKB1-K78M. E, RET/PTC-dependent activation of STAT3 in LKB1$^{-/-}$ MEF cells. LKB1$^{-/-}$ MEF and MEF cells were transfected with 0.5 μg RET/PTC3. F, Effect of down-regulation of LKB1 on activation of STAT3. HEK293 cells were transiently cotransfected with plasmid expressing RET/PTC3 (0.1 μg) and 20 nM *LKB1* siRNA.

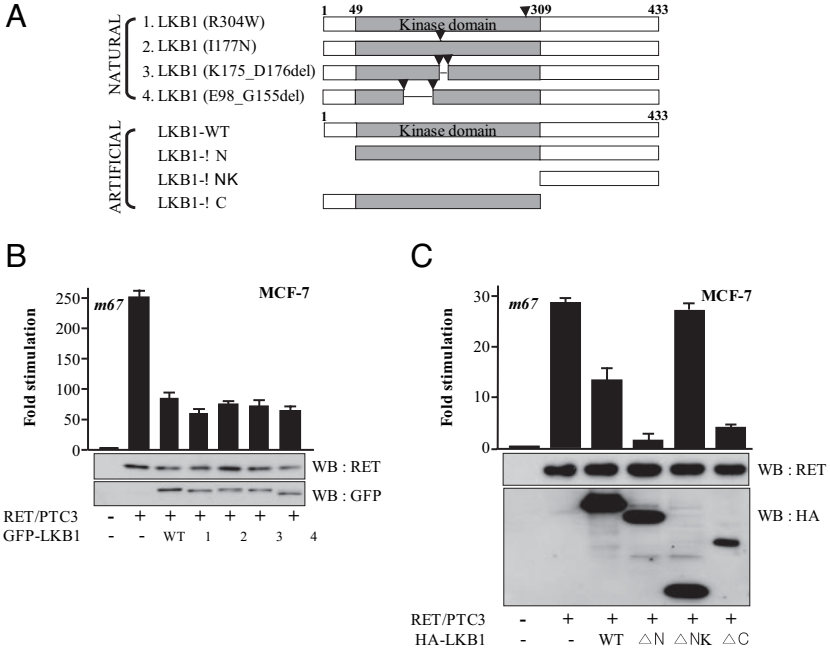

Fig. 2. Wild-Type and Kinase-Deficient Forms of LKB1 Suppress STAT3-Mediated Transcriptional Activity

A, Schematic diagram of constructs expressing truncated forms of LKB1. B and C, The LKB1 kinase domain is critical for suppression of STAT3 transcriptional activity. MCF-7 cells were transiently cotransfected with 0.1 μg of the following plasmids, as indicated: *m*67-Luc and plasmids expressing naturally occurring LKB1 kinase-deficient mutants (1–4; B) or recombinant LKB1 deletion mutants (LKB1-ΔN, LKB1-ΔNK, and LKB1-ΔC; C). Luciferase activity was measured as described in *Materials and Methods*. WB, Western blot; WT, wild type.

tivation activity of STAT3. Cells expressing RET/PTC, or RET/PTC-activated STAT3, exhibit elevated rates of transformation, proliferation, migration, and invasion (23). Taken together, these findings suggest that the tumor suppressor function of LKB1 may be mediated by its ability to suppress RET/PTC-dependent activation of STAT3 and STAT3-mediated oncogenesis.

RESULTS

LKB1 Suppresses RET/PTC-Dependent Activation of STAT3

Previous studies showed that RET/PTC is a constitutively activated tyrosine kinase expressed in papillary thyroid cancer cells (25) that phosphorylates STAT3 Y705, thus stimulating dimerization of STAT3 and expression of its downstream transcriptional targets (23). These observations were confirmed in ARO cells transfected with plasmids expressing wild-type or kinase-deficient K284M RET/PTC. As expected, wild-type RET/PTC3 induced phosphorylation of endogenous STAT3 Y705, whereas the kinase-deficient mutant of RET/PTC3 did not (Fig. 1A). Using a luciferase reporter gene linked to the STAT3-regulated m67 promoter (*m67-Luc*), it was also confirmed that the transcriptional activity of STAT3 increased in cells transfected with wild-type RET/PTC3 but not in cells transfected with RET/PTC3-K284M (Fig. 1B, lanes 3 and 5). Transcriptional activation of STAT3 required Y705, because the luciferase reporter gene activity did not increase when cells were cotransfected with non-phosphorylatable STAT3-Y705F (Fig. 1B, lane 4). These observations confirm that RET/PTC3 activates STAT3 via phosphorylation of Y705 and show that activation of STAT3 can be monitored in transiently transfected cells with the *m67-Luc* reporter plasmid.

The ability of LKB1 to regulate activation of STAT3 was examined in transiently transfected MCF-7 cells carrying the *m67-Luc* reporter plasmid. MCF-7 cells were transiently transfected with plasmids expressing RET/PTC3 (Fig. 1C) and STAT3, in the absence or presence of vectors expressing Myc-tagged LKB1. Expression of wild-type LKB1 suppressed RET/PTC3-dependent activation of STAT3. Unexpectedly, Myc-LKB1-K78M, which lacks serine/threonine kinase activity, also suppressed activation of STAT3 (Fig. 1C). Similarly, induction of luciferase activity stimulated by IL-6, STAT3c (the constitutively active form of STAT3), and TEL-JAK2 (26) was equivalently reduced by both wild-type and kinase-deficient Myc-LKB1 proteins (data not shown).

Because MCF-7 cells express endogenous LKB1, the above experiments were repeated in LKB1$^{-/-}$ mouse embryo fibroblasts (MEF) (10) (Fig. 1D) and in the LKB1-deficient cell line NPA (data not shown). The results confirmed our previous results, indicating that

wild-type LKB1 and Myc-LKB1-K78M suppressed RET/PTC-dependent activation of STAT3 in LKB1$^{-/-}$ MEF (Fig. 1D). In addition, RET/PTC-dependent activation of STAT3 was significantly higher in LKB1$^{-/-}$ MEF than in wild-type MEF (Fig. 1E), and small interfering RNA (siRNA)-mediated down-regulation of LKB1-stimulated transcriptional transactivation by STAT3 (Fig. 1F). These results indicate that LKB1 negatively regulates RET/PTC-dependent activation of STAT3 and that this effect does not require LKB1 kinase activity.

The Kinase Domain of LKB1 Is Required to Suppress Activation of STAT

Several point mutations and deletions in LKB1 mutants have been implicated in PJS (27). The effect of these LKB1 mutants, all of which lack kinase activity, on STAT3 transactivation was determined using the reporter gene system described above (Fig. 2A, 1–4). All of these naturally occurring LKB1 mutants suppressed activation of STAT3 (Fig. 2B). Similar experiments were then carried out using two N-terminally truncated (LKB1-ΔN and LKB1-ΔNK) forms and one C-terminally truncated (LKB1-ΔC) form of recombinant LKB1. LKB1-ΔN and LKB1-ΔC retain the kinase domain and inhibit activation of STAT3 (Fig. 2C), whereas LKB1-ΔNK, which lacks the N-terminal and kinase domains, did not inhibit activation of STAT3. These findings suggest that the kinase domain, but not the kinase activity, of LKB1 is required to suppress RET/PTC3-dependent activation of STAT3.

LKB1-Mediated Suppression of STAT3 Transcriptional Activity Is Independent of STAT3 Phosphorylation

Dual phosphorylation of the residues Y705 and S727 is required for STAT3 dimerization, nuclear translocation, and binding of STAT3 to its target promoters (21). In NIH3T3 cells transiently transfected with STAT3 Y705F, RET/PTC3-induced activation of STAT3 was not observed (Fig. 3A). Although STAT3-dependent reporter gene activity was observed in the presence of STAT3-Y705F after induction of RET/PTC3 in these cells, this was due to activation of endogenous (wild type) STAT3. In cells expressing RET/PTC3 and STAT3 or STAT3-Y705F, wild-type and kinase-deficient LKB1 suppressed activation of STAT3. In cells expressing RET/PTC and STAT3-S727A, somewhat reduced but still significant levels of STAT3 activity were induced by RET/PTC, and this activity was suppressed by both wild-type and kinase-deficient LKB1. Thus, the effect of LKB1 on RET/PTC-dependent activation of STAT3

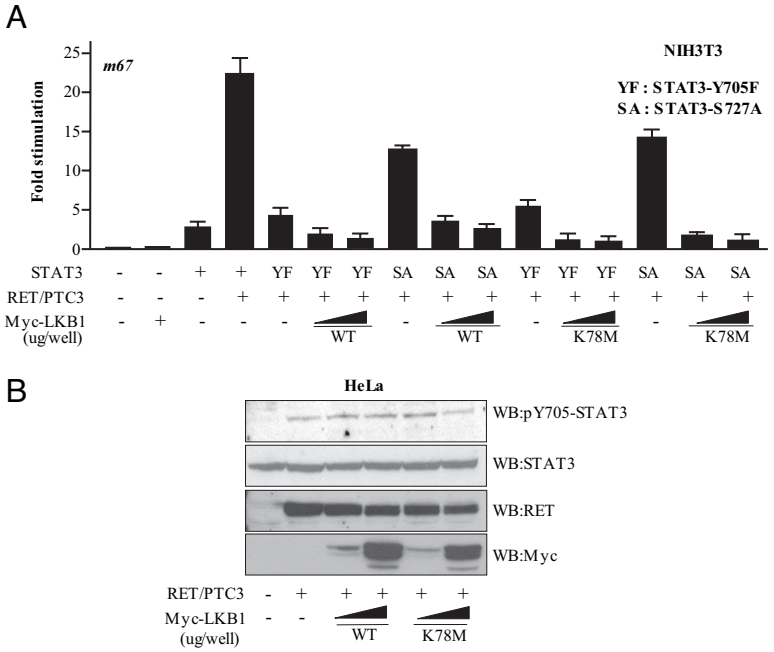

Fig. 3. LKB1 Suppression of STAT3 Transcriptional Activity Is Independent of STAT3 Phosphorylation Status

A, NIH3T3 cells were transiently cotransfected with 0.1 μg *m*67-Luc and plasmids expressing STAT3-Y705F (YF) or STAT3-S727A (SA) as well as 0.1 or 0.2 μg plasmids expressing Myc-LKB1 or Myc-LKB1-K78M. Cells were lysed and luciferase activity measured. B, LKB1 does not affect STAT3 tyrosine phosphorylation. HeLa cells were transiently cotransfected with plasmids expressing RET/PTC3 (0.5 μg) and Myc-LKB1 or Myc-LKB1-K78M (0.3 or 1.0 μg). Cells were lysed and extracts were immunoblotted with anti-pY705-STAT3 antibody to detect endogenous STAT3 phosphorylation levels. Anti-STAT3, anti-RET, and anti-Myc antibodies were used to detect total STAT3, RET/PTC3, and LKB1 levels, respectively. WB, Western blot; WT, wild type.

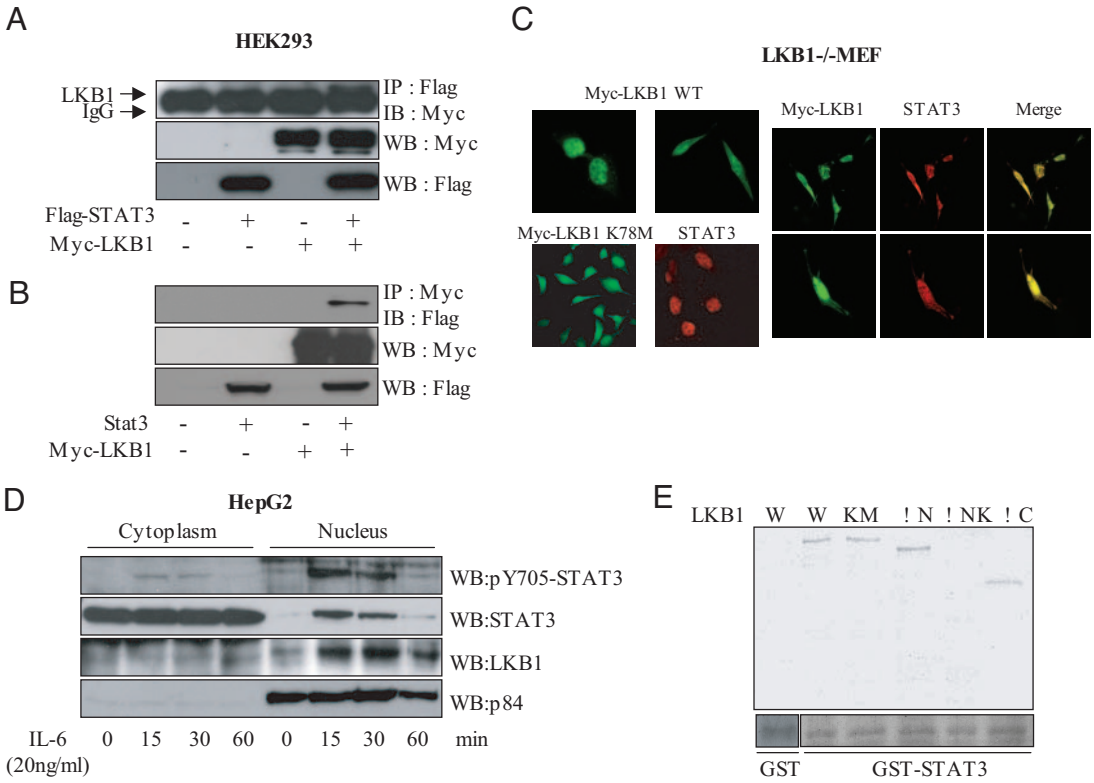

Fig. 4. The Kinase Domain of LKB1 Interacts with the Linker (LK) Domain of STAT3

A and B, LKB1 binds STAT3. HEK293 cells were grown in 60 mm dishes and transiently cotransfected with plasmid expressing Flag-STAT3 (1 μg), Myc-LKB1 (1 μg), or control vector (2 μg), after which proteins (500 μg) were immunoprecipitated with anti-Flag (A) and anti-Myc (B) antibodies and separated by SDS-PAGE followed by immunoblotting. C, Colocalization of LKB1 and STAT3 to the nucleus. LKB1$^{-/-}$ MEF cells were grown on curved glass and transiently transfected with plasmids expressing Myc-LKB1 or Myc-LKB1-K78M. Cells were fixed and observed by laser scanning confocal microscopy. Myc-LKB1-WT, Myc-LKB1-K78M, and STAT3 were detected by indirect immunostaining with anti-Myc or anti-STAT3 antibodies, respectively. D, IL-6 induces the translocation of LKB1 and STAT3 to the nucleus. HepG2 cells were serum starved for 12 h and then treated with IL-6 (20 ng/ml) for the indicated amount of time. Cells were lysed and fractionated. Anti-p84 antibody was used to identify the nuclear fraction. E, The Promega TNT-coupled transcription-translation system was used to produce [^{35}S]methionine-labeled LKB1-WT, LKB1-K78M(KM), LKB1-ΔN(ΔN), LKB1-ΔNK(ΔNK), and LKB1-ΔC(ΔC). For the binding assays, 20 μl *in vitro*-translated LKB1-WT, LKB1-K78M, or LKB1 deletion mutants was mixed with purified GST-STAT3. WB, Western blot.

may be independent of the phosphorylation status of tyrosine 705 and serine 727.

To test this hypothesis, wild-type and kinase-deficient Myc-LKB1 and RET/PTC were expressed in HeLa cells, which lack endogenous LKB1, and the effect on endogenous STAT3 phosphorylation was examined (7). In these cells, expression of Myc-LKB1 did not alter the phosphorylation status of endogenous STAT3 Y705 (Fig. 3B). Taken together, these results suggest that LKB1-mediated suppression of the activation of STAT3 is independent of STAT3 tyrosine or serine phosphorylation.

The LKB1 Kinase Domain Interacts with the STAT3 Linker Domain *in Vivo*

The above results suggest that LKB1 may inhibit STAT3 by direct binding, independent of its kinase function. This idea was tested by immunoprecipitation (IP) of extracts of HEK293 cells cotransfected with plasmids expressing Flag-tagged STAT3 and Myc-tagged LKB. IP analyses were carried out with anti-Flag (Fig. 4A) and anti-Myc (Fig. 4B) antibodies. Flag IP products were analyzed by Western blot using anti-Myc antibodies, and Myc-IP products were analyzed by Western blot with anti-Flag antibodies. LKB1 and STAT3 were detected in both immunoprecipitates, indicating that LKB1 and STAT3 interact physically with each other (Fig. 4, A and B). Furthermore, by using confocal microscopy, we observed that Myc-LKB1 and endogenous STAT3 colocalize in the nucleus (Fig. 4C). This observation was confirmed by treating cells with IL-6 to induce translocation of STAT3 to the nucleus. Figure 4D shows that IL-6 also induced nuclear localization of LKB1. This result suggests that STAT3

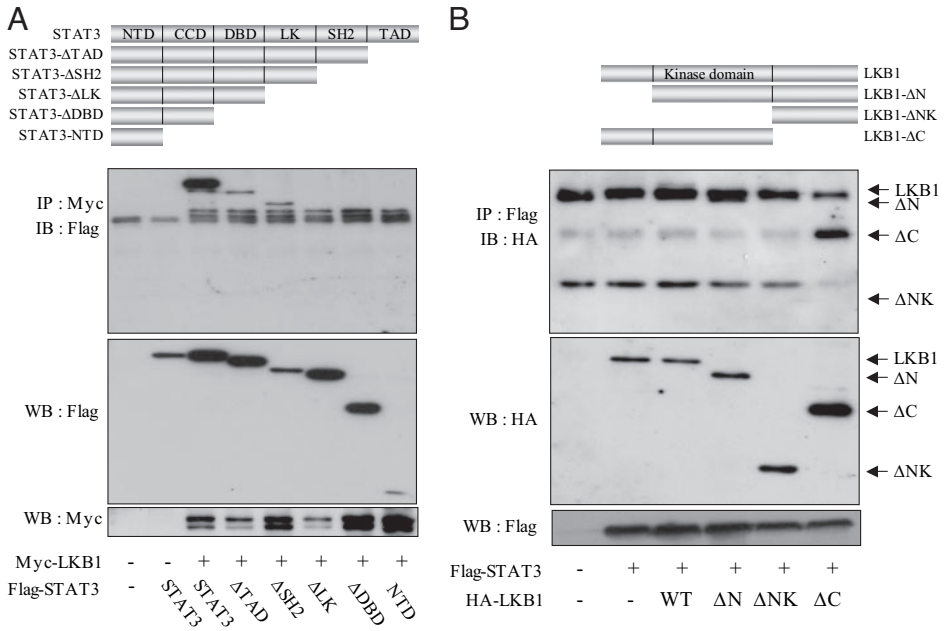

Fig. 5. The LKB1 Kinase Domain Interacts with the STAT3 Linker Domain

A, HEK293 cells were grown in 100-mm dishes and transiently cotransfected with plasmids expressing STAT3 deletion constructs (2 μg), Myc-LKB1 (2 μg), and control vector (4 μg). Protein (1 mg) was immunoprecipitated with anti-Myc antibody and separated by SDS-PAGE, followed by immunoblotting. Blots were probed with anti-Flag antibody. Total LKB1 expression (*bottom*) and the STAT3 deletions (*middle*) were detected using the antibodies indicated. DBD, DNA-binding domain; LK, linker domain; NTD, N-terminal domain; SH2, Src homology 2 domain; TAD, transactivating domain. B, STAT3 interacts with the LKB1 kinase domain. HEK293 cells were grown in 100-mm dishes and transiently cotransfected with plasmids expressing Flag-STAT3 (2 μg) and LKB1 deletion constructs (2 μg) or control vector (4 μg). Proteins (1 mg) were immunoprecipitated with the anti-Flag antibody and separated by SDS-PAGE followed by immunoblotting. Blots were probed with the anti-Myc antibody. Levels of STAT3 (*bottom*) and LKB1 (*middle*) were detected with the antibodies indicated. ΔC, C-terminal deletion; ΔN, N-terminal deletion; ΔNK, N-terminal and kinase domain deletion.

and LKB1 form a complex after migrating to the nucleus and that the activity of STAT3 in the nucleus is suppressed by LKB1.

To know whether LKB1 directly interacts with STAT3, we performed glutathione S-transferase (GST) pull-down assays using GST-STAT3 and ^{35}S-labeled LKB1 proteins synthesized in an *in vitro* translation system using rabbit reticulocytes (Promega). Figure 4E shows that LKB1-WT, LKB1-K78M, LKB1-ΔN, and LKB1-ΔC but not LKB1-ΔNK bind to STAT3, supporting the possibility that LKB1 directly interacts with STAT3. Taken together, our results suggest that LKB1 suppresses the transcriptional activities of STAT3 through a direct interaction with STAT3.

To identify the domains required for the protein-protein interaction between STAT3 and LKB1, IP experiments were performed using truncated forms of STAT3 and LKB1 (Fig. 5, A and B). In cells expressing Myc-LKB1 and wild-type or truncated STAT3, wild-type STAT3, STAT3-ΔTAD, and STAT3-ΔSH2 were competent to bind Myc-LKB1, but STAT3-ΔLK, STAT3-ΔDBD, and STAT3-NTD were not (Fig. 5A). In cells expressing Flag-STAT3 and wild-type or truncated LKB1, wild-type LKB1, HA-LKB1-ΔN, and HA-LKB1-ΔC were competent to bind Flag-STAT3, but HA-LKB1-ΔNK, which lacks both the N-terminal and kinase domains, were not (Fig. 5B). These results suggest that the kinase domain of LKB1 interacts with the linker domain of STAT3.

LKB1 Represses Binding of STAT3 to Its Target Promoters

STAT3 binds to GAS consensus sites in the promoter regions of STAT3 target genes including *c-myc*, *socs3*, and *c-fos* (28–30). Chromatin IP (ChIP) assays were performed to determine whether LKB1 suppresses the binding of STAT3 to these target promoters. LKB1$^{-/-}$ MEF cells were grown and transfected with vectors expressing Myc-LKB1 or Myc-LKB1-K78M. Cells were stimulated with oncostatin M (OSM) and cross-linked with formaldehyde *in vivo*. Soluble chromatin was extracted and immunoprecipitated with anti-STAT3 antibodies. DNA was recovered from the immunoprecipitate and PCR amplified using GAS site-specific primers. OSM stimulated STAT3 binding to its target promoters at 0.5 and 1 h, but binding was significantly reduced in cells expressing LKB1 or kinase-deficient LKB1 (Fig. 6).

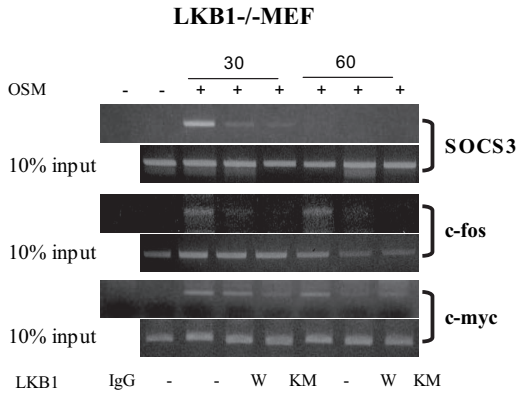

Fig. 6. LKB1 Inhibits Binding of STAT3 to Target Promoters

A, LKB1 suppresses the binding of STAT3 to its target promoters. MCF-7 cells were grown in 100-mm dishes and transiently cotransfected with plasmids (2 μg) expressing Myc-LKB1, Myc-LKB1-K78M, or control vector and incubated for 24 h. Cells were treated with OSM for the indicated amount of time and then analyzed by ChIP using anti-STAT3 antibody. PCR was performed using primers for the promoter regions of *socs3*, *c-fos*, and *c-myc*.

LKB1 Suppresses STAT3-Mediated Gene Expression

LKB1 appears to suppress binding of STAT3 to its target promoters (Fig. 6) and STAT3-dependent transcription of the *m67-Luc* reporter gene (Figs. 1–3). Thus, experiments were performed to test the effect of LKB1 on expression of STAT3 target genes and on cell proliferation. The promoters of genes encoding cyclin D1 and VEGF are induced by STAT3 (12, 14, 31, 32); thus, cyclin D1- and VEGF-*Luc* reporter gene constructs were used to measure activation of STAT3. In NIH3T3 cells, STAT3-dependent transcriptional activation was significantly higher in cells expressing RET/PTC (Fig. 7, A and B). However, RET/PTC-dependent activation of STAT3 was suppressed by wild-type and kinase-deficient LKB1 (Fig. 7, A and B, bars 4 and 5). The effect of LKB1 overexpression on expression of endogenous cyclin D1 and Bcl-xL was examined in MDA-MB-435 cells, in which STAT3 is activated constitutively (33). In these cells, overexpression of LKB1 significantly suppressed expression of endogenous cyclin D1 and Bcl-xL protein.

The effect of siRNA-mediated knockdown of LKB1 on cellular proliferation was examined in TPC-1 human thyroid cancer cells (34, 35). TPC-1 cells have a rearrangement of the endogenous RET/PTC1 gene, and the growth of these cells is dependent on activation of STAT3 by RET/PTC (36). As shown in Fig. 7D, transfection with LKB1 targeted siRNA-stimulated proliferation of TPC-1 cells, whereas control siRNA did not. The stimulated cell proliferation of TPC-1 cells by LKB1 siRNA is abrogated by cotransfection of STAT3 siRNA. These results suggest that LKB1 modulates RET/PTC-and STAT3-dependent proliferation of TPC-1 cells.

DISCUSSION

LKB1 is a tumor suppressor with intrinsic serine/threonine kinase activity, defects in which cause PJS, an autosomal dominant human disease characterized by high incidence of benign and malignant intestinal tumors (2, 37). The gene expression signature of LKB1-null fibroblasts includes significantly elevated expression of STAT3 target genes such as *MMP2*, *MMP9*, *VEGF*, *IGFBP5*, and *COX-2* (12–16, 32). Previous studies reported that RET/PTC tyrosine kinase phosphorylates and activates STAT3 (23). This study demonstrates that the ability of RET/PTC to activate endogenous and exogenous STAT3 is suppressed by wild-type and kinase-deficient LKB1. It is unlikely that LKB1 acts through an upstream activator of STAT3, because it suppresses STAT3 transcriptional activity in the presence of Tel-JAK2 (26) and IL-6 (17), and it also stimulates the constitutively active STAT3c (21). This study also shows that LKB1-mediated suppression of STAT3 transcriptional activity is independent of the phosphorylation status of STAT3.

LKB1 has been reported to cause G1 growth arrest in cultured cells via induction of $p21^{WAF1/CIP1}$ (7) and stimulation of Brg1 ATPase (8). LKB1 kinase activity is required for $p21^{WAF1/CIP1}$ induction, and overexpression of LKB1 causes $p21^{WAF1/CIP1}$-induced growth arrest in cells with undetectable or low levels of endogenous LKB1 (7). In contrast, although Brg1-associated growth arrest also requires LKB1 kinase activity, wild-type LKB1 and the kinase-deficient form LKB1-SL26 both stimulate Brg1 ATPase (8). This suggests that some regulatory functions of LKB1 may not require its kinase activity. This is consistent with our observation that several naturally occurring and recombinant kinase-deficient forms of LKB1 retain the ability to suppress activation of STAT3. One exception to this is LKB1-ΔNK, a recombinant form of LKB1 that lacks the N-terminal region and the kinase domain and that does not suppress RET/PTC-dependent activation of STAT3. These results support the conclusion that the LKB1 kinase domain, but not its kinase activity, is essential for suppressing activation of STAT3.

STAT3 is also regulated by the phosphorylation status of tyrosine 705 and serine 727, although tyrosine 705 appears to be more important in this regard than serine 727 (17, 23). Nevertheless, some regulators of STAT3, including PIAS3 and Grim-19, suppress STAT3 transcriptional activity via direct binding (38–40) and have no effect on tyrosine phosphorylation of STAT3. Similarly, wild-type and kinase-deficient forms of LKB1 bind to STAT3 and modulate its activity independent of tyrosine 705 phosphorylation. Furthermore, the LKB1 kinase domain and the STAT3 linker domain are required for binding of LKB1 and STAT3. Thus, there are similarities in the mechanism by which LKB1, PIAS3, and Grim-19 interact with STAT3.

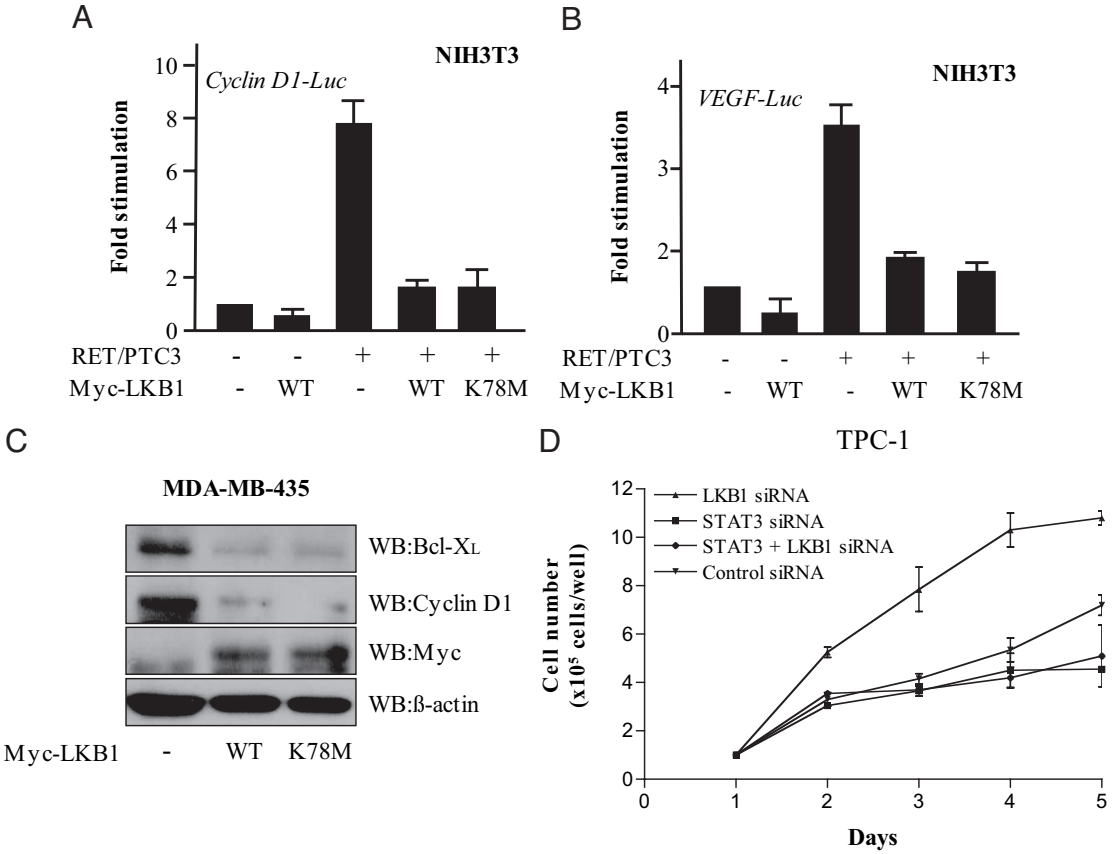

Fig. 7. LKB1 Suppresses STAT3-Mediated Gene Expression

A and B, LKB1 suppresses transcription of *Cyclin D1*-Luc and *VEGF*-Luc. NIH3T3 cells were grown in 12-well plates and transiently cotransfected with plasmids (0.1 μg) expressing Cyclin D1-Luc (A) VEGF1-Luc (B), RET/PTC3, and Myc-LKB1 or Myc-LKB1-K78M. Cells were lysed 24 h after transfection and luciferase activity measured. C and D, LKB1 decreases Cyclin D1 and Bcl-xL protein levels. C, MDA-MB-435 cells were transiently transfected with plasmids (0.5 μg) expressing Myc-LKB1 or Myc-LKB1-K78M and control vector. D, TPC-1 cells were transfected with LKB1 siRNA, STAT3 siRNA, or control siRNA. Cell numbers were counted daily.

STAT3 regulates transcription of many downstream target genes, including many oncogenesis-related genes (28–30, 41), by binding to and activating the promoter of the target genes. Here, ChIP experiments were performed to determine the effect of LKB1 on binding of STAT3 to the promoter regions of *socs3*, *c-fos*, and *c-myc*, all of which contain GAS consensus sites (21, 28, 29, 41). The results showed that wild-type and kinase-deficient forms of LKB1 decreased binding of STAT3 to its target promoters. Furthermore, expression of exogenous LKB1 reduced expression of cyclin D1 and Bcl-xL protein and transcription of a luciferase reporter gene driven by the cyclin D1 or VEGF promoters. Lastly, siRNA-mediated knockdown of LKB1 siRNA stimulated proliferation of TPC-1 cells.

In conclusion, this study shows that LKB1 suppresses RET/PTC-dependent activation of STAT3 by interacting with the STAT3 linker domain, leading to decreased expression of downstream targets of STAT3. Thus, we propose here a novel mechanism by which LKB1 acts as tumor suppressor and counteracts oncogenic STAT3.

MATERIALS AND METHODS

Materials

Cell culture reagents were purchased from Life Technologies Inc. (Gaithersburg, MD), Sigma Chemical Co. (St. Louis, MO), Fisher Scientific (Fairlawn, NJ), and Corning Inc. (Corning, NJ). Antibodies against RET, Bcl-xL, and cyclin D1 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-STAT3, anti-pY705-STAT3, anti-Myc, and anti-HA antibodies were purchased from New England Biolabs Inc. (Ipswich, MA). Anti-Flag antibody was purchased from Stratagene (La Jolla, CA). IL-6 and OSM were purchased from R&D Biosystems (Abingdon, UK).

Plasmids

pcDNA3-RET/PTC3, pcDNA3-RET/PTC3 K284M, pRC/CMV-STAT3, pRC/CMV-STAT3-Y705F, pRC/CMV-STAT3c, pRC/CMV-STAT3-S727A, m67-Luc, cyclin D1-Luc, pcDNA3-TEL-JAK2, and VEGF-Luc were described previously (23, 42). pEGFP-LKB1-WT, pEGFP-LKB1-R304W, pEGFP-LKB1-I-177N, pEGFP-LKB1-K175-D176del, and GFP-LKB1-E98-G155del were provided (27). pcDNA3.1-flag-STAT3, pCMV2-flag-LKB1-ΔNK, pCMV2-flag-STAT3-ΔTAD, pCMV2-flag-

STAT3-ΔSH2, pCMV2-flag-STAT3-ΔLK, pCMV2-flag-STAT3-ΔDBD, and pCMV2-flag-NTD were provided (40). Myc-tagged LKB1-WT was generated by PCR using pCMV6-HA-LKB1 as a template; and the point mutant LKB1-K78M was constructed using the QuikChange II XL Site-Directed Mutagenesis kit (Stratagene) and the following primers: sense, 5′-CGCAGGG-CGGTCATGATCCTCAAGAAG-3′, and antisense, 5′-CTTCTT-GAGGATCATGACCGCCCTGCG-3′. pCMV6-HA-LKB1-ΔN, pCMV6-HA-LKB1-ΔNK, and pCMV6-HA-LKB1-ΔC were constructed by PCR amplification of the respective regions from pCMV6-LKB1. The amplified fragments were digested with *Xba*I and *Eco*RI and ligated to the expression vector pCMV6-HA. All plasmid constructs generated in this study were confirmed by automated DNA sequencing.

Cell Culture

Murine fibroblasts (NIH3T3), human kidney fibroblasts (HEK293), human breast cancer cells (MCF-7 and MDA-BA-435), human cervical adenoma cells (HeLa), LKB1 knockout murine embryo fibroblasts (LKB1$^{-/-}$ MEF) (10) and papillary thyroid carcinoma (TPC-1) were cultured in DMEM. Human thyroid carcinoma cells (ARO and NPA) and human hepatocellular carcinoma cells (HepG2) were cultured in RPMI 1640. Media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 g/ml streptomycin in a humidified chamber containing a 5% CO_2 atmosphere at 37 C.

Immunoblot Analysis

Cells were centrifuged and the pellet was lysed with SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 125 mM dithiothreitol (DTT), and 0.03% bromophenol blue]. Total cell lysates were denatured by boiling for 5 min, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were blocked for 1 h in Tris-buffered saline containing 5% milk and 0.1% Tween and then incubated for 2 h with primary antibody diluted in blocking buffer. Blots were developed using a horseradish peroxidase-conjugated secondary antibody kit (Phototope-Horseradish Peroxidase Western Blot Detection Kit; New England Biolabs).

IP

All IP procedures were performed at 4 C. Before lysis, cells were washed twice with PBS and then lysed for 30 min in IP assay buffer containing protease inhibitors. Lysates were collected, triturated, and centrifuged at 3000 × *g* for 10 min. To preclear the lysates, supernatants were mixed with 20 μl protein A/G beads (Santa Cruz Biotechnology), agitated for 30 min, and centrifuged for 5 min at 3000 × *g*. Precleared samples were incubated with the indicated primary antibody for 3 h with agitation. Protein A/G beads were added to the mixture, which was incubated for 1 h and centrifuged at 3000 × *g*. Immunoprecipitates were collected and washed three times with IP assay buffer.

Confocal Microscopy

LKB1$^{-/-}$ MEFs were grown on coverslips and transfected with Myc-LKB1-WT and Myc-LKB1-K78M using Lipofectamine Twenty-four hours after transfection, cells were washed three times with cold PBS and fixed in 3.7% formaldehyde for 30 min. Cells mounted on glass slides were permeabilized with 2 ml PBS containing 0.1% Triton X-100 and 0.1 M glycine at room temperature, incubated for 15 min, washed three times with 1× PBS, and blocked with 3% BSA in PBS for 10 min at RT. Cells were incubated with primary anti-Myc and anti-STAT3 antibody for 1 h at 37 C, washed three times with 1× PBS, and incubated for 1 h at 37 C with fluorescein isothiocyanate-conjugated antimouse and rhodamine-conjugated antirabbit secondary antibodies for LKB and STAT3, respectively (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Cells were mounted on glass slides and observed using a laser-scanning confocal microscope (Olympus Corp., Lake Success, NY).

ChIP Assay

The ChIP assay was performed using the commercially available EZ ChIP kit (Upstate Biotechnology Inc., Lake Placid, NY). In brief, LKB1$^{-/-}$ MEF cells were grown in 100-mm dishes and transfected with Myc-LKB1-WT and Myc-LKB1-K78M. Twenty-four hours after transfection, cells were stimulated with OSM (20 ng/ml) for 30 or 60 min and then cross-linked with formaldehyde (27 μl/ml growth medium) and gently swirled to mix. Reactions were incubated at room temperature for 10 min. Unreacted formaldehyde was quenched by incubation with 1 ml 10× glycine for 5 min, after which samples were rinsed twice with ice-cold PBS containing protease inhibitors. Cells were scraped and spun down at 700 × *g* for 5 min. The cell pellet was resuspended in 1 ml SDS lysis buffer at 2 × 10^7 cells/ml and sonicated with three sets of 10-sec pulses on wet ice (Heat Systems Ultrasonic Processor, Farmingdale, NY; 550-W model with a 2-mm tip, set to 10% maximal power). To preclear the cell lysate, 60 μl protein G agarose was added, and the mixture was incubated at 4 C for 1 h and then centrifuged at 3000 × *g* for 1 min. Precleared samples were incubated with antimouse IgG (negative control) and anti-STAT3 antibodies at 4 C overnight. Protein G agarose was added, after which the mixture was incubated for 1 h and centrifuged at 3000 × *g*. Protein G agarose and chromatin complexes were washed with low-salt, high-salt, LiCl, and Tris-EDTA buffers for 5 min each and then recovered with elution buffer containing 1 M $NaHCO_3$ and 20% SDS. To reverse formaldehyde cross-linking, eluted protein/DNA complexes were incubated in 5 M NaCl at 65 C for 5 h in the presence of proteinase K. DNA was purified using spin columns, and PCR was conducted using the following gene-specific primers: *c-myc* sense, 5′-AAAAATAG-AGAGAGGTGGGGAAG-3′, and antisense, 5′-TGGAATTAC-TACAGCGAGTCAGAA-3′; *socs3* sense, 5′-CAGGCGAGTG-TAGAGTCAGAGTT-3′, and antisense, 5′-CACAGCCTTTCA-GTGCAGAGTAG-3′; and *c-fos* sense, 5′-CTGCGTACTTGC-TTCTCCTAATAC-3′, and antisense, 5′-GAGTGTT-CACATTTGGGATCTT-3′.

Luciferase Assay

The dual-luciferase reporter assay system was used. Cells were transfected with *m*67-Luc, *Cyclin D1-Luc*, or *VEGF-Luc*, along with 50 ng pRL-SV40, encoding *Renilla* luciferase (Promega Corp., Madison, WI) using Lipofectamine OptiMEM (Invitrogen, Carlsbad, CA). Firefly and *Renilla* luciferase activities were measured using a dual-luciferase reporter assay system (Promega). Firefly luciferase activities were measured and normalized to that of *Renilla* luciferase.

Cytoplasmic and Nuclear Extracts

HepG2 cells were grown in RPMI 1640 supplemented with 10% FBS. Cells were exposed to IL-6, after which cytoplasmic and nuclear extracts were prepared. After centrifugation at 500 × *g*, cells were resuspended in five pellet volumes of 0.3 M sucrose and 2% Tween 40 in buffer A [10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml pepstatin A]. After freezing, thawing, and gentle homogenization, nuclei were isolated by centrifugation at 25,000 × *g* in buffer A using a 1.5 M sucrose cushion. The

supernatant was used as the cytoplasmic fraction. Isolated nuclei were lysed in buffer B [10 mM HEPES-KOH (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 10% glycerol, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml pepstatin A] and centrifuged at 100,000 $\times$ *g* for 1 h. The supernatant was dialyzed against buffer C [10 mM Tris (pH 7.9), 1 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA and 5% glycerol] and then aliquoted and stored at −70 C.

Stealth RNA and Cell Counts

Lipofectamine (Invitrogen) was used to transfect cells with 20 nM Stealth RNA (Invitrogen) targeting the 25-nucleotide human LKB1 sequences UCUACAACAUCACCACGGGUCUGUA and UAACCUCCUCAGUAGUUGAAUUUCC and human STAT3 sequence CCGCUUCCUGCAAGAGUCGAAUGUU. For cell growth-curve analysis, TPC-1 cells were cultured and transfected with LKB1 siRNA, STAT3 siRNA, or control siRNA, respectively. These cells were split and plated into 60-mm dish plates at the densities of 1 $\times$ 10^5 (1 d), and cell numbers were counted every day.

GST Pull-Down Assay

GST-STAT3 fusion proteins were expressed in BL21 (Amersham Bioscience, Inc., Pittsburgh, PA). GST fusion proteins were analyzed on 10% SDS-PAGE gels to confirm their integrity and to normalize the amounts of each protein. The Promega TNT-coupled transcription-translation system was used to produce [^{35}S]methionine-labeled LKB1-WT, LKB1-K78M, and LKB1 deletion mutants, which were visualized by SDS-PAGE. *In vitro* binding assays were performed with glutathione-agarose beads (Amersham) coated with 500 ng GST fusion protein and 20 μl [^{35}S]methionine-labeled protein in 200 μl of a binding buffer containing 100 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% Nonidet P-40, 5 μg ethidium bromide, and 100 μg BSA. The reaction was allowed to proceed for 1–2 h at 4 C with constant agitation. The beads were then collected by centrifugation and washed five times with 1 ml binding buffer lacking ethidium bromide and BSA, resuspended in 20 μl SDS-PAGE sample buffer, and boiled for 5 min. The eluted proteins were fractionated by SDS-PAGE, and then the gel was treated with Amersham Amplify Fluor, dried at −70 C, and autoradiographed.

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