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Research Article

The Tumor Suppressor LKB1 Regulates Lung Cancer Cell Polarity by Mediating cdc42 Recruitment and Activity

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Abstract

The tumor suppressor LKB1 is mutated in 30% of non–small cell lung cancer (NSCLC) tumors and cell lines and is proposed to be a key regulator of epithelial cell polarity; however, how LKB1 regulates cancer cell polarity is not known. The experiments described herein show for the first time that LKB1 is a dynamic, actin-associated protein that rapidly polarizes to the leading edge of motile cancer cells. LKB1 proves to be essential for NSCLC polarity, because LKB1 depletion results in classic cell polarity defects, such as aberrant Golgi positioning, reduced lamellipodia formation, and aberrant morphology. To probe how LKB1 regulates these events, we show that LKB1 colocalizes at the cellular leading edge with two key components of the polarity pathway — the small rho GTPase cdc42 and its downstream binding partner p21-activated kinase (PAK). Importantly, LKB1 functionality is required for cdc42 polarization to the leading edge, maintaining active cdc42 levels, and downstream PAK phosphorylation. To do this, LKB1 interacts only with active form of cdc42 and PAK, but not with inactive cdc42. Taken together, these results show that LKB1 is a critical mediator of the NSCLC polarity program in lung cancer cells through a novel LKB1-cdc42-PAK pathway. [Cancer Res 2008;68(3):740–8]

Introduction

Cell polarization is essential for a broad range of cellular processes, such as mitosis, morphogenesis, and motility. In most eukaryotic cells, this cell polarity program is regulated by a complex network of signaling molecules, cytoskeletal elements, and extracellular cues that ultimately create a functionally and spatially distinct polarized region within the cell (1). One master regulator of this network is the small rho GTPase cdc42 (2); like other Rho GTPases, cdc42 cycles between a GTP-bound active state and GDP-bound inactive state. In its active GTP-bound state, cdc42 regulates key events of cell polarity that include lamellipodia formation (protrusive structure at the cellular leading edge of motile cells), Golgi reorientation, centrosome reorientation, and tight junction formation (2). To do this, active cdc42 relies on a series of downstream effectors that includes its direct binding partner p21-activated kinase (PAK; refs. 3, 4). Upon binding of active cdc42 to PAK, phosphorylation of PAK occurs and cell polarization is triggered (5).

Defects in cell polarity have been linked to cancer progression. It is proposed that the disruption of cell polarity within the epithelial cell lining serves as an initiator for cancer cell invasion into the surrounding environment (6). One potential regulator of the cancer polarity program is LKB1 (also known as STK11; ref. 7). LKB1 is a serine/threonine kinase that contains two nuclear localization sequences, a central kinase domain and a C-terminal farnesylation motif (8). The LKB1 gene is located on chromosome 19p13.3 and produces a 3.1-kb transcript that is expressed in many adult and fetal tissues (7). The N-terminal and C-terminal noncatalytic regions of LKB1 share no relatedness to other proteins. LKB1 was recently shown to be both a lung cancer tumor suppressor that serves as a repressor for the mTOR pathway of biosynthesis (9) and as a regulator of normal epithelial cell polarity (10). Approximately, 30% of non–small cell lung cancer (NSCLC) cell lines and tumors harbor an LKB1 mutation (11, 12). These mutations are primarily point mutations that were either nonsense or frameshift mutations (11).

Importantly, a landmark study showed that LKB1 activation causes complete polarization of single intestinal cells, even in the absence of junctional cell-to-cell contacts, traditionally a prerequisite for polarization (13). Because several LKB1 mutants are incapable of polarizing normal epithelial cells (14), it is intriguing to consider the possibility that one consequence of LKB1 mutation is aberrant cancer cell polarity. Nevertheless, the molecular details of how LKB1 mediates these cell polarity events is not well understood.

Here, we wanted to determine if LKB1 functions in NSCLC polarity, and if so, how LKB1 regulates these events. To address this, we performed a comprehensive analysis of endogenous LKB1 function in NSCLC cell lines using a wounding model of cell polarity and motility. We now show that LKB1 behaves as a dynamic, actin-associated protein that rapidly polarizes to the leading edge of motile cells. LKB1 is essential for maintaining cancer cell polarity because LKB1 defects affect a variety of cell polarity events. To do this, LKB1 regulates the activity and recruitment of cdc42 via its association with the active form of cdc42 and its binding partner PAK. Based upon these studies, we propose a novel LKB1-cdc42-PAK pathway that oversees cancer polarity.

Materials and Methods

Cell culture. All cells were maintained at 37°C in a humidified chamber as previously described (15).

Wounding assays. Cells were grown to confluency on either plastic dishes or coverslips (for microscopy studies) and then wounded using a 1-μL to 10-μL pipette tip. Over time cells will polarize and migrate into the wound. Cells were then processed for immunofluorescence, Western blotting, or immunoprecipitation as described below.

Transfections. Lipofectamine 2000 (Invitrogen) was used to transfect cell lines according to the manufacturer's protocol. Transfections were performed 24 h before wound induction. Oligofectamine (Invitrogen) was

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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used for small interfering RNA (siRNA) transfection according to manufacturer's protocol. LKB1 siRNA was used at 200 nmol/L 72 h before wound induction.

Immunofluorescence and confocal imaging. Immunofluorescence was performed as described previously (16). Antibodies against LKB1 (Abcam), cdc42 (Cytoskeleton), PAK (Santa Cruz), phosphorylated PAK (Biosource), GM-130 (Golgi; Calbiochem), or actin-phalloidin 488 (Invitrogen) were incubated with cells at 4°C overnight. Either Alexa 488, 563, or 633 secondary antibodies (Invitrogen) were used at a dilution of 1:500 and were incubated for 1 h at room temperature. Nuclear staining was performed by incubating cells with 0.4 μmol/L 4′,6-diamidino-2-phenylindole (DAPI) to mounting slides. Cells were imaged on a Zeiss LSM 510 META as described in ref. (15). In all cases, either a 63× or 100× Zeiss Plan-Apo oil objective was used (numerical aperature of 1.3 and 1.4, respectively). To quantitate fluorescence intensity, grayscale images were first thresholded and subjected to mean intensity analysis, with a designated region of interest in Metamorph 6.2 (Molecular Devices). The lamellipodia was defined as the region within 5 μm from the leading edge of the cell. Colocalization analysis was performed by using the "percentage colocalization" feature in Metamorph, which compared the amount of LKb1 colocalized with the protein of interest (e.g., cdc42, PAK) This analysis measured the percentage of LKB1 associated with the protein of interest by comparing signal overlap in thresholded images on a pixel-by-pixel basis. All images have contrast expansion performed in Adobe Photoshop.

Golgi reorientation polarity assays. To image Golgi positioning cells were fixed at different time points postwounding and stained for Golgi, LKB1, and DAPI as described above. Wound edge cells were divided into three 120° regions, with one region facing the wound edge (Supplementary Fig. S2). All cells with the Golgi facing the wound front were scored positive. For each time point, at least 20 cells were examined. Cell orientation was measured as shown in Supplementary Fig. S2. To do this, the angle was calculated between a line dropped from the midpoint of the cellular edge facing the wound and a line tracing the wound front. For examples, cells aligned perpendicular to the leading edge had a nearly 90° orientation, whereas cells aligned parallel to the wound front had a 0° orientation.

Western blotting. Western blotting was performed as previously described (16). Protein concentrations were determined by the bicinchoninic acid protein assay kit (Pierce). Equal amounts of protein from whole-cell lysates were solubilized in SDS sample buffer and separated on SDS 12.5% polyacrylamide gels. The same primary antibodies were used as described above with the appropriate secondary horseradish peroxidase–conjugated antibody (1:2,000).

Cdc42 activation assay. Cdc42 activation assays was performed with the cdc42 activation assay kit from Cell Biolabs, Inc. (STA-402) and used according to the manufacturer's protocol. Cell lysates were prepared from confluent monolayers of LKB1 wild-type H1299 cells or cells transfected 72 h prior with LKB1 siRNA. All the cells were scratched 200 times with a multitip pipette. Cells were washed with cold PBS twice and lysed with 700 μL of lysis buffer [25 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1% NP40, 10 mmol/L MgCl, 1 mmol/L EDTA, and 2% glycerol]. For each time point, 750 μg of protein was incubated with PAK-PBD conjugated agarose beads. Samples were incubated for 3 h at 4°C, washed, centrifuged, resuspended in 40 μL of 2× reducing SDS-PAGE sample buffer, and processed for Western blotting with an anti-Cdc42 mouse monoclonal antibody (described above). Blots were reprobed with mouse anti-LKB1 antibody to visualize the status of the LKB1/PAK-PBD interaction over time.

Immunoprecipitation. Immunoprecipitation was performed with the Catch and Release Reversible Immunoprecipitation System (Upstate) and used according to the manufacturer's protocol. Cells were first seeded into 10 cm^2 dishes then transfected with myc-PAK, FLAG-LKB1, and GFP-tagged cdc42 plasmids as described above. The GFP-cdc42-T17N is a dominant negative inactive cdc42, whereas the GFP-cdc42-Q61L is a constitutively active cdc42. Cells were lysed 24 h after transfection at 70% confluency in 300 μL kit supplied lysis buffer. Cell lysates (500 μg) were incubated with rabbit IgG or antirabbit flag antibody with 10 μL affinity ligand into a bead-coated column. The column was incubated at 4°C for 1 h and washed, and beads were resuspended into 70 μL of 1× denaturing elution buffer containing βME (5%). The samples were boiled for 5 min, centrifuged, and processed for Western blotting.

Results

LKB1 polarizes to the leading edge and associates with actin in motile NSCLC cells. To investigate LKB1 function in NSCLC, we assessed endogenous LKB1 localization in H1703 NSCLC cells (LKB1 wild-type). Cell motility and polarity were induced with a wounding assay that generates a cell free gap bordered by a confluent monolayer of cells. In this assay (17), cells that border the wound will polarize, generate a lamellipodia, and move into the gap over time. In confluent cells (i.e., stationary cells not bordering the wound), LKB1 was found throughout the cytoplasm and nucleus (Fig. 1*A*); however, in cells bordering and migrating into the wound, LKB1 drastically relocalized to or near the plasma membrane, at the leading edge of the cell, facing the direction of movement (Fig. 1*A* and *B*). This pattern of LKB1 localization was observed in all wild-type LKB1 NSCLC cell lines tested, including H1299 and H520 NSCLC cells (Supplementary Fig. S1). Furthermore, a time course showed that LKB1 polarization to the leading edge was rapid and occurred within 5 min postwounding (Fig. 1*C*). Quantitative fluorescent intensity analysis confirms these observations and showed that the percentage of motile cells with polarized LKB1 significantly increased in motile cells within 5 min ($P < 0.05$), as well did LKB1 fluorescent intensity at or near the plasma membrane (Fig. 1*D*). To our knowledge, this is the first time endogenous LKB1 has been successfully visualized and shows a distinct and rapid polarization to the leading edge of motile NSCLC cells.

The actin cytoskeleton is one of the primary components governing cell motility and polarity and is enriched in the lamellipodia at the leading edge; therefore, we next determined whether LKB1, which polarizes to the leading edge (Fig. 1), is associated with actin. To do this, cells migrating into the wound were fixed and costained for LKB1 and actin. LKB1 showed a highly significant colocalization with actin only at the leading edge of motile cells but not in other cellular locales (Fig. 2*A*). Depolymerization of the actin cytoskeleton with the actin inhibitor cytochalasin D (5 μg/mL) removed LKB1 from the leading edge, but LKB1 still seemed associated with a depolymerized actin cytoskeleton (Fig. 2*B*). Further temporal studies showed that LKB1 associated with actin within 5 min postwounding (Fig. 2*C*), indicating that this event is rapid and closely tied to its leading edge polarization, which also occurred within 5 min (Fig. 1*C*).

Lastly, we also examined LKB1 localization in Madin-Darby canine kidney (MDCK) cells, which are noncancerous cell lines that polarize to form an apical and basolateral region in tissue culture dishes when grown to confluency. In nonpolarized cells, endogenous LKB1 is primarily localized in the nucleus and perinuclear region (Supplementary Fig. S1); however, in confluent polarized cells, LKB1 drastically relocalizes to the plasma membrane (Supplementary Fig. S1). Moreover, in the polarized cells, LKB1 colocalizes with actin at the cellular edges (Supplementary Fig. S1), similar to our previous results in NSCLC (Fig. 2). Thus, like the NSCLC cells tested above, LKB1 relocalizes near the plasma membrane and associates with actin when the cell polarity program is triggered in MDCK cells.

LKB1 loss leads to defective Golgi positioning, inhibition of lamellipodia formation, and aberrant lung cancer polarity. Next, we asked the question, does LKB1 regulate NSCLC polarity?

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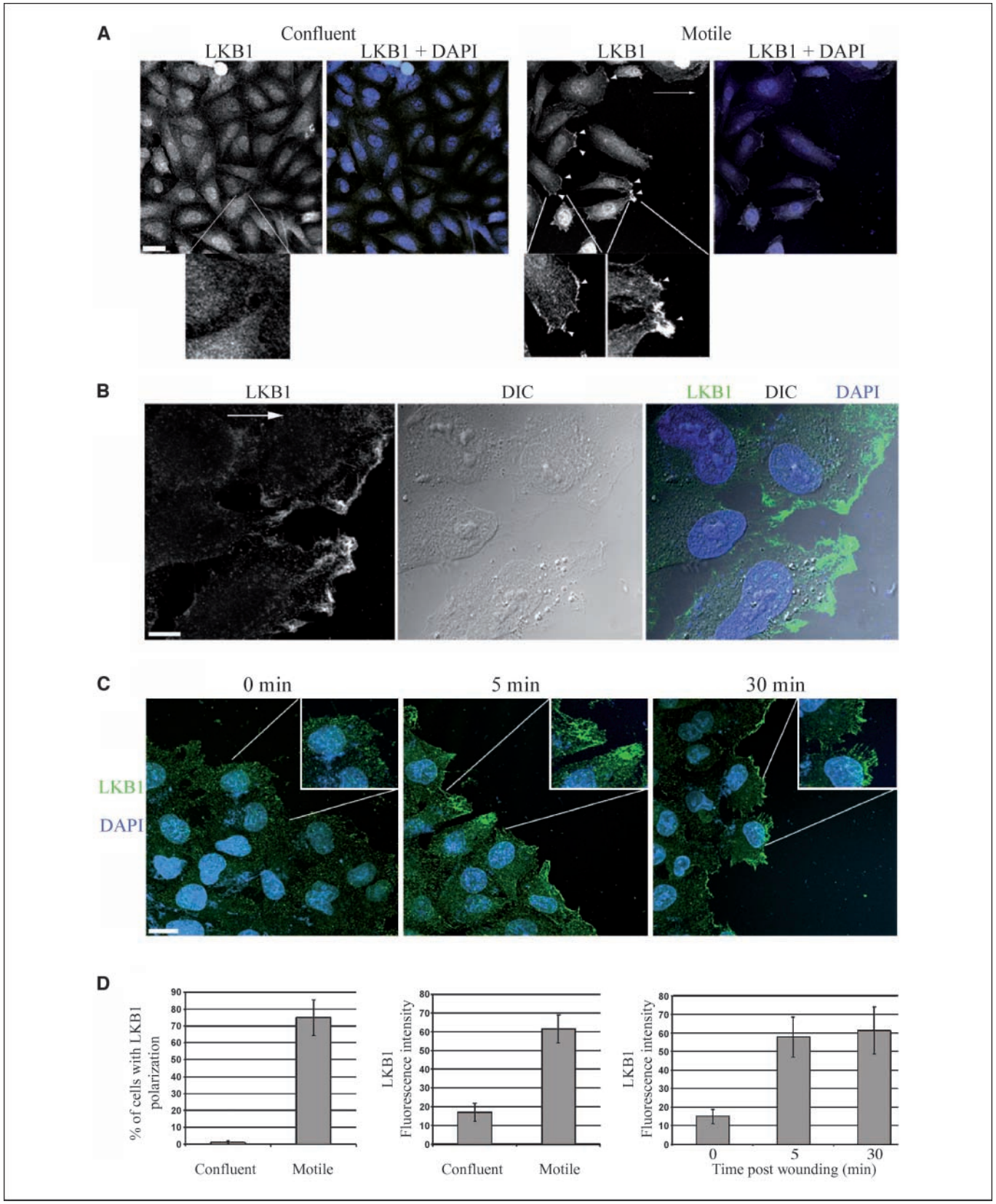

Figure 1. Endogenous LKB1 rapidly polarizes to the leading edge of motile NSCLC cells (*A–C*). Confocal images of endogenous LKB1 immunofluorescence in H1703 NSCLC cells. *Arrow,* direction of movement due to wounding. *A,* LKB1 and DAPI (nuclear) staining in confluent and motile cells. *Arrowheads,* regions of LKB1 polarization. *Bar,* 20 μm. *B,* higher magnification of LKB1 polarization and its relationship to the plasma membrane as revealed by DIC staining. *Blue,* DAPI staining. *Scale bar,* 5 μm. *C,* time course of LKB1 polarization. *Scale bar,* 10 μm. *D,* left bar graph shows the percentage of cells with LKB1 polarization after wounding. Center and right bar graphs show LKB1 mean fluorescent intensity; *n* = 20 cells per experimental group; *bars,* SD.

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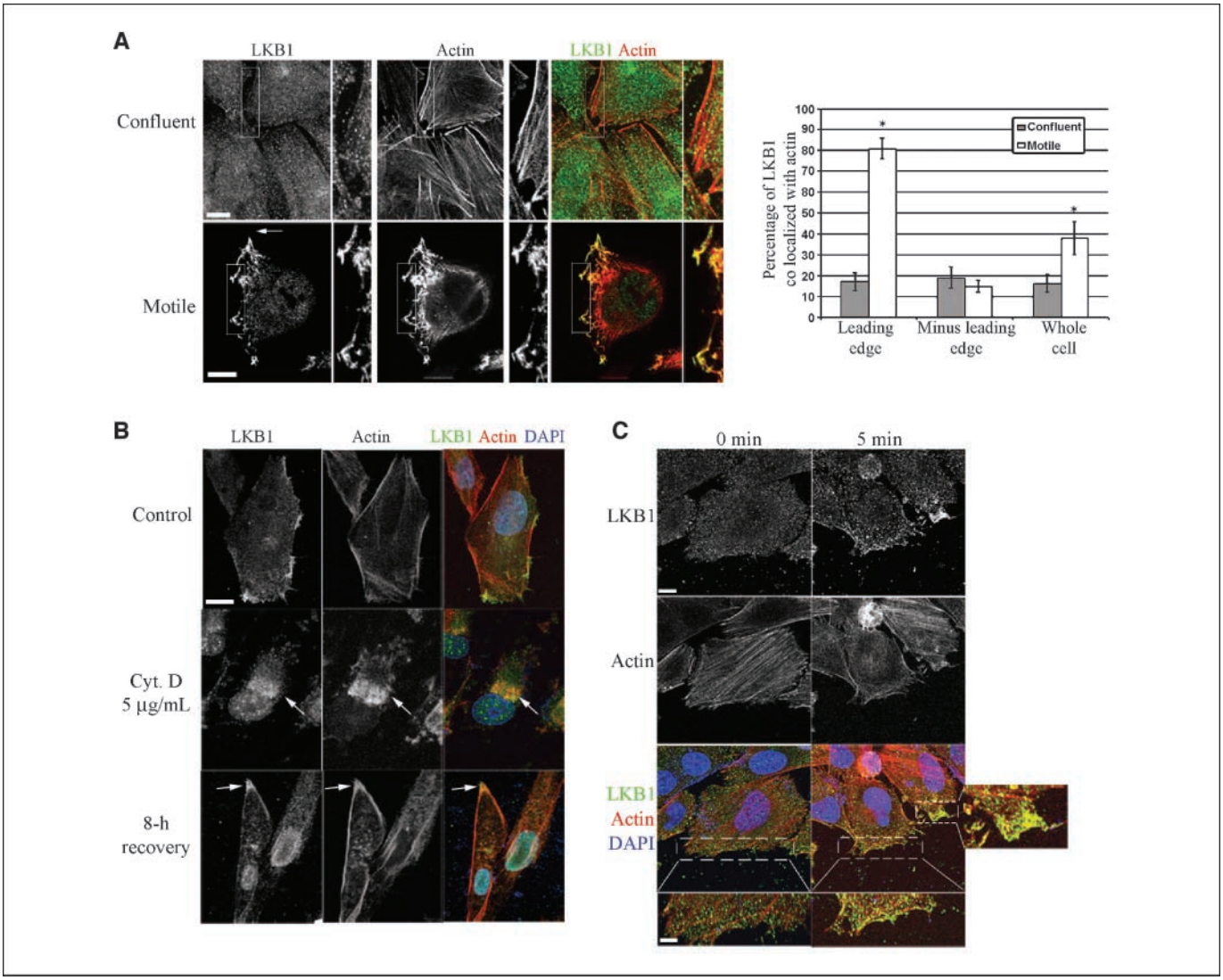

Figure 2. LKB1 associates with actin in motile NSCLC cells (*A–C*). Confocal images of LKB1, actin, and DAPI immunofluorescence. *A,* LKB1 and actin staining in confluent and motile cells. Insets show magnified view of boxed region. Bar graph shows percentage colocalization of LKB1 and actin in the various cellular regions (n = 10 cells). *Scale bar,* 10 μm. *Bars,* SD. *B,* LKB and actin staining in cytochalasin D–treated cells and after an 8-h recovery from treatment. *Scale bar,* 10 μm. *C,* LKB1 and actin staining at 0 and 5 min postwounding. *Scale bars,* 5 μm. Insets show magnified field of boxed region; *scale bar,* 2 μm.

To do this, we used siRNA to deplete LKB1 and subsequently examined the effect on cell polarity. Due to the Golgi's role in protein trafficking to the leading edge, it serves as a marker of cell polarization and reorients between the nucleus and the leading edge when cell polarity is stimulated (18, 19). We therefore examined Golgi reorientation in a quantifiable manner by dividing cells into three regions (120° in each region), such that in unpolarized cells the Golgi had an equal likelihood of being in any of the three regions (example in Supplementary Fig. S2), but in polarized cells, the Golgi realigns to the 120° region facing the direction of movement (18, 19). To assess this, staining of the Golgi, LKB1, and nucleus was performed in control siRNA–transfected and LKB1 siRNA–transfected cells (Fig. 3*A*). In cells transfected with control siRNA, the Golgi realigned by 6 h, with nearly 70% of cells having proper Golgi orientation. In contrast, LKB1 depleted cells showed only 35% of cells with proper Golgi positioning after 6 h and only 45% after 16 h, indicating defective cell polarity in the absence of LKB1 (Fig. 3*B* and *C*). Additional defects in cell polarity were also observed when cell shape and lamellipodia formation were analyzed. Control cells were elongated perpendicular to the wound front and had a clear lamellipodia by 6 h, whereas LKB1-depleted cells remained randomly oriented and lacked a distinctive lamellipodia (Fig. 3*B*). Image quantitation (example in Supplementary Fig. S2) showed that controls cells repositioned nearly 70° relative to the wound by 6 h and maintained this position for at least 16 h (Fig. 3*D, top and bottom*). In contrast, LKB1-depleted cells had a 30° orientation relative to the wound at 6 h and 15° orientation by 16 h. Interestingly, LKB1-depleted cells ultimately aligned parallel to the wound instead of the normal perpendicular orientation observed in control cells (Supplementary Fig. S2), further indicating defective cell polarity. Similar results were also obtained in other NSCLC cell lines with wild-type LKB1 (data not shown), indicating that this is not a cell line–specific event. Taken together, these data indicate that LKB1 is necessary for NSCLC polarity.

LKB1 colocalizes with the polarity proteins cdc42 and PAK and regulates cdc42 recruitment. Cdc42 is a master regulator of

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cell polarity and also polarizes to the leading edge of motile cells (2); therefore, we next determined the relationship between LKB1 and cdc42 in this region. Immunofluorescence colocalization analysis of endogenous LKB1 and cdc42 in H1299 cells revealed that a significant portion of LKB1 colocalized with cdc42 within 15 min postwounding at the leading edge (Fig. 4*A*). Interestingly, the two proteins were not associated in any other regions within the cytoplasm (Fig. 4*A*) and LKB1 only colocalized with cdc42 in motile but not confluent cells (data not shown). Similar colocalization between LKB1 and cdc42 were also observed in H1703 NSCLC cells (Supplementary Fig. S3).

We next examined LKB1 colocalization with the cdc42 binding partner PAK. When active, cdc42 binds to PAK causing PAK phosphorylation and eliciting a variety of downstream cell polarity events, including Golgi reorientation and actin remodeling (4, 20). Colocalization analysis showed that, indeed, LKB1 colocalizes with PAK only at the leading edge (Fig. 4*A*), suggesting that LKB1, cdc42, and PAK are possibly linked in the cell polarity pathway. Moreover, LKB1 also colocalizes at the leading edge with phosphorylated PAK (Ser^{144}; Supplementary Fig. S3), which represents the active form of PAK. To exclude the possibility of a putative cell adhesion role for LKB1, we stained for phosphorylated focal adhesion kinase (FAK), a functional marker of the cell adhesion pathway. In this case, LKB1 did not colocalize phosphorylated FAK, rather it was excluded from these adhesive sites (Supplementary Fig. S3), suggesting that LKB1 is not involved in the cell adhesion pathway or localizes to adhesive sites within the motile cell.

To further examine the spatiotemporal relationship between LKB1 and cdc42, we performed a 10-min short-time course of LKB1 and cdc42 localization to determine when these proteins colocalize. These results showed that LKB1 accumulates near the plasma membrane as early as 1 min postwounding, and this accumulation precedes cdc42 arrival (Fig. 4*B*). By 5 min, cdc42 begins to accumulate at the leading edge, at LKB1-marked sites (Fig. 4*B*). This trend continues, and by 10 min, both proteins are colocalized (Fig. 4*B*). Thus, LKB1 polarization precedes that of cdc42, but ultimately cdc42 colocalizes to the LKB1-designated sites. Notably, we did not observe any cells where cdc42 was localized to the leading edge without LKB1 already there; however, there were cells that had neither LKB1 nor cdc42 at the leading edge at the early

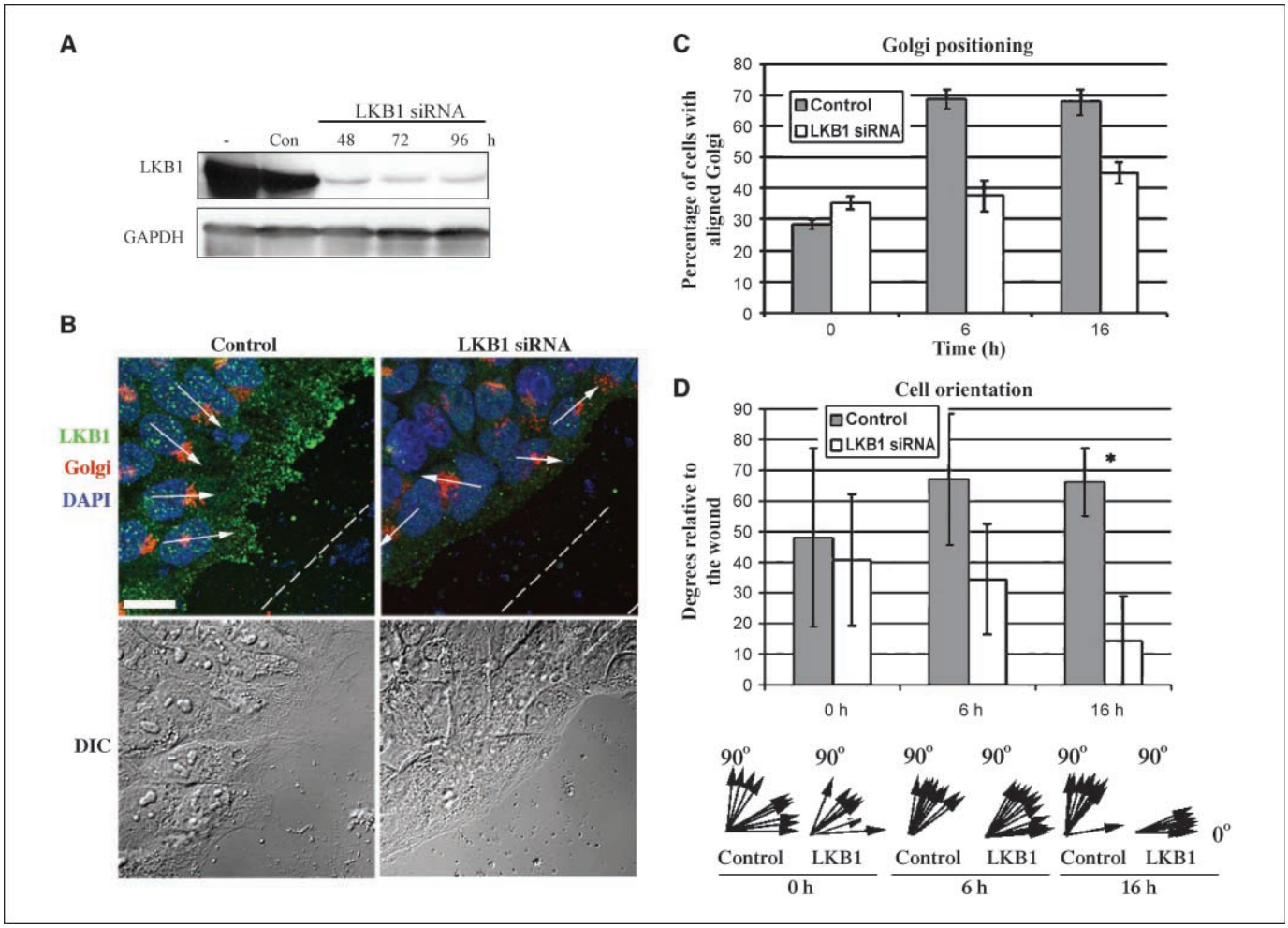

Figure 3. LKB1 depletion causes defective cell polarity. *A,* Western blot showing siRNA depletion of LKB1 in H1792 NSCLC cells. *Dash,* no transfection; *Con,* control siRNA. *B,* LKB1, Golgi, and DAPI immunofluorescence in H1792 NSCLC cells. *Arrows,* Golgi positioning relative to the wound designated with a dashed line. DIC images below show cellular morphology and lamellipodia of the same cells as above. *Scale bar,* 10 μm. *C,* bar graph quantitating the percentage of cells with aligned Golgi in control and LKB1-depleted cells ($n = 20$ cells per experimental group). *D, top,* bar graph depicting cell positioning in degrees relative to the wound ($n = 20$ cells per experimental group; *bottom*). *Arrows,* graphical representation of cellular positioning relative to the wound in control and LKB1-depleted cells.

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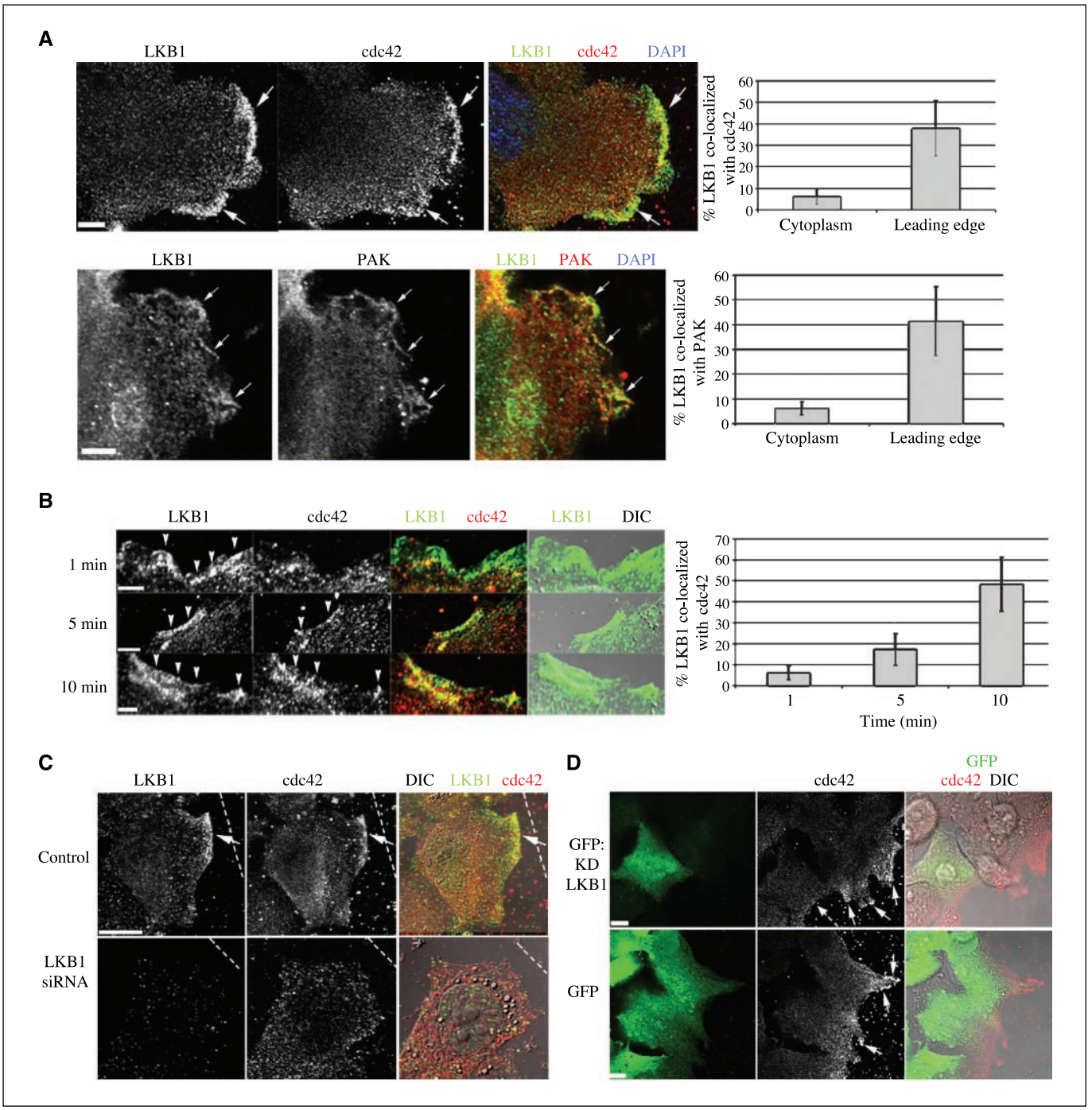

Figure 4. LKB1 colocalizes with cdc42, PAK, and p-PAK and precedes cdc42 recruitment (*A–D*). Confocal images of LKB and cdc42 or PAK immunofluorescence in H1299 NSCLC cells. Bar graphs show percentage LKB1 colocalized with the respective protein. In all cases, error bars are SD. *A,* LKB1 and cdc42 staining at the leading edge (*top*). *Arrows,* regions of colocalization. LKB1 and PAK staining at the leading edge (*bottom*). *Arrows,* regions of colocalization. *Scale bars,* 5 μm. *B,* short-interval time course of LKB1 and cdc42 localization. DIC staining shows LKB1 localization at or near the plasma membrane. *Scale bar,* 2 μm. *C,* LKB1 and cdc42 staining in control and LKB1 siRNA knockdown cells. *Arrows,* cdc42 polarization to the leading edge; *dashed lines,* the wound front. *Scale,* 10 μm. *D,* confocal images of cdc42 staining in a cell expressing either a dominant-negative kinase dead LKB1 (GFP:KD LKB1) or GFP control (*bottom*). *Arrow,* cdc42 polarization; *dashed arrow,* lack of cdc42 polarization. Insets show magnified view. *Scale,* 10 μm. For all bar graphs, *n* = 10 cells per experimental group.

time points. This same pattern of LKB1-cdc42 colocalization was observed in H1703 NSCLC cells (Supplementary Fig. S3). Taken together, these results suggest that LKB1 serves as an intracellular marker for future cdc42 recruitment.

The above results suggest that LKB1 may regulate cdc42 recruitment. To test this, LKB1 was depleted in H1299 cells with siRNA and cdc42 recruitment to the leading edge was assayed. In cells transfected with control siRNA, LKB1 and cdc42 were colocalized at the leading edge after 15 min postwounding in nearly all cells observed as expected (Fig. 4*C*). In contrast, in cells transfected with LKB1 siRNA, cdc42 did not polarize to the leading edge but rather displayed a punctate appearance throughout the

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cytoplasm (Fig. 4*C*). As an alternative approach, we overexpressed a kinase dead mutant of LKB1 fused to GFP (GFP:KD LKB1; ref. 21) in LKB1 wild-type cells. This GFP:KD LKB1 lacks LKB1 kinase activity and therefore serves as an excellent probe for understanding the specific role of LKB1 kinase activity. In GFP:KD LKB1–positive cells, cdc42 did not polarize to the leading edge (Fig. 4*D*), whereas neighboring cells that were not expressing the kinase dead LKB1 did, indeed, have cdc42 polarization (Fig. 4*D*). Furthermore, these cells did not have a lamellipodia, consistent with the idea that LKB1 kinase activity is required for its role in cell polarity. Transfections with a control GFP-only vector did not alter cdc42 recruitment or lamellipodia formation (Fig. 4*D*). Thus, when LKB1 expression is depleted or endogenous LKB1 activity is inhibited with a kinase dead dominant-negative LKB1, cdc42 cannot polarize to the leading edge. These results therefore support the hypothesis that LKB1 is required for cdc42 recruitment to the leading edge.

LKB1 mediates cdc42 activity by complexing with PAK and active cdc42, but not inactive cdc42. Previous reports show that cdc42 is recruited and activated at the leading edge (22, 23) by a series of GTPase-activating proteins (GAP) and guanine exchange factors (GEF; ref. 22). Because we show that LKB1 is essential for cdc42 leading edge recruitment, we assayed for active cdc42 levels in control siRNA–transfected and LKB1 siRNA–transfected cells prewounding and postwounding, using a coimmunoprecipitation approach with PAK-coated beads. This immunoprecipitation-based approach is based upon the principle that active cdc42 (GTP bound) binds specifically to the PBD domain of PAK. Thus, this assay uses PAK-PBD agarose beads to selectively isolate and pull down active cdc42 (cdc42-GTP) from lysates. Subsequently, the precipitated GTP-cdc42 is detected by Western blot analysis using a mouse anti-cdc42 antibody. To activate cdc42, multiple wounds were made to confluent H1299 NSCLC cells on 10 cm^2 dishes; this is the standard approach for cdc42 activation (Fig. 5*A*; ref. 19). Control H1299 LKB1 wild-type NSCLC cells initially have low prewounding levels of active cdc42, but after 10 min postwounding, active cdc42 substantially increases as predicted (Fig. 5*B*). In contrast, in LKB1-depleted cells, active cdc42 levels were significantly lower, in both prewounding and postwounding samples relative to their control counterparts (Fig. 5*B*). However, cdc42 activation itself was not impaired, because we observed a nearly 2-fold increase in cdc42 levels in control and LKB1-depleted cells (Fig. 5*B*). These data show that LKB1 depletion significantly reduces active cdc42 levels, but not cdc42 activation per se. Total cdc42 levels did not change throughout the experiment (Fig. 5*B*). Similar experiments were performed with the kinase dead GFP:KD LKB1 and showed that the levels of active cdc42 postwounding were significantly reduced in the population expressing the kinase dead LKB1 compared with the wild-type LKB1 (Fig. 5*C*). Thus, this result is consistent with LKB1 mediating active cdc42 levels.

Because these results suggest that LKB1 can mediate cdc42 activity levels, we wanted to determine if LKB1 complexes with cdc42 and the downstream cdc42 binding partner PAK. Moreover, we also wanted to examine the possibility that a putative association is dependent upon the activity state of cdc42. Therefore, we performed coimmunoprecipitation assays in H1299 cells with cotransfected FLAG-LKB, myc-PAK, and three different GFP-cdc42 constructs — a constitutively active cdc42 (Cdc42-Q61L), dominant-negative cdc42 (Cdc42-T17N), or wild-type cdc42 (23, 24). The activity of the Cdc42-Q61L and inactivity of cdc42-T17N was validated in our wounding assay (Supplementary Fig. S4). Importantly, these results show that LKB1 has an enhanced association with the constitutively active cdc42-Q61L mutant compared with inactive cdc42-T17N or wild-type cdc42 (presumably a mixed population of active and inactive cdc42; Fig. 5*C*). Specifically, the cdc42-Q61L mutant coimmunoprecipitated with LKB1, whereas the cdc42-T17N and wild-type cdc42 showed minimal association. In addition, myc-PAK showed an enhanced coimmunoprecipitation with LKB1 in the presence of the constitutively active cdc42-Q61L mutant, suggesting that LKB1, PAK, and active cdc42 are complexed together within the cell in a cdc42 activity–dependent manner (Fig. 5*D*).

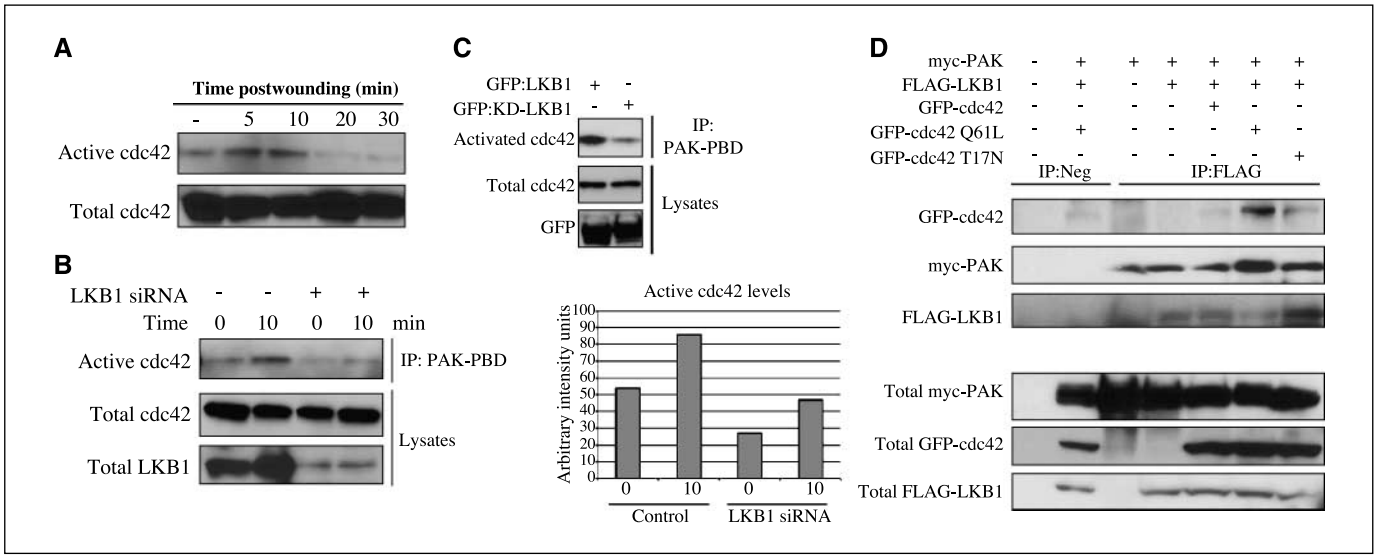

Figure 5. LKB1 mediates active cdc42 levels and associates with the active cdc42-PAK complex. *A,* Western blot showing a time course of cdc42 activation in our wounding model. *B,* Western blot showing cdc42 activation in control and LKB1-depleted cells at 0 min (prewounding) and 10 min (postwounding). *C,* Western blot of a cdc42 activation assay in H1299 NSCLC cells overexpressing a dominant-negative GFP:KD LKB1 after 15 min postwounding compared with wild-type GFP:LKB1. *D,* immunoblotting of FLAG-LKB1 immunoprecipitation in H1299 cells transfected with the various constructs shown. Immunoblots of total lysates are shown below.

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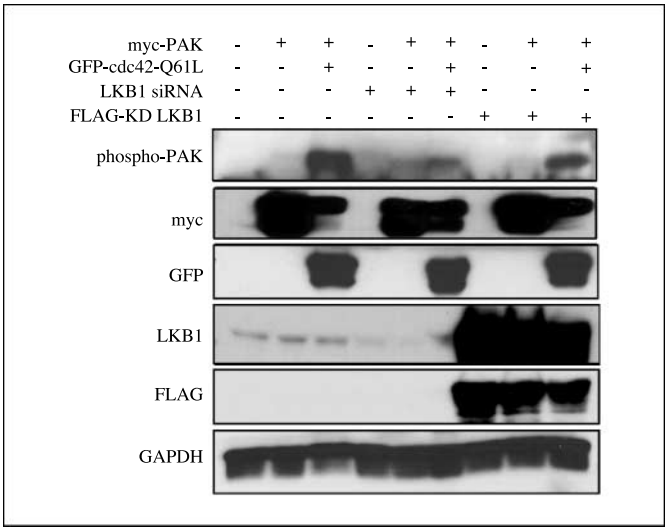

Figure 6. Compromised LKB1 functionality affects downstream PAK phosphorylation. Western blot of phosphorylated PAK levels in control, LKB1-depleted cells, and cells transfected with a dominant-negative FLAG-KD LKB1. A GFP-labeled constitutively active cdc42 mutant (GFP-cdc42-Q61L) was overexpressed to activate myc-PAK phosphorylation.

Compromised LKB1 functionality affects downstream PAK phosphorylation. Binding of active cdc42 to PAK stimulates its phosphorylation and triggers various cell polarity event (4). To determine the effect of LKB1 on the downstream activation of PAK, levels of phosphorylated PAK in control and LKB1-depleted cells were examined. In control cells, phosphorylated PAK levels are initially low, but are stimulated in the presence of constitutively active cdc42-Q61L mutant as expected (Fig. 6*A*). In contrast, the cdc42-Q61L–induced stimulation of PAK phosphorylation was suppressed in LKB1-depleted cells or cells overexpressing a kinase dead LKB1 mutant (GFP:KD LKB1; Fig. 6*A*). Taken together, these results indicate that LKB1 activity is required for active cdc42 to induce PAK phosphorylation. Furthermore, LKB1 also colocalizes with phosphorylated PAK sites at the leading edge but not throughout the cytoplasm (Supplementary Fig. S3). This is similar to LKB1 localization with both cdc42 and total PAK (Supplementary Fig. S3); however, phosphorylated PAK seems to form well-defined puncta within the LKB1-stained regions.

Discussion

LKB1 behaves as a dynamic protein that rapidly migrates to the cellular leading edge within 5 min and associates with actin. This pattern of localization is similar to other cell polarity proteins, such as the small Rho GTPase rac, β-catenin, and GSK3β (17, 25, 26). Moreover, a polarized LKB1 localization was recently observed in some human lung cancer tissues by immunohistochemistry (27). LKB1 polarization and activity seem to be regulated by the cell polarity stimulus because LKB1 has a diffused cytoplasmic and nuclear pattern before wounding, but upon cell polarity stimulation, LKB1 relocalizes to the leading edge and colocalizes with actin filaments. LKB1 rarely colocalizes with actin stress fibers that span the cytoplasm, suggesting that the mechanism of LKB1 translocation to the leading edge is not actin-based; however, it requires actin to maintain its localization to the leading edge because actin inhibition removes LKB1 from this region.

LKB1 is essential for NSCLC polarity in our wounding model because LKB1 depletion results in classic cell polarity defects, such as aberrant Golgi positioning, reduced lamellipodia formation, and aberrant morphology. Previous studies show that these cell polarity events are regulated, at least in part, by the small rho GTPase cdc42 and its downstream binding partner PAK (2). Importantly, our data now show that LKB1 is a critical member of this cdc42-PAK pathway. LKB1 protein expression and kinase activity are essential for both the recruitment and maintenance of active cdc42 levels. Specifically, when LKB1 function is compromised, cdc42 does not polarize to the leading edge and active cdc42 levels are significantly diminished. Cdc42 activation involves a series of GAPs and GEFs (22) As cdc42 activation takes place at the leading edge (23), any defect in its polarization to the leading edge would be expected to affect levels of cdc42 activation. Our data are most consistent with the idea that LKB1 is necessary for the translocation of cdc42 rather than its direct activation as wounding induces a similar 2-fold activation of cdc42 in both control and LKB1-depleted cells.

LKB1 forms a complex with cdc42 and PAK in NSCLC, and this interaction is dependent on the activation status of cdc42. Consistent with this idea, LKB1 colocalizes with cdc42, PAK, and phosphorylated PAK at the cellular leading edge when the cell polarity program is stimulated but does not colocalize with any of these proteins before cell polarity stimulation or in other regions throughout the cytoplasm. Furthermore, LKB1 depletion or inhibition of its kinase activity suppresses PAK phosphorylation at Ser^{144}, a site critical for activation of PAK kinase activity (4). Active cdc42 is known to stimulate PAK phosphorylation, leading to downstream induction of cell morphogenesis, changes in actin/microtubule dynamics, and cell motility (4). Thus, the cell polarity defects observed in the absence of LKB1 are thus likely due to impaired translocation and activation of cdc42 and consequently diminished PAK phosphorylation. Interestingly, initial results show that cell lines naturally defective in LKB1 (e.g., A549) have reduced cdc42-dependent PAK phosphorylation (data not shown), suggesting that natural loss of LKB1 function impairs PAK phosphorylation.

Taken together, we propose an LKB1-cdc42-PAK pathway such that, when cell motility and polarity are stimulated, LKB1 polarizes to the leading edge where it associates with actin to serve as a scaffold for the subsequent recruitment and activation of cdc42. Once this occurs, a complex between LKB1, active cdc42, and PAK assembles, resulting in PAK phosphorylation and downstream activation of cell polarity events, such as lamellipodia formation and Golgi reorientation. We believe that LKB1 is an essential component of this process, because LKB1 depletion leads to reduced cdc42 activation and recruitment, reduced PAK phosphorylation, and negatively affects several hallmarks of normal cell polarity.

It is interesting to note that PAK is a member of the STE20 family of kinases (28), as well as the LKB1 cofactor binding partner STRAD, although the kinase domain of STRAD lacks several residues indispensable for intrinsic catalytic activity and is, thus, catalytically inactive (29). Because the interaction between STRAD and LKB1 is necessary for LKB1 kinase activity and localization (30), this structural relatedness between PAK and STRAD may provide additional interesting clues on the functional consequences of the LKB1-PAK association. Lastly, LKB1 is also is an upstream kinase to AMP-activated kinase (AMPK), which serves as a sensor for energy stress (31). Recent studies show that AMPK also functions in cell polarity (32–34) and AMPK mutants lose their polarity and overproliferate under energetic stress (32). Thus, AMPK may

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indeed be a downstream substrate of the LKB1-cdc42-PAK pathway, and future studies will investigate this.

More than 30% of NSCLC tumors harbor mutations in LKB1 (11, 12). This high LKB1 mutation rate, in combination with the data presented herein, provides the intriguing possibility that LKB1 loss in human tumors could cause aberrant lung cancer cell polarity. In fact, C-terminal LKB1 mutations affect the ability of intestinal epithelial cells and migrating astrocytes to establish and maintain polarity (14). Because a loss of epithelial cell polarity is linked to increased cancer invasion via EMT (6, 35), LKB1 mutations in patients may serve as a trigger for cancer cell invasion. This idea is supported by recent work, showing that a somatically activatable mutant Kras-driven model of mouse lung cancer showed that homozygous inactivation of LKB1 resulted in shorter latency and more frequent metastasis compared with tumors lacking p53 or Ink4a/Arf (36). Moreover, cells expressing mutant LKB1 possessed greater invasive potential compared with wild-type LKB1 cells (37). Thus, future experiments will address this possibility and determine if a compromised LKB1-cdc42-PAK pathway triggers cancer cell invasion.

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