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LKB1 Controls Human Bronchial Epithelial Morphogenesis through p114RhoGEF-Dependent RhoA Activation

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LKB1 is a Ser/Thr kinase that plays an important role in controlling both energy metabolism and cell polarity in metazoan organisms. LKB1 is also a tumor suppressor, and homozygous, inactivating mutations are found in a wide range of human cancers. In lung cancer, inactivating mutations are found in 10 to 50% of cases, but the consequences of functional loss in this context are poorly understood. We report here that LKB1 is required for the maturation of apical junctions in the human bronchial epithelial cell line 16HBE14o- (16HBE). This activity is dependent on an interaction with the Rho guanine nucleotide exchange factor p114RhoGEF but is independent of LKB1 kinase activity. Together, LKB1 and p114RhoGEF control RhoA activity in these cells to promote apical junction assembly.

Germ line mutations in *LKB1* (*STK11*), a gene on the short arm of chromosome 19 encoding a serine/threonine kinase, result in Peutz-Jeghers syndrome (PJS), characterized by intestinal hamartomas and an increased risk of developing malignant epithelial cancers (1). Somatic, inactivating mutations in LKB1 are frequently found in human tumors, including cervical and lung cancers (2, 3). About 20% of cervical cancers harbor biallelic *LKB1* mutations, and these are associated with disease progression and poor survival (2). Restoration of LKB1 in the cervical cancer-derived line HeLa-S3 leads to cell cycle arrest and cytoskeleton reorganization, indicating that loss of the LKB1 tumor suppressor may be advantageous for both tumor cell proliferation and migration (4, 5). The frequency of *LKB1* mutations reported in non-small-cell lung cancers (NSCLCs) ranges from 10 to 50%, and *LKB1* ranks as the third most commonly altered gene after *TP53* and *KRAS* in this disease (6, 7). Loss of LKB1 often coexists with KRAS activation. *LKB1* mutations in human lung cancers include those that delete the C-terminal regulatory region yet retain kinase activity, although more frequently, mutations lead to deletion of both kinase and C-terminal regions (8).

LKB1 contains an N-terminal nuclear localization signal and a C-terminal CAAX box (where C is cysteine, A is an aliphatic amino acid, and X is any amino acid), in addition to its central Ser/Thr kinase domain. When expressed in mammalian cells, it is farnesylated on the CAAX box Cys but found predominantly in the nucleus (9). LKB1 forms a complex with STRAD, an inactive pseudokinase, and MO25, an armadillo repeat scaffold-like protein. Binding of LKB1 to STRAD activates LKB1 kinase activity and promotes relocalization to the cytosol, while MO25 binds to the C terminus of STRAD and further enhances kinase activity (10, 11). Among the many reported LKB1 substrates, AMP-activated protein kinase (AMPK) is the best studied (12). Under conditions of stress, such as nutrient deprivation, LKB1 phosphorylates and activates AMPK, which in turn regulates a signal transduction pathway leading to the inactivation of mTOR, a key promoter of cell growth. LKB1 is therefore recognized as an important regulator of energy homeostasis. However, LKB1 is also a major player in the control of cell polarity (13). Its ortholog in *Caenorhabditis elegans*, PAR-4, is required for establishing the anterior-posterior (A-P) axis during the first cell division, at least in part by controlling the polarized activity of cortical nonmuscle myosin (14, 15). In *Drosophila*, LKB1 is essential for establishing the early A-P polarity of the oocyte and polarization of the oocyte cytoskeleton. Farnesylation of *Drosophila* LKB1 is essential for this polarity function, although, interestingly, *C. elegans* PAR-4 lacks a prenylation site (16). In mammals, conditional deletion of LKB1 in the mouse mammary gland compromises epithelial cell integrity, although this alone is insufficient to promote tumorigenesis, while in the pancreas, LKB1 loss disrupts acinar polarity (17, 18). Most strikingly, coexpression of LKB1 and STRAD was found to promote a polarized actin cytoskeleton in single, intestinal cells in culture (19). Further analysis revealed that this polarity pathway requires Rap2A-mediated activation of the Mst4 kinase and phosphorylation of ezrin (20, 21). A connection between LKB1 and actomyosin filament assembly has also been reported in HeLa-S3 cells, where LKB1 activates the RhoA GTPase (5).

The functional consequences of LKB1 inactivation in the context of lung cancer are not well understood. Depletion of LKB1 in immortalized human small airway epithelial cells induces an epithelial-mesenchymal-like transition (EMT), accompanied by increased expression of ZEB1, a transcriptional repressor for E-cadherin and an EMT inducer (22). Gene expression and microarray analysis to compare expression patterns in lung cancer cells harboring either wild-type or mutated LKB1 identified increased expression of COX-2 and PEA3, two known regulators of EMT and cancer invasion, in the absence of LKB1 (23). A significant upregulation of lysyl oxidase (LOX) was also reported in lung tumors derived from KRAS/LKB1$^{-/-}$ mice compared with the regulation in lung tumors derived from mice with KRAS knockout alone, and this was associated with enhanced cell proliferation and invasion through β1 integrin signaling (24). Finally, a comparison of KRAS and KRAS/LKB1$^{-/-}$ primary lung tumors and KRAS/LKB1$^{-/-}$ distant metastases reported increased levels of EMT markers in the

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metastatic cells compared to primary tumors and increased activity of FAK and SRC tyrosine kinases in both primary tumors and metastases lacking LKB1 (25). Mouse models of lung cancer in which mice harbor KRAS mutations have revealed strong cooperation with the loss of LKB1 compared to that with the loss of p53 or Ink4a/Arf in terms of shorter tumor latency and greater levels of metastasis (26).

The loss of bronchial epithelial cell polarity is a defining feature during lung cancer progression toward an invasive and metastatic phenotype. To explore the potential role of LKB1 in lung epithelial morphogenesis, we have made use of an immortalized, human bronchial epithelial cell line, 16HBE14o- (16HBE). In culture, 16HBE cells form well-developed, mature apical junctions, consisting of tight junctions and adherens junctions intimately associated with actomyosin filaments. We recently showed the importance of two Rho family GTPases, RhoA and Cdc42, and three GTPase-regulated kinases, PAK4, protein kinase C (PKC)-related kinase 2 (PRK2), and Par6B/atypical PKC (aPKC), in maintaining the integrity of apical junctions in 16HBE cells. We report here that LKB1, acting together with RhoA, is also required for apical junction assembly in 16HBE cells.

MATERIALS AND METHODS

Cell culture and transfection. 16HBE14o- (16HBE) cells (a gift of Dieter Gruenert, California Pacific Medical Center, San Francisco, CA) were cultured in minimal essential medium with GlutaMAX (41090; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; BenchMark; Gemini Bio-Products, West Sacramento, CA) and penicillin-streptomycin (Invitrogen, Carlsbad, CA) at 37°C in 5% CO_2. For calcium switch experiments, cells were washed in phosphate-buffered saline three times and incubated in calcium-free medium for 4 h, and then normal growth medium containing calcium was added. Calcium-free medium was Dulbecco modified Eagle medium (DMEM) without calcium chloride (21068; Invitrogen, Carlsbad, CA) supplemented with 10% FBS pretreated with Chelex 100 resin (Bio-Rad, Hercules, CA). Small interfering RNA (siRNA) was transfected (50 nM) in medium without antibiotics and with 5 μl Lipofectamine LTX (15338030; Invitrogen, Carlsbad, CA) per 1.0×10^5 16HBE cells. HEK293T cells (ATCC, Manassas, VA) were cultured in high-glucose DMEM supplemented with 10% FBS and penicillin-streptomycin at 37°C in 5% CO_2. For HEK293T cell transfection with plasmid DNA, 3×10^5 cells were transfected with 1 μg plasmid DNA and 5 μl Lipofectamine 2000 (11668-027; Invitrogen, Carlsbad, CA).

Virus production and cell infection. For retroviral production, HEK293T cells were plated at a density of 3.8×10^6 in 100-mm dishes and 1 day later transfected with 5 μg vesicular stomatitis virus glycoprotein (VSV-G), 5 μg Gag Pol, and 5 μg retrovirus vector using Lipofectamine 2000, and at 6 h after transfection, the medium was replaced with fresh medium. The medium containing the virus was collected 2 to 3 days after transfection and filtered through a 0.45-μm-pore-size filter. For lentivirus production, HEK293T cells were transfected with 20 μg of a short hairpin RNA (shRNA) DNA construct, 5 μg of VSV-G, and 15 μg of pDeltaR8.9 using a calcium phosphate transfection kit (Invitrogen, Carlsbad, CA). Medium was removed after overnight incubation and replaced with fresh medium. A total of 10^5 16HBE cells were seeded per 6-well dish and infected with 1 to 1.5 ml of viral suspension plus 8 μg/ml Polybrene by centrifugation at 2,250 rpm for 30 min. The viral suspension was removed and replaced with fresh medium; where indicated, puromycin selection was initiated 2 days later. Experiments were performed with short-term cultures of selected, stable pools.

Antibodies. Primary antibodies used were ZO-1 (clone 1A12; 339100; Invitrogen, Carlsbad, CA), ZO-1 (617300; Invitrogen, Carlsbad, CA) E-cadherin (clone ECCD-2; 131900; Invitrogen, Carlsbad, CA), E-cadherin (clone 34; 610404; BD Transduction, Lexington, KY), α-tubulin (clone YL1/2; AbD Serotec, Raleigh, NC), hemagglutinin (HA; clone 3F10; Roche, Indianapolis, IN), green fluorescent protein (GFP; rabbit polyclonal; Invitrogen, Carlsbad, CA), myc (clone 9E10; Cancer Research UK, London, United Kingdom), phospho-PRK (p-PRK; 2611; Cell Signaling, Beverly, MA), PAK4 (3242; Cell Signaling, Beverly, MA), p114RhoGEF (EB06163; Everest Biotech Ramona, CA), LKB1 (27D10; Cell Signaling, Beverly, MA), and RhoA (sc-418; Santa Cruz, Santa Cruz, CA). Alexa Fluor 488- and 568-conjugated secondary antibodies and Alexa Fluor 488-phalloidin were from Invitrogen (Carlsbad, CA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Dako (Carpinteria, CA).

DNA constructs and shRNA sequences. Mouse LKB1 (amino acids [aa] 1 to 436) and mutants were cloned into either pRK5-myc (XbaI and PstI), pEGFP vectors (SalI and ApaI), or pQCXIN (AgeI and EcoRI); human LKB1 (hLKB1; aa 1 to 433) and mutants were cloned into pLL3.7-GFP (NheI and EcoRI); and mouse p114RhoGEF (aa 1 to 1021aa) and mutants were cloned into pBabe-HA (BamHI and SalI) or pRK5-Flag (BamHI and SalI) vectors. All mutations were introduced by QuikChange site-directed mutagenesis. Human STRAD cDNA from Addgene was subcloned into pQCXIP-HA (BamHI and EcoRI).

The p114RhoGEF siRNA was from Thermo Fisher Scientific (Lafayette, CO). The p114RhoGEF siRNA sequence (D-009654-02) was UCAG GGCGCUUGAAAGAUA.

The LKB1 shRNA was from Sigma-Aldrich (St. Louis, MO). The sequences used in this study included GAGTGTGCGGTCAATAT TTAT (LKB1 shRNA1; TRCN0000000407), GATCCTCAAGAAGAAGA AGTT (LKB1 shRNA2; TRCN0000000409), GAAGAAGAAGTTGCGAA GGAT (LKB1 shRNA3; TRCN0000000410), and CATCTACACTCAGG ACTTCAC (LKB1 shRNA4; TRCN0000000411).

The sequences of p114RhoGEF shRNAs used in this study were GAA GCTGTTAGTCATTACA (p114RhoGEF shRNA1), CAAGAGCGGTTG AGCATGA (p114RhoGEF shRNA2), and GGCTACGACTGCACA AACA (p114RhoGEF shRNA3).

Immunoprecipitation and Western blotting. 16HBE cell lysates were prepared in protein sample buffer (2% SDS, 100 mM dithiothreitol, 50 mM Tris-HCl, pH 6.8, 10% glycerol, 0.1% bromophenol blue) and boiled for 5 min at 100°C. For immunoprecipitation, transfected HEK293T cells were lysed in immunoprecipitation buffer (0.5% NP-40, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl) with 2 mM phenylmethylsulfonyl fluoride and a complete protease inhibitor tablet (Roche, Indianapolis, IN) and centrifuged for 10 min at 13,000 rpm and 4°C to pellet cell debris. The soluble fraction was incubated with primary antibody for 2 h, followed by protein G-Sepharose beads (Sigma-Aldrich, St. Louis, MO) for 1 h at 4°C with tumbling. Beads were washed with immunoprecipitation buffer and boiled in sample buffer. Proteins were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA), and incubated with the appropriate primary antibodies. Proteins were visualized using HRP-conjugated secondary antibodies and enhanced chemiluminescence detection reagents (GE Healthcare, Waukesha, WI).

PLA. The interaction between LKB1 and p114RhoGEF in 16HBE cells was analyzed using a Duolink II *in situ* proximity ligation assay (PLA; Olink Bioscience, Uppsala, Sweden), according to the manufacturer's instructions. The rabbit anti-LKB1 or rabbit anti-myc antibodies were used together with mouse anti-HA antibody. Fluorescence spots generated were automatically counted, and the average number of spots per cell was calculated using Volocity software (Volocity Software, Waltham, MA).

GTPase activation assays. 16HBE cells stably expressing HA-tagged STRAD were transfected with siRNA for 3 days, followed by infections with pLL3.7-LKB1 viruses. Cells were harvested 1 day after infection. Pulldown assays were carried out using 20 μg of the Rho binding domain of rhotekin (rhotekin-RBD) as a glutathione *S*-transferase (GST) fusion protein bound to glutathione-Sepharose (Sigma-Aldrich, St. Louis, MO). Cell lysates and pulldown samples were run on 10% SDS-polyacrylamide

gels, and proteins were transferred to PVDF membranes and subjected to Western blotting with anti-RhoA antibody.

Apical junction quantification. At 2 days after infection, cells were replated on coverslips and grown to confluence. Cells were fixed in 3.7% (vol/vol) formaldehyde for 12 min and permeabilized in 0.5% (vol/vol) Triton X-100 for 5 min. Primary and secondary antibodies were incubated for 1 h at room temperature (RT). Coverslips were mounted with fluorescent mounting medium and visualized using a Zeiss AxioImager microscope (Zeiss, Oberkochen, Germany). A fluorescence microscope with ×40 (numerical aperture [NA], 0.75) and ×63 (NA, 1.4) objectives (Thornwood, NY), a Hamamatsu ORCA-ER 1394 C4742-80 digital camera (Bridgewater, NJ), and AxioVision software (Zeiss, Oberkochen, Germany) were used. For each sample, 6 random nonoverlapping images were taken, and apical junction formation was quantified using Metamorph image analysis software. Cells with a continuous ring of ZO-1 at cell-cell contacts were counted as having intact apical junctions. Cells with punctate or discontinuous ZO-1 at cell-cell contacts were defined as not having apical junctions. Results were analyzed and statistical analysis was done using MetaMorph software (Molecular Devices, Downingtown, PA).

RESULTS

LKB1 is required for apical junction formation in 16HBE cells independently of its kinase activity. To investigate the role of LKB1 in human lung cell morphogenesis, LKB1 was depleted from cells of an immortalized human bronchial epithelial cell line, 16HBE. Cells were infected with lentiviral vectors harboring either control or LKB1-targeted shRNAs. At 2 days after infection, cells were replated on coverslips and grown to confluence over a period of 2 days. Under these conditions, control cells formed mature, apical junctions, seen as a continuous ring of ZO-1 and of E-cadherin at cell contacts. A wider region of E-cadherin could also be seen at the periphery, representing the lateral membrane. However, in LKB1-depleted cells, ZO-1 and E-cadherin formed discrete puncta at cell-cell contacts (Fig. 1A). Multiple, nonoverlapping shRNAs were tested to control for possible off-target effects. Three out of the 4 shRNAs (shRNA2, shRNA3, and shRNA4) tested resulted in significant LKB1 depletion and apical junction disruption, whereas shRNA1 had little effect on LKB1 expression and had no significant effect on ZO-1 localization (Fig. 1A and B). The correlation between LKB1 depletion and phenotype supports a specific role for LKB1 in apical junction formation in 16HBE cells.

To investigate the contribution of LKB1 to apical junction formation, RNA interference (RNAi) rescue experiments were performed using mouse wild-type LKB1 (mLKB1WT), kinase-dead (K78M) LKB1 (mLKB1KD), and two additional mutants, mLKB1SL26 and mLKB1C433A. LKB1SL26 was originally isolated from a PJS family (27) and contains an intact kinase domain but a 9-bp, in-frame deletion C terminal to the kinase domain that abrogates interaction with STRAD (10). mLKB1C433A encodes a point mutation in the CAAX box, preventing farnesylation. The level of endogenous phospho-AMPK (p-AMPK) was used to confirm that LKB1 kinase activity requires STRAD binding and is blocked by the K78M mutation. Upon transient expression, an increase in p-AMPK was observed only with mLKB1WT (Fig. 1C, top).

Stable pools of 16HBE cells expressing RNAi-resistant, myc-tagged mLKB1WT, mLKB1KD, mLKB1SL26, or mLKB1C433A were infected with the shRNA2 lentiviral vector to deplete endogenous LKB1 protein. A parallel infection of 16HBE cells stably selected with an empty vector revealed efficient depletion of endogenous LKB1 (Fig. 1C, bottom). As expected, expression of mLKB1WT fully rescued normal apical junction formation after depletion of endogenous LKB1 (Fig. 1D and E). Intriguingly, mLKB1KD also rescued junction formation, indicating that this junctional activity is independent of LKB1 kinase activity. However, neither mLKB1SL26 nor mLKB1C433A was able to rescue normal apical junctions after depletion of endogenous LKB1 (Fig. 1D and E). mLKB1WT and mLKB1KD showed an indistinguishable localization; i.e., they were predominantly in the nucleus, but with a fraction found in the cytosol. mLKB1SL26, which cannot interact with endogenous STRAD, was exclusively nuclear, while mLKB1C433A localization was similar to that of wild type (Fig. 1D, top). LKB1WT, LKB1KD, LKB1SL26, and LKB1C433A localization patterns were similar to those previously reported (28–30). We have also used a different rescue vector that leads to the expression of GFP-tagged mLKB1WT, mLKB1KD, mLKB1SL26, or mLKB1C433A at levels comparable to those of endogenous hLKB1, and the results from rescue experiments were similar to those just described (data not shown). Together, these data demonstrate that LKB1 is required for apical junction maturation in 16HBE bronchial epithelial cells and that this is independent of its kinase activity. The data are consistent with a role for STRAD, although this may be due to its role in sequestering LKB1 from the nucleus to the cytosol. Farnesylation is also required for apical junction formation.

LKB1 promotes RhoA activity and enhances recruitment of phospho-PRK to apical junctions. LKB1 is best known as a master kinase regulating the activity of several downstream kinases, notably, AMPK. However, since the role of LKB1 in 16HBE cell apical junction formation is independent of kinase activity, we considered other possible signaling pathways. We previously reported that LKB1 can affect RhoA activity in a kinase-independent manner in HeLa cells (5). Furthermore, RhoA is required for apical junction assembly in 16HBE cells (31). To explore this potential link, 16HBE cells stably expressing HA-tagged STRAD were infected with lentiviral vectors encoding GFP-tagged LKB1WT, LKB1KD, or LKB1SL26. One day later, a Rho.GTP pulldown assay was performed on cell lysates using a GST-rhotekin fusion protein attached to agarose beads. Expression of either wild-type or kinase-dead LKB1 led to a 6- to 8-fold increase in RhoA.GTP levels compared to the level for control cells, whereas expression of the LKB1SL26 had no detectable effect (Fig. 2A).

To determine whether STRAD is required only to promote the cytosolic accumulation of LKB1 or whether it is also required for LKB1-dependent activation of RhoA, we generated two additional LKB1 mutants. In LKB1P38A, the nuclear localization sequence (NLS) PRRKRA (aa 38 to 43) of human LKB1 has been altered to AAAKRA, while LKB1SL26/P38A contains a disrupted NLS and a disrupted STRAD binding region. When expressed in 16HBE cells stably expressing HA-tagged STRAD, both mutants predominantly localized in the cytosol (Fig. 2B). To determine whether the STRAD interaction is required for Rho activation, Rho.GTP pulldown assays were performed using these two mutants. Expression of LKB1P38A led to a robust increase in RhoA.GTP levels similar to that for wild-type and kinase-dead LKB1, whereas expression of LKB1SL26/P38A induced no significant increase in Rho.GTP (Fig. 2A). These data indicate that a cytoplasmic LKB1/STRAD complex is required for RhoA activation but that this is kinase independent.

Since RhoA controls apical junction formation through its ef-

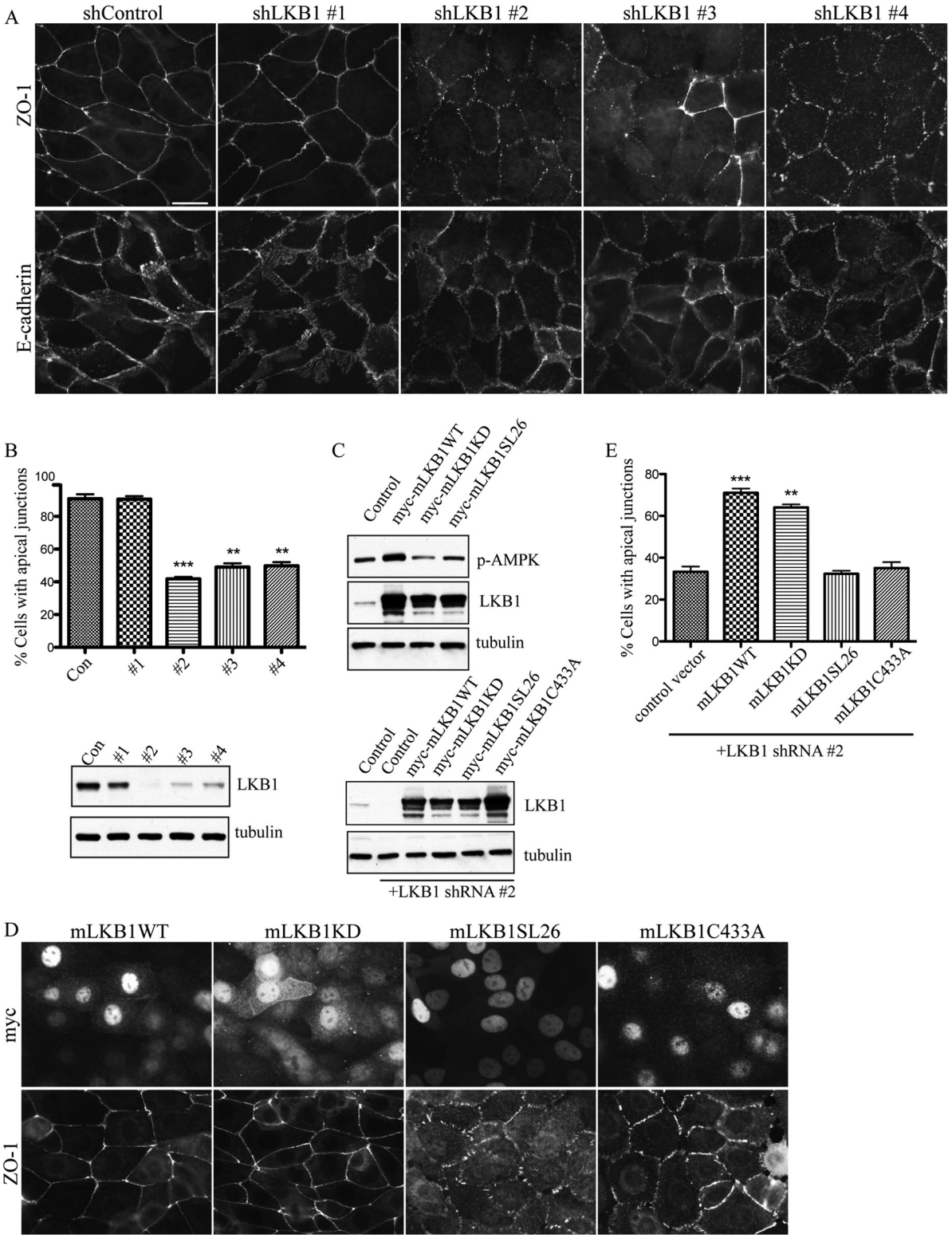

FIG 1 LKB1 controls apical junction formation in 16HBE cells. (A) At 2 days after infection with lentiviral vectors harboring control or 4 distinct shRNAs targeting LKB1 (shLKB1 to shLKB4), 16HBE cells were seeded on coverslips. At 2 days after plating, confluent cells were fixed and stained with anti-ZO-1 (top) and anti-E-cadherin (bottom). Bar, 20 μm. (B) (Top) Quantification of apical junction formation from three independent experiments. Error bar, SEMs. **, $P < 0.01$; ***, $P < 0.001$. (Bottom) At 5 days after infection, cells were lysed and analyzed by Western blotting with the indicated antibodies. Con, control. (C) (Top) Myc-tagged LKB1WT, LKB1KD (K78M), or LKB1SL26 was transiently transfected into 16HBE cells stably expressing HA-STRAD. One day later, cells were lysed and analyzed by Western blotting with the indicated antibodies. (Bottom) 16HBE cells stably expressing mLKB1WT, mLKB1KD (K78M), mLKB1SL26, or mLKB1C433A were infected with shRNA targeting LKB1 lentiviral vector, and 2 and 5 days later cells were lysed and analyzed by Western blotting with the indicated antibodies. (D) Cells described for panel C were replated, grown to confluence, and fixed and stained with anti-ZO-1 (bottom) and anti-myc (top). (E) Quantification of apical junction formation as described for panel D from three independent experiments. Error bars, SEMs. **, $P < 0.01$; ***, $P < 0.001$.

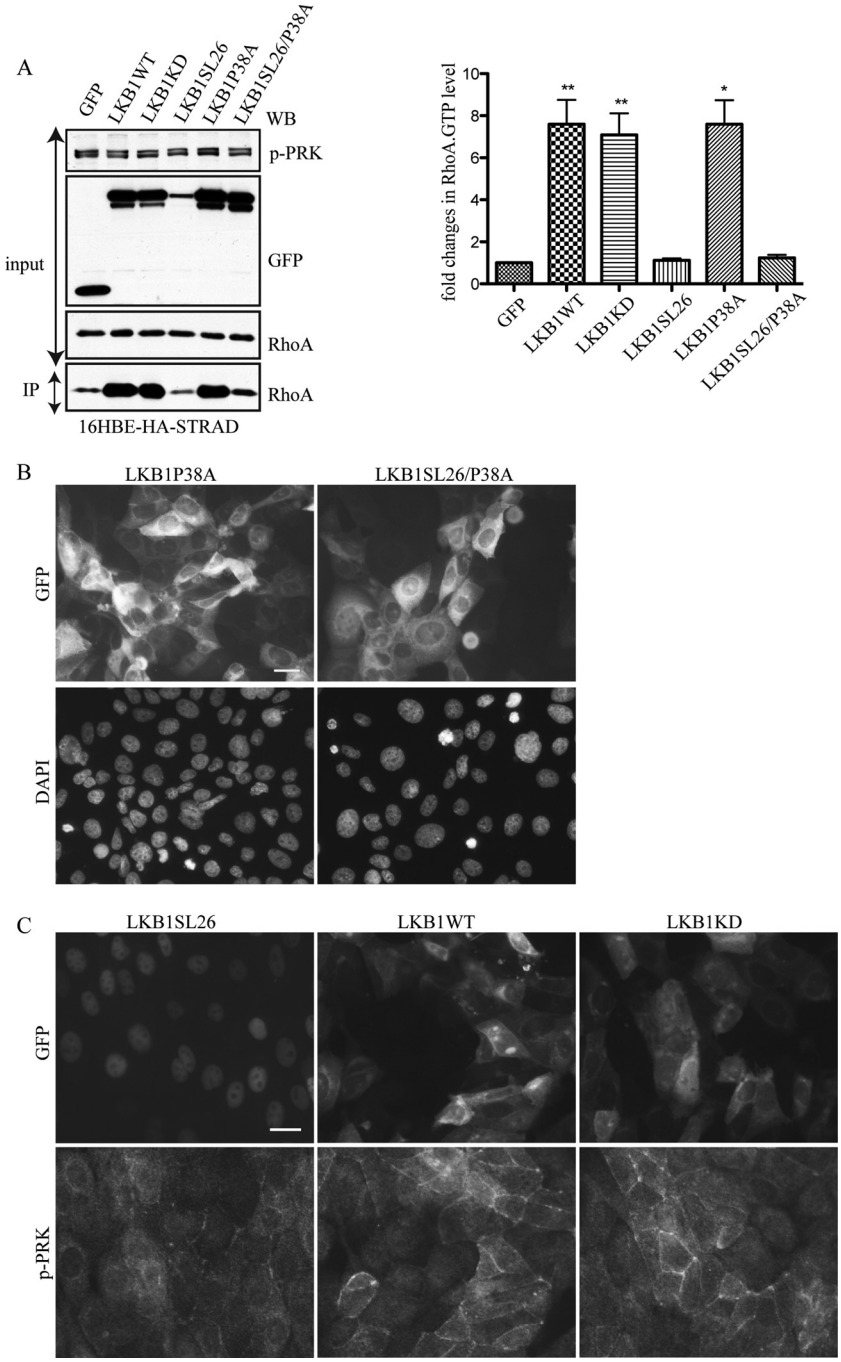

FIG 2 LKB1 and STRAD activate RhoA and enhance phospho-PRK recruitment to apical junctions. (A) 16HBE cells stably expressing HA-tagged STRAD were infected with pLL3.7 lentiviral vectors encoding GFP-tagged LKB1 constructs. Twenty-four hours later, cells were harvested and Rho activity (Rho.GTP) was determined using a standard pulldown assay. LKB1WT, LKB1KD (K78M), and LKB1P38A induced 6- to 8-fold increases in the levels of active RhoA.GTP relative to the level for the control, LKB1SL26, or LKB1SL26/P38A. Total phospho-PRK was determined in the input cell lysates. Error bars, SEMs. **, $P < 0.01$; *, $P < 0.05$. WB, Western blotting. (B) 16HBE cells expressing GFP-tagged hLKB1P38A and hLKB1SL26/P38A were fixed and stained with DAPI (4′,6-diamidino-2-phenylindole). GFP signals were visualized directly. Bar, 20 μm. (C) At 1 day after infection, cells were stained for anti-phospho-PRK. GFP signals were visualized directly. GFP indicated infected cells. Bar, 20 μm.

fector, PRK2, we investigated whether PRK2 recruitment to junctions was enhanced by coexpression of LKB1 and STRAD (Fig. 2C) (31). Cells expressing STRAD and GFP-tagged wild-type or kinase-dead LKB1 showed an increase in phospho-PRK staining at apical junctions compared with that for neighboring cells lacking GFP expression (Fig. 2C). Both wild-type and kinase-dead LKB1 showed a largely cytosol localization due to coexpression of STRAD (Fig. 2C, top). In the cells coexpressing STRAD and LKB1SL26, only a basal level of phospho-PRK was observed at apical junctions (Fig. 2C, bottom). The total levels of phospho-

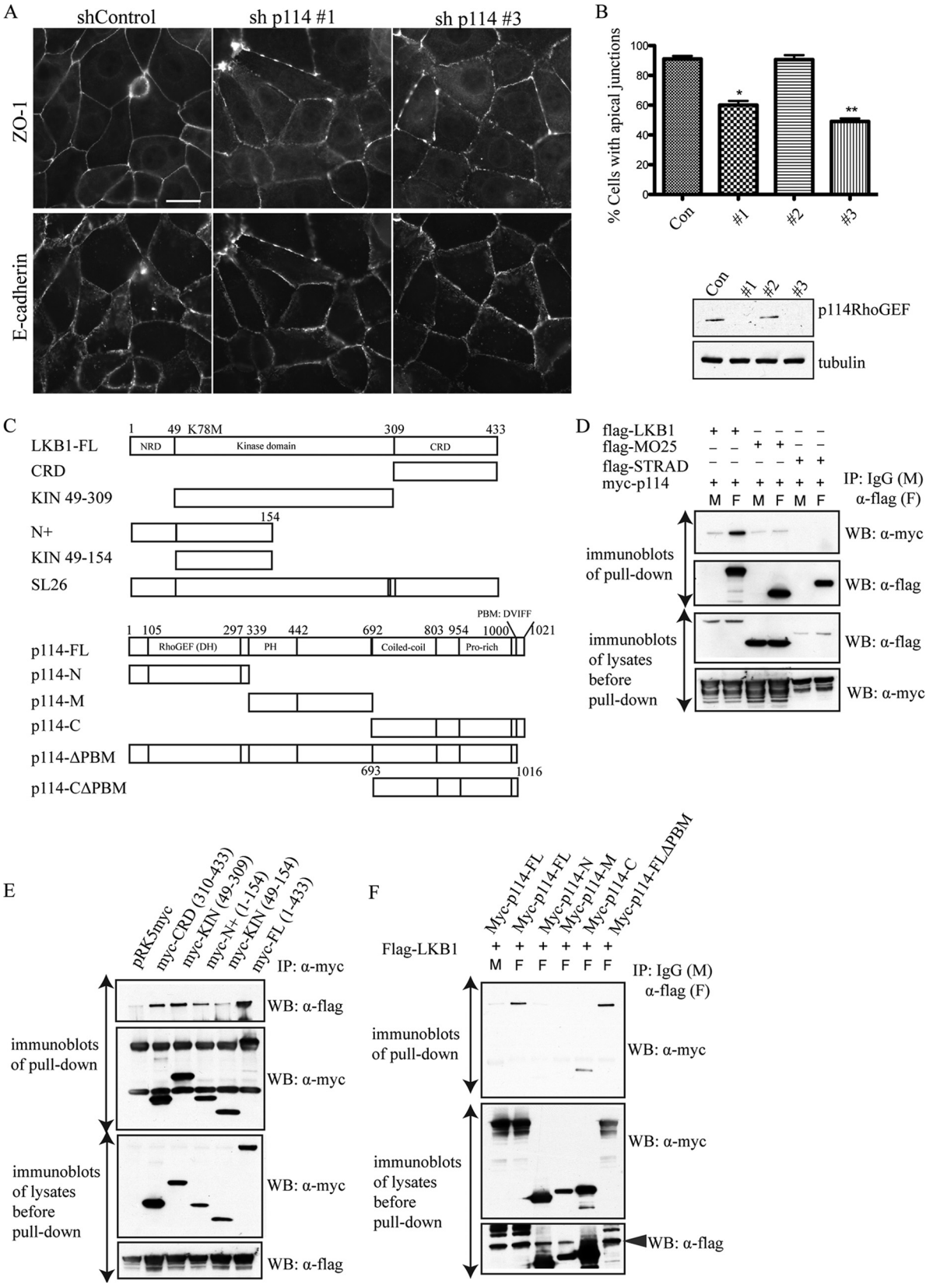

FIG 3 p114RhoGEF controls apical junction formation in 16HBE cells and interacts with LKB1. (A) At 2 days after infection with lentiviral vectors harboring shRNAs targeting p114RhoGEF, 16HBE cells were seeded on coverslips. Two days later, confluent cells were fixed and stained with anti-ZO-1 (top) and anti-E-cadherin (bottom). Bar, 20 μm. (B) (Top) Quantification of apical junction formation from three independent experiments. Error bars, SEMs.

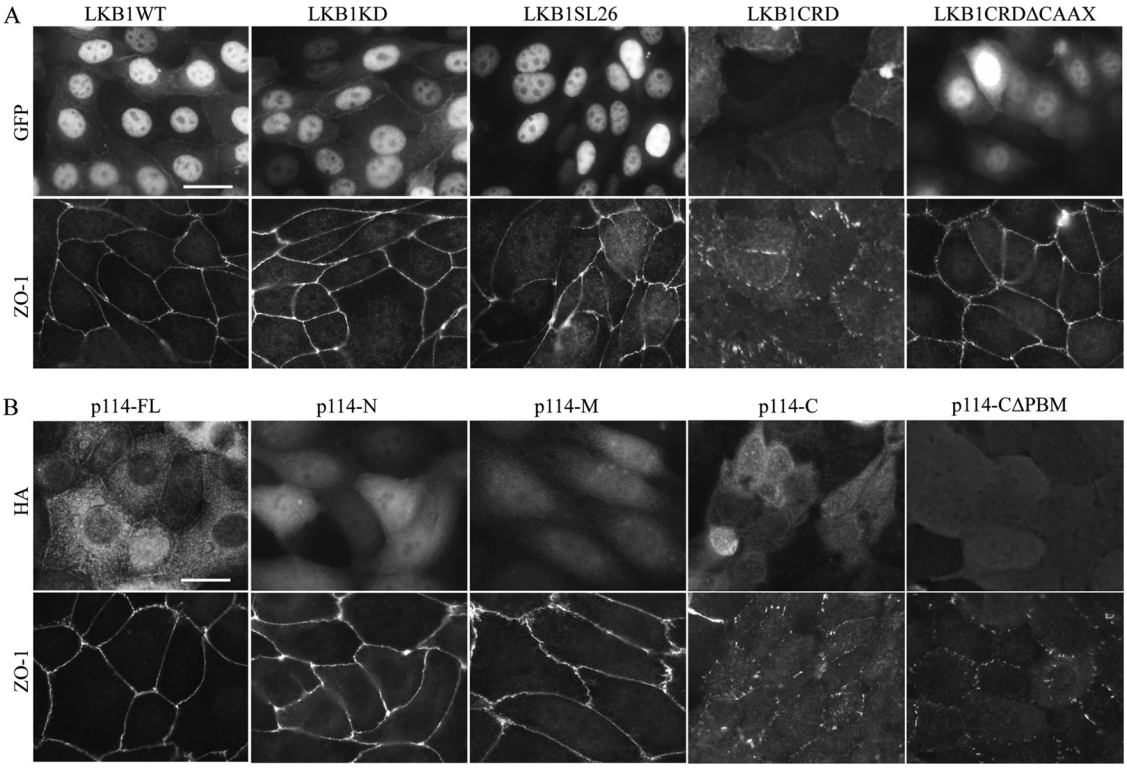

FIG 4 LKB1 and p114RhoGEF C-terminal domains have a dominant negative effect on apical junction formation in 16HBE cells. (A) 16HBE cells stably expressing GFP-tagged LKB1WT, LKB1KD, LKB1SL26, LKB1CRD (aa 309 to 433), or LKB1CRDΔCAAX (aa 309 to 429) were stained with anti-ZO-1 (bottom) and visualized directly with GFP signaling (top). (B) 16HBE cells stably expressing HA-tagged p114RhoGEF-FL (p114-FL), p114RhoGEF-N (p114-N), p114RhoGEF-M (p114-M), p114RhoGEF-C (p114-C), or p114RhoGEF-CΔPBM (p114-CΔPBM) were stained with anti-ZO-1 and anti-HA antibodies. Bars, 20 μm.

PRK were not, however, significantly changed after LKB1 expression (Fig. 2A). We previously showed that Cdc42 and its downstream effector, PAK, are required for apical junction formation in 16HBE cells (32). Overexpression of LKB1 and STRAD did not lead to enhanced recruitment of PAK4 to apical junctions (data not shown), consistent with the effect of LKB1 being specific to the RhoA pathway.

p114RhoGEF is required for apical junction formation and interacts with LKB1. Rho GTPases are typically activated by guanine nucleotide exchange factors (GEFs), and we previously reported that LKB1 can interact with the GEF Dbl in HeLa-S3 cells (5). Depletion of endogenous Dbl in 16HBE cells, however, had no detectable effect on junction formation (data not shown). Recently, another Rho GEF, p114RhoGEF (ARHGEF18), was reported to be a junction-associated protein that regulates tight junction assembly in epithelial cells (33, 34). Depletion of p114RhoGEF in 16HBE cells led to an apical junction defect similar to that seen in LKB1-depleted cells, with ZO-1 forming puncta along regions of cell-cell contact (Fig. 3A, top). Two (of 3) shRNAs (shRNA1, shRNA3) were able to deplete p114RhoGEF, and both resulted in a junctional phenotype, whereas shRNA2 was inefficient in depleting p114RhoGEF and had no significant effect on apical junctions (Fig. 3B).

To explore a potential relationship between LKB1 and p114RhoGEF, a series of human LKB1 and p114RhoGEF fragments was coexpressed in HEK293T cells (Fig. 3C). Immunoprecipitation of full-length LKB1 resulted in coprecipitation of p114RhoGEF (Fig. 3D). However, neither STRAD nor MO25 directly interacted with p114RhoGEF (Fig. 3D). Further analysis revealed that LKB1CRD (residues 310 to 433) and part of it kinase domain (residues 155 to 309) strongly interacted with p114RhoGEF. p114RhoGEF residues (residues 692 to 1021; p114-C) interacted with LKB1 (Fig. 3C, E, and F). The C-terminal PDZ binding motif (PBM) of p114RhoGEF (residues 1017 to 1021), however, was not required for interaction with LKB1 (Fig. 3C and F).

To explore whether the interaction between p114RhoGEF and LKB1 is functionally important, C-terminal fragments of LKB1 (residues 309 to 433; LKBCRD) or p114RhoGEF (residues 692 to 1021; p114-C) were expressed in 16HBE cells and the effects on apical junctions were visualized. Strikingly, expression of the C-terminal regions of either LKB1 or p114RhoGEF caused a severe

*, $P < 0.05$; **, $P < 0.01$. (Bottom) At 5 days after infection, cells were lysed and analyzed by Western blotting with the indicated antibodies. (C) Schematic organization of hLKB1 and mouse p114RhoGEF (numbers represent amino acids). hLKB1 possesses a farnesylation site at aa 430. SL26 represents a 9-bp, in-frame deletion in the kinase domain. p114RhoGEF has a potential PBM at its C terminus. NRD, N-terminal regulatory domain; DH, Dbl homology domain. (D to F) HEK293T cells cotransfected with the indicated combinations of constructs. Proteins were immunoprecipitated (IP) from cell lysates using anti-mouse IgG or anti-Flag antibody. Input and immunoprecipitated lysates were analyzed by Western blotting.

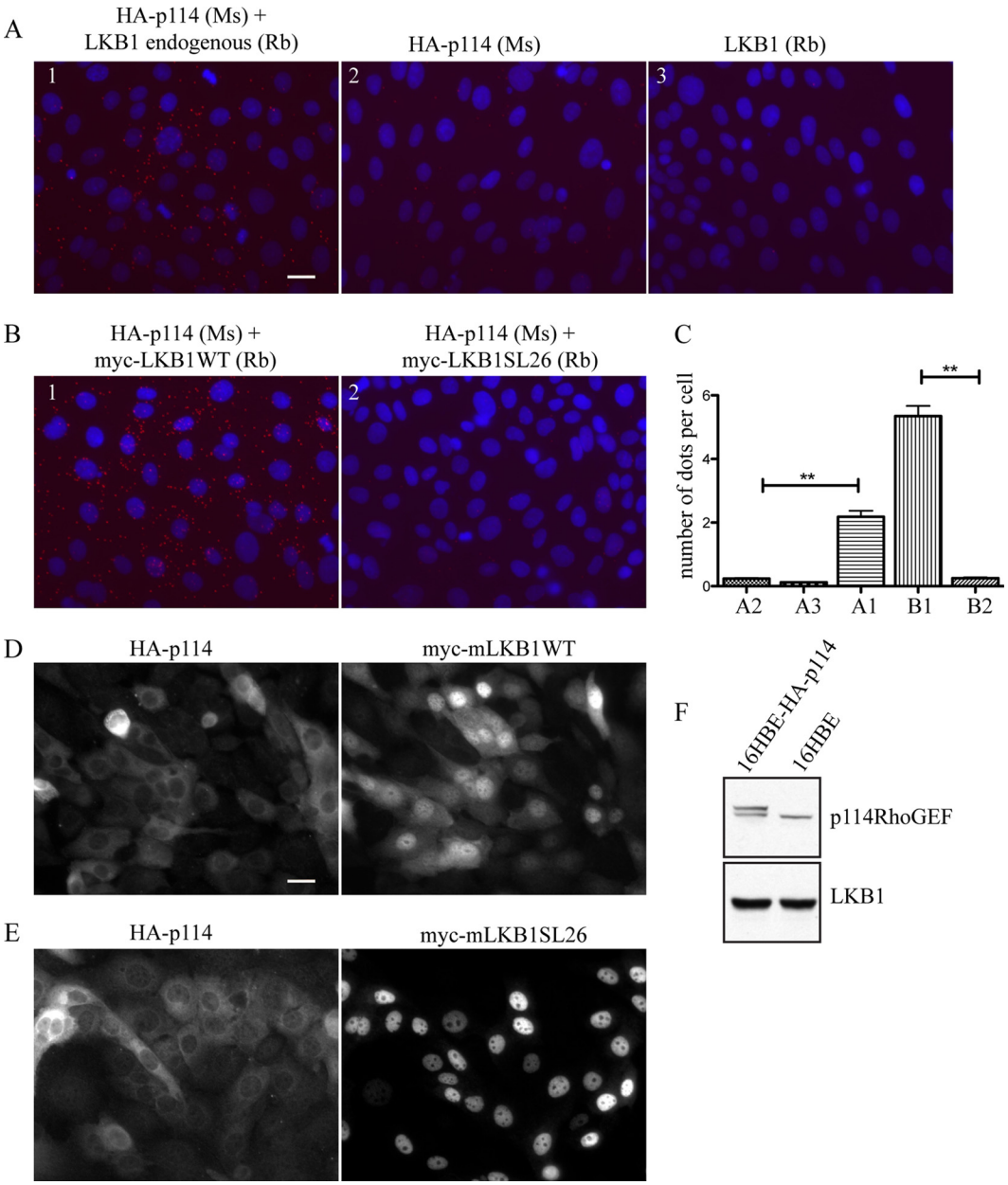

FIG 5 PLA to visualize the interaction between LKB1 with p114RhoGEF in 16HBE cells. (A) 16HBE cells stably expressing HA-tagged p114RhoGEF were fixed and stained with a mixture of mouse (Ms) anti-HA and rabbit (Rb) anti-LKB1 (A1), with anti-HA alone (A2), or with anti-LKB1 alone (A3). PLA signals were visualized by addition of a mixture of anti-mouse and anti-rabbit antibody reagents provided by the company (see Materials and Methods). Bar, 20 μm. (B) 16HBE cells stably expressing HA-tagged p114RhoGEF and either myc-LKB1WT (B1) or myc-LKB1SL26 (B2) were fixed and stained with a mixture of mouse anti-HA and rabbit anti-myc antibodies (left). PLA signals were visualized by addition of a mixture of anti-mouse and anti-rabbit antibody reagents provided by the company (see Materials and Methods). (C) Quantification of PLA signals. Data represent the number of positive dots per cell. Six random nonoverlapping images were taken, and signals were quantified using Volocity image analysis software. Error bars, SEMs. **, $P < 0.01$. (D) Fluorescent images of stable cells expressing HA-p114RhoGEF and myc-mLKB1WT stained with anti-HA antibody (left) and anti-myc antibody (right). Bar, 20 μm. (E) Fluorescent images of stable cells expressing HA-p114RhoGEF and myc-mLKB1SL26 stained with anti-HA antibody (left) and anti-myc antibody (right). (F) Lysates from 16HBE cells stably expressing HA-tagged p114RhoGEF and control cells were examined on Western blots with anti-p114RhoGEF or anti-LKB1 antibodies.

disruption of apical junctions, consistent with both acting in a dominant negative manner (Fig. 4A and B, bottom). Both constructs localized predominantly to the cytosol (Fig. 4A and B, top). The C-terminal region of LKB1 lacking a CAAX box (LKB1CRDΔCAAX) did not show dominant negative activity (Fig. 4A, top right), suggesting that farnesylation is required. The C-terminal region of p114RhoGEF with a PBM deletion (p114-CΔPBM) also disrupted apical junctions, showing that the dominant negative effect is not mediated via a PDZ interaction.

LKB1 and STRAD have both been reported to localize at adherens junctions in fully polarized Caco-2 and MDCK cells but not at apical, tight junctions (35). We were not able to show endogenous LKB1 localization in 16HBE cells with the antibodies available. However, GFP-tagged LKB1WT and LKB1KD both showed

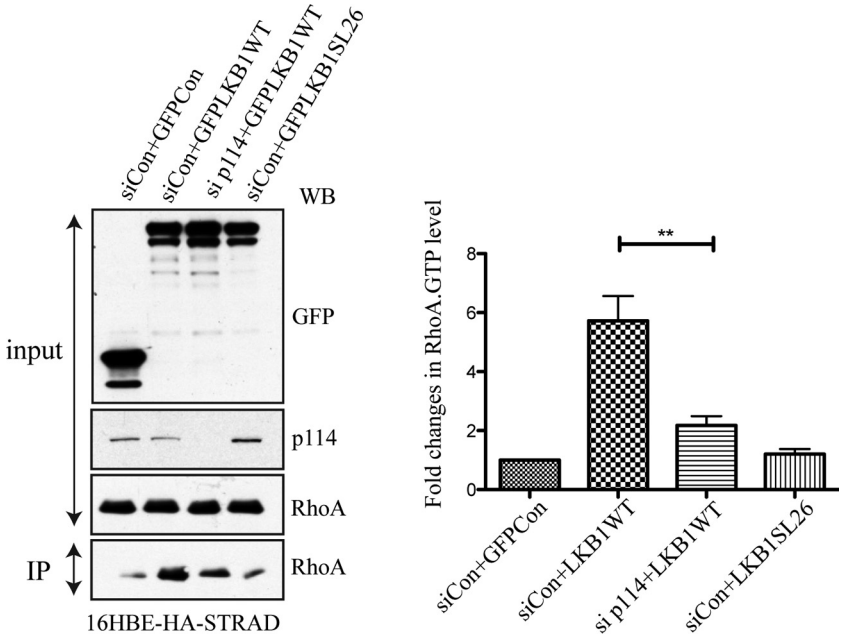

FIG 6 p114RhoGEF is required for LKB1-dependent RhoA activation. (Left) 16HBE cells stably expressing HA-tagged STRAD cells were transfected with siRNA control (siCon) or siRNA oligonucleotide targeting p114RhoGEF. Three days later, cells were infected with lentiviral vectors encoding LKB1WT, LKB1KD, and LKB1SL26. Twenty-four hours later, cells were harvested and the levels of activated Rho (Rho.GTP) were determined using a pulldown assay, followed by Western blotting. (Right) Quantification of the data from three experiments is shown. Error bars, SEMs. **, $P < 0.01$.

strong localization in the nucleus, but with some signal in the cytosol and at the membrane, with the signal at the latter colocalizing with ZO-1 in apical junctions (Fig. 4A). p114RhoGEF has been reported to localize to apical junctions in Dld-1, Caco-2, and MDCK cells (33, 34). p114RhoGEF also colocalized with ZO-1 at apical junctions in 16HBE cells (not shown) and in a stable 16HBE cell line expressing HA-p114RhoGEF at a level similar to that of endogenous protein (Fig. 4B and 5F).

Attempts to coprecipitate endogenous LKB1 and endogenous p114RhoGEF from 16HBE cell lysates were unsuccessful, and so we made use of a proximity ligation assay (PLA) to visualize HA-tagged p114RhoGEF (stably expressed at endogenous levels; Fig. 5F) together with either endogenous LKB1 or ectopically expressed myc-tagged LKB1. The PLA assay, based on a PCR between overlapping oligonucleotides attached to two antibodies, allows the detection of endogenous complexes with minimal cell disruption. A PLA signal was detected when appropriate antibodies to HA-p114RhoGEF and to endogenous LKB1 were used but not with either antibody alone (Fig. 5A and C). Expression of myc-tagged LKB1WT with HA-p114RhoGEF led to a stronger PLA signal (Fig. 5B and C), even though a significant amount of LKB1 resided in the nucleus (Fig. 5D). Expression of myc-tagged LKB1SL26 with HA-p114RhoGEF, on the other hand, gave no signal (Fig. 5B and C), since LKB1SL26 was essentially exclusively in the nucleus (Fig. 5E). These results support an interaction between p114RhoGEF and LKB1 in 16HBE cells.

p114RhoGEF mediates RhoA activation by LKB1. To investigate a possible role for p114RhoGEF in the LKB1/STRAD-dependent activation of RhoA, 16HBE cells stably expressing STRAD were transfected with control or p114RhoGEF siRNA for 3 days and then infected with lentiviral vectors encoding LKB1. As shown earlier, expression of LKB1 induces activation of RhoA in cells transfected with control siRNA. However, prior depletion of p114RhoGEF by siRNA inhibited activation of RhoA by LKB1 (Fig. 6).

LKB1 and p114RhoGEF are required for the maturation of primordial junctions. We previously used a calcium-switch assay to characterize the effect of RhoA depletion on junction assembly in 16HBE cells and showed that it is required for maturation of primordial junctions into mature apical junctions (31, 32). During the first hour after calcium addition, 16HBE cells form primordial junctions, consisting of punctate accumulations of E-cadherin and ZO-1 at cell contacts (Fig. 7A). At 6 h after calcium addition, junction maturation occurs with the formation of apical tight and adherens junctions, seen as continuous staining of ZO-1 and E-cadherin (Fig. 7A). At 1 h after calcium switch, 16HBE cell monolayers depleted of LKB1 (Fig. 7B) or p114RhoGEF (Fig. 7C) look similar to monolayers depleted of RhoA (not shown) or control cells (Fig. 7A), with the formation of primordial junctions. However, at 6 h after calcium switch, control cells showed mature apical junctions (Fig. 7A), whereas cells depleted of LKB1 (Fig. 7B) or p114RhoGEF (Fig. 7C) were largely indistinguishable from cells at the 1-h time point with no maturation to apical junctions. We conclude that both LKB1 and p114RhoGEF are required for the transition from primordial junctions to mature apical junctions.

DISCUSSION

LKB1 inactivation is considered a major factor in the etiology of human lung cancer, but its exact functional contribution to the development of the disease is unclear. LKB1 is a key player in energy homeostasis and cell polarity, but it has many substrates, and its loss likely has pleiotropic effects. To understand better the relationship between LKB1 and lung epithelial cell behavior, we

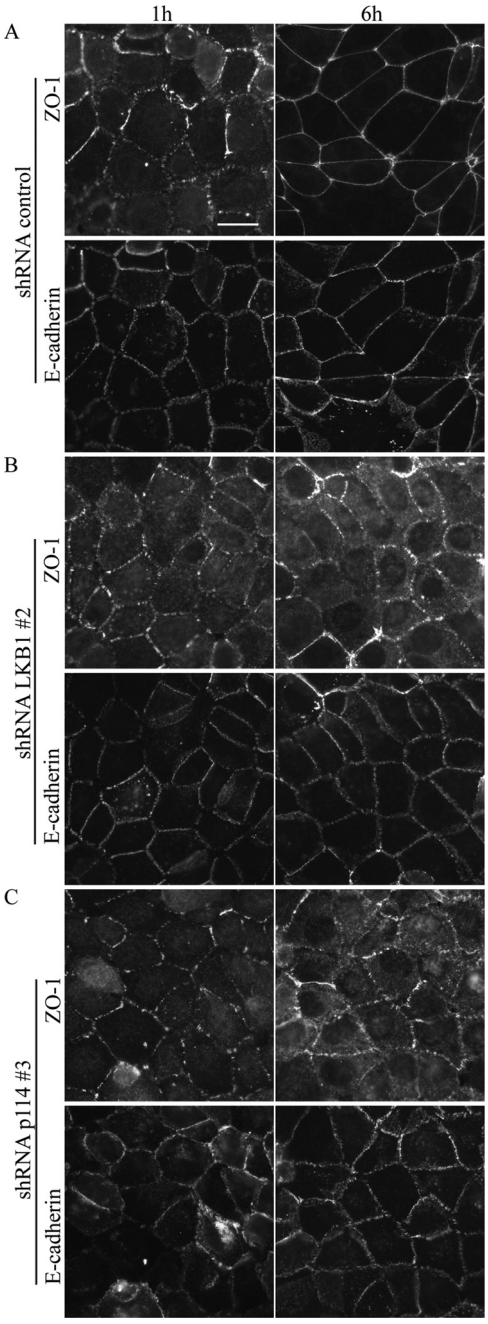

FIG 7 LKB1 and p114RhoGEF regulate the maturation of primordial junctions to apical junctions. 16HBE cells infected with lentiviral vectors harboring control shRNA (A), shRNA2 targeting LKB1 (B), and shRNA3 targeting p114RhoGEF (C) were tested. At 5 days after infection, confluent monolayers were subjected to a calcium-switch assay. After calcium readdition, cells were fixed at 1 h and 6 h and stained with anti-ZO-1 (top) and anti-E-cadherin (bottom). Bar, 20 μm.

have chosen to examine its role in the immortalized human bronchial epithelial cell line 16HBE. We report here that RNAi-mediated depletion of LKB1 disrupts the assembly of apical junctions in these cells. We recently published the finding that RhoA has a similar effect in 16HBE cells, and in the course of examining a possible relationship between LKB1 and RhoA, we identified an interaction between LKB1 and a Rho guanine nucleotide exchange factor, p114RhoGEF. LKB1 and p114RhoGEF are required for RhoA activity in these cells, leading to the assembly of mature apical junctions.

Most surprisingly, the effect of LKB1 on RhoA activation and apical junction formation appears to be independent of its kinase activity. An interaction with STRAD is essential, and interestingly, in addition to relocalizing LKB1 from the nucleus to the cytosol, STRAD binding is also required for junctional activity. It appears that LKB1 is acting as a scaffold-like protein rather than a kinase in this context. This is far from unprecedented; many other examples of a nonkinase role for kinases have been found, ranging from PBS2 in *Saccharomyces cerevisiae* through to Src and Chk1 in mammalian cells (36, 37). A recent report using a knock-in mouse with a Shokat mutation in LKB1 found that while kinase activity is required (together with AMPK) for branching morphogenesis in the lung, kinase activity is not required for the formation of apical basal polarity and apical junctions (38). This level of analysis was made possible through the use of mouse explants; whether complete loss of LKB1 would disrupt apical junctions in this context has, however, not been addressed. In intestinal epithelial cells and migrating astrocytes, LKB1 kinase activity and its C-terminal regulatory domain (CRD) were required to establish and maintain polarity (39). Two groups have used MDCK epithelial cell monolayers and reported that AMPK is required for the assembly of tight junctions in this cell type (40, 41). AMPK was activated during a calcium-switch assay, and a kinase-dead version of AMPK inhibited junction formation in MDCK cells. However, even though AMPK phosphorylation was mediated by LKB1 in this context, neither group directly examined whether LKB1 is required for junction formation. We have depleted LKB1 in the colon cancer-derived cell line Caco-2 and found no effect on junctions (data not shown), and together with work reported in *Drosophila*, it seems likely that its role in different epithelial cell types may be different (42).

We previously reported that RhoA regulates the formation of apical junctions in 16HBE cells, in part at least through the Ser/Thr kinase PRK2 (31). In this case, RNAi rescue experiments showed that the kinase activity of PRK2 is essential. Data from two groups, as well as the work reported here, suggest that the Rho exchange factor p114RhoGEF plays an important role in regulating Rho activity in several epithelial cell types, in the context of apical junction formation. How p114RhoGEF itself is regulated is, however, not known. Several proteins have been reported to interact with this GEF, including Gβγ subunits, a complex of myosin II/cingulin/ROCKII, Lulu, Patj, and Par3 (33, 34, 43). Interestingly, the myosin II/cingulin/ROCKII complex interacts with the pleckstrin homology (PH) domain (residues 338 to 440), while Patj/Par3 interact with the C-terminal PDZ binding motif. It seems unlikely, therefore, that these interactions could account for the strong, dominant negative phenotype that we see after expressing the C-terminal region of p114RhoGEF with a PBM deletion (p114-CΔPBM) in 16HBE cells. Lulu does interact with p114RhoGEF through C-terminal residues, similar to LKB1, and we are currently performing fine mapping to analyze these interactions in more detail. Lulu has also been reported to activate p114RhoGEF, and we show here that expression of LKB1 also leads to p114RhoGEF-dependent activation of RhoA (34). The relationship between Lulu and LKB1 clearly deserves further investigation.

In conclusion, we have identified a kinase-independent role for the LKB1 tumor suppressor in an RhoA pathway regulating the assembly of apical junctions in human bronchial epithelial cells. Loss of LKB1 activity is a common feature of human lung cancer, and the work suggests that one consequence of this may be disruption of apical junctions, leading to loss of epithelial cell integrity.

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