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Cdc42 Regulates Apical Junction Formation in Human Bronchial Epithelial Cells through PAK4 and Par6B

Sean W. Wallace, Joanne Durgan, Dan Jin, and Alan Hall

Cell Biology Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10065

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Cdc42 has been implicated in numerous biochemical pathways during epithelial morphogenesis, including the control of spindle orientation during mitosis, the establishment of apical-basal polarity, the formation of apical cell–cell junctions, and polarized secretion. To investigate the signaling pathways through which Cdc42 mediates these diverse effects, we have screened an siRNA library corresponding to the 36 known Cdc42 target proteins, in a human bronchial epithelial cell line. Two targets, PAK4 and Par6B, were identified as necessary for the formation of apical junctions. PAK4 is recruited to nascent cell–cell contacts in a Cdc42-dependent manner, where it is required for the maturation of primordial junctions into apical junctions. PAK4 kinase activity is essential for junction maturation, but overexpression of an activated PAK4 mutant disrupts this process. Par6B, together with its binding partner aPKC, is necessary both for junction maturation and for the retention of PAK4 at sites of cell–cell contact. This study demonstrates that controlled regulation of PAK4 is required for apical junction formation in lung epithelial cells and highlights potential cross-talk between two Cdc42 targets, PAK4 and Par6B.

INTRODUCTION

Tight junctions and adherens junctions are found at the apical margin of the lateral membrane in epithelial cells (Farquhar and Palade, 1963). Their formation is initiated through transmembrane proteins, whose extracellular domains interact with neighboring cells, and whose intracellular domains associate with numerous junctional proteins and filamentous actin. Adherens junctions are principally involved in cell–cell adhesion and are composed of E-cadherin, a transmembrane, homophilic adhesion molecule, and the associated catenin family of cytoplasmic adaptor proteins (Pokutta and Weis, 2007). Tight junctions provide a barrier function to control permeability through the paracellular space by the formation of selective pores. They are composed of the transmembrane proteins occludin and claudin and associated cytoplasmic adaptor proteins of the ZO family (Aijaz *et al.,* 2006). Tight junctions are generally thought to contribute to the establishment and maintenance of cell polarity through the separation of apical and basolateral plasma membrane domains (Shin *et al.,* 2006).

Epithelial junctions are dynamic structures, whose assembly, maintenance, and disassembly are regulated during development. Numerous pathways have been reported to influence the behavior of these two junctional complexes, including the expression and trafficking of junctional proteins to the plasma membrane, organization of the actin cytoskeleton, and posttranslational modifications of junctional proteins (Fujita and Braga, 2005; Gonzalez-Mariscal *et al.,* 2008; Miyoshi and Takai, 2008; Wirtz-Peitz and Zallen, 2009). Many signaling pathways have been implicated in regulating epithelial junctions including Rho, Rac, and Cdc42, three members of Rho family of small GTPases (Braga *et al.,* 1997; Gonzalez-Mariscal *et al.,* 2008; Samarin and Nusrat, 2009).

The role of Cdc42 in epithelial cells has been of particular interest as one of its targets is the Par6–aPKC complex, a major regulator of cell polarity in a wide variety of contexts (Joberty *et al.,* 2000; Lin *et al.,* 2000; Iden and Collard, 2008). Cdc42 regulates asymmetric cell division in the *Caenorhabditis elegans* zygote by localizing Par6 to the anterior cortex (Gotta *et al.,* 2001) and regulates apical-basal polarization of *Drosophila* epithelial cells by localizing Par6 to the apical membrane after cellularization of the embryo (Hutterer *et al.,* 2004). In Caco-2 cells, Cdc42 and Par6/aPKC regulate the orientation of the mitotic spindle to promote symmetric cell divisions in epithelial monolayers (Jaffe *et al.,* 2008; J. Durgan, A. Hall, and N. Kaji, unpublished data). Evidence that Cdc42 regulates junction formation in vertebrate epithelial cells has come from both in vivo and in vitro studies. Inhibiting Cdc42 prevents adherens junction assembly in MDCK and Caco-2 cells, though not in cultured keratinocytes (Erasmus *et al.,* 2009; Fukuhara *et al.,* 2003; Mertens *et al.,* 2005; Otani *et al.,* 2006). Other reports, however, have concluded that Cdc42 is not required for junction formation in Madin-Darby canine kidney (MDCK) cells (Takaishi *et al.,* 1997; Gao *et al.,* 2002). Murine embryonic stem cells lacking Cdc42 or atypical protein kinase C (aPKC) activity exhibit defects in polarity and cell–cell junctions during pre-implantation, and in vivo deletion of Cdc42 in mouse keratinocytes inhibits maturation of their cell–cell junctions (Wu *et al.,* 2007; Du *et al.,* 2009). The Par6–aPKC complex localizes to vertebrate tight junctions, and studies using RNAi knockdown and expression of dominant-negative mutants in cul-

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Address correspondence to: Alan Hall (halla@mskcc.org).

tured cells have shown this complex to be required for tight junction formation (Suzuki *et al.*, 2001, 2002, 2004; Yamanaka *et al.*, 2001).

In addition to Par6, Cdc42 interacts with more than 30 target (effector) proteins. IQGAP1, a target for Rac and Cdc42, localizes at adherens junctions in MDCK cells, and RNA interference (RNAi)-mediated knockdown of IQGAP1 impairs adherens junction formation (Noritake *et al.*, 2004). IQGAP1 binds to actin filaments and might regulate adherens junction formation by stabilizing actin at sites of cell–cell adhesion. N-WASP is a Cdc42 target that stimulates actin polymerization by activating the Arp2/3 complex (Adams *et al.*, 1998; Millard *et al.*, 2004). RNAi-mediated depletion of N-WASP in Caco-2 cells resulted in a delay in E-cadherin recruitment during junction formation (Otani *et al.*, 2006), whereas pharmacological inhibition of N-WASP in T84 cells prevented apical junction formation (Ivanov *et al.*, 2005). However the contribution of Cdc42 targets in the context of a specific epithelium has not been systematically studied.

We report here the analysis of Cdc42 function in 16HBE, an immortalized, but nontransformed cell line derived from ciliated human bronchial epithelial cells that line the airway of the lung (Cozens *et al.*, 1994). The integrity of the epithelium in the lung is particularly critical, because it is exposed to the external environment over a very large surface area. Lung disease is one of the greatest causes of death worldwide, and the loss of epithelial integrity is a defining feature of chronic obstructive pulmonary disease (COPD) and cancer (World Health Organization, 2003). Understanding the signaling pathways that regulate the architecture and integrity of the lung epithelium is therefore of great interest. In this study we report the systematic analysis of Cdc42 target proteins regulating junction formation in bronchial epithelial cells and identify PAK4 and Par6B.

MATERIALS AND METHODS

Reagents and Antibodies

Primary antibodies used: occludin (rabbit polyclonal, Invitrogen), ZO-1 (clone 1A12, Invitrogen, Carlsbad, CA), ZO-1 (rabbit polyclonal, Invitrogen), E-cadherin (clone ECCD-2, Invitrogen), E-cadherin (clone 34, BD Transduction, Lexington, KY), Cdc42 (clone 44, BD Transduction), α-tubulin (clone YL1/2, AbD Serotec, Raleigh, NC), β-actin (clone AC-74, Sigma-Aldrich, St. Louis, MO), PAK4 (rabbit polyclonal, Cell Signaling, Beverly, MA), Par6B (rabbit polyclonal H-64, Santa Cruz Biotechnology, Santa Cruz, CA), aPKC (rabbit polyclonal C-20, Santa Cruz Biotechnology), hemagglutinin (HA; clone 3F10, Roche), green fluorescent protein (GFP; rabbit polyclonal, Invitrogen), myc (clone 9E10, Cancer Research UK London, UK). Alexa488- and 568-conjugated secondary antibodies and Alexa488-phalloidin were from Invitrogen. AMCA-, FITC- and Cy3-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Dako (Carpinteria, CA). Gö6983 and BIM1 inhibitors (Calbiochem, La Jolla, CA) were used at concentrations of 6 and 1 μM, respectively.

Cell Culture and Transfection

16HBE14o− cells (gift of Dr. Dieter Gruenert, California Pacific Medical Center, San Francisco) were cultured in MEM + GlutaMAX (Invitrogen, 41090) supplemented with 10% FBS (BenchMark, Gemini Bio-Products, West Sacromento, CA) and penicillin (100 U/ml)-streptomycin (100 μg/ml; Invitrogen) at 37°C in 5% CO_2. For calcium-switch experiments cells were washed in PBS without calcium and incubated in low-calcium medium for 4 h, and then normal growth medium containing calcium was added. Low-calcium medium: DMEM without calcium chloride (Invitrogen, 21068) and 10% FBS pretreated with Chelex 100 resin (Bio-Rad, Hercules, CA). For transfection, cells were seeded at 1.5×10^4 cells/cm^2 (10–20% confluency) and allowed to adhere overnight. Small interfering RNA (siRNA) was transfected (final concentration, 50 nM) in medium without antibiotics, using 100 pmol siRNA and 5 μl Lipofectamine LTX (Invitrogen) per 1.2×10^5 cells. For DNA transfection, 5 μl Lipofectamine LTX and 200 ng plasmid DNA were used per 1.2×10^5 cells. For retroviral infection, cells were seeded at 1.5×10^4 cells/cm^2, and allowed to adhere overnight, and then incubated overnight in growth medium containing retroviral particles, produced in HEK293T cells, supplemented with 8 μg/ml polybrene (hexadimethrine bromide). Two days after infection, stable pools were selected using 1.5 μg/ml puromycin (Invitrogen).

HEK293T cells (ATCC, Manassas, VA) were cultured in DME HG + sodium pyruvate supplemented with 10% FBS and penicillin (100 U/ml)-streptomycin (100 μg/ml; Invitrogen) at 37°C in 5% CO_2. For transfection with plasmid DNA, cells were seeded at 3×10^4 cells/cm^2 and allowed to adhere overnight. 3×10^5 cells were transfected with 1 μg plasmid DNA using 5 μl Lipofectamine 2000 (Invitrogen). For retroviral production, cells were triply transfected with VSV-G, GagPol, and pBABE vector of interest, and 6 h after transfection the medium was changed to 16HBE growth medium for viral collection.

siRNA Reagents

siRNAs were from Dharmacon/Thermo Fisher Scientific (Lafayette, CO). Unless stated otherwise, products are siGENOME SMARTpool or siGENOME individual duplexes. Cdc42 SMARTpool M-005057-01; Cdc42 duplex1 D-005057-01; Cdc42 duplex2 D-005057-02, Cdc42 duplex3 D-005057–03; Cdc42 duplex4 D-005057-04; PAK4 duplex1 D-003615-07; PAK4 duplex2 D-003615–06; Par6B duplex1 D-010681-01; Par6B duplex2 D-010681-02; Par6B duplex3 D-010681–03; Par6B duplex4 D-010681-04; siControl, custom sequence GGAAAUUAUACAAGACC AA; aPKC(ι/ζ) duplex1, custom sequence GGAAAUUAUACAAGACCAA; aPKC(ι/ζ) duplex2, custom sequence GUGUUUGAGCAGGCAUCCA. Additional SMARTpool reagents used for screening are listed in Supplemental Table 1.

DNA Constructs

Human PAK4, PAK4(K350M), and PAK4(S450N,S474E) were a kind gift of Dr. Audrey Minden (Rutgers, Piscataway, NJ). PAK4*CRIB (I11A,S12A,P14A) was generated by single-step PCR mutagenesis using the following primers: forward: GAAGAAGCGGGTGGAGGCCGCCGCGGCGTCCAACTTCGAG; reverse: GAAGTTGGACGCCGCGGCGGCCTCCACCCGCTTCTTCCTC. Three silent mutations were also introduced in to the PAK4 ORF to make it resistant to PAK4 siRNA duplex2, by single-step PCR mutagenesis using the following primers: forward: CCAGCACGAAAACGTCGTGGAGATGTACAACAGCTACCTGGTG; reverse: GCTGTTGTACATCTCCACGACGTTTTCGTGCTGGTAGTCCCTC. Primers were from Sigma-Genosys. Inserts were subcloned in to pBABE-HA or pRK5myc vectors, using PCR amplification to introduce BamHI (5′) and EcoRI (3′) restriction sites using the following primers: forward: GCGCGGGATCCATGTTTGGGAAGAGGAAGAAG; reverse: GCGCGGAATTCTCATCTGGTGCGGTTCTGGCG.

Immunoprecipitation and Western Blotting

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

Microscopy

16HBE cells grown on glass coverslips were fixed in 3.7% (vol/vol) formaldehyde for 15 min and permeabilized in 0.5% (vol/vol) Triton X-100 for 5 min. For staining with anti-PAK4, cells were fixed in ice-cold methanol at −20°C for 5 min. Primary and secondary antibody incubations were carried out for 1 h at room temperature (RT). Coverslips were mounted with fluorescent mounting medium (Dako) and visualized using a Zeiss AxioImager.A1 fluorescence microscope with 40× NA 0.75 and 63× NA 1.4 objectives (Thornwood, NY), using a Hamamatsu ORCA-ER 1394 C4742-80 digital camera (Bridgewater, NJ) and AxioVision software (Zeiss).

Tight Junction Quantification

For each sample 12 random nonoverlapping images were taken at 40× magnification (~400 cells), and tight junction formation was quantified using the Metamorph image analysis software manual count function (Universal Imaging, West Chester, PA). Cells with a continuous ring of occludin or ZO-1 at cell–cell contacts were defined as having intact tight junctions. Cells with punctate or discontinuous occludin or ZO-1 at cell–cell contacts were defined as not having tight junctions. Graphical presentation of results and statistical analysis were carried out using Prism (GraphPad Software, San Diego, CA). Error bars are SEM, and significance values have been calculated using a two-tailed unpaired *t* test at 95% confidence interval.

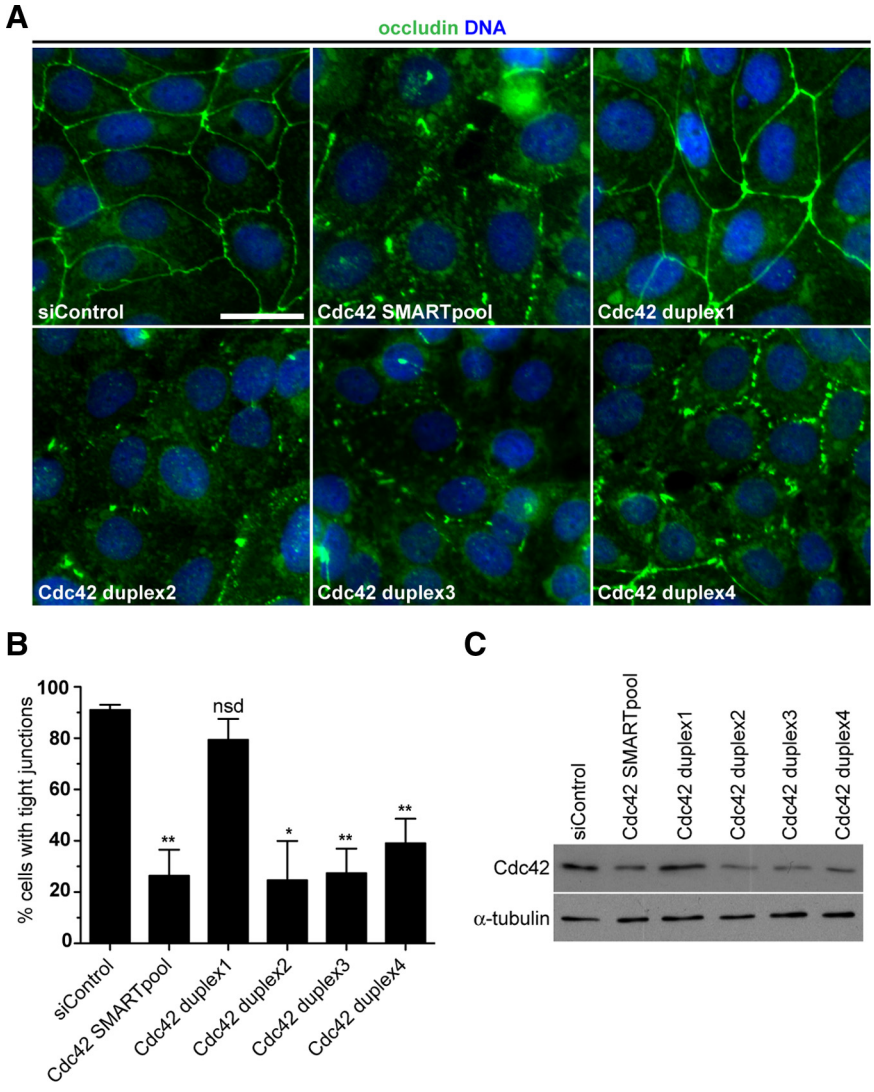

Figure 1. Cdc42 regulates tight junction formation in 16HBE cells. 16HBE cells were seeded at low density on glass coverslips and transfected with the indicated siRNA. (A) Three days after transfection cells were fixed and analyzed by immunofluorescence microscopy with anti-occludin (green) and Hoechst (blue). Scale bar, 20 μm in all images. (B) Quantification of tight junction formation from three independent experiments (see *Materials and Methods* for details); error bars, SEM; nsd, no significant difference, $^{**}p < 0.01$, $^{*}p < 0.02$. (C) Three days after transfection cells were lysed and analyzed by Western blot with the indicated antibodies.

RESULTS

Cdc42 Is Required for Tight Junction Formation in Human Bronchial Epithelial Cells

To investigate whether Cdc42 is required for junction formation in 16HBE cells, we used an RNAi approach to down-regulate Cdc42 expression. 16HBE cells were seeded at low density and transfected with a SMARTpool mixture of four distinct siRNAs targeting Cdc42 or with a control siRNA (siControl). Three days after transfection, cells were close to confluence, and the majority of control cells had formed tight junctions, defined as a continuous ring of occludin and ZO-1 at cell–cell contacts (Figure 1 and Supplemental Figure 1A). In contrast, cells depleted of Cdc42 showed punctate occludin and ZO-1 at cell–cell contacts (Figure 1 and Supplemental Figure 1A). This phenotype did not result from loss of expression of junctional proteins (Supplemental Figure 1C). To determine whether this phenotype is a specific consequence of Cdc42 depletion, the effect of the four individual siRNA duplexes comprising the Cdc42 SMARTpool was determined. Three of the four siRNA duplexes down-regulated Cdc42 expression and resulted in a defect in tight junction formation, whereas duplex 1 was inefficient at down-regulating Cdc42 expression and had no significant effect on tight junctions (Figure 1). These results show that Cdc42 is required for tight junction formation in 16HBE cells.

Two Cdc42 Target Proteins, PAK4 and Par6B, Are Required for Tight Junction Formation in 16HBE Cells

Thirty-six potential target proteins have been reported that interact with Cdc42 in a GTP-dependent manner. To identify target proteins acting downstream of Cdc42 during tight junction formation in 16HBE cells, a library of SMARTpool siRNAs corresponding to each of these targets (Supplemental Table 1) was screened. Two proteins, PAK4 and Par6B, were identified as required for tight junction formation in 16HBE cells (Figure 2 and Supplemental Figure 1B). Two of the four individual PAK4 siRNA duplexes down-regulated protein expression and perturbed tight junction formation (Figure 2, A–C), whereas all four individual Par6B siRNA duplexes down-regulated protein expression and perturbed tight junction formation (Figure 2, A, D and E), indicating that the effects are specific.

Like other Par6 isoforms, Par6B is thought to function through an associated aPKC. To determine whether aPKC is required for tight junction formation in 16HBE cells, two siRNA duplexes that simultaneously target both isoforms of

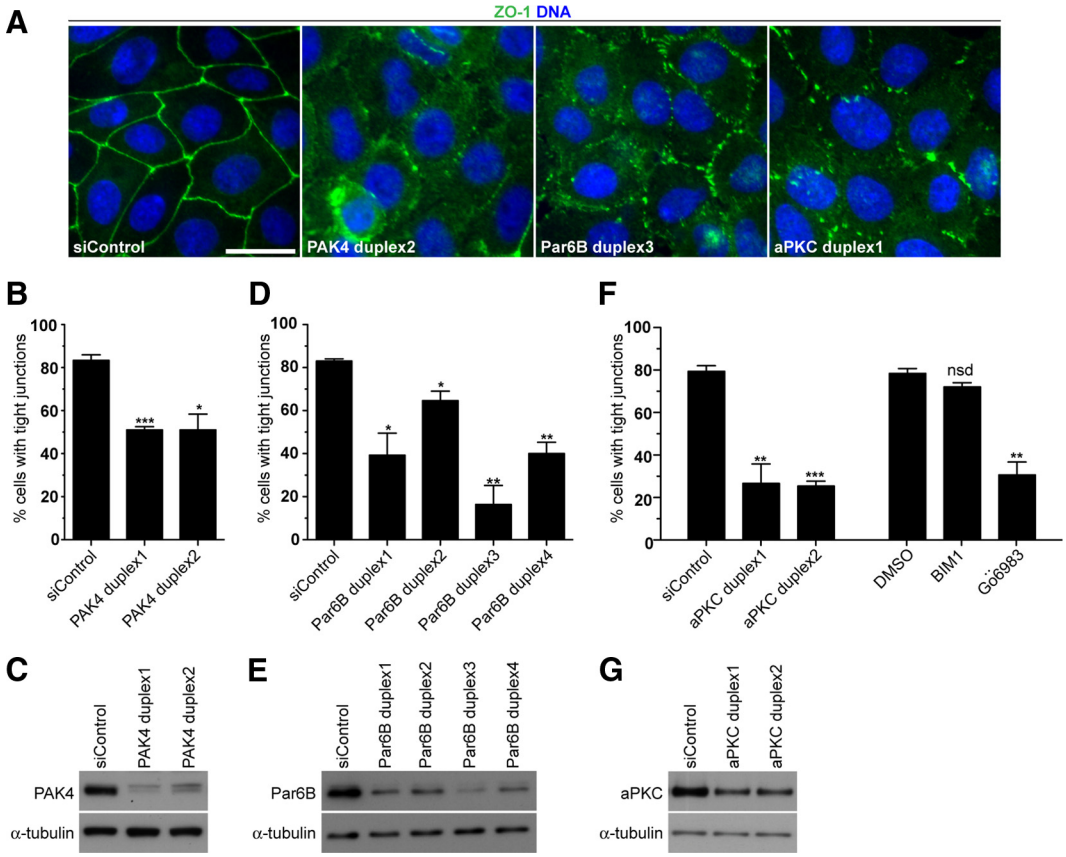

Figure 2. PAK4, Par6B, and aPKC regulate tight junction formation in 16HBE cells. (A) 16HBE cells were transfected with the indicated siRNAs. Three days after transfection, cells were fixed and analyzed by immunofluorescence microscopy with anti-ZO-1 (green) and Hoechst (blue). Scale bar, 20 μm in all images. (B, D, and F) Quantification of tight junction formation from three independent experiments after transfection of the indicated siRNAs or treatment with the indicated inhibitors (see *Materials and Methods* for details); error bars, SEM; nsd, no significant difference, ***$p < 0.001$, **$p < 0.01$, *$p < 0.02$. Note that in F siRNA treatments are compared with siControl and drug treatments are compared with DMSO. (C, E, and G) Western blot analysis of siRNA transfected cell lysates with the indicated antibodies.

aPKC (aPKCι/ζ) were transfected. Both duplexes efficiently down-regulated aPKC expression and phenocopied the depletion of Par6B, resulting in defective tight junction formation (Figure 2, A, F, and G). To determine whether the kinase activity of aPKC is required, cells were seeded at low density and treated with either Gö6983, an inhibitor of classical, novel and atypical PKCs, or BIM1, an inhibitor of classical and novel PKCs only, for 3 d (Martiny-Baron *et al.*, 1993). Gö6983, but not BIM1, inhibited tight junction formation (Figure 2F), suggesting that the kinase activity of aPKC is required.

PAK4 Function During Apical Junction Formation Is Cdc42- and Kinase-dependent

To determine whether PAK4 acts as a target of Cdc42 function during junction formation, a triple amino acid substitution (I11A, S12A, and P14A) was introduced into the CRIB (Cdc42/Rac interactive binding) motif of PAK4, to generate a mutant protein (PAK4*CRIB) unable to bind Cdc42 (Figure 3A). PAK4*CRIB is no longer able to interact with a constitutively activated version of Cdc42 (Cdc42L61), as determined by coimmunoprecipitation experiments after expression in HEK293T cells (Figure 3B). To determine whether the Cdc42/PAK4 interaction is necessary for junction formation, a rescue experiment was performed using a PAK4*CRIB mutant resistant to siRNA duplex2 (see *Materials and Methods*). 16HBE cells were infected with pBABE retroviral vectors containing no insert (control), or N-terminal HA-tagged versions of PAK4 or PAK4*CRIB and stable pools selected with puromycin. Cells were seeded at low density and transfected with PAK4 siRNA duplex2 or siControl. Western blot analysis showed that exogenous PAK4 and PAK4*CRIB were expressed at similar levels to each other and at much higher levels than endogenous PAK4 (Figure 3, C and D). As expected, PAK4 siRNA duplex2 inhibited tight junction formation in cells infected with control virus, while wild-type PAK4 completely rescued the siRNA-induced phenotype (Figure 3, E and F). However, PAK4*CRIB did not rescue the phenotype (Figure 3, E and F).

To determine whether PAK4 function is kinase-dependent, a kinase-dead mutant PAK4(K350M) was used (Abo *et al.*, 1998). PAK4(K350M), despite being expressed at higher levels than the endogenous protein (Figure 3D), was unable to rescue tight junction formation after depletion of endogenous PAK4 (Figure 3, E and F). We conclude that PAK4 is a direct target of Cdc42 in tight junction formation and that its function is kinase-dependent.

Expression of Constitutively Activated PAK4 Disrupts Apical Junctions

To determine whether hyperactivation of PAK4 can overcome the requirement for Cdc42 in junction assembly, 16HBE cells were transfected with a constitutively activated

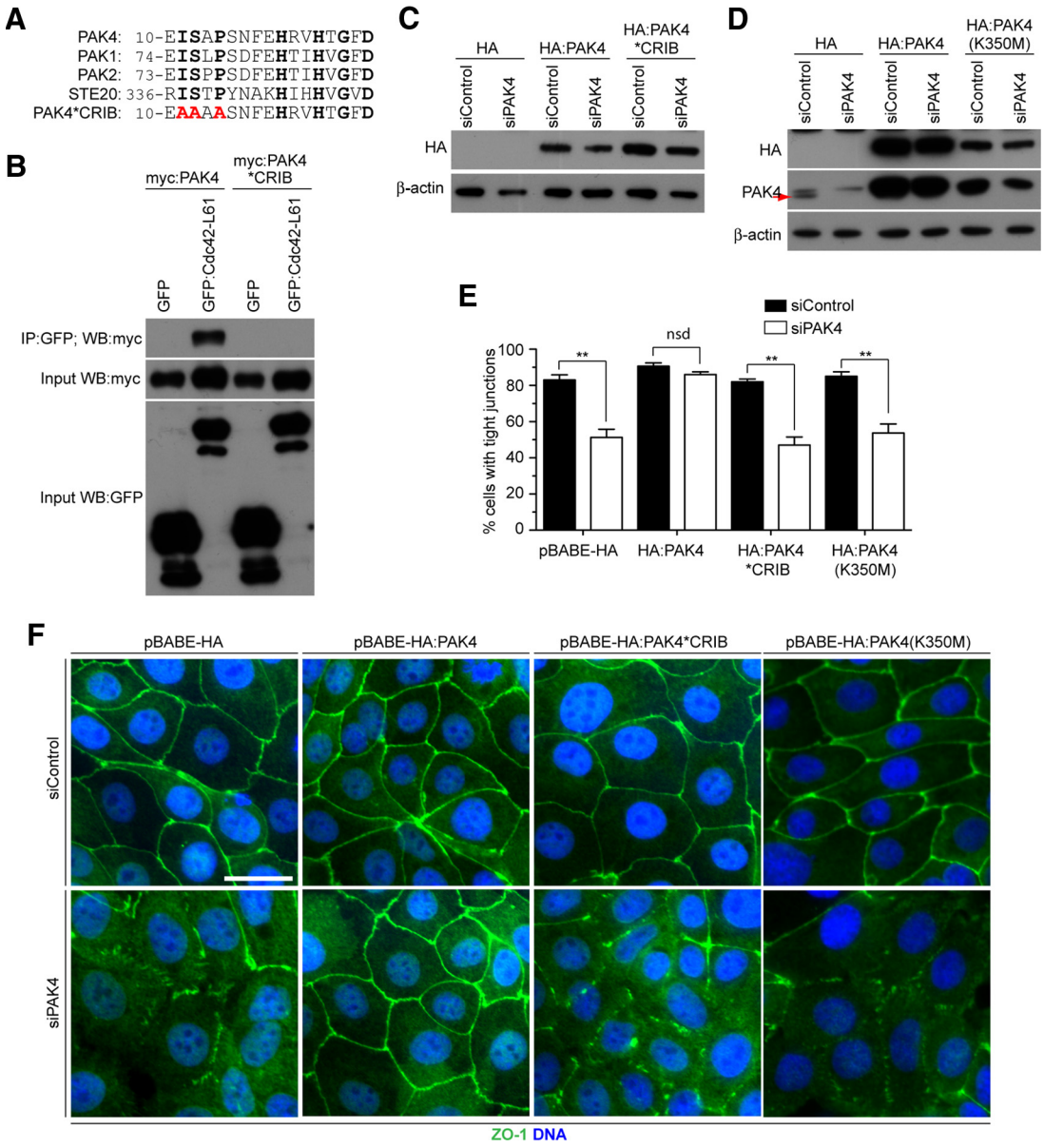

Figure 3. PAK4 function during tight junction formation is Cdc42- and kinase-dependent. (A) Sequence alignment of the CRIB motif of PAK family kinases with conserved residues in bold. Mutations introduced in to PAK4 to generate PAK4*CRIB are indicated in red. (B) myc-PAK4, but not myc-PAK4*CRIB, coimmunoprecipitates with GFP-Cdc42-L61 when expressed in HEK293T cells. (C and D) Western blot analysis, using the indicated antibodies, of lysates from 16HBE cells described in F. (E) Quantification of tight junction formation from F. Three independent experiments were quantified (see *Materials and Methods*); error bars, SEM; nsd, no significant difference; $^{**}p < 0.01$. (F) 16HBE cells stably expressing pBABE-HA empty vector control or RNAi-resistant versions of HA-PAK4, HA-PAK4*CRIB, and HA-PAK4(K350M) were transfected with siControl or PAK siRNA duplex2 and stained three days after transfection with anti-ZO-1 (green) and Hoechst (blue). Scale bar, 20 μm in all images.

mutant, PAK4 (S450N, S474E; Qu *et al.*, 2001). In contrast to wild-type PAK4, which does not affect apical junction formation even when expressed at about 20-fold higher levels than endogenous protein (Figure 3D), activated PAK4 inhibited tight junction formation (Figure 4), suggesting that PAK4 activity must be tightly regulated.

Cdc42, PAK4, and Par6B/aPKC Are Required for the Maturation of Primordial Junctions in to Apical Junctions

In polarized epithelial cells the apical junctional complex consists of tight junctions, adherens junctions, and the associated perijunctional actin ring. To analyze the assembly of junctions in more detail, 16HBE cells were grown to confluence and incubated in calcium-depleted medium for 4 h to induce junction disassembly. The addition of normal growth medium containing calcium stimulates relatively synchronous junction assembly (a calcium switch). Cells cultured in calcium-depleted medium showed weak cytoplasmic staining of E-cadherin and ZO-1 and a diffuse array of cortical actin filaments, indicative of epithelial cells lacking junctions (Figure 5, low calcium). One hour after calcium switch, control cells formed primordial junctions, consisting of E-cadherin and ZO-1 puncta oriented in a radial manner at cell–cell contacts, with some fine actin filaments appearing

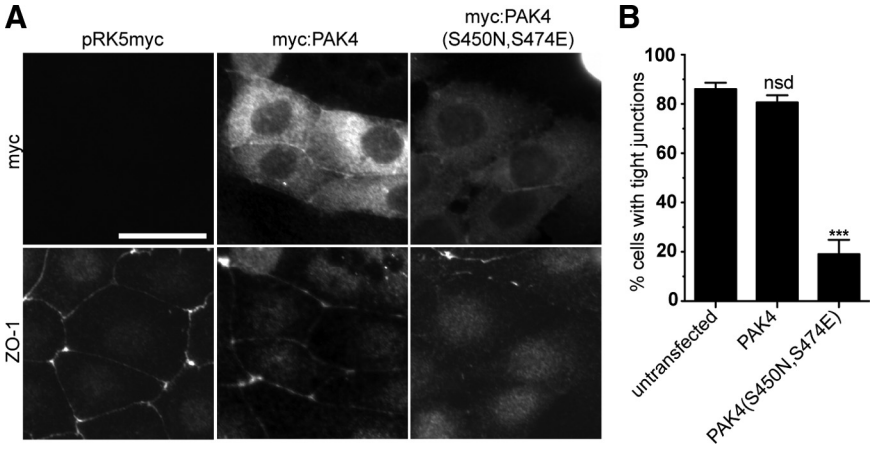

Figure 4. Constitutively activated PAK4 inhibits tight junction formation. 16HBE cells were seeded at low density and transfected with pRK5myc control vector, myc-tagged wild-type PAK4, or myc-tagged PAK4 (S450N, S474E). (A) Two days after transfection, cells were fixed and stained with the indicated antibodies. Scale bar, 20 μm in all images. (B) Quantification of tight junction formation in three independent experiments (see *Materials and Methods*); error bars, SEM; nsd ,no significant difference; ***$p < 0.001$.

to associate with E-cadherin puncta (Figure 5, 1 h siControl). Two hours after calcium switch, E-cadherin and ZO-1 were more continuous along cell–cell contacts, and cortical actin had rearranged to form loosely associated filaments running along the length of the cell–cell contact (Figure 5, 2 h siControl). By 6 h, apical junction formation was complete, with a continuous line of ZO-1 and E-cadherin at the apical margin of cell–cell contact regions, corresponding to the tight junction and adherens junction, respectively, and prominent, perijunctional actin filaments (Figure 5, 6 h siControl). Apical junction formation in 16HBE cells thus follows a similar series of steps as described in other cell types, in which cell–cell contact initiates the formation of primordial, or spot-like junctions, which then mature in to apical tight junctions and adherens junctions (Suzuki *et al.*, 2002; Zhang *et al.*, 2005).

16HBE cells depleted of Cdc42 or PAK4 were indistinguishable from control cells 1 h after calcium switch, both showing accumulation of E-cadherin and ZO-1 in radial puncta at nascent cell–cell contacts (Figure 5, 1 h). However, at 2 and 6 h after calcium switch, cells depleted of Cdc42 or PAK4 remained indistinguishable from cells at the 1-h time point (Figure 5). Similar results were seen in 16HBE cells depleted of Par6B or aPKC (Supplemental Figure 2). We conclude that Cdc42 and its target proteins PAK4 and Par6B/aPKC are required specifically for the maturation of primordial junctions into apical junctions found in polarized epithelial monolayers.

Cdc42 Regulates PAK4 During Junction Formation by Controlling Its Localization

It has been proposed that Cdc42 regulates PAK4 function primarily through affecting its subcellular localization (Abo *et al.*, 1998). Visualization of PAK4 in 16HBE cells by immunofluorescence showed apical junction and nuclear staining (Figure 6A). The apical junction staining is specific, because the signal is lost after siRNA-mediated PAK4 depletion (Figure 6B), and exogenously expressed HA-tagged PAK4 localizes at apical junctions (Figure 6C). The nuclear PAK4 staining is nonspecific, because the signal did not diminish after PAK4 depletion (Figure 6B), and exogenously expressed HA-tagged PAK4 did not localize to the nucleus (Figure 6C).

The localization of PAK4 during calcium-induced junction formation was analyzed. In calcium-depleted medium, PAK4 did not show membrane localization (Figure 6A, low calcium). On addition of calcium, PAK4 accumulated at nascent cell–cell contacts (Figure 6B, 1 h), consistent with a role in promoting the subsequent maturation of primordial junctions. PAK4 remained localized at apical junctions after junction maturation (Figure 6B, 6 h). To determine whether Cdc42 regulates PAK4 localization during junction formation, 16HBE cells were transfected with Cdc42 siRNA. Under these conditions, PAK4 did not localize to primordial junctions even after cells were left for up to 6 h (Figure 6B). To confirm that PAK4 localization is Cdc42-dependent, the localization of PAK4*CRIB was analyzed. Although HA-tagged wild-type PAK4 localized at primordial junctions (Figure 6C, 1 h) and mature apical junctions (Figure 6C, 6 h), HA-tagged PAK4*CRIB showed only cytoplasmic localization (Figure 6C). We conclude that the recruitment of PAK4 to cell–cell contacts during junction formation is Cdc42-dependent.

Par6B Is Not Required for PAK4 Recruitment, But Is Required for PAK4 Stabilization at Primordial Junctions

To determine whether PAK4 and Par6B localize independently to junctions, 16HBE cells were depleted of Par6B using siRNA. One hour after calcium switch, Par6B-depleted cells formed primordial junctions, and PAK4 was recruited similar to control cells (Figure 7, 1 h). As shown earlier, 6 h after calcium switch, Par6B-depleted cells still show primordial junctions; however, PAK4 no longer localized at these primordial junctions (Figure 7, 6 h). Similar results were seen with aPKC-depleted cells (data not shown). We conclude that the recruitment of PAK4 to primordial junctions is Cdc42-dependent, but that the maintenance of PAK4 at later times during junction maturation is Par6B/aPKC-dependent.

DISCUSSION

Epithelial apical junctions, including tight junctions and adherens junctions, are regulated by Rho GTPases in a variety of cell types. In this study we sought to investigate the signaling pathways through which Cdc42 regulates apical junction formation in human bronchial epithelial cells. Using an siRNA screen of all 36 known Cdc42 targets, two ser/thr kinases, PAK4 and the Par6B/aPKC complex, were found to be required for apical junction formation. Other Cdc42 targets, such as IQGAP and N-WASP, which have been implicated in junction formation in other cell types, were not found. It is possible that their effects are cell type–dependent, but in addition, because the efficacy of each of

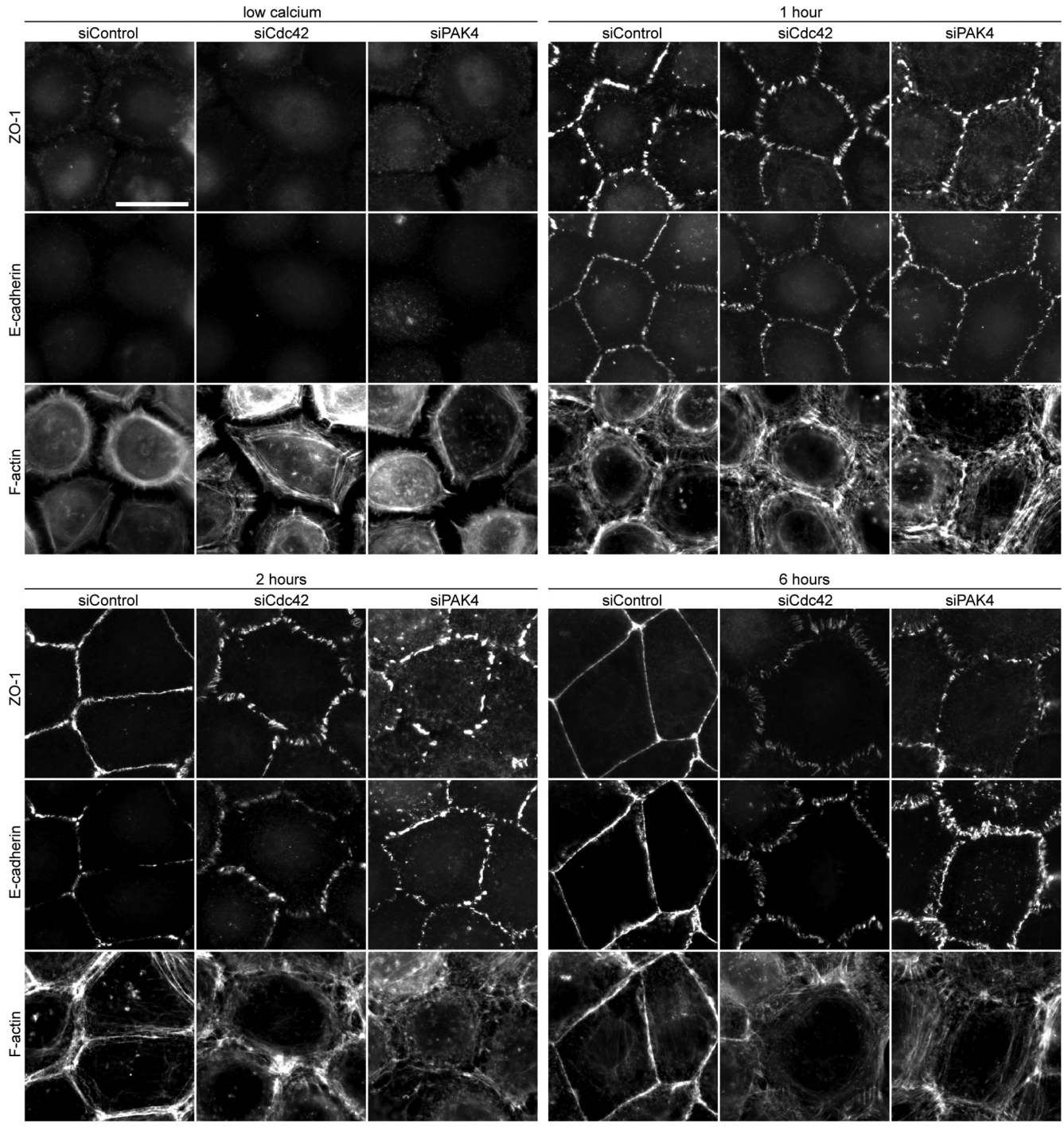

Figure 5. Cdc42 and PAK4 regulate the maturation of primordial junctions into apical junctions. Three days after transfection confluent monolayers of 16HBE cells transfected with siControl, Cdc42 siRNA duplex2, or PAK4 siRNA duplex2 were subjected to calcium switch, fixed at the indicated time points, and stained with anti-ZO-1, anti-E-cadherin, and Alexa488-phalloidin. Scale bar, 20 μm in all images.

the library SMARTpools is not known, it is possible they were not efficiently depleted in the screen.

Par6 is one of the Par (partitioning-defective) polarity genes, which were discovered in a screen to identify genes required for asymmetric cell division in the *C. elegans* zygote and which have since been shown to have conserved roles in establishing cell polarity in higher organisms (Goldstein and Macara, 2007). Par6 is a scaffold protein that is found in a complex with aPKC (Joberty *et al.*, 2000; Lin *et al.*, 2000). The interaction of Cdc42, with a CRIB motif in Par6, regulates the activity of aPKC. In epithelial cells the Par6-aPKC complex localizes to tight junctions and regulates tight junction formation (Suzuki *et al.*, 2001, 2002, 2004; Yamanaka *et al.*, 2001; Wu *et al.*, 2007; Du *et al.*, 2009). In particular, the kinase activity of aPKC has been shown to be required for the maturation of nascent cell–cell contacts into mature apical junctions in a mouse tumor-derived cell line and in vivo in mouse keratinocytes (Suzuki *et al.*, 2002; Du *et al.*, 2009).

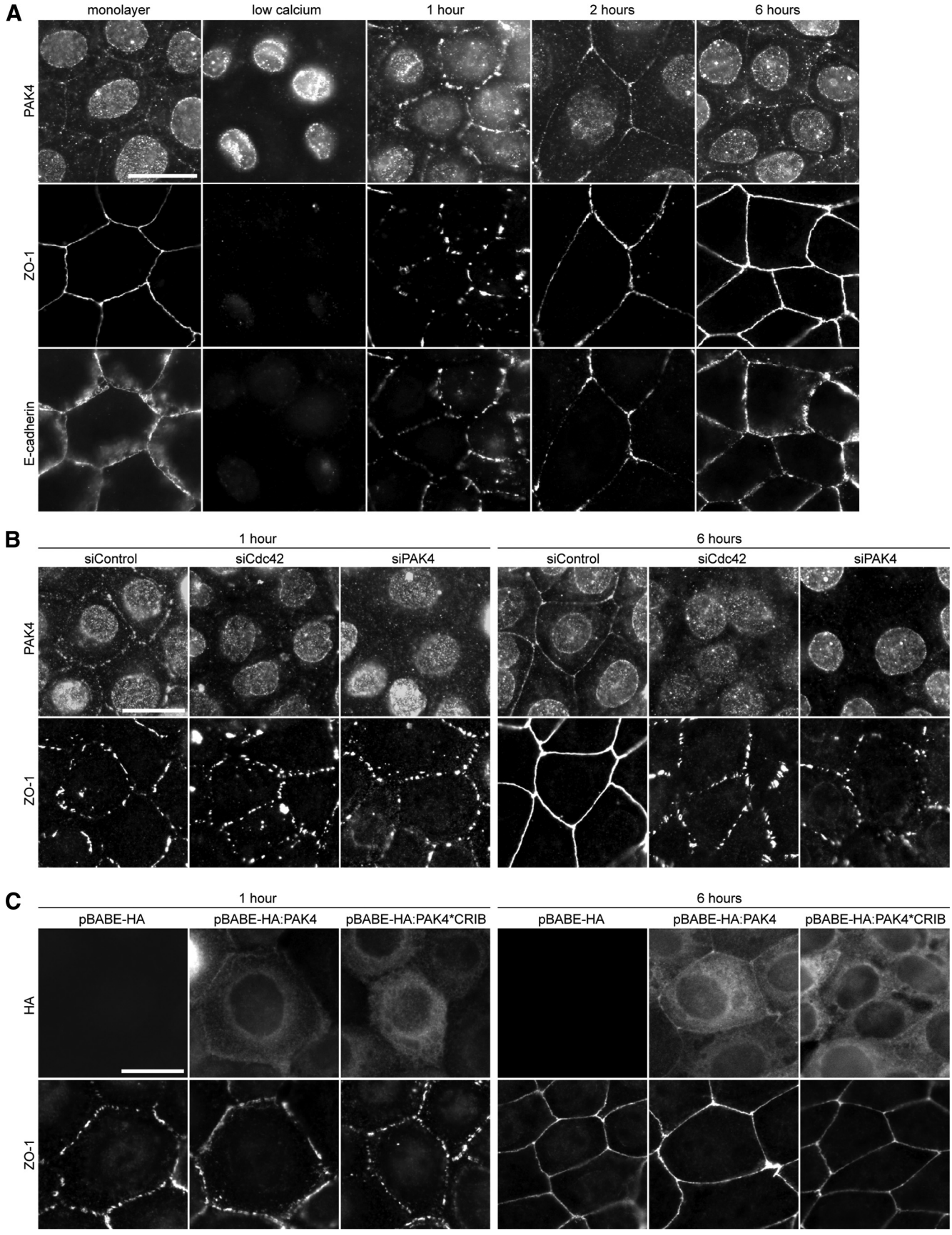

Figure 6. PAK4 recruitment to cell–cell contacts during junction formation is Cdc42-dependent. (A) Confluent 16HBE monolayers were subject to calcium switch, fixed at the indicated times, and stained with anti-PAK4, anti-ZO-1, and anti-E-cadherin. (B) Three days after

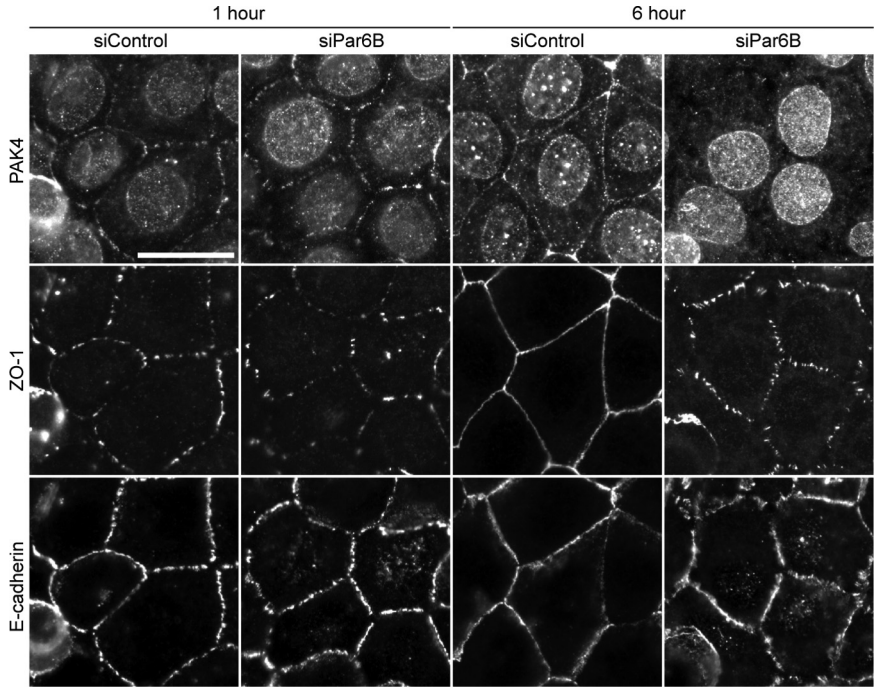

Figure 7. Par6B is required for the maintenance, but not recruitment of PAK4 at junctions. Three days after transfection confluent monolayers of 16HBE cells transfected with siControl or Par6B siRNA duplex3 were subjected to a calcium switch, fixed at the indicated time points, and stained with the indicated antibodies. Scale bar, 20 μm in all images.

Consistent with these previous studies, we find that the Par6B/aPKC complex is also required for apical junction maturation in 16HBE cells.

PAK4 belongs to a family of kinases consisting of six members in mammals. Based on sequence similarity and mode of regulation, they have been subdivided into two subfamilies, type I (PAK1,2,3) and type II (PAK4,5,6; Jaffer and Chernoff, 2002). In contrast to PAK1, which is an autoinhibited kinase that is activated by direct binding to Rac or Cdc42 (Lei *et al.,* 2000), PAK4 lacks the autoinhibitory region and in vitro at least, shows relatively high kinase activity, which is not enhanced by Cdc42. For this reason, it has been proposed that role of Cdc42 is to regulate the subcellular localization of PAK4 (Abo *et al.,* 1998). We find that PAK4 is recruited to nascent cell–cell contacts during junction formation in 16HBE cells in a Cdc42-dependent manner. The initiation of epithelial cell–cell contact activates a number of signaling pathways, including Rho GTPases, and activation Cdc42 has been reported in MCF7 and Caco-2 cells (Kim *et al.,* 2000; Otani *et al.,* 2006). On the basis of our findings, we conclude that the activation of Cdc42 at nascent cell–cell contacts results in recruitment of PAK4, which in a kinase-dependent pathway promotes further maturation of primordial junctions into apical junctions. It has been reported that the activation of Cdc42 after cell–cell contact is transient, lasting around 90 min in MCF7 cells (Kim *et al.,* 2000). This might suggest that after its initial recruitment, the retention of PAK4 is Cdc42-independent. Interestingly, Par6B, which is not required for the initial recruitment of PAK4, is required to maintain PAK4 at maturing junctions.

Figure 6 (cont). transfection confluent monolayers of 16HBE cells transfected with siControl, Cdc42 siRNA duplex2, or PAK4 siRNA duplex2 were subjected to calcium switch, fixed at the indicated time points, and stained with the indicated antibodies. (C) 16HBE cells stably expressing HA-PAK4, HA-PAK4*CRIB, or pBABE-HA empty vector, were subjected to calcium switch, fixed at the indicated time points, and stained with the indicated antibodies. Scale bar, 20 μm in all images.

Mammalian PAK4 has not previously been studied in the context of epithelial junction formation. PAK4 knockout mice die during embryogenesis, with defects in multiple tissues, making it difficult to draw conclusions about function (Qu *et al.,* 2003). PAK4 is overexpressed in many cancer cell lines, including lung cancer (Callow *et al.,* 2002), and previous studies have implicated a role in transformation, migration, and invasion (Li *et al.,* 2010; Wells *et al.,* 2010; Qu *et al.,* 2001; Paliouras *et al.,* 2009). Interestingly, although overexpression of wild-type PAK4 had no effect on junction formation in 16HBE cells, an activated version of PAK4 prevented junction formation. Our results suggest that PAK4 activity must be tightly regulated in epithelial cells to ensure junctional integrity. PAK4 orthologues have been described in *Drosophila* and *Xenopus* and reported to play a role in epithelial morphogenesis. The *Drosophila* ortholog, Mbt, localizes to adherens junctions in differentiating photoreceptor cells in the eye imaginal disk and Mbt mutants show morphological defects in the eye (Schneeberger and Raabe, 2003). The localization and function of Mbt in photoreceptor cells are Cdc42-dependent. During development, the *Xenopus* ortholog, X-PAK5, localizes to adherens junctions in animal cap cells and dorsal marginal zone cells and expression of kinase-dead X-PAK5 interferes with convergence extension movements in these cells, a process that requires dynamic rearrangements of cell–cell junctions (Faure *et al.,* 2005).

The mechanism by which PAK4 and Par6/aPKC promote the maturation of primordial junctions to apical junctions is not known. Primordial E-cadherin puncta contain many of the proteins found in mature adherens junctions, as well the tight junction protein ZO-1 (Adams *et al.,* 1998; Asakura *et al.,* 1999; Vasioukhin *et al.,* 2000). Additional tight junction proteins are later recruited to primordial junctions, which mature into apical junctions, consisting of distinct tight and adherens junctions, a process that is accompanied by elongation of cells along the apical-basal axis and the establishment of cell polarity (Suzuki *et al.,* 2002). During apical-basal polarization, actin filaments reorganize to form the charac-

teristic perijunctional actin ring found associated with apical junctions, a process that requires actomyosin contractility (Ivanov *et al.*, 2005; Zhang *et al.*, 2005). PAK4 could promote junctional maturation by a number of mechanisms, including regulation of membrane trafficking pathways, modulation of the actin cytoskeleton, or direct regulation of junctional protein activities. Several PAK4 substrates have been described, including slingshot phosphatase (SSH) and LIM kinase (LIMK), which regulate actin filament assembly through cofilin (Dan *et al.*, 2001; Soosairajah *et al.*, 2005). However, there are conflicting reports as to whether cofilin contributes to the disassembly or to the assembly of epithelial junctions (Ivanov *et al.*, 2004; Chen and Macara, 2006). Another PAK4 substrate of interest is armadillo, the *Drosophila* ortholog of β-catenin (Menzel *et al.*, 2008). Phosphorylation of armadillo by Mbt disrupts its interaction with DE-cadherin and could, therefore, be a potential target in the disassembly of junctions seen after expressing the constitutively activated PAK4. Future work will require a systematic identification of PAK4 and aPKC substrates during junction maturation.

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