THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 27, pp. 18812–18820, July 4, 2008

Krüppel-like Factor 5 Controls Keratinocyte Migration via the Integrin-linked Kinase*[S]

Received for publication, February 21, 2008, and in revised form, April 30, 2008 Published, JBC Papers in Press, May 1, 2008, DOI 10.1074/jbc.M801384200

Yizeng Yang, Marie-Pier Tetreault, Yuliya A. Yermolina, Bree G. Goldstein, and Jonathan P. Katz[1]

From the Department of Medicine, Gastroenterology Division, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Migration of epithelial cells is critical for normal homeostasis in gut and skin, but the factors regulating this process are not completely understood. The zinc finger transcription factor Klf5 (IKLF; BTEB2) is highly expressed in proliferating cells of esophagus, skin, and other organs. We hypothesized that Klf5 regulates keratinocyte migration via the integrin-linked kinase (ILK), which, like Klf5, is localized to basal keratinocytes. We stably transduced mouse primary esophageal keratinocytes to overexpress *Klf5* or small interfering RNA against *Klf5*. *Klf5* overexpression in keratinocytes increased migration and correlated directly with ILK expression and activation. *ILK* expression restored migratory capacity in keratinocytes with suppression of *Klf5*, whereas *ILK* small interfering RNA blocked the increased migration resulting from *Klf5* overexpression. By chromatin immunoprecipitation, electromobility shift assay, and luciferase reporter assays, we confirmed that *ILK* was a direct target for Klf5. In addition, Klf5 induced the activation of the ILK targets Cdc42 and myosin light chain, which are critical for cell migration and motility but not Rac1, AKT, or GSK3β. Overall, these results demonstrate that Klf5 is a key regulator of cell migration via ILK and provide new insight into the regulation of epithelial cell migration.

Spatial separation of proliferating and differentiating cells is a common theme throughout the epithelia of the luminal gastrointestinal tract, the skin, and several other organs (1–3). Migration of cells within these epithelia is critical for differentiation and normal homeostasis. A number of different epithelial types are found throughout the human body, of which the stratified squamous epithelium, which lines the esophagus, skin, oral cavity, and several other organs, is the most common. In stratified squamous epithelia, the basal layer is composed of both epithelial stem cells and transit-amplifying cells (4–6). These transit-amplifying cells are partially committed, rapidly proliferating cells that originate from asymmetric divisions of the stem cells and then migrate, both within the basal layer and from the basal layer, toward the surface (7, 8). While migrating within the basal layer, the transit-amplifying cells undergo several rounds of rapid proliferation and clonal expansion. Keratinocytes then undergo additional differentiation during migration through the suprabasal and superficial layers, losing their proliferative capacity.

A number of pathways are known to be critical for keratinocyte migration (reviewed in Ref. 9). Interactions of keratinocytes with the extracellular matrix are especially important for migration, and the integrins and the integrin-linked kinase (ILK)[2] play key roles in transducing signals from the extracellular matrix. ILK is an adaptor protein that couples integrins and growth factor receptors to a variety of downstream signaling events, influencing cell adhesion, proliferation, migration, differentiation, and survival (10–12). ILK binds to the cytoplasmic tails of integrin β1 and β3 and forms a complex with a number of other proteins, including PINCH and members of the Parvin family (12–14). Through interactions with these and other proteins, ILK mediates the actions of a large number of downstream effectors, including Cdc42, Rac, myosin light chain (MLC_{20}), GSK-3β, and AKT. Of note, activation of the small Rho GTPases Cdc42 and Rac is critical for cell migration and motility (11, 15), and phosphorylation of MLC_{20}, the 20-kDa regulatory light chain of myosin II, is important for cell motility and contractility (16, 17). Nonetheless, the targets for ILK are clearly cell type- and tissue-specific; for example, the well established ILK targets GSK-3β and AKT are not ILK targets in chondrocytes (18). Thus, the role of ILK in regulating its downstream effectors must be understood within a specific context. In squamous epithelia, such as those of the skin and esophagus, ILK is localized to basal cells, and expression is lost as cells enter the suprabasal layer (19). Within keratinocytes, ILK function is critical and is required for normal skin morphogenesis (20).

Although many substrates for ILK have been identified (14), the transcriptional regulation of *ILK* is still not well established. Moreover, many of the key transcriptional regulators of keratinocyte migration have not been identified. In stratified squamous epithelia such as skin and esophagus, the zinc finger tran-

* This work was supported, in whole or in part, by National Institutes of Health Grants R21 DK073888 from NIDDK, P30 DK050306 from NIDDK (to the University of Pennsylvania Center for Molecular Studies in Digestive and Liver Disease) through the Morphology Core and the Molecular Biology Core, and P01 A098101 from NCI ("Mechanisms of Esophageal Carcinogenesis"). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–5.

[1] To whom correspondence should be addressed: 600 Clinical Research Bldg., 415 Curie Blvd., Philadelphia, PA 19104-6144. Tel.: 215-746-7780; Fax: 215-573-2024; E-mail: jpkatz@mail.med.upenn.edu.

[2] The abbreviations used are: ILK, integrin-linked kinase; Klf5, *Krüppel*-like factor 5; MLC_{20}, myosin light chain; siRNA, small interfering RNA; ChIP, chromatin immunoprecipitation; EMSA, electromobility shift assay; PBS, phosphate-buffered saline; EGF, epidermal growth factor; bis-tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; MEK, MAPK/ERK kinase.

scription factor *Krüppel*-like factor 5 (Klf5; IKLF; BTEB2), like ILK, localizes to basal keratinocytes (21). Members of the KLF family are linked to the regulation of cell migration (22), as well as numerous other key cellular processes (23). Klf5 was initially identified in proliferating crypt cells of the intestine (24). In the developing gut and epidermis, Klf5 is expressed beginning at embryonic day 10.5 and persists in the adult in cells within the proliferative compartments (25). In vascular cells, Klf5 mediates the response to injury, and heterozygous knock-out mice have abnormalities in cardiovascular remodeling in response to stress (26, 27). Klf5 also regulates adipocyte differentiation (28). In keratinocytes, Klf5 transcriptionally regulates *EGFR*, activates MAPK signaling, and promotes rapid cell proliferation, such as that seen in transit-amplifying cells of the basal layer (21, 29). Thus Klf5 exhibits many features necessary for the transcriptional control of normal epithelial homeostasis.

We hypothesized that in addition to promoting proliferation in transit-amplifying cells, Klf5 might also regulate migration of these cells, a critical step for keratinocyte differentiation. We further hypothesized that Klf5 might regulate keratinocyte migration via ILK. Here, using mouse primary esophageal keratinocytes as a model, we identify a critical positive regulatory role for Klf5 in keratinocyte migration. We also demonstrate that *ILK* is a transcriptional target of Klf5, that the effect of Klf5 on cell migration occurs via ILK, and that Cdc42 and MLC_{20} are key specific mediators of the effects of Klf5 and ILK on keratinocyte migration. Overall, these studies provide important new insights into the regulation of epithelial cell migration and the mechanisms of normal epithelial homeostasis.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment—The isolation and culture of mouse primary esophageal keratinocytes have been described elsewhere (29), and all animal studies were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. Once isolated, mouse esophageal epithelial cells were grown in keratinocyte serum-free medium (K-SFM, Invitrogen) supplemented with 40 μg/ml bovine pituitary extract (Invitrogen), 1.0 ng/ml epidermal growth factor (EGF) (Invitrogen), 100 units/ml penicillin, and 100 μg of streptomycin (Invitrogen). All experiments were begun when cells reached 70% confluence except as noted.

Retroviral Vectors and Infection—For retroviral expression of *Klf5*, we utilized full-length mouse *Klf5* cDNA subcloned into the pFB-neo retroviral vector (Stratagene, La Jolla, CA), as described previously (29). To suppress *Klf5* in mouse esophageal keratinocytes, we employed the pSR-siKlf5 retroviral construct (29), which generates double-stranded RNA directed against nucleotides 676–694 of mouse *Klf5*. Empty vector served as control for *Klf5* overexpression. For siRNA experiments, we generated a mismatch control (30), pSR-siKlf5Δ, which is identical to pSR-siKlf5 except for mutations in 6 of the 19 targeted nucleotides (ACATTATCATATTACTACC, mismatches underlined) and contains no similarities to other mouse sequences by BLAST analyses. The constructs were packaged in Phoenix-Ampho cells (Stanford University, Stanford, CA) by transfection with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, and mouse primary keratinocytes were infected with culture supernatants from individual Phoenix-Ampho cells at a 1:6 dilution in K-SFM. Cells were passaged after 24 h and then selected with 300 μg/ml G418 for 14 days.

Cell Migration and Adhesion Assays—Cell migration assays were performed as described previously (31). In brief, after culturing cells without EGF for 48 h, single cell suspensions containing 4×10^4 cells/well in 0.5 ml of K-SFM (without EGF) were plated in triplicate into 24-well inserts (Falcon cell culture inserts, 8 μm pore size). The lower chamber was filled with 0.5 ml of K-SFM containing 0.5 μg/ml hydrocortisone (Sigma) and 10 ng/ml EGF (BD Biosciences). After incubation for 16 h at 37 °C, the cells on the upper side of the transwell membrane were removed by cotton swab and rinsed with PBS. Cells migrating to the lower side of membrane were fixed in 4% paraformaldehyde for 20 min at room temperature, stained with crystal violet (Sigma), and counted. To perform migration assays in cells with *ILK* overexpression, we utilized an *ILK*-expressing adenovirus, Ad-ILK (gift of Dr. Gregory Hannigan, University of Toronto) or an empty vector control (32). Adenovirus was amplified in 293T cells and used to infect 1×10^6 primary esophageal keratinocytes per well in each well of a 6-well plate (BD Biosciences) at a multiplicity of infection of 1:15. After 48 h of infection, migration assays were performed as described above. For studies of cell adhesion, 24-well plates coated with Matrigel (BD Biosciences) were seeded with 6×10^4 cells/well in K-SFM and incubated for 2 h at 37 °C. After washing three times with PBS to remove nonadherent cells, the remaining cells were dissociated with 0.05% trypsin/EDTA and counted. Data represented the average number of cells from triplicate wells.

Immunofluorescence—Cells were plated into 8-well Lab-Tek chamber slides (Nunc) and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. F-actin was stained with Alexa Fluor 546 phalloidin (Invitrogen) at 1:40 dilution in PBS containing 1% bovine serum albumin (Sigma) for 20 min at room temperature. Images were captured on a Nikon Eclipse E600 microscope with a Photometrics CoolSNAP CCD camera (Roper Scientific). Lamellipodia and filopodia were counted, as described (33), in five ×200 fields for each experimental condition. For detection of Klf5 in mouse esophagus, we utilized 1:5000 rabbit anti-Klf5 (29) and 1:200 anti-rabbit Cy3 (Invitrogen), and for ILK we used 1:400 rabbit anti-ILK (Millipore) and 1:200 anti-rabbit Alexa Fluor 488 (Invitrogen).

Western Blotting—Western blots were performed as described previously (29). For each sample, 30 μg of total protein was separated on a NuPAGE 4–12% bis-tris acrylamide gel (Invitrogen) and transferred onto polyvinylidene difluoride membrane (Millipore). After blocking, membranes were incubated overnight at 4 °C with the following primary antibodies: 1:5000 rabbit anti-Klf5 (29); 1:1000 rabbit anti-ILK (Millipore); 1:1000 rabbit anti-Akt (Cell Signaling Technology); 1:1000 rabbit anti-phospho-Akt (Ser^{473}) (Cell Signaling Technology); 1:1000 rabbit anti-GSK3β (Cell Signaling Technology); 1:1000 anti-phospho-GSK3β (Ser^9) (Cell Signaling Technology); 1:1000 rabbit anti-Rac1 (Cell Signaling Technology); 1:1000 rabbit anti-Cdc42 (Cell Signaling Technology); 1:1000 rabbit anti-phospho-MLC (Thr^{18}/Ser^{19}) (Cell Signaling Technology);

and 1:2000 mouse anti-α-tubulin (Sigma). Membranes were then incubated with a 1:3000 dilution of anti-rabbit/horseradish peroxidase or anti-mouse/horseradish peroxidase (Amersham Biosciences) and developed with the enhanced chemiluminescence plus Western blot analysis kit (Amersham Biosciences).

Quantitative PCR—Total RNA was isolated with the RNeasy micro kit (Qiagen, Valencia, CA), and cDNA was synthesized with Superscript II reverse transcriptase (Invitrogen). Quantitative real time PCR was performed in triplicate on three samples for each experimental condition using an ABI Prism 7000 sequence detection system (Applied Biosystems) and SyBr Green PCR master mix (Applied Biosystems). TATA box-binding protein was used as the internal control. Primer sequences are available on request.

ILK Kinase Assay—ILK kinase assays were performed as described previously (32). In brief, equivalent protein concentrations of cell lysates were pre-cleared with nonspecific IgG bound to protein A-Sepharose, and supernatants were immunoprecipitated with 1.5 μg of rabbit anti-ILK antibody (Millipore) overnight at 4 °C with rotation. Protein A-Sepharose (Sigma), pre-swollen in Nonidet P-40 lysis buffer, was added for 2 h at 4 °C to capture the antibodies. Following two washes with Nonidet P-40 lysis buffer and two washes with kinase wash buffer (10 mM $MgCl_2$, 10 mM $MnCl_2$, 50 mM HEPES, pH 7.5, 0.1 mM sodium orthovanadate, 1 mM dithiothreitol), kinase assays were performed directly on the protein A beads in a 25-μl volume containing 10 mM $MgCl_2$, 10 mM $MnCl_2$, 50 mM HEPES, pH 7.5, 1 mM sodium orthovanadate, 2 mM sodium fluoride, 5 mCi of [γ-^{32}P]ATP (Amersham Biosciences), and 12.5 μg of myelin basic protein as substrate (Millipore). The reaction was incubated for 30 min at 30 °C and stopped with 10 μl of SDS-PAGE nonreducing stop buffer and heated for 5 min at 95 °C. Phosphorylated myelin basic protein bands were visualized by 10% SDS-PAGE and autoradiography with analysis of five independent experiments on a Storm 840 PhosphorImager (GE Healthcare).

Cdc42 and Rac1 Activation Assay—Cdc42 and Rac1 activation assays were performed with the Rac/Cdc42 assay reagent (Millipore) following the manufacturer's instructions. Briefly, cells were lysed with a magnesium-containing lysis buffer (MLB), and active complexes were immediately precipitated with 10 μg of PAK-1 PBD using 300 μg of protein lysate in a 1-ml total volume at 4 °C for 60 min with rotation. PAK-1 PBD corresponds to the p21 binding domain (PBD, residues 67–150) of human PAK-1. After collecting and washing with MLB three times with pulsing centrifugation, agarose beads were resuspended in 20 μl of Laemmli sample buffer and boiled for 5 min. Rac1-GTP and Cdc42-GTP were detected by Western blotting with Rac1 or Cdc42 antibodies as described above.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were performed in triplicate with the ChIP assay kit (Millipore) as described previously (29). Cells were treated with 1% formaldehyde for 10 min to cross-link associated protein to DNA, lysed, and sonicated. After a 10-fold dilution, the samples were pre-cleared with protein A-agarose/salmon sperm DNA for 30 min at 4 °C and incubated overnight at 4 °C with 1:500 anti-Klf5 or 1:500 anti-mouse IgG (Sigma), as a negative control. Cells were then precipitated with protein A-agarose for 1 h, heated at 65 °C for 4 h, treated with proteinase K, and DNA-extracted with phenol/chloroform. Primers were designed to amplify the region from −322 to −82 and the region from −829 to −671 in the 5′ regulatory region of the *ILK* gene, and PCR was performed with puReTaq Ready-to-Go PCR beads (Amersham Biosciences) for 25 cycles at 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. PCR products were separated on a 2% agarose gel and visualized by a Gel Doc XR system (Bio-Rad).

Electrophoretic Mobility Shift Assay—A double-stranded DNA probe with the sequence 5′-ATAGGAGCTGGCCGGGCGGGGCCGGGCGGGGCCGGGCGGCGGGCGCGGCCCGGA-3′, which corresponded to a region of triple Klf5-binding sites in the 5′ regulatory region of *ILK*, was labeled with [^{32}P]dCTP, as was a similar probe containing mutations in 12 bases of the triple Klf5-binding sites (5′-ATAGGAGCTGGTCGAGTGAGTCAGGTGAGACTGAGTGGCGGGCGCGGCCCGGA-3′, mutated bases are underlined). Klf5 *in vitro* translated protein was made using the TNT Quick-Coupled transcription/translation kit (Promega), and 60,000 cpm of probe was incubated with 3.0 μg of nuclear extract or differing amounts of *in vitro* translated Klf5 protein in a 25-μl volume of binding buffer, containing 20 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, 150 mM NaCl, 20 M KCl, 5 μM $ZnCl_2$, 0.5 mM dithiothreitol, 5% glycerol, and 1 μg of poly(dI-dC), for 20 min at 4 °C. Supershifts were performed with 5 μg of anti-Klf5 antibody per lane. The DNA-protein complexes were separated on a native 6% polyacrylamide gel and visualized on a Storm 840 PhosphorImager.

Cell Transfection and Reporter Assays—A DNA fragment corresponding to the 5′ regulatory region of the mouse *ILK* gene from −938 to +496, immediately upstream of the translational start site, was amplified by PCR and subcloned into a pGL3-Luc basic reporter vector (Promega). We constructed a reporter vector with mutation of 12 bases in the 5′ regulatory region of *ILK*, between −240 and −206 (GGTCGAGTGAGTCAGGTGAGACTGAGTGGCGGGC, mutated bases underlined), the region corresponding to the putative triple Klf5-binding site, using the GeneEditor *in vitro* site-directed mutagenesis system (Promega). Cells were co-transfected with pIRES-*Klf5*-GFP, containing the complete *Klf5* coding sequence in pIRES-GFP (Clontech) or with pIRES-GFP empty vector at 50% confluence in triplicate on 24-well plates using FuGENE 6 transfection reagent (Roche Applied Science). After 48 h, cells were lysed with Cell Lysis Buffer (Pharmingen), and luciferase reporter activity was analyzed using luciferase assay reagent (Promega) with a microtiter plate luminometer (Dynex Technologies). Luciferase activity was normalized to β-galactosidase and expressed as relative luciferase activity.

RESULTS

Klf5 Increases Keratinocyte Migration—Cell migration is important in such diverse processes as embryonic development, wound healing, and tumor progression and metastases (34) and in the maintenance of normal epithelial homeostasis (1). To evaluate the role of Klf5 in cell migration, we stably transduced mouse primary esophageal keratinocytes with retroviral vectors to overexpress *Klf5* or to suppress *Klf5* with

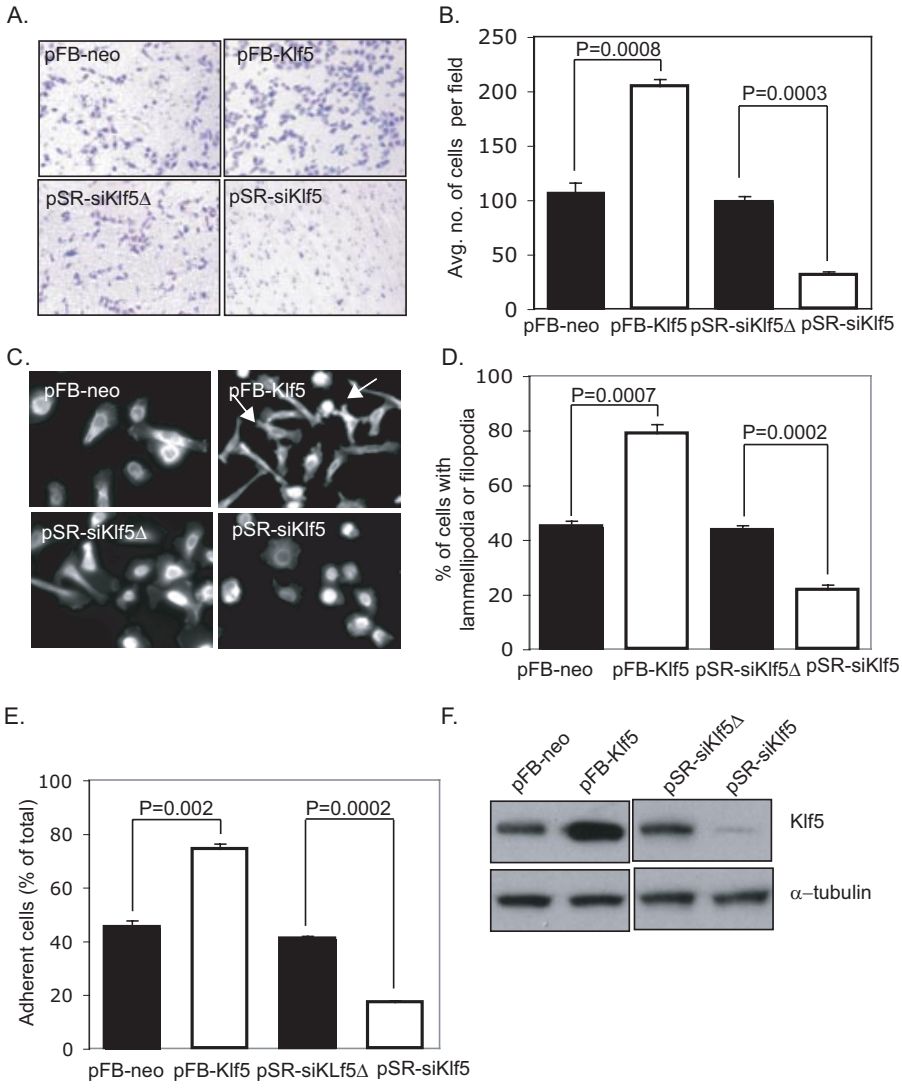

FIGURE 1. **Klf5 increased keratinocyte migration and adhesion.** *A,* Transwell assays demonstrated that Klf5 increased migration of mouse primary esophageal keratinocytes across the membrane. Cells migrating across the membrane were stained *purple*. *B,* overall, *Klf5* overexpression increased keratinocyte migration 2-fold whereas *Klf5* suppression with siRNA decreased migration by 70% ($n = 5$). Results were expressed as mean ± S.E. *C,* keratinocytes with overexpression of *Klf5* appeared larger with more prominent cellular protrusions (*arrows*) compared with control cells. In contrast, keratinocytes with *Klf5* suppression by siRNA appeared more compacted and had fewer protrusions. Cells were stained with phalloidin for F-actin. *D,* overexpression of *Klf5* resulted in a 75% increase in the number of keratinocytes with filopodia or lamellipodia, whereas inhibition of *Klf5* produced a 50% decrease in the number of keratinocytes with these protrusions, which are characteristic of migrating cells ($n = 5$). Results were expressed as mean ± S.E. *E,* Klf5 increased adherence of keratinocytes to Matrigel by more than 60%, and *Klf5* suppression resulted in a 60% decrease in cell adherence. The number of adherent cells was expressed as a percentage of total cells ($n = 3$), and results represent the mean ± S.E. *F,* Western blots confirmed that retroviral infection of primary esophageal keratinocytes with pFB-Klf5 increased Klf5 expression, compared with pFB-neo control, whereas infection with pSR-siKlf5 decreased Klf5 expression, compared with pSR-siKlf5Δ mismatch control. Student's *t* test was used for statistical analyses.

siRNA (29). We quantitated the effects of Klf5 on keratinocyte migration using Transwell assays. Cells migrating across the membrane were stained purple (Fig. 1*A* and supplemental Fig. 1*B*) and counted. As demonstrated in Fig. 1*B* and supplemental Fig. 1*C*, *Klf5* overexpression increased keratinocyte migration across the membrane, whereas *Klf5* suppression inhibited cell migration significantly. Notably, suppression of *Klf5* did not significantly alter apoptosis in primary esophageal keratinocytes (supplemental Fig. 2).

The early stages of cell migration require adhesion and the formation of cell protrusions, the broad lamellipodia or the "spike-like" filopodia, which provide the traction sites for cells (34). To understand the effects of Klf5 on the processes of migration, we examined the formation of lamellipodia and filopodia. Grossly, cells with *Klf5* overexpression appeared to have more of these cell protrusions, whereas keratinocytes with *Klf5* inhibition by siRNA had fewer protrusions (Fig. 1*C*). As indicated in Fig. 1*D*, *Klf5* expression increased the percentage of primary esophageal keratinocytes with lamellipodia and filopodia, whereas inhibition of *Klf5* markedly decreased lamellipodia and filopodia formation. Adhesion of cells to the extracellular matrix, another key component of migration, is mediated in large part by integrins and integrin signaling (35). Overexpression of *Klf5* resulted in a statistically significant increase in cell adhesion, whereas *Klf5* suppression inhibited adhesion (Fig. 1*E*). Fig. 1*F* and supplemental Fig. 1*A* confirm the successful overexpression or suppression of Klf5 in these stably infected cell lines. Overall, these data support an important role for Klf5 in the regulation of keratinocyte migration.

Klf5 Up-regulates ILK Expression and Kinase Activity—As Klf5 is a DNA-binding transcriptional regulator (36), we sought to identify the mediators of the effects of Klf5 on keratinocyte migration. Signals from the extracellular matrix are critical for cell migration (37), and we hypothesized that Klf5 might impact integrin signaling, specifically by regulating the key adaptor protein ILK (10–12). Following scratch wounding, Klf5 and ILK are both expressed in keratinocytes migrating across the wound, consistent for a role for both these proteins in keratinocyte migration (supplemental Fig. 3). In addition, both Klf5 and ILK are expressed in the basal layer of the esophagus *in vivo* (Fig. 2*A*). Cells of the basal layer include the stem cells and transit-amplifying cells, daughters of the stem cells that migrate within the basal layer, undergoing several rounds of rapid proliferation before migrating further, undergoing additional differentiation, and losing their proliferative capacity (6, 38).

Utilizing mouse primary esophageal keratinocytes with overexpression of *Klf5* or *Klf5* suppression with siRNA, we examined whether Klf5 altered *ILK* mRNA and protein expression.

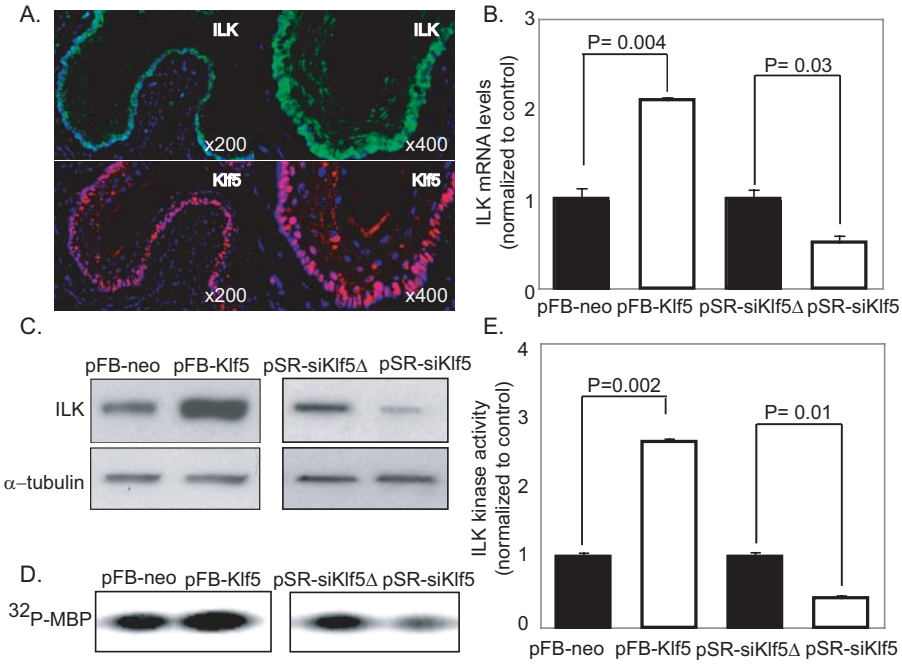

FIGURE 2. **Klf5 increased ILK expression and kinase activity.** *A,* both ILK (*green*) and Klf5 (*red*) were expressed in the basal layer of the esophagus in mice. 4′,6-Diamidino-2-phenylindole (*blue*) stained the cell nuclei. *B,* by quantitative PCR, *Klf5* overexpression increased levels of *ILK* mRNA by 2-fold, whereas *Klf5* suppression decreased *ILK* expression by 50%. Values were normalized to controls and expressed as mean ± S.E. *C,* Western blots revealed that *Klf5* overexpression increased ILK protein levels, whereas inhibition of *Klf5* decreased ILK protein expression. α-Tubulin served as a loading control. *D* and *E,* assays of ILK kinase activity (*D*) revealed a 2.5-fold increase in kinase activity with *Klf5* overexpression and a 60% decrease in activity (*E*), when band density was quantitated on a PhosphorImager ($n = 3$). Kinase activities were normalized to controls and expressed as mean ± S.E. Student's *t* test was used for statistical analyses.

Quantitative real time PCR revealed that *Klf5* overexpression increased *ILK* mRNA levels, whereas siRNA against *Klf5* reduced *ILK* expression (Fig. 2*B*). By Western blot, overexpression of *Klf5* increased ILK protein levels, whereas ILK protein was reduced by inhibition of *Klf5* (Fig. 2*C*). Although some have argued that ILK is a "pseudokinase" (39), a number of studies support an important role for the kinase activity of ILK (40, 41). To ensure that this increased *ILK* expression correlated with an increase in ILK kinase activity, we performed ILK kinase assays (32). A representative blot is shown in Fig. 2*D*. Overall, *Klf5* overexpression produced a 2.5-fold increase in ILK kinase activity, whereas *Klf5* inhibition yielded a 60% decrease in activity (Fig. 2*E*). Although we had shown previously that Klf5 upregulates MEK/ERK signaling (29), inhibition of MEK with the MEK inhibitor PD98059 did not alter ILK expression or ILK kinase activity (supplemental Fig. 4), indicating that the regulation of ILK by Klf5 was not mediated by MEK/ERK signaling.

ILK Is a Transcriptional Target for Klf5—To determine whether *ILK* was a direct transcriptional target for Klf5, we examined the 5′ regulatory region of *ILK* for putative Klf5-binding sites, using the computational program TESS (56). We identified a putative triple Klf5-binding site from −240 to −206 and hypothesized that Klf5 might bind to *ILK* in this region. Using ChIP assays, we demonstrated binding of Klf5 to *ILK* in the vicinity of the triple Klf5 site (Fig. 3*A*). Next, to confirm that Klf5 bound to the region between −240 and −206, we performed electromobility shift assays (EMSA) with an oligonucleotide probe corresponding to the triple Klf5 site. By EMSA, Klf5 bound to the triple Klf5 site on *ILK* (Fig. 3*B*). Addition of increasing amounts of unlabeled probe competed away this binding, and the Klf5 band was shifted upward with anti-Klf5 antibody. Klf5 did not bind to a probe containing mutations in 12 bases of the triple Klf5 site, and inclusion of 200× cold mutant probe did not compete away binding of Klf5 to labeled probe that did not contain the mutations (Fig. 3*C*). These results demonstrated the sequence specificity of Klf5 binding to this region of *ILK*.

To examine the transcriptional regulation of *ILK* by Klf5, we cloned a DNA fragment of *ILK* from −938 to +496, immediately upstream of the translational start site, into the pGL3-Luc basic reporter vector to create the pILK-Luc reporter. We co-transfected mouse primary esophageal keratinocytes with pILK-Luc and with the bicistronic *Klf5* expression plasmid pIRES-*Klf5*-GFP or the pIRES-GFP empty vector. As shown in Fig. 3*D*, expression of *Klf5* increased *ILK* promoter activity by 4-fold. To determine whether Klf5 regulated *ILK* transcription via the Klf5-binding site identified on ChIP and EMSA, we created a mutant reporter construct, pILK-LucMT, containing mutations in the same 12 nucleotides within the triple Klf5-binding site of pILK-Luc as for the EMSA probe. When pIRES-*Klf5*-GFP was co-transfected with pILK-LucMT, the responsiveness of the reporter to Klf5 was lost. Moreover, basal *ILK* promoter activity, likely related to endogenous *Klf5* expression in primary esophageal keratinocytes, was reduced by 50%. These results confirm that Klf5 transcriptionally regulates ILK by binding to the triple Klf5-binding site in the 5′ regulatory region of *ILK*.

ILK Mediates the Effects of Klf5 on Keratinocyte Migration—To demonstrate that ILK was downstream of Klf5 and mediated the effects of Klf5 on cell migration, we infected primary esophageal keratinocytes stably infected with pSR-siKlf5Δ or pSR-siKlf5 using an adenovirus that expresses *ILK* (Ad-ILK) or an empty vector control (32). Infection with Ad-ILK produced markedly increased expression of ILK, both in control cells and in cells with *Klf5* suppression with siRNA (Fig. 4*A*). In contrast, infection with pSR-ILK, which generates siRNA against *ILK*, reduced ILK levels both in control and *Klf5*-overexpressing keratinocytes, compared with infection with pSR-siILKΔ control (Fig. 4*B* and supplemental Fig. 1*D*). As seen in Fig. 4, *C* and *D*, transwell assays revealed that *ILK* overexpression increased keratinocyte migration in control cells. Moreover, in cells with *Klf5* suppression, *ILK* overexpression produced a 7-fold increase in migration of cells across the membrane, thus restoring migratory capacity in primary esophageal keratinocytes with suppression of *Klf5*.

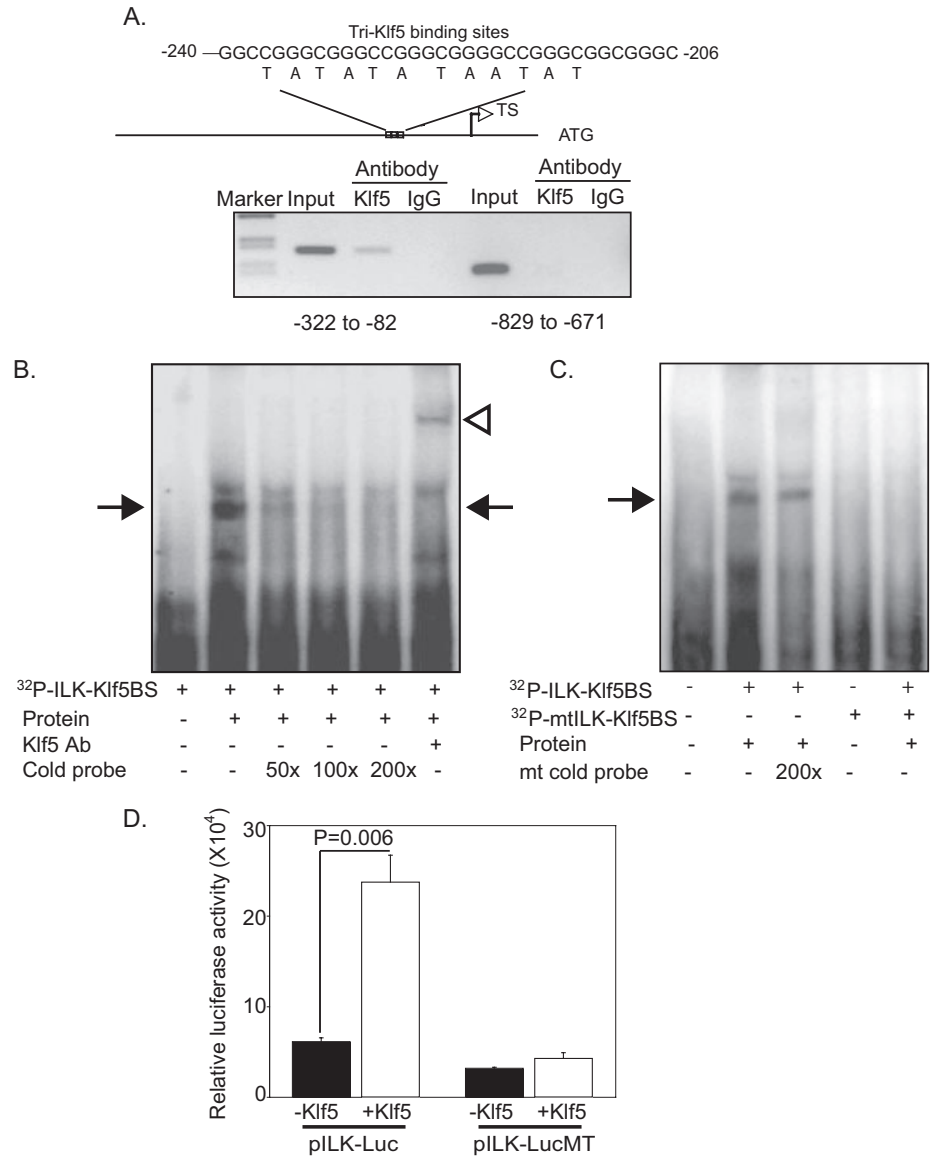

FIGURE 3. ***ILK* was a direct transcriptional target for Klf5.** *A,* 5′ regulatory region of *ILK* contained a putative triple Klf5-binding site from −240 to −206. ChIP assays ($n = 3$) demonstrated that Klf5 bound to *ILK* within the region −322 to −82, which includes the triple Klf5-binding site, but not to the region from −829 to −671, which was used as a control. Input was total DNA prior to immunoprecipitation. DNA was precipitated with anti-IgG antibody as an additional negative control. *B,* EMSA revealed binding of Klf5 (*black arrows*) to a probe corresponding to the triple Klf5-binding site from *ILK*. This binding could be competed away with increasing amounts of unlabeled probe. Upon addition of 5 μg of anti-Klf5 antibody (*Ab*), a new band (*white arrowhead*) appeared at an apparent higher molecular mass, and the lower Klf5 band disappeared, confirming that this band represented Klf5 binding to the probe. *C,* by EMSA, Klf5 binding to the probe (*black arrow*) was not altered by the addition of 200× mutant probe, which contained mutations in 12 bases within the triple Klf5-binding site from *ILK* (mutated bases are shown *below* the sequence from −240 to −206 in *A*). In addition, Klf5 did not bind to labeled mutant probe, confirming the sequence-specific binding of Klf5 to this region of *ILK*. *D,* co-transfection of mouse primary esophageal keratinocytes with the bicistronic *Klf5* expression vector pIRES-*Klf5*-GFP (or pIRES-GFP control) and an *ILK* reporter, pILK-Luc, which contained the region −938 to +496, revealed a 4-fold induction of *ILK* transcriptional activity with Klf5. No promoter activation was seen when a 12-nucleotide mutation (pILK-LucMT) was introduced in the triple Klf5-binding site of *ILK* ($p = 0.004$ for mutant *versus* control, $n = 4$). Results were normalized to β-galactosidase and expressed as mean of relative luciferase activity × 10^4 ± S.E. Student's *t* test was used for statistical analyses.

Next, to confirm these findings, we performed the opposite experiment, inhibiting *ILK* in primary esophageal keratinocytes with overexpression of *Klf5*. When we examined keratinocyte migration by transwell assay, we observed that *ILK* inhibition significantly reduced migration of keratinocytes with *Klf5* overexpression (Fig. 4*E* and supplemental Fig. 1, *E* and *F*). These results demonstrated that Klf5 regulated cell migration in primary esophageal keratinocytes via ILK.

Cdc42 and MLC_{20} Are Targets for ILK in Keratinocytes—To identify the mechanism by which ILK regulated migration in primary esophageal keratinocytes, we investigated the expression of several known ILK targets. Akt and GSK3β, as well as the small Rho GTPases Rac1 and Cdc42, which are critical for cell motility, contractility, and migration, have all been shown to be ILK targets in other cell types (40, 42, 43). *Klf5* overexpression activated the ILK target Cdc42, and *Klf5* inhibition decreased Cdc42 activation (Fig. 5, *A* and *B*), whereas activation of Rac1 was not affected. In addition, levels of phosphorylated Akt and GSK3β (Fig. 5, *C* and *D*) as well as total Cdc42, Rac1, Akt, and GSK3β were unchanged. Confirming that increases in Cdc42 upon induction of *Klf5* were mediated by *ILK*, suppression of *ILK* with siRNA in *Klf5*-overexpressing cells markedly decreased Cdc42 activation (Fig. 5*E*). MLC_{20}, which also plays a critical role in cell motility, has been identified both as a direct target for ILK (41) and as a downstream target of Cdc42 (44). MLC_{20} phosphorylation at Thr^{18} and Ser^{19} was increased in keratinocytes with *Klf5* overexpression and decreased by *Klf5* suppression (Fig. 5*F*). Suppression of *Cdc42* in *Klf5*-overexpressing keratinocytes produced a significant decrease in cell migration (supplemental Fig. 5). Thus, Cdc42 and MLC_{20} were downstream targets of ILK in primary keratinocytes, providing a link between Klf5 and two critical regulators of cell migration.

DISCUSSION

Cell migration is a fundamental process for organogenesis during development, for normal homeostasis of adult tissues, and for tissue regeneration and repair after injury (34). In the past few years, enormous advances have been achieved in our understanding of the complexities and subtleties underlying the regulation of cell migration, which includes the spatio-temporal controls of cell adhesion, asymmetric polarization, and individual or layered cell motility. Using primary esoph-

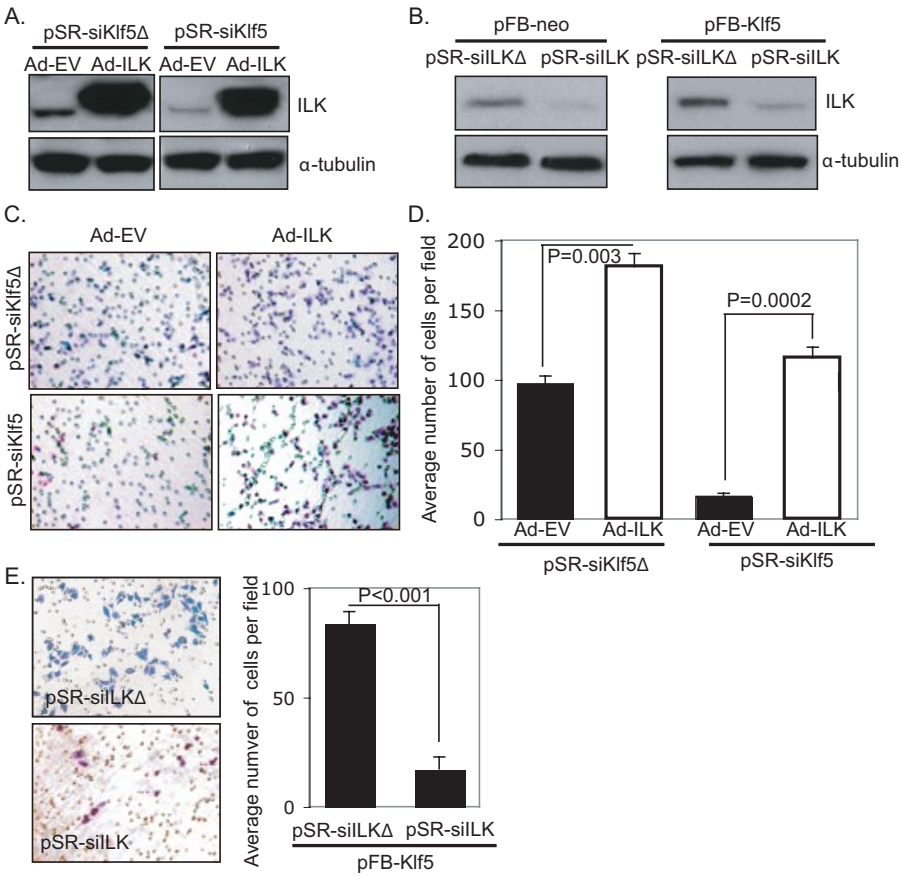

FIGURE 4. **ILK mediated the effects of Klf5 on keratinocyte migration.** *A,* ILK was overexpressed by the adenovirus Ad-ILK in cells stably infected with pSR-siKlf5Δ control or the pSR-siKlf5 retrovirus, which expresses siRNA against *Klf5*. *B,* in cells with stable overexpression of *Klf5*, *ILK* was effectively suppressed by infection with pSR-siILK, compared with cells infected with pSR-siILKΔ mismatch control. *C,* overexpression of *ILK* increased migration in cells infected with pSR-siILKΔ or pSR-siILK. Cells migrating across the membrane were stained *purple*. *D,* overall, migration was increased by 2-fold in control cells with *ILK* overexpression; in cells with *Klf5* suppression, migration was increased 7-fold by *ILK* overexpression ($n = 5$). Results were expressed as mean ± S.E. *E,* inhibition of *ILK* with siRNA reduced keratinocyte migration by 80% ($n = 5$). Results were expressed as mean ± S.E. Student's *t* test was used for statistical analyses.

ageal keratinocytes, we now identify a role for the pro-proliferative *Krüppel*-like factor Klf5 as a regulator of cell migration.

Epithelial stem cells often reside within a stem cell "niche," and migration of epithelial cells out of this niche, even within the proliferative compartment, is critical for differentiation into transit-amplifying cells (5, 7). In stratified squamous epithelia, such as those of the skin and esophagus, proliferative cells in the basal layer periodically detach from the underlying basement membrane, move outward, undergo terminal differentiation, and eventually die (1). Loss of contact with the extracellular matrix contributes to the terminal differentiation of keratinocytes and is mediated by changes in integrin signaling (45). Thus, migration of keratinocytes within the basal layer and out of the basal layer is essential for proliferation and differentiation and for the maintenance of epithelial integrity (8, 38, 46). Perturbations of the processes of keratinocyte migration result in abnormal wound healing, as is seen in gastroesophageal reflux disease, which affects nearly one-half of the population of the United States (47), and esophageal cancer, the sixth most common cause of cancer deaths worldwide (48).

Here, we identify Klf5, which is expressed within the basal layer of the esophagus and skin, as a novel transcriptional activator of *ILK*. Furthermore, we describe an important role for Klf5 in the regulation of normal keratinocyte migration via the adaptor protein ILK, a central mediator of signaling from the extracellular matrix via the integrins (11, 14), and we identify Cdc42 and MLC_{20} as downstream targets. Normally, ILK forms a complex at focal adhesions, binding to the cytoplasmic tail of integrin β1, as well as a number of other adaptor proteins, such as PINCH, paxillin, and the parvins CH-ILKBP/α-parvin and affixin/β-parvin (14). Yet the role of ILK in the activation of its targets is controversial. Although a number of studies implicate the kinase activity of ILK in the phosphorylation of target proteins (40, 41), others argue that ILK is a pseudokinase (39). Thus, it is not clear whether Cdc42 and MLC_{20} are directly phosphorylated by ILK in keratinocytes. Other proteins, such as the focal adhesion kinase, are recruited to focal adhesions at the leading edge of migrating cells (49), and Rac and Cdc42 have been reported to be regulated by ILK via the guanine-nucleotide exchanger α-PIX (43). Thus it may be valuable in the future to identify additional intermediates for the activation of Cdc42 and MLC_{20} by Klf5 in keratinocytes.

Although the inhibition of both cell adhesion and cell migration by suppression of *Klf5* may seem counterintuitive, these findings are consistent with the established role for adhesion and especially integrin-mediated adhesion in cell migration (34, 35). Moreover, the cell adhesion assays that we performed measure adhesion of cells to the matrix, rather than cell-cell adhesion. Of note, similar findings of both decreased adhesion and decreased migratory capacity were seen in keratinocytes from *ILK* knock-out mice (20).

Overexpression of ILK restores migratory capacity in keratinocytes with suppression of *Klf5*, and siRNA against *ILK* decreases keratinocyte migration by 80% in cells with *Klf5* overexpression, indicating that the primary means of regulation of keratinocyte migration by Klf5 is directly through ILK. Nonetheless, other factors might also be involved in the regulation of keratinocyte migration by Klf5 and ILK. For example, Klf5 may regulate other binding partners for ILK, such as the integrins, PINCH, α-PIX, or the parvins. Notably, the adaptor protein Nck-2 associates with PINCH and is regulated by EGF receptor signaling (50), providing a potential link between another Klf5 target, EGF receptor (29), and the ILK complex. In addition, other Rho GTPases, in addition to Cdc42, may contribute to the

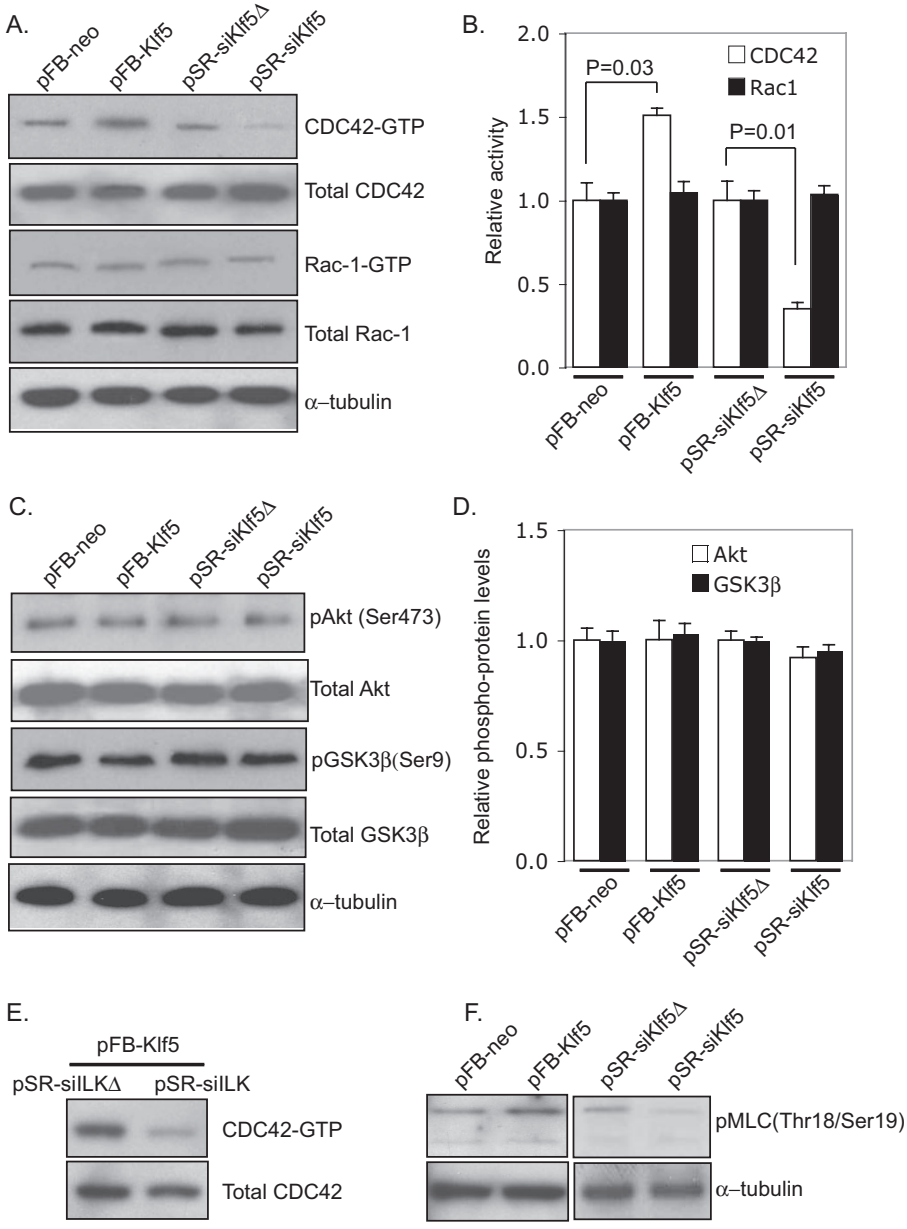

FIGURE 5. **MLC_{20} and the small Rho GTPase Cdc42 were targets for ILK in keratinocytes.** *A,* by Western blot, Cdc42 activation was increased by *Klf5* expression in mouse primary esophageal keratinocytes and decreased by *Klf5* inhibition, whereas activation of Rac1 was not changed. *B,* quantitation of assays performed in triplicate confirmed a statistically significant effect of Klf5 on Cdc42 but not on Rac1. Results were normalized to controls and expressed as mean ± S.E. *C,* activation of Akt and GSK3β was also unchanged by overexpression or suppression of *Klf5* in mouse primary esophageal keratinocytes. α-Tubulin served as a loading control. Notably, Rac1, Akt, and GSK3β have been identified as ILK targets in other contexts. *D,* quantitation of triplicate assays on Akt and GSK3β demonstrated no change in expression or activation. Results were normalized to controls and expressed as mean ± S.E. *E,* suppression of *ILK* with siRNA blocked the activation of Cdc42 by *Klf5* overexpression in keratinocytes, indicating that Klf5 activated Cdc42 via ILK. *F,* phosphorylation of MLC_{20}, which plays a key role in cell motility, was increased by *Klf5* overexpression and decreased by *Klf5* suppression. α-Tubulin was a loading control. Student's *t* test was used for statistical analyses.

processes of keratinocyte migration. In fact, Rho-associated kinase and RhoA have been implicated in ILK-mediated regulation of osteosarcoma cell spreading and motility (51). Activation of pathways such as these may be downstream or may occur in parallel to ILK signaling in keratinocytes.

We have previously identified Klf5 as part of a feedback loop critical for the induction of rapid keratinocyte proliferation (29). By identifying a role for Klf5 as an important mediator of keratinocyte migration, we demonstrate that Klf5 confers two essential properties of transit-amplifying cells. In contrast to its effects in primary esophageal keratinocytes, KLF5 inhibits proliferation and invasion in esophageal squamous cancer cells and also promotes anoikis (31). A context-dependent effect of KLF5 has also been described in intestinal cells (52) and has been reported for the KLF family member KLF4 (53), so this may be a common theme both for KLF5 and for other KLF family members.

Interestingly, ILK has been shown to suppress anoikis through Akt and to promote an invasive phenotype by up-regulation of MMP-9 via GSK3β and AP-1 (54, 55). Thus, it will be interesting to examine the effects of Klf5 on the regulation of ILK, Cdc42, MLC_{20}, and other ILK targets such as AKT and GSK3β, in nontransformed *versus* transformed esophageal epithelial cells, as well as to determine the role of Klf5 on migration and proliferation in other epithelial cells.

In sum, we have identified a novel role for the *Krüppel*-like factor Klf5 in regulating keratinocyte migration via the integrin-linked kinase, ILK. Klf5 binds to and transcriptionally activates *ILK*, as shown by quantitative PCR, ChIP assays, EMSA, and promoter reporter constructs. In addition, we have identified Cdc42 and MLC_{20} as specific targets for Klf5 via ILK in keratinocytes. These studies provide a means to understand the processes of cell migration in skin, esophagus, and other stratified squamous epithelia, as well as offering a potential mechanism for migration in other epithelia. Moreover, these data establish a framework for future investigations of altered migration in diseases such as gastroesophageal reflux disease and of tumor invasion and metastases in esophageal squamous cell cancers.

Acknowledgments—We thank Dr. Gregory Hannigan for providing the ILK-expressing adenovirus, Dr. Anil Rustgi for reagents and discussions, and Dr. Cameron Johnstone and Dr. Hiroshi Nakagawa for discussions.

REFERENCES

1. Karam, S. M. (1999) *Front. Biosci.* **4,** D286–D298
2. Crosnier, C., Stamataki, D., and Lewis, J. (2006) *Nat. Rev. Genet.* **7,** 349–359
3. Fuchs, E., and Raghavan, S. (2002) *Nat. Rev. Genet.* **3,** 199–209
4. Blanpain, C., Horsley, V., and Fuchs, E. (2007) *Cell* **128,** 445–458
5. Watt, F. M., and Hogan, B. L. (2000) *Science* **287,** 1427–1430
6. Seery, J. P. (2002) *J. Cell Sci.* **115,** 1783–1789
7. Ohlstein, B., Kai, T., Decotto, E., and Spradling, A. (2004) *Curr. Opin. Cell Biol.* **16,** 693–699
8. Lechler, T., and Fuchs, E. (2005) *Nature* **437,** 275–280
9. Raja, Sivamani, K., Garcia, M. S., and Isseroff, R. R. (2007) *Front. Biosci.* **12,** 2849–2868
10. Dedhar, S., Williams, B., and Hannigan, G. (1999) *Trends Cell Biol.* **9,** 319–323
11. Hannigan, G., Troussard, A. A., and Dedhar, S. (2005) *Nat. Rev. Cancer* **5,** 51–63
12. Wu, C., and Dedhar, S. (2001) *J. Cell Biol.* **155,** 505–510
13. Hannigan, G. E., Leung-Hagesteijn, C., Fitz-Gibbon, L., Coppolino, M. G., Radeva, G., Filmus, J., Bell, J. C., and Dedhar, S. (1996) *Nature* **379,** 91–96
14. Legate, K. R., Montanez, E., Kudlacek, O., and Fassler, R. (2006) *Nat. Rev. Mol. Cell Biol.* **7,** 20–31
15. Schmitz, A. A., Govek, E. E., Bottner, B., and Van Aelst, L. (2000) *Exp. Cell Res.* **261,** 1–12
16. Sellers, J. R. (2000) *Biochim. Biophys. Acta* **1496,** 3–22
17. Watanabe, T., Hosoya, H., and Yonemura, S. (2007) *Mol. Biol. Cell* **18,** 605–616
18. Grashoff, C., Aszodi, A., Sakai, T., Hunziker, E. B., and Fassler, R. (2003) *EMBO Rep.* **4,** 432–438
19. Xie, W., Li, F., Kudlow, J. E., and Wu, C. (1998) *Am. J. Pathol.* **153,** 367–372
20. Lorenz, K., Grashoff, C., Torka, R., Sakai, T., Langbein, L., Bloch, W., Aumailley, M., and Fassler, R. (2007) *J. Cell Biol.* **177,** 501–513
21. Goldstein, B. G., Chao, H. H., Yang, Y., Yermolina, Y. A., Tobias, J. W., and Katz, J. P. (2007) *Am. J. Physiol.* **292,** G1784–G1792
22. Carlson, C. M., Endrizzi, B. T., Wu, J., Ding, X., Weinreich, M. A., Walsh, E. R., Wani, M. A., Lingrel, J. B., Hogquist, K. A., and Jameson, S. C. (2006) *Nature* **442,** 299–302
23. Bieker, J. J. (2001) *J. Biol. Chem.* **276,** 34355–34358
24. Conkright, M. D., Wani, M. A., Anderson, K. P., and Lingrel, J B. (1999) *Nucleic Acids Res.* **27,** 1263–1270
25. Ohnishi, S., Laub, F., Matsumoto, N., Asaka, M., Ramirez, F., Yoshida, T., and Terada, M. (2000) *Dev. Dyn.* **217,** 421–429
26. Nagai, R., Shindo, T., Manabe, I., Suzuki, T., and Kurabayashi, M. (2003) *Adv. Exp. Med. Biol.* **538,** 57–66
27. Shindo, T., Manabe, I., Fukushima, Y., Tobe, K., Aizawa, K., Miyamoto, S., Kawai-Kowase, K., Moriyama, N., Imai, Y., Kawakami, H., Nishimatsu, H., Ishikawa, T., Suzuki, T., Morita, H., Maemura, K., Sata, M., Hirata, Y., Komukai, M., Kagechika, H., Kadowaki, T., Kurabayashi, M., and Nagai, R. (2002) *Nat. Med.* **8,** 856–863
28. Oishi, Y., Manabe, I., Tobe, K., Tsushima, K., Shindo, T., Fujiu, K., Nishimura, G., Maemura, K., Yamauchi, T., Kubota, N., Suzuki, R., Kitamura, T., Akira, S., Kadowaki, T., and Nagai, R. (2005) *Cell Metab.* **1,** 27–39
29. Yang, Y., Goldstein, B. G., Nakagawa, H., and Katz, J. P. (2007) *FASEB J.* **21,** 543–550
30. Cullen, B. R. (2006) *Nat. Methods* **3,** 677–681
31. Yang, Y., Goldstein, B. G., Chao, H. H., and Katz, J. P. (2005) *Cancer Biol. Ther.* **4,** 1216–1221
32. Leung-Hagesteijn, C., Hu, M. C., Mahendra, A. S., Hartwig, S., Klamut, H. J., Rosenblum, N. D., and Hannigan, G. E. (2005) *Mol. Cell. Biol.* **25,** 3648–3657
33. Dise, R. S., Frey, M. R., Whitehead, R. H., and Polk, D. B. (2008) *Am. J. Physiol.* **294,** G276–G285
34. Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T., and Horwitz, A. R. (2003) *Science* **302,** 1704–1709
35. Hynes, R. O. (2002) *Cell* **110,** 673–687
36. McConnell, B. B., Ghaleb, A. M., Nandan, M. O., and Yang, V. W. (2007) *BioEssays* **29,** 549–557
37. Berrier, A. L., and Yamada, K. M. (2007) *J. Cell. Physiol.* **213,** 565–573
38. Seery, J. P., and Watt, F. M. (2000) *Curr. Biol.* **10,** 1447–1450
39. Boudeau, J., Miranda-Saavedra, D., Barton, G. J., and Alessi, D. R. (2006) *Trends Cell Biol.* **16,** 443–452
40. Delcommenne, M., Tan, C., Gray, V., Rue, L., Woodgett, J., and Dedhar, S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 11211–11216
41. Deng, J. T., Van Lierop, J. E., Sutherland, C., and Walsh, M. P. (2001) *J. Biol. Chem.* **276,** 16365–16373
42. Raftopoulou, M., and Hall, A. (2004) *Dev. Biol.* **265,** 23–32
43. Filipenko, N. R., Attwell, S., Roskelley, C., and Dedhar, S. (2005) *Oncogene* **24,** 5837–5849
44. Wilkinson, S., Paterson, H. F., and Marshall, C. J. (2005) *Nat. Cell Biol.* **7,** 255–261
45. Watt, F. M., Kubler, M. D., Hotchin, N. A., Nicholson, L. J., and Adams, J. C. (1993) *J. Cell Sci.* **106,** 175–182
46. Kirfel, G., and Herzog, V. (2004) *Protoplasma* **223,** 67–78
47. Locke, G. R., III, Talley, N. J., Fett, S. L., Zinsmeister, A. R., and Melton, L. J., III (1997) *Gastroenterology* **112,** 1448–1456
48. Parkin, D. M., Bray, F., Ferlay, J., and Pisani, P. (2005) *CA-Cancer J. Clin.* **55,** 74–108
49. Zaidel-Bar, R., Ballestrem, C., Kam, Z., and Geiger, B. (2003) *J. Cell Sci.* **116,** 4605–4613
50. Tu, Y., Li, F., and Wu, C. (1998) *Mol. Biol. Cell* **9,** 3367–3382
51. Khyrul, W. A., LaLonde, D. P., Brown, M. C., Levinson, H., and Turner, C. E. (2004) *J. Biol. Chem.* **279,** 54131–54139
52. Bateman, N. W., Tan, D., Pestell, R. G., Black, J. D., and Black, A. R. (2004) *J. Biol. Chem.* **279,** 12093–12101
53. Rowland, B. D., Bernards, R., and Peeper, D. S. (2005) *Nat. Cell Biol.* **7,** 1074–1082
54. Attwell, S., Roskelley, C., and Dedhar, S. (2000) *Oncogene* **19,** 3811–3815
55. Troussard, A. A., Costello, P., Yoganathan, T. N., Kumagai, S., Roskelley, C. D., and Dedhar, S. (2000) *Oncogene* **19,** 5444–5452
56. Schug, J. (2008) *Curr. Protoc. Bioinform.* **21,** 2.6.1–2.6.15