STE20-related kinase adaptor protein alpha (STRADα) Regulates Cell Polarity and Invasion through PAK1 Signaling in LKB1 Null Cells*

Carrie M. Eggers, Erik R. Kline, Diansheng Zhong, Wei Zhou, and Adam I. Marcus

From the Department of Hematology and Oncology, Winship Cancer Institute
Emory University, Atlanta, GA 30322

*Running title: STRADα regulates cell polarity

To whom correspondence should be addressed: Adam Marcus, Department of Hematology and Oncology, Winship Cancer Institute, Emory University, 1365 Clifton Rd, Atlanta, GA, USA 30322, Tel.: (404) 778-4597; Fax: (404)-778-5530; E-mail: aimarcu@emory.edu

Keywords: STRADα; PAK1; LKB1; polarity; invasion; metastasis

Background: STRADα is the co-factor of the tumor suppressor LKB1; however, it is unclear if STRADα has LKB1-independent roles.

Results: STRADα complexes with the kinase PAK1 to modify PAK1 phosphorylation likely via rac1 and control cell motility when LKB1 is null.

Conclusion: STRADα regulates PAK1 in LKB1-null cells to oversee cancer cell polarity and invasion.

Significance: This shows an undiscovered role of STRADα distinct from the LKB1 pathway.

SUMMARY

LKB1 is a ser/thr kinase and its activity is regulated by the pseudokinase, STE20-related adaptor alpha (STRADα). The STRADα-LKB1 pathway plays critical roles in epithelial cell polarity, neuronal polarity, and cancer metastasis. Though much attention is given to the STRADα-LKB1 pathway, the function of STRADα itself, including a role outside of the LKB1 pathway, has not been well-studied. Data in C. elegans suggest that STRADα has an LKB1-independent role in regulating cell polarity, and therefore we tested the hypothesis that STRADα regulates cancer cell polarity and motility when wild-type LKB1 is absent. These results show that STRADα protein is reduced in LKB1-null cell lines (mutation or homozygous deletion) and this partial degradation occurs through the Hsp90-dependent proteasome pathway. The remaining STRADα participates in cell polarity and invasion, such that STRADα depletion results in mis-aligned lamellipodia, improper Golgi positioning, and reduced invasion. To probe the molecular basis of this defect, we show that STRADα associates in a complex with PAK1, and STRADα loss disrupts PAK1 activity via thr423 PAK1 phosphorylation. When STRADα is depleted, PAK1-induced invasion could not occur, suggesting that STRADα is necessary for PAK1 to drive motility. Furthermore, STRADα overexpression caused increased activity of the PAK1-activating protein, rac1, and a constitutively active rac1 mutant (Q61L) rescued pPAKthr423 and STRADα invasion defects. Taken together, these results show that a STRADα-rac1-PAK1 pathway regulates cell polarity and invasion in LKB1-null cells. It also suggests that while the function of LKB1 and STRADα undoubtedly overlap, they may also have mutually exclusive roles.

INTRODUCTION

LKB1 is a serine/threonine kinase (also known as STK11:(1)) that contains two nuclear localization sequences, a central kinase domain, and a C-terminal farnesylation motif (2). LKB1 ranks as the 3rd highest mutated gene in lung adenocarcinoma (3-5) and functions in epithelial cell polarity, neuronal polarity, energy stress, and cancer metastasis. LKB1 is localized to the cytoplasm and nucleus in mammalian cells and its localization is regulated by its co-factor STRAD (6-9).

STRAD is a pseudokinase that activates LKB1 in an allosteric manner where ATP binding
to STRAD transitions the LKB1 kinase domain to an active-like kinase conformation (10) resulting in phosphorylation of LKB1 and STRAD (7). There are two known isoforms of STRAD, STRADα and STRADβ, and while both enhance LKB1’s kinase activity, only STRADα is involved in LKB1 nucleo-cytoplasmic shuttling (11). Studies show that STRADα and another LKB1-regulating protein, MO25α, influence the subcellular localization of wild-type, but not mutant LKB1, such that STRADα overexpression induces LKB1 nuclear export into the cytoplasm (7). Mutational analysis of human cancers shows that out of 34 LKB1 point mutants, 12 of these mutants fail to interact with STRADα-MO25, suggesting that the LKB1-STRADα-MO25 interaction is functionally significant (12).

Studies investigating STRADα-LKB1 function have linked it to two major pathways—the AMP kinase (AMPK) energy stress pathway and the cell polarity program (reviewed in (13)). In the latter, LKB1 activation via STRADα binding causes cell autonomous polarization such that LKB1-activated cells fully polarize even in the absence of junctional cell-cell contacts, traditionally a prerequisite for polarization (6). In lung cancer, LKB1 is critical for cell polarity where LKB1 rapidly translocates to the cellular leading edge in motile cells to regulate cdc42 activity, a small Rho GTPase in the cell polarity pathway and activator of p21-activated kinase 1 (PAK1) (14,15). PAK1 is a ser/thr kinase that associates with actin and lamellipodia proteins to mediate actin rearrangements, polarity, and adhesion (16-20). Mouse knockout studies support a role for LKB1 in lung cancer invasion and metastasis, since in a mutant k-ras driven mouse model of lung cancer, LKB1 inactivation led to lung carcinomas with more frequent metastasis compared to tumors lacking p53 or Ink4a/Arf (21). Thus, these data support a role for STRADα-LKB1-inactivation in the invasion and metastasis of lung tumors.

To date, most studies have focused on LKB1 function and its role in cancer progression, motility and metastasis. However, the molecular details of how STRADα itself functions and how its function is regulated have not been well described. A report in C. elegans shows an LKB1-independent function of STRADα (22), suggesting that STRADα could have roles outside of the LKB1 pathway in human cancer. Therefore, we tested the hypothesis that STRADα can function in cancer cell motility when wild-type LKB1 is absent. To do this, STRADα expression, regulation, and functionality were examined in LKB1 wild-type and null cell lines. These data show that STRADα protein is reduced in all LKB1 mutant cell lines tested compared to LKB1 wild-type cells, and this partial degradation occurs through the canonical Hsp90-dependent proteasome pathway. The remaining STRADα participates in maintaining proper cell polarity during cancer cell motility through a rac1-PAK1 cell polarity/motility pathway. Based upon these data, we conclude that STRADα regulates PAK1 in LKB1-null cells to oversee cancer cell polarity and invasion.

EXPERIMENTAL PROCEDURES

Cell Culture – All cell lines were maintained at 37°C in a humidified chamber with 5% CO₂. ATCC-recommended media (RPMI 1640 or DMEM) were used for each cell line, supplemented with 10% FBS and 1% penicillin/streptomycin. Keratinocyte Serum Free Media supplemented with Bovine Pituitary Extract (BPE) and Epidermal Growth Factor (EGF) was used for Beas-2b epithelial lung cells. Human plasma fibronectin (Chemicon/Millipore, Billerica, MA, USA) at 5 ug/cm² diluted in RPMI 1640 (supplemented with 10% FBS) was added to the cell culture dish and incubated at 37°C for 20 minutes prior to plating cells for experiments.

Antibodies – Antibodies against LKB1 (Abcam, Cambridge, MA, USA), STRADα (Santa Cruz Biotechnology, Santa Cruz, CA, USA), ubiquitin (Cell Signaling/Millipore, Billerica, MA, USA), HA (Invitrogen, Carlsbad, CA, USA), GAPDH (Cell Signaling), actin (Sigma), tubulin (Millipore, Billerica, MA, USA), and pPAKα423 (Cell Signaling), anti-GFP (Covance, Princeton, NJ), Hsp90 (Cell Signaling), myc (Santa Cruz) antibodies were used for western blotting and immunofluorescence.

Transfections and Drug Treatments – Lipofectamine 2000 (Invitrogen) was used to transfect cells with the GFP-LKB1, GFP-LKB1 K78M, racQ61L, FLAG-STRADα and the myc-PAK1 according to the manufacturer’s protocol. Cells were harvested 48 hours post-transfection. Oligofectamine (Invitrogen) was used for small
interfering RNA (siRNA) transfections according to the manufacturer’s protocol. LKB1 siRNA and STRADα siRNA were used at 100 nmol/L in two successive 24-hour transfections. The LKB1 siRNA was a different sequence than the LKB1 shRNA. To inhibit protein translation, cells were treated with 100 uM cycloheximide (Acros Organics, Morris Plains, New Jersey, USA) solubilized in water, at various time points. Proteasome inhibition was accomplished using MG-132 (Peptide Institute, Osaka, Japan) dissolved in DMSO. MG-132 was added to cells at 50 uM and incubated at 37°C for 6 hours, or 5-10 uM for 24 hours. The Hsp90 inhibitor geldanamycin (Enzo Life Sciences, Farmingdale, NY, USA) was used at 10 µM.

**Western blotting** – Cells were harvested and lysed in TNES solution (50 mM Tris pH 7.5, 100 mM NaCl, 2mM EDTA, 1% NP-40, Roche Complete Protease Inhibitor Cocktail) at 4°C. When preparing soluble and insoluble fractions, a high-salt lysis buffer (1% NP-40, 10% glycerol, 20 mM HEPES pH 7.6, 150 mM NaCl, 2 mm Na3VO4, 2 mM Sodium Molybdnate, 2 mM Na4P2O7, and Complete Protease Inhibitors) was added to cells and incubated for 30 minutes at 37°C. The soluble fraction was collected after a 30 min at 2100g spin at 4°C, and the pellet (insoluble fraction) was sonicated in an equivalent volume of high salt lysis buffer to disrupt the aggregate. Cell lysates were boiled for 5 minutes with protein loading buffer prior to SDS-PAGE. Protein concentrations were determined by the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Equal concentrations of protein from whole-cell lysates were solubilized in SDS sample buffer and separated on SDS 12.5% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membrane and the resultant membrane was blocked in 10% milk, then probed with primary antibodies diluted in 5% BSA for a minimum of 1 hour at room temperature up to overnight at 4°C. This was followed with the appropriate secondary horseradish peroxidase–conjugated antibody and visualized by chemiluminescence.

**Immunofluorescence, confocal microscopy, and image analysis** – Cells were fixed on coverslips with PHEMO buffer (68 mmol/L PIPES, 25 mmol/L HEPES, 15 mmol/L EGTA, 3 mmol/L MgCl2, 10% DMSO) with 3.7% formaldehyde, 0.05% glutaraldehyde, and 0.5% Triton X-100. After washing in PBS, coverslips were blocked in 10% BSA for 30 minutes and processed according to (14). Cells were imaged using a Zeiss LSM 510 META confocal microscope using a 20x Plan-Apo objective (NA = 0.75). Image analysis of the LKB1 and STRADα signal was performed on confocal images using Metamorph software. DAPI fluorescence was used to identify the nucleus and used to create an object of the nucleus. A region of interest was then created by the software outlining the nucleus, which was transferred to the corresponding LKB1 or STRADα image. The average fluorescence intensity was then quantified within this region of interest from thresholded images to generate nuclear intensity. To determine cytoplasmic intensity the nuclear object was subtracted from the corresponding LKB1 or STRADα image (to leave the cytoplasm) then the cytoplasmic mean fluorescence intensity was quantified from the thresholded image. All images in the same cell line were acquired with identical settings and thresholded the same.

**Cell Polarity measurements** – Cell polarity measurements were done as previously described in (14).

**Quantitative Real-Time PCR** – Total RNA was isolated from cells using QIAGEN’s RNeasy Mini Kit and subsequently reverse-transcribed with M-MLV Reverse Transcriptase (Invitrogen). The resultant cDNA was amplified using primers specific for LKB1 and STRADα, and analyzed by quantitative real-time PCR using SYBR Green detection. Reactions (25 ul) contained 1 ul of DNA, 0.2 um primers and 12.5 ul of IQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). The reaction protocol began with a 3 minute hot start at 95°C and followed with cycles of 95°C, 10 seconds; 55 °C, 60 seconds. Melt curve analysis verified a single product. Relative quantities were calculated, standardized by comparison to 18s rRNA (small ribosomal subunit)(23). Primers used were as follows: STRADα (forward – GCCATGTCCCCTTTAAGGAT, reverse – TCATGGTCAGCTCCTCAGC), 18s (forward – GAGGGAGCCTGAGAAACGG, reverse – GTCGGGAGTGGGTAATTTGC).

**Invasion Assays** – BD Biocoat Matrigel invasion chambers (BD Biosciences, Sparks, MD, USA) were loaded with 0.5 x 105 cells in serum-
free media that had been treated with control or STRADα siRNA, or transfected with PAK1 or a rac1 Q61L mutant as described above. The well surrounding the Matrigel insert was partially filled with complete media (RPMI 1640 with 10% FBS and 1% penicillin/streptomycin) supplemented with fibronectin. The cells were incubated for 18-24 hours at 37°C to allow invasion to occur. Membranes from the inserts were fixed and stained according to the manufacturer’s protocol, and mounted on slides for imaging with a Zeiss Axioplan upright microscope using a 10X objective.

Co-immunoprecipitation – Cells were lysed in buffer containing protease and phosphatase inhibitors (50 mM Tris, 400 mM NaCl, 5 mM EDTA, 5% glycerol, 1% triton x-100, 25 mM NaF, 2 mM Na2VO4, Na molybdate, Roche Complete Protease Inhibitor Cocktail), incubated for 30 minutes on ice and spun down at 14,000g at 4° C for 15 minutes. After centrifugation, the protein concentration of the supernatant was determined and 500 ug of protein were incubated rotating with 10 ug of the appropriate antibody overnight at 4° C. The following day, each sample was bound to Dynabeads (Invitrogen #100.03D) by incubating on a rotator for 2 hours at 4° C. After three, 30-minute washes with TBS (with protease and phosphatase inhibitors) at 4° C, the bound protein was eluted in 2x Laemmli buffer for 10 minutes at 100° C and beads removed by placing tubes on a magnet.

Small rho GTPase activation assays The G-LISA rac1 and cdc42 Activation Assay luminescence-based kits (Cytoskeleton, Denver, CO, USA) were used to ascertain rac1 and cdc42 activity in HeLa and H157 cells according to general kit protocol. Cells were either transfected with FLAG-STRADα or with FLAG-pcDNA3 as previously described, then lysed in kit lysis buffer. Lysates were diluted to 1 mg/ml and assayed using anti-rac1 or anti-cdc42 primary antibody dilution of 1/250 and secondary HRP labeled antibody at 1/200. Readings were obtained using a SpectraMax luminometer.

RESULTS

LKB1 regulates STRADα protein levels independent of LKB1 kinase activity – To test the hypothesis that STRADα could have LKB1 independent roles in cancer motility, STRADα protein levels were first assessed in LKB1 wild-type, and LKB1-null (mutation causing an early stop codon or homozygously deleted) cancer cell lines. STRADα western blotting showed that LKB1 null cells (24-27) have reduced STRADα levels compared to LKB1 wild-type cell lines (Fig. 1A). Similar results were observed when LKB1 expression was reduced using different LKB1-targeted shRNA and siRNA in LKB1 wild-type lung cancer cell lines (H1299 and H1703) and normal lung epithelial cells (BEAS-2b); Fig 1B). These results were confirmed by immunofluorescence imaging, which showed that STRADα signal intensity decreased in H1299-LKB1-shRNA lung cancer cells relative to isogenic control H1299 pLKO.1 cells (Fig. 1C, top). Within the cell line we observed some heterogeneity such that neighboring cells have different levels of both LKB1 and STRADα, which could be due to the dynamic localization of LKB1 (14,28). Similar results were also observed in H1703 pLKO.1 and H1703 LKB1 shRNA cells (Fig 1C, bottom). Furthermore, quantitative image analysis showed that LKB1, which has both a nuclear and cytoplasmic localization (14), was successfully depleted in both the nucleus and cytoplasm in both cell lines (Fig. 1D). This resulted in corresponding decrease in STRADα nuclear and cytoplasmic intensity (Fig. 1D).

To determine if reduced STRADα protein expression is mediated at the transcriptional level, quantitative real-time PCR was performed for STRADα in control H1299 pLKO.1 cells and H1299-LKB1-shRNA cells. In this case, STRADα mRNA levels remained unchanged (Fig. 1E); therefore, STRADα transcription is unaffected by LKB1 depletion, suggesting that these effects are mediated at the protein level and not transcriptionally.

To determine if LKB1 re-expression in LKB1-null cell lines restores STRADα protein expression, wild-type FLAG-LKB1 and a FLAG-LKB1 K78M kinase dead mutant were transfected into LKB1 null cells. In the four lung cancer cell lines (H23, H460, H1944, and A549) and Hela cells tested, restoration of both wild-type FLAG-LKB1 and the K78M kinase dead LKB1 mutant restored soluble STRADα levels to varying degrees (Fig. 2). Taken together, LKB1 re-expression restores STRADα levels in a kinase-
STRADα regulates cell polarity

independent manner, suggesting that LKB1 kinase activity is not critical for maintaining STRADα protein levels, and that LKB1-STRAD oligomerization serves as a protective mechanism for STRADα depletion.

**STRADα stability is regulated by Hsp90-dependent proteasome degradation** – To assess how STRADα protein stability is regulated, protein synthesis was halted with cycloheximide and STRADα levels were assessed in LKB1 wild-type H1299 lung cancer cells. Western blotting shows that STRADα is rapidly degraded and 45 minutes post-cycloheximide treatment, STRADα is nearly absent. In contrast, LKB1 is more stable and shows minor degradation beginning at around 75 minutes (Fig. 3A). The rate of STRADα degradation was similar in LKB1 wild-type pLKO.1 cells compared to H1299 LKB1 shRNA cells; however, overall STRADα levels were significantly less (Fig S1).

To determine if STRADα degradation is mediated by Hsp90, the Hsp90 inhibitor, geldanamycin was used. Dose-dependent STRADα degradation was observed (Fig. 3B), suggesting that STRADα is degraded when Hsp90 is inhibited. Furthermore, geldanamycin reduced the re-expression of STRADα when FLAG-LKB1 was transfected in H1297-deficient H157 cells (Fig. 3C), showing that a rescue of STRADα induced by LKB1 re-expression is Hsp90-dependent. To determine if this occurs through the proteasome, the proteasome inhibitor MG-132 was used alone and in combination with geldanamycin. Both the lysis buffer-soluble and insoluble fractions were obtained, since degraded protein often is insoluble after lysis. Geldanamycin-induced degradation of STRADα could be partially reversed by co-treatment with the proteasome inhibitor MG-132 as evidenced by the re-appearance of the STRADα protein band in the soluble and insoluble fraction (Fig. 3D). This suggests that Hsp90 drives STRADα degradation through the proteasome and when the proteasome is inhibited, both soluble and insoluble STRADα accumulate. MG-132 treatment also resulted in a higher molecular weight STRADα band in both the insoluble and soluble fractions (Fig. 3D) that would be consistent with ubiquitinated STRADα, since MG-132 treatment leads to an accumulation of ubiquitinated protein (Fig. 3E ubiquitin blot). The basal levels of this potentially ubiquitinated STRADα may also vary between cell lines since some cell lines show a prominent higher molecular weight STRADα band (Fig. 2).

To determine if STRADα degradation occurs in a similar manner when LKB1 levels are reduced, LKB1 was depleted in the H1299 lung cancer cell line and BEAS-2B lung epithelial cell line in the presence and absence of MG-132. Western blotting shows that when LKB1 is depleted, STRADα levels decrease as shown previously; however, this can be partially reversed with MG-132 treatment predominantly resulting in a higher molecular weight STRADα band (Fig. 3E). This suggests that when LKB1 is absent, STRADα is partially degraded through the proteasome.

**STRADα regulates cell polarity in LKB1 null cell lines** – The results show that STRADα is partially degraded when LKB1 is absent; however, a significant fraction of STRADα still remains (Figs. 1-3). Therefore, we wanted to determine if the remaining STRADα has a functional role in LKB1-null cells. We investigated the role of STRADα in cancer cell polarity since STRADα regulates cell polarity independently of LKB1 in *C. elegans* (22). To test this, a Golgi polarization assay was performed to quantitate cell polarity defects (14,29,30) in control and STRADα-depleted cells. This assay uses Golgi re-alignment as a functional marker of the cell polarity program, where the Golgi re-aligns between the nucleus and the leading edge of the cell when polarity is intact. To quantitate this, the region adjacent to the nucleus is sub-divided into three 120° regions (see Materials and Methods), such that random alignment would occur 33% of the time (Fig. 4A) and in general proper alignment occurs in greater than 60% of cells. To determine how STRADα impacts cell polarity in LKB1 null cells, STRADα was successfully depleted in all cell lines using STRADα-specific siRNA (Fig 4B). Control siRNA-treated cells show intact polarity such that 63% of H157 cells have properly aligned Golgi, 81% of A549 cells have proper polarity, and 60% of HeLa and H460 cells have proper polarity (Fig. 4C,D). In STRADα depleted cells, the Golgi were unable to properly align in H157 and Hela cells, with only 35%, and 37% of cells having proper alignment, respectively. In A549 cells, only moderate alignment was observed, with 54% of cell showing proper Golgi positioning, and in
H460 only 37% had proper polarity. In all cell lines where STRADα was depleted, mis-directed and shorter lamellipodia were also observed (Fig. 4D), which is consistent with defective cell polarity. These results suggest that STRADα functions autonomously from LKB1 in LKB1 null cell lines to regulate cell polarity.

**STRADα regulates cancer cell invasion in LKB1-null cell lines** – Since cell polarity is necessary to establish directionality during cancer cell invasion, invasion assays were performed to determine if STRADα depletion affects cancer cell invasion. The data showed that in all four cell lines tested, STRADα depletion significantly reduced invasion, whereby cells lacking STRADα had significantly less invaded cells than control (Fig. 5A). Next, the converse experiment was performed to determine how STRADα overexpression (Fig. 5B) impacted cancer cell invasion. In these cases, FLAG-STRADα overexpression led to increased invasion in all cell lines tested. Specifically, in H460 and HeLa cells, STRADα overexpression significantly increased invasion 2.0 fold and 4.0 fold, respectively (Fig. 5C). In A549 and H157 cells, the increase in invasion was not significant; however, both cases clearly trended to increased invasion. Thus, taken together, these results show that in cell lines deficient of wild-type LKB1, STRADα depletion reduced cancer cell invasion, whereas its overexpression increased invasion.

**STRADα regulates PAK1^{thr423} phosphorylation in the absence of LKB1 and complexes with PAK1** – To determine how STRADα regulates cell polarity and invasion, we focused on one of the key regulators of the cell polarity and motility program, PAK1. When activated by the small rho GTPases cdc42 or rac1, PAK1 undergoes a conformational change leading to the^{thr423} PAK1 phosphorylation (18,31). We wanted to determine if STRADα regulates PAK1 phosphorylation when wild-type LKB1 is absent. These results showed that STRADα depletion caused decreased thr^{423} PAK1 phosphorylation in both LKB1 mutant (H157) and deleted (HeLa) cells lines (Fig 6A,B). Total PAK1 levels did not change in either case. Therefore, based upon these data we conclude that STRADα regulates thr^{423} PAK1 phosphorylation in LKB1 null cell lines.

Next, we determined whether STRADα can complex with PAK1 using a co-immunoprecipitation approach. To do this, myc-PAK1 was expressed in H157 cells and endogenous STRADα was immunoprecipitated. Western blotting shows that endogenous STRADα is capable of co-immunoprecipitating with PAK1 (Fig. 6C), suggesting that STRADα forms a complex with PAK1. Based upon the data in Fig 3, we also tested whether STRADα associated with Hsp90 and this result showed that STRADα was not associated with Hsp90 in LKB1 deficient cells (Fig. 6C). An IgG immunoprecipitation is shown as a negative control, which did not result in a PAK1 signal.

To determine if this STRADα-PAK1 pathway is linked to cancer invasion, PAK1-driven invasion was assessed in control and STRADα-depleted cells. In control HeLa and H157 cells, PAK1 overexpression resulted in increased invasion as expected (Fig 6D); however, wild-type PAK1 overexpression in STRADα depleted cells led to no significant change in invasion (Fig 6D), suggesting that STRADα is necessary for PAK1 to enhance invasion.

**STRADα regulates rac1 activity during cancer cell invasion**

Since we show that STRADα regulates pPAK1^{thr423} in the absence of LKB1 and associates in a complex with PAK1 (Fig 6), we next wanted to determine how this pathway is mediated. Since PAK1 is primarily activated by the rac1 or cdc42 small Rho GTPases, activation assays were done to determine if FLAG-STRADα overexpression induced activation of cdc42 or rac1. FLAG-STRADα overexpression did not result in a significant increase in cdc42 activation (not shown); however, FLAG-STRADα overexpression in both HeLa and H157 cells led to a significant increase in GTP bound rac1 (active rac1; Fig 7A). This result suggests that STRADα regulates PAK1 activity through rac1.

We then tested whether rac1 activation could rescue pPAK1^{thr423} and invasion defects in STRADα depleted cells by utilizing a rac1 Q61L mutant that is always in the active state (32,33). Transfection of rac1 Q61L mutant rescued pPAK1^{thr423} defects induced by STRADα depletion (Fig. 7B). Furthermore, it also rescued cell invasion in STRADα-depleted cells (Fig. 7C). Specifically, STRADα depletion resulted in decreased invasion as expected; however,
transfection of rac1 Q61L into STRADα-depleted cells rescued defective invasion (Fig. 7C). Therefore, this result showed that an active rac1 can restore pPAK thr423 levels and STRADα invasion defects in STRADα-depleted cells, further suggesting that STRADα signals via rac1 to activate PAK1.

**DISCUSSION**

Motile cells polarize and generate functionally distinct cellular compartments to drive directional movement. These morphological changes are due to a dynamic and coordinated interplay of signaling molecules and the cytoskeleton (34,35). We show that STRADα loss in LKB1-null cell lines disrupts cell polarity during motility, where cells fail to properly align their Golgi and have mis-directed lamellipodia (Fig. 4). On the molecular level, this observation is associated with reduced PAK1 thr423 phosphorylation. PAK1 thr423 is the critical site regulating PAK1 activity (18), allowing PAK1 to associate with actin and lamellipodial proteins to mediate actin rearrangements, polarity, and adhesion through downstream signaling cascades (36). We show that STRADα complexes with PAK1, suggesting that this STRADα-PAK1 complex is regulating PAK1 thr423 phosphorylation (Fig. 6). It is likely that STRADα regulates PAK1 activity via rac1, since overexpression of STRADα causes increased rac1 activity and a constitutively active rac1 mutant restores STRADα-depletion signaling and invasion defects (Fig. 7). Interestingly, LKB1 itself is implicated in regulating pPAK1 in cancer cell polarity and also complexes with PAK1 (14,37); however, since these cell lines are LKB1 null, these results show that STRADα functions independently of LKB1 to regulate cancer cell invasion and polarity via PAK1.

Studies in *C. elegans* support this result by showing that STRADα can function in an LKB1-independent manner to oversee neuronal polarity (22). In this case, STRD-1 (STRADα) and PAR-4 (LKB1) work independently, whereby STRADα directly interacts with SAD-1 but PAR-4 does not (22). Despite different model systems used by our group and by Kim et al., in both cases aberrant polarity is observed when STRADα functionality alone is compromised. Additional data also from *C. elegans*, show that LKB1 can function autonomously from STRADα, such that PAR-4 (LKB1) requires STRD-1 (STRADα) to phosphorylate AMPK under energy stress conditions but PAR-4 can promote phosphorylation of PAR-1 (MARKs) in a STRD-1 independent manner (38). Taken together, these results show that STRADα and LKB1 are not always essential binding partners, and while their functions undoubtedly overlap, they also have mutually exclusive roles.

Cancer invasion is impeded when STRADα is depleted, but is enhanced when STRADα is overexpressed (Fig. 6). This reduced invasion in STRADα-depleted cells may be a consequence of the aberrant polarity observed in these cells (Fig. 4) and is likely tied to the PAK pathway, since PAK1 can only induce invasion when STRADα is present (Fig. 6D). Furthermore, STRADα is likely regulating PAK through rac1 since overexpression of STRADα led to activation of rac1 (Fig. 7) and constitutively active rac1 rescues STRADα-depletion pPAK thr423 and invasion defects (Fig. 7). Based upon these data, we conclude that STRADα is required for lung cancer invasion likely through rac1-PAK1 signaling, and thus could serve a pro-metastatic role when wild-type LKB1 is absent. This is in contrast to LKB1, which when depleted or knocked-out in a mouse model induces metastasis (39,40), thereby serving as a metastasis suppressor. The question then arises, could STRADα be playing dual roles, such that it represses invasion when associated with LKB1, but stimulates invasion when LKB1 is null. Other proteins can play seemingly opposing roles, such as the growth factor TGFβ, which can promote invasion and metastasis, but also induce growth suppression depending on the cell type and environment (41). If this is indeed the case with STRADα, this result suggests that metastasis induced by wild-type LKB1 loss or mis-localization in patients (27,28,40) could be driven by, in part, a pro-invasive role of STRADα.

The data presented here also show that STRADα protein stability is LKB1-dependent, such that loss of LKB1 results in reduced STRADα protein levels (Figs. 1-3). In LKB1 wild-type cells, STRADα is degraded through the proteasome in an Hsp90-dependent manner but turnover is enhanced when LKB1 is mutant. This pathway is similar to the LKB1 degradation...
pathway where LKB1 interacts with the molecular chaperones Hsp90 and Cdc37/p50 to regulate LKB1 stability and proteasomal degradation (42,43). Based upon our data, we propose a model that when LKB1 is present, STRADα is degraded via the proteasome in an Hsp90-dependent manner but when LKB1 is mutant or absent, the rate of STRADα degradation through this pathway is increased.

Overall, these data show that STRADα can function independently of LKB1 to regulate cell polarity and invasion via PAK1 when LKB1 function is compromised. The question of whether an LKB1-independent, STRADα-rac1-PAK1 pathway exists when wild-type LKB1 function is intact is difficult to determine since LKB1 itself can alter PAK1 phosphorylation (14,37); thus, discerning the effects of LKB1-STRADα-PAK1 from the effects of a separate STRADα-PAK1 pathway is difficult with standard approaches. Nevertheless, future studies will attempt to determine if these signaling events unfold in LKB1 wild-type cells and whether LKB1 loss triggers a pro-metastatic STRADα signaling pathway.

REFERENCES

1. Jenne, D. E., Reimann, H., Nezu, J., Friedel, W., Loff, S., Jeschke, R., Muller, O., Back, W., and Zimmer, M. (1998) *Nat Genet* **18**, 38-43
2. Alessi, D. R., Sakamoto, K., and Baydascas, J. R. (2006) *Annu Rev Biochem*
3. Carretero, J., Medina, P. P., Pio, R., Montuenga, L. M., and Sanchez-Cespedes, M. (2004) *Oncogene* **23**, 4037-4040
4. Sanchez-Cespedes, M., Parrella, P., Esteller, M., Nomoto, S., Trink, B., Engles, J. M., Westra, W. H., Herman, J. G., and Sidransky, D. (2002) *Cancer Res* **62**, 3659-3662
5. Ding, L., Getz, G., Wheeler, D. A., Mardis, E. R., McLellan, M. D., Cibulskis, K., Sougnez, C., Greulich, H., Muzny, D. M., Morgan, M. B., Fulton, L., Fulton, R. S., Zhang, Q., Wendl, M. C., Lawrence, M. S., Larson, D. E., Chen, K., Dooling, D. J., Sabo, A., Hawes, A. C., Shen, H., Jhangiani, S. N., Lewis, L. R., Hall, O., Zhu, Y., Mathew, T., Ren, Y., Yao, J., Scherer, S. E., Clerc, K., Metcalf, G. A., Ng, B., Milosavljevic, A., Gonzalez-Garay, M. L., Osborne, J. R., Meyer, R., Shi, X., Tang, Y., Koboldt, D. C., Lin, L., Abbott, R., Miner, T. L., Pohl, C., Fewell, G., Haieck, C., Schmidt, H., Dunford-Shore, B. H., Kraja, A., Crosby, S. D., Sawyer, C. S., Vickery, S., Tander, S., Robinson, J., Winckler, W., Baldwin, J., Chirieac, L. R., Dutt, A., Fennell, T., Hanna, M., Johnson, B. E., Onofrio, R. C., Thomas, R. K., Tonon, G., Weir, B. A., Zhao, X., Ziaugra, L., Zody, M. C., Giordano, T., Orringer, M. B., Roth, J. A., Spitz, M. R., Wistuba, Ii, Ozenberger, B., Good, P. J., Chang, A. C., Beer, D. G., Watson, M. A., Ladanyi, M., Broderick, S., Yoshizawa, A., Travis, W. D., Pao, W., Province, M. A., Weinstock, G. M., Varmus, H. E., Gabriel, S. B., Lander, E. S., Gibbs, R. A., Meyerson, M., and Wilson, R. K. (2008) *Nature* **455**, 1069-1075
6. Baas, A. F., Kuipers, J., van der Wel, N. N., Batlle, E., Koerten, H. K., Peters, P. J., and Clevers, H. C. (2004) *Cell* **116**, 457-466
7. Baas, A. F., Boudeau, J., Sapkota, G. P., Smit, L., Medema, R., Morrice, N. A., Alessi, D. R., and Clevers, H. C. (2003) *Embo J* **22**, 3062-3072
8. Boudeau, J., Baas, A. F., Deak, M., Morrice, N. A., Kieloch, A., Schutkowski, M., Prescott, A. R., Clevers, H. C., and Alessi, D. R. (2003) *Embo J* **22**, 5102-5114
9. Hawley, S. A., Boudeau, J., Reid, J. L., Mustard, K. J., Udd, L., Makela, T., P., Alessi, D. R., and Hardie, D. G. (2003) *J Biol* **2**, 28
10. Zeqiraj, E., Filippi, B. M., Deak, M., Alessi, D. R., and van Aalten, D. M. (2009) *Science* **326**, 1707-1711
11. Dorfman, J., and Macara, I. G. (2008) *Mol Biol Cell* **19**, 1614-1626
12. Boudeau, J., Scott, J. W., Resta, N., Deak, M., Kieloch, A., Komander, D., Hardie, D. G., Prescott, A. R., van Aalten, D. M., and Alessi, D. R. (2004) *J Cell Sci* **117**, 6365-6375
13. Marcus, A. I., and Zhou, W. (2010) J Thorac Oncol 5, 1883-1886
14. Zhang, S., Schafer-Hales, K., Khuri, F. R., Zhou, W., Vertino, P. M., and Marcus, A. I. (2008) Cancer Res 68, 740-748
15. Herbst, R. S., Heymach, J. V., and Lippman, S. M. (2008) N Engl J Med 359, 1367-1380
16. Manser, E., Leung, T., Salihuddin, H., Zhao, Z. S., and Lim, L. (1994) Nature 367, 40-46
17. Kim, A. S., Kakalis, L. T., Abdul-Manan, N., Liu, G. A., and Rosen, M. K. (2000) Nature 404, 151-158
18. Bokoch, G. M. (2003) Annu Rev Biochem 72, 743-781
19. Dummler, B., Ohshiro, K., Kumar, R., and Field, J. (2009) Cancer Metastasis Rev 28, 51-63
20. Kumar, A., Molli, P. R., Pakala, S. B., Bui Nguyen, T. M., Rayala, S. K., and Kumar, R. (2009) J Cell Biochem 107, 579-585
21. Ji, H., Ramsey, M. R., Hayes, D. N., Fan, C., McNamara, K., Kozlowski, P., Torrice, C., Wu, M. C., Shimamura, T., Perera, S. A., Liang, M. C., Cai, D., Naumov, G. N., Bao, L., Contreras, C. M., Li, D., Chen, L., Krishnamurthy, J., Koivunen, J., Chirieac, L. R., Padera, R. F., Bronson, R. T., Lindeman, N. I., Christiani, D. C., Lin, X., Shapiro, G. I., Janne, P. A., Johnson, B. E., Meyerson, M., Kwiatkowski, D. J., Castrillon, D. H., Bardeesy, N., Sharpless, N. E., and Wong, K. K. (2007) Nature
22. Kim, J. S., Hung, W., Narbonne, P., Roy, R., and Zhen, M. (2010) Development 137, 93-102
23. Lucas, M. E., Crider, K. S., Powell, D. R., Kapoor-Vazirani, P., and Vertino, P. M. (2009) J Biol Chem 284, 1498-14709
24. Ikediobi, O. N., Davies, H., Bignell, G., Edkins, S., Stevens, C., O'Meara, S., Santarius, T., Avis, T., Barthorpe, S., Brackenbury, L., Buck, G., Butler, A., Clements, J., Cole, J., Dicks, E., Forbes, S., Gray, K., Halliday, K., Harrison, R., Hills, K., Hinton, J., Hunter, C., Jenkinson, A., Jones, D., Kosmidou, V., Lugg, R., Menzies, A., Mironenko, T., Parker, A., Perry, J., Raine, K., Richardson, D., Shepherd, R., Small, A., Smith, R., Solomon, H., Stephens, P., Teague, J., Tofts, C., Varian, J., Webb, T., West, S., Widaa, S., Yates, A., Reinhold, W., Weinstein, J. N., Stratton, M. R., Futreal, P. A., and Wooster, R. (2006) Mol Cancer Ther 5, 2606-2612
25. Zhong, D., Guo, L., de Aguirre, I., Liu, X., Lamb, N., Sun, S. Y., Gal, A. A., Vertino, P. M., and Zhou, W. (2006) Lung Cancer 53, 285-294
26. McCabe, M. T., Powell, D. R., Zhou, W., and Vertino, P. M. (2010) Cancer Genet Cytogenet 197, 130-141
27. Wingo, S. N., Gallardo, T. D., Akbay, E. A., Liang, M. C., Contreras, C. M., Boren, T., Shimamura, T., Miller, D. S., Sharpless, N. E., Bardeesy, N., Kwiatkowski, D. J., Schorge, J. O., Wong, K. K., and Castrillon, D. H. (2009) PLoS One 4, e5137
28. Kline, E. R., Muller, S., Pan, L., Tighiouart, M., Chen, Z. G., and Marcus, A. I. (2010) Head Neck
29. Chacko, A. D., Hyland, P. L., McDade, S. S., Hamilton, P. W., Russell, S. H., and Hall, P. A. (2005) J Pathol 206, 458-465
30. Etienne-Manneville, S., and Hall, A. (2001) Cell 106, 489-498
31. Lei, M., Lu, W., Meng, W., Parrini, M. C., Eck, M. J., Mayer, B. J., and Harrison, S. C. (2000) Cell 102, 387-397
32. Sells, M. A., Knaus, U. G., Bagrodia, S., Ambrose, D. M., Bokoch, G. M., and Chernoff, J. (1997) Curr Biol 7, 202-210
33. Sells, M. A., Pfaff, A., and Chernoff, J. (2000) J Cell Biol 151, 1449-1458
34. Jiang, P., Enomoto, A., and Takahashi, M. (2009) Cancer Lett
35. Etienne-Manneville, S. (2008) Oncogene 27, 6970-6980
36. Edwards, D. C., Sanders, L. C., Bokoch, G. M., and Gill, G. N. (1999) Nat Cell Biol 1, 253-259
37. Deguchi, A., Miyoshi, H., Kojima, Y., Okawa, K., Aoki, M., and Taketo, M. M. (2010) J Biol Chem 285, 18283-18290
38. Narbonne, P., Hyenne, V., Li, S., Labbe, J. C., and Roy, R. (2010) Development 137, 661-670
Acknowledgements – This work was supported by an American Cancer Society Research Scholar Award (RSG-08-035-01-CSM), the National Lung Cancer Partnership, and an RO1 (1RO1CA142858) awarded to A.I.M. This work was also supported by a lung cancer program project grant (5PO1 CA116676) awarded to A.I.M. and W.Z. A.I.M. is a Georgia Cancer Coalition Distinguished Scholar. Imaging was performed in the Winship Cell Imaging and Microscopy shared resource.

FIGURE 1. LKB1-dependent loss of STRADα protein (A) Western blot of LKB1 wild-type and null cell lines where all LKB1 mutant cell lines show reduced STRADα levels compared to wild-type cell lines. (B) Western blot showing that depletion with LKB1-targeted shRNA or siRNA also results in reduced STRADα levels. (C) Immunofluorescence for LKB1 and STRADα in H1299 pLKO.1 control and H1299-LKB1shRNA cells showing that LKB1 depletion results in less cellular STRADα. scale bar = 50µm for H1299 and 20 µm for H1703. (D) Bar graphs show image analysis of LKB1 and STRADα signal in the nucleus and cytoplasm of control siRNA and LKB1 depleted H1299 (top) and H1703 (bottom) cells. (E) Real time PCR of STRADα in H1299 pLKO.1 control cells and H1299-LKB1shRNA cells.

FIGURE 2. Re-expression of full length LKB1 or kinase dead LKB1 rescues STRADα levels in LKB1 mutant cells (A) Western blots showing that re-expression of a full length FLAG-LKB1 or a FLAG-LKB1 K78M kinase dead mutant increases STRADα levels in LKB1 mutant cell lines. GAPDH is shown as a loading control.

FIGURE 3. STRADα is degraded via the proteasome in an Hsp90 dependent manner. (A) Western blot showing that STRADα is rapidly degraded in LKB1 wild-type H1299 cells after inhibition of protein translation with cycloheximide. (B) Hsp90 inhibition with geldanamycin results in both STRADα and LKB1 degradation as shown by western blotting. (C) Western blot of FLAG-LKB1 transfection into LKB1-deficient H157 cells with and without geldanamycin. (D) Western blot of both the soluble and insoluble fraction in H1299 LKB1 wild-type cells where geldanamycin-induced degradation of STRADα can be partially reversed with the proteasome inhibitor MG-132. Tubulin and GAPDH are shown as loading controls. (E) Western blot in Beas-2b and H1703 LKB1 cells after LKB1 siRNA plus the proteasome inhibitor MG-132 shows that MG-132 can partially restore STRADα levels in LKB1 siRNA-depleted cells. Ubiquitin is shown as a marker for proteasome inhibition.
FIGURE 4. STRADα regulates cell polarity in the absence of wild-type LKB1. (A) Representative confocal image of Golgi, nucleus, and actin as an example of normal cell polarity. Arrow shows direction of movement. (B) Western blot showing successful siRNA depletion of STRADα in LKB1 deficient cell lines. (C) Bar graphs quantitating cell polarity during motility in control and STRADα depleted H157 (LKB1 mutant), A549 (LKB1 mutant), H460 (LKB1 mutant) and HeLa (LKB1 deleted) cell lines. Time = time post-wounding. (*p value <0.05, **p-value < 0.005 between control and STRADα-depleted cells) (D) Confocal images showing representative examples of cell polarity in control siRNA and STRADα siRNA transfected cells. Red=Actin, Green=Golgi, Blue=DAPI. Arrows show examples of polarized cells and arrowheads show mis-polarized cells as assessed by Golgi alignment.

FIGURE 5. STRADα regulates cancer invasion in LKB1 null cell lines (A) Matrigel invasion assays show that STRADα depletion (STRAD-) leads to significantly reduced invasion compared to control siRNA (Cntl) in four LKB1 null cell lines. *** = p<0.005 and ** = p<0.01 (B) Western blots of various cell lines transfected with FLAG-STRADα (C) Matrigel invasion assays show that FLAG-STRADα overexpression increases invasion in all cell lines tested.

FIGURE 6. STRADα regulates PAK1Thr423 phosphorylation and PAK1 induced invasion Western blots showing siRNA depletion of STRADα results in reduced pPAKThr423 in (A) LKB1 deleted HeLa cells and (B) LKB1 mutant H157 cells. Total myc-PAK is shown as a control and tubulin as a loading control. (C) Western blot showing IP of STRADα with subsequent blotting for PAK1. IgG is shown as a negative control. Input lanes shown on right. (D) Representative bar graphs of a Matrigel invasion assay where PAK overexpression increased invasion in control cells but does not increase invasion in STRADα – depleted cells.

FIGURE 7 STRADα regulates rac1 activity during cancer cell invasion (A) Bar graph showing results from rac1 activation assay in control (FLAG-onlym) and FLAG-STRADα transfected cells. (Error bars = SD; *p<0.05) (B) Western blot showing that pPAKThr423 levels are restored by the rac1 Q61L mutant, and confirming depletion of STRADα and overexpression of rac1 Q61L constitutively active mutant. (C) Matrigel invasion assays showing invasion in control and STRADα-depleted cells overexpressing a rac1 Q61L mutant. (**=p<0.01; *=p<0.1)
Figure 2

|       | H23          | H460         | H1944        | A549          | Hela         |
|-------|--------------|--------------|--------------|---------------|--------------|
| +FLAG | +FLAG-LKB1   | +FLAG-LKB1   | +FLAG-LKB1   | +FLAG-LKB1   | +FLAG-LKB1  |
|       | +FLAG-K78M LKB1 | +FLAG-K78M LKB1 | +FLAG-K78M LKB1 | +FLAG-K78M LKB1 | +FLAG-K78M LKB1 |

LKB1, STRADα, GAPDH
Figure 3
Figure 4
Figure 6
**Figure 7**

(A) Quantitative analysis of active Rac1 levels in HeLa and H157 cells. **Bold bars** represent a statistically significant increase compared to the control.

(B) Control siRNA | STRADα siRNA | rac1 Q61L | Empty vector
---|---|---|---
| + | - | - | + | - | + | - | + | + | - | - |

Western blot images show expression levels of STRADα, pPAK$, thr423$, GFP:rac1 Q61L, and GAPDH.

(C) Analysis of cell invasion in H157 cells. **Bold bars** indicate a statistically significant increase compared to the control.

Control siRNA | STRADα siRNA | rac1 Q61L | Empty vector
---|---|---|---
| + | - | - | + | - | + | - | + | + | - | - |
