

Vimentin Regulates Scribble Activity by Protecting It from Proteasomal Degradation

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Submitted February 25, 2008; Revised March 17, 2009; Accepted April 13, 2009

Monitoring Editor: M. Bishr Omary

Scribble (Scrib), Discs large, and Lethal giant larvae form a protein complex that regulates different aspects of cell polarization, including apical–basal asymmetry in epithelial cells and anterior–posterior polarity in migrating cells. Here, we show that Scrib interacts with the intermediate filament cytoskeleton in epithelial Madin-Darby canine kidney (MDCK) cells and endothelial human umbilical vein endothelial cells. Scrib binds vimentin via its postsynaptic density 95/disc-large/zona occludens domains and in MDCK cells redistributes from filaments to the plasma membrane during the establishment of cell–cell contacts. RNA interference-mediated silencing of Scrib, vimentin, or both in MDCK cells results in defects in the polarization of the Golgi apparatus during cell migration. Concomitantly, wound healing is delayed due to the loss of directional movement. Furthermore, cell aggregation is dependent on both Scrib and vimentin. The similar phenotypes observed after silencing either Scrib or vimentin support a coordinated role for the two proteins in cell migration and aggregation. Interestingly, silencing of vimentin leads to an increased proteasomal degradation of Scrib. Thus, the upregulation of vimentin expression during epithelial to mesenchymal transitions may stabilize Scrib to promote directed cell migration.

INTRODUCTION

Cell polarization is characterized by the asymmetrical distribution of cellular components such as proteins, lipids, and organelles to particular regions within a cell. The establishment of apical–basal polarity in epithelial cells is among the best-studied models of cell polarization (reviewed in Yeaman *et al.*, 1999). The generation of cellular asymmetry, however, is also observed during cell proliferation, migration, and cell–cell or cell–matrix interactions, among others. Accordingly, cell polarity can be classified into, apical–basal (exemplified by epithelia), anterior–posterior (observed for example during migration; reviewed in Dow and Humbert, 2007), and planar or tissue polarity (found in the cochlea or the fly wing; reviewed in Zallen, 2007). Importantly, deregulation of cell polarity plays a central role in human diseases, in particular cancer (Wodarz and Nathke, 2007).

Three evolutionarily conserved complexes of interacting proteins, the Par/atypical protein kinase C (aPKC) (Par6, Baz, and aPKC; reviewed in Suzuki and Ohno, 2006), the Crumbs (Crb, Sdt, and PATJ; reviewed in Assémat *et al.*, 2007), and the Scribble (Scrib, Discs large [Dlg], and Lethal giant larvae [Lgl]; reviewed in Humbert *et al.*, 2006; Dow and

Humbert, 2007) complex coordinately regulate, in conjunction with reorganization of the cytoskeleton and directed vesicle trafficking, cell polarization. In apical–basal polarity, a current model proposes that the Par and Crb complexes provide apical specification, whereas the Scrib complex confers basal identity. Par/Crb and Scrib complexes then act to repress each other's activity on the apical or basal domain, respectively (Dow and Humbert, 2007).

In *Drosophila* epithelial cells, Scrib and Dlg localize to septate junctions and Lgl to cortical junctions. Loss of these three proteins affects adherens junctions (AJs) and results in a disruption of apical–basal polarity (reviewed in Bilder, 2003, 2004). Several lines of evidence indicate that the Scrib complex also plays a role in regulating apical–basal polarization in vertebrates. Similar to *Drosophila* Lgl, the two mammalian homologues bind Par6/aPKC to suppress aPKC kinase activity and inhibit apical identity (Plant *et al.*, 2003; Yamanaka *et al.*, 2003). Phosphorylation of Lgl by aPKC in turn inhibits its basal activity in the apical domain (Yamanaka *et al.*, 2003).

Although the importance for Scrib and Dlg in the formation of septate junctions and AJ in flies and worms is well established (Woods *et al.*, 1996; Bilder and Perrimon, 2000), their role in apical–basal polarization of mammalian epithelial cells is controversial. In one study, Dlg was required for proper AJ assembly (Laprise *et al.*, 2004), whereas in another, E-cadherin–mediated adhesion was a requisite for recruitment of Scrib to the lateral membrane (Navarro *et al.*, 2005). Furthermore, silencing of Lgl2 (Yamanaka *et al.*, 2006), but not Scrib (Qin *et al.*, 2005; Dow *et al.*, 2007), was recently reported to affect apical–basal polarity of mammalian epithelial cells. These apparently conflicting observations may be partly reconciled by the type of extracellular matrix (e.g., matrigel or collagen I) used in the different experiments,

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E08-02-0199>) on April 22, 2009.

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Abbreviations used: AJ, adherens junction; Dlg, Discs large; IF, intermediate filament; Lgl, Lethal giant larvae; PCR, polymerase chain reaction; PDZ, postsynaptic density 95/disc-large/zona occludens; Scrib, Scribble; siRNA, small interfering RNA; WT, wild-type; ZO, zonula occludens.

shown to also influence the effect of Crb3 on apical–basal polarity (Roh *et al.*, 2003; Lemmers *et al.*, 2004).

In addition to this role in apical–basal polarity, the Scrib complex regulates anterior–posterior cell polarization such as during asymmetric cell division of neuroblasts or the migration of epithelial cell sheets during dorsal closure in *Drosophila* (Bildler *et al.*, 2000) and wound closure in mammals (Dow *et al.*, 2007). Scrib regulates the recruitment of Rho to the leading edge (Osmani *et al.*, 2006; Dow *et al.*, 2007) and, like Dlg (Cau and Hall, 2005; Gomes *et al.*, 2005), is required for repositioning of the microtubule-organizing center (MTOC) and the Golgi apparatus. Although it is still debated whether Scrib restricts or facilitates cell movement, it is clear that it is required for directionality of migration (Qin *et al.*, 2005; Dow *et al.*, 2007).

The roles of the actin and microtubule cytoskeleton in different types of cell polarization are well established. However, the importance of the intermediate filaments (IFs) in these processes is less well characterized. Up-regulation of vimentin and increased cell motility are hallmarks of the conversion from an epithelial into a mesenchymal phenotype (Lee *et al.*, 2006). In wound healing assays, vimentin expression is up-regulated at the wound edge where cells migrate into the wounded area (Gilles *et al.*, 1999), and wound healing is impaired in mice lacking vimentin (Eckes *et al.*, 2000). Furthermore, cell motility is reduced in vimentin-deficient fibroblasts (Eckes *et al.*, 1998) or if vimentin (McInroy and Määttä, 2007) or keratin 8 (Long *et al.*, 2006) are down-regulated. In contrast, vimentin promotes cell motility (Ivaska *et al.*, 2005). These data support a critical role of IFs during cell migration, although the molecular mechanism is unknown (Ivaska *et al.*, 2007).

In the present study, we characterize an unexpected association of Scrib with the IF cytoskeleton. Silencing of either Scrib or vimentin results in similar phenotypes, consistent with a functional link between the two proteins. Furthermore, we provide evidence that vimentin stabilizes Scrib by protecting it from proteasomal degradation. Our results thus reveal an important role for vimentin in the regulation of Scrib protein levels and function.

MATERIALS AND METHODS

Plasmid Constructs and Small Interfering RNAs (siRNAs)

Full-length cDNA encoding human Scribble, hScrib wild type (WT) (National Center for Biotechnology Information [NCBI] accession NM_015356) cloned into N-terminal tagged pEGFP-C1 (Clontech, Mountain View, CA) was described previously (Dow *et al.*, 2003). hScrib deletion constructs Δ Cter (amino acids 1–1194), leucine-rich repeats (LRR) (amino acids 1–727), postsynaptic density 95/disc-large/zona occludens (PDZ) (amino acids 728–1630), 4PDZ (amino acids 728–1194), and Cter (amino acids 1195–1630) were polymerase chain reaction (PCR) amplified from hScrib WT template and inserted into pEGFP-C1. hScrib WT was also subcloned into C-terminal tagged pEGFP-N1. The mouse Scribble, mScrib WT construct was created by subcloning the cDNA clone mKIAA0147 (NCBI accession AK122211), which encodes full-length mouse Scribble, into N-terminal hemagglutinin (HA)-tagged pcDNA3 vector (Invitrogen, Carlsbad, CA). Using mScrib WT as a template, mScrib LRR (amino acids 1–713) and PDZ (amino acids 714–1638) were PCR amplified and cloned into HA-pcDNA3. hScrib PDZ1, PDZ2, PDZ3, and PDZ4 cloned into pGEX-6p-2 (GE Healthcare, Chalfont St. Giles, Buckinghamshire, United Kingdom) was a gift from Sachdev S. Sidhu (Department of Protein Engineering, Genentech, South San Francisco, CA). Enhanced green fluorescent protein (EGFP)-tagged rat vimentin and enhanced cyan fluorescent protein (ECFP)- and enhanced yellow fluorescent protein (EYFP)-tagged human keratin 8 and 18 cDNAs were generously provided by Ronald Liem (Department of Pathology and Cell Biology, Columbia University College of Physicians and Surgeons, New York, NY) and Rudolf Leube (Department of Anatomy, Johannes Gutenberg University, Mainz, Germany), respectively. Using EGFP-rat vimentin as a template, full-length rat vimentin was subcloned into FLAG-pcDNA3. Full-length canine ZO-2 (NCBI accession NM_001003204) was kindly provided by Manuela Reichert (Reichert *et al.*, 2000). This was

substituted at the C-terminal three amino acids TEL with AAA to create a PDZ binding mutant, i.e., ZO2-PBM and was cloned into FLAG-pcDNA3.

Custom SMARTpool PLUS of four siRNAs directed against canine Scribble (catalog no. Q-120233-00) based on Ensembl transcript ENSCAFT00000002152, vimentin (catalog no. Q-120187-00) based on NCBI accession XM_535175 and keratin 18 (catalog Q-120323-00) based on NCBI accession XM_534794, XM_854026, and XM_854071 were designed by and purchased from Thermo Fisher Scientific (Waltham, MA). Dharmacon RNA Technologies (Lafayette, CO). ON-TARGETplus SMARTpool of four siRNAs directed against human vimentin (catalog no. L-003551-00) based on NCBI accession NM_003380 and keratin 18 (catalog no. L-010604-00) based on NCBI accession NM_199187 were also purchased from Dharmacon RNA Technologies as was the siControl nontargeting siRNA #1.

Cell Culture and Transfection

Madin-Darby canine kidney (MDCK) strain II and COS-1 (monkey transformed kidney fibroblast) cells were cultured in DMEM (glucose, 1000 mg/l) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, and 2 mM sodium pyruvate (Invitrogen) and maintained at 37°C in 5% CO₂. HeLa (human cervical adenocarcinoma) cells were cultured likewise but in DMEM (glucose, 4500 mg/l) instead. MCF-10A (human nontumorigenic mammary gland epithelial) cells were cultured in DMEM/F12 (Invitrogen) supplemented with 5% horse serum (Invitrogen), 10 μ g/ml human insulin (Sigma-Aldrich, St. Louis, MO), 20 ng/ml epidermal growth factor (Millipore, Billerica, MA), 100 ng/ml cholera toxin (Calbiochem, San Diego, CA), 0.5 μ g/ml hydrocortisone (Calbiochem), 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, and 2 mM sodium pyruvate (Invitrogen) and maintained at 37°C in 5% CO₂. Human umbilical vein endothelial cells (HUVECs) were cultured in E-STIM endothelial cell culture medium supplemented with 10 ng/ml epidermal growth factor, 0.2 mg/ml endothelial cell growth supplement (BD Biosciences, San Jose, CA), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen), and maintained at 37°C in 5% CO₂. All plasmid constructs were transfected using Lipofectamine and PLUS reagent (Invitrogen) according to the manufacturer's instructions. MDCK cell lines stably expressing pEGFP-hScrib constructs were selectively maintained in 0.5 mg/ml G418 sulfate (Calbiochem), pooled, and enriched using the fluorescence activated cell sorter FACSVantage SE (BD Biosciences). siRNAs were transiently transfected using DharmaFECT 1 (Thermo Fisher Scientific) according to the manufacturer's protocol.

Antibodies and Reagents

Primary polyclonal antibodies used in this study were rabbit anti-Scrib H-300, goat anti-Scrib C-20, rabbit anti-ZO-2 H110, and rabbit anti-ubiquitin FL-76 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Erbin, and goat anti-green fluorescent protein (GFP) (Abcam, Cambridge, United Kingdom) and rabbit anti-actin (Sigma-Aldrich). Monoclonal antibodies were mouse anti-GM130 clone 35 and anti- β -Catenin clone 14 (BD Biosciences Transduction Laboratories, Lexington, KY), mouse anti-vimentin clone V9 and anti- α -tubulin clone GTU-88 (Sigma-Aldrich), rat anti-HA clone 3F10 (Roche Diagnostics, Indianapolis, IN), mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) clone 6C5 (Millipore Bioscience Research Reagents, Temecula, CA), mouse anti-keratin 18 clone C-04 (Abcam), and mouse anti-keratin 18 clone LDK18, a gift from Birgit E. Lane (Institute of Medical Biology, Immunos, Singapore). Control antibodies used in immunoprecipitations were normal mouse and goat immunoglobulin G (IgG) (Santa Cruz Biotechnology). Secondary antibodies used for immunofluorescence were donkey anti-mouse IgG Alexa Fluor 488 and 594, anti-goat IgG Alexa Fluor 488 and 594, and anti-rabbit IgG Alexa Fluor 488 (Invitrogen) and donkey anti-mouse IgG 7-amino-4-methylcoumarin-3-acetic acid (Jackson ImmunoResearch Laboratories, West Grove, PA). For Western blots, horseradish peroxidase (HRP)-coupled goat antibodies to mouse, rabbit (Bio-Rad Laboratories, Hercules, CA), or rat IgG (Pierce Chemical, Rockford, IL) or HRP-coupled donkey antibodies to goat IgG (Jackson ImmunoResearch Laboratories) were used. Actin was labeled with BODIPY 558/568 phalloidin, and nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Cytochalasin D (10 μ g/ml) and nocodazole (10 μ g/ml) (Sigma-Aldrich) were used to disrupt actin and microtubule filaments, respectively.

Immunofluorescence Labeling

Poly-D-lysine or collagen type I (Sigma-Aldrich)-coated glass coverslips or 0.4- μ m permeable polycarbonate filters (Corning Life Sciences, Lowell, MA) were used as a platform for cell growth. Cells were fixed with either cold methanol at –20°C for 2.5 min or 3.7% paraformaldehyde at ambient temperature for 30 min. Paraformaldehyde-fixed cells were quenched with 50 mM ammonium chloride and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS). Cells were then blocked in 1% bovine serum albumin in 0.1% Triton X-100 in PBS. Primary antibodies were then applied and subsequently labeled with appropriate fluorescent dye-conjugated secondary antibodies. Images were acquired using either an LSM 510 META laser scanning confocal microscope or an Axio Imager.D1 upright microscope coupled to an AxioCam HR or a MRm digital camera, respectively (Carl Zeiss, Jena, Germany).

Cell Lysis, Immunoprecipitations, and Binding Assays

Cell lysates were prepared in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS, and supplemented with a protease inhibitor cocktail). Soluble fractions were obtained by centrifugation at 13,200 rpm for 20 min. In immunoprecipitation assays, appropriate antibodies were applied to precleared lysates for 16 h at 4°C and immunoprecipitated with protein G-Sepharose 4 Fast Flow (GE Healthcare) for 2 h at 4°C. Immunoprecipitates were washed with lysis buffer, fractionated by SDS-polyacrylamide gel electrophoresis, Western transferred onto nitrocellulose membrane Hybond-C Extra (GE Healthcare), blocked with 5% skimmed milk in 0.1% Tween 20 in PBS, and incubated with appropriate primary and secondary antibodies in 1% skimmed milk in 0.1% Tween 20 in PBS. Membranes were visualized by chemiluminescence (Super Signal West Pico (Pierce Chemical) or enhanced chemiluminescence detection reagents (GE Healthcare).

In the *in vitro* binding assays, constructs in pcDNA3 were *in vitro* translated using TNT T7 Quick Coupled Transcription/Translation system with cold methionine or Transcend NonRadioactive Translation Detection System (Promega, Madison, WI). For the *in vitro* vimentin binding assays, polymerized vimentin was obtained using the Vimentin Filament Biochem kit (Cytoskeleton, Denver, CO), in which lyophilized recombinant Syrian hamster vimentin protein was reconstituted in polymerization buffer [5 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 7.0, 1 mM dithiothreitol (DTT), and 150 mM NaCl] and processed according to the manufacturer's instructions. Nonpolymerized vimentin was obtained by reconstitution of the vimentin protein in subunit buffer (5 mM Tris, pH 7.4, 5 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS). Reconstituted vimentin was subjected to ultracentrifugation at 100,000 × *g* for 30 min. Supernatant and pellet fractions were recovered and analyzed by Western blot to monitor vimentin assembly. In cosedimentation assays, polymerized vimentin was incubated with HA-mScrib gene products in polymerization buffer at 4°C for 16 h, subjected to ultracentrifugation at 100,000 × *g* for 30 min, and the supernatant and pellet fractions were analyzed by Western blot. For *in vitro* immunoprecipitation assays, HA-mScrib gene products were incubated with the reconstituted nonpolymerized or polymerized vimentin at 35°C for 2 h in subunit buffer or RIPA buffer, respectively. This was then precleared, immunoprecipitated, and subjected to Western blot analysis as mentioned previously.

Glutathione transferase (GST) pull-down assays were performed using purified GST-hScrib PDZ1, -2, -3, and -4 recombinant proteins provided by Sachdev S. Sidhu (Genentech). These were incubated with soluble MDCK cell lysates or *in vitro*-translated gene products for 16 h at 4°C in binding buffer (25 mM Tris, pH 7.4, 50 mM NaCl, 20 mM MgCl₂, 0.1% Triton X-100, and 1 mM DTT, and supplemented with a protease inhibitor cocktail). GST-recombinant proteins were pulled down using glutathione-Sepharose 4B (GE Healthcare), washed with washing buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 20 mM MgCl₂, 0.1% Triton X-100, and 1 mM DTT), and analyzed by Western blot as mentioned previously.

Wound Healing Assay

MDCK cells were grown to confluence in the previously described culture conditions. Wounds were created with a P1000 micropipette tip and allowed to recover for 16 h before analysis. Subsequently, wounded monolayers were either fixed and analyzed by immunofluorescence, or tracked over time using time-lapse videomicroscopy. Fixed cells were analyzed for their morphology and Golgi orientation. The Golgi position relative to the nucleus and wound was scored in cells of the leading edge according to Kupfer *et al.*, 1982. Briefly, Golgi orientation relative to the nucleus and the migration front was quantified by dividing the cell into three 120° sectors with the nucleus at the center. One sector faces the wound edge and is bisected perpendicular to this edge. Correct orientation was scored when at least 50% of the Golgi fell within this sector. Based on this assay, a score of 33% denotes a random orientation. For live cell tracking, wound closure was either tracked statically using an Eclipse TE2000-S (Nikon, Tokyo, Japan) inverted microscope or continually by time-lapse videomicroscopy using an Axiovert 200M inverted microscope (Carl Zeiss) in a controlled humidified chamber at 37°C in 5% CO₂. Images were captured digitally with a Nikon DS-5Mc or an AxioCam HRC, respectively (Carl Zeiss). Time-lapse images were analyzed using the AxioVision software (Carl Zeiss).

Cell Aggregation Assay

Assay conditions were described previously (Redfield *et al.*, 1997; Kim *et al.*, 2000). Briefly, trypsinized MDCK cells were resuspended at 1.2×10^6 cells/ml in culture medium and 20 μ l (2.4×10^5 cells) drops were placed onto the inner surface of a 10-cm tissue culture dish lid. The lid was then placed onto the dish containing 10 ml of PBS in the bottom to prevent evaporation of the drops. After subsequent incubation, drops were directly analyzed for cell aggregation by inverting the lid and viewing under an Eclipse TE2000-S inverted microscope. Alternatively, cell drops were replated onto poly-D-lysine-coated glass coverslips, allowed to adhere, and later studied for cell spreading.

Proteasome Inhibitor Assay

MDCK cells treated with appropriate siRNAs for 3 d were incubated at 37°C in 5% CO₂ with 10 μ M proteasome inhibitor II (Z-Leu-Leu-Phe-aldehyde; A.G. Scientific, San Diego, CA) in culture medium from 0 to 9 h. Subsequently, cells were lysed and analyzed by Western transfer or immunofluorescence microscopy as described previously.

RESULTS

Scrib and Intermediate Filaments Partially Colocalize in MDCK Cells and HUVECs

In sparse cultured epithelial MDCK cells (Figure 1A), endogenous Scrib localization was observed along vimentin filaments concentrated at the perinuclear region and spanning across the cytoplasm toward the cell plasma membrane periphery (Figure 1A, a–c). This colocalization was particularly exhibited as punctate Scrib staining aligned along the length of the perinuclearly emanating vimentin bundles (Figure 1A, a–c, blue inset, and d–f, arrowheads). At nascent cell–cell contacts, Scrib seemed fragmented at the membrane and partially coincided with vimentin-positive fibrils at the cell periphery (Figure 1A, a–c, yellow inset, and g–i, arrowheads). When cells grew to full confluence (Figure 1B), Scrib displayed a clear membrane localization, whereas vimentin filaments lined the cell periphery (Figure 1B, a–c). Colocalization of both proteins was apparent in particular regions along the membrane, in which Scrib membrane localization seemed punctate (Figure 1B, a–c, yellow inset, and d–f, arrowheads). A similar colocalization was also observed in HUVECs. HUVECs grown sparsely (Figure 1C and Supplemental Figure 1A) demonstrated a punctate Scrib decoration of vimentin bundles spanning the cytoplasm, reminiscent of that observed in MDCK cells (Figure 1C, a–c, blue inset, and d–f, arrowheads). Interestingly, spreading HUVECs showed an apparent partial colocalization of Scrib and vimentin at membrane protrusions (Figure 1C, a–c, yellow inset, and g–i, arrowheads). This is consistent with the presence of Scrib at membrane protrusions of migrating astrocytes and mammary epithelial cells (Osmani *et al.*, 2006; Dow *et al.*, 2007). In confluent HUVEC cultures, Scrib localized to the membrane borders between cells. Surprisingly, this localization partially coincided with a similar nonfilamentous vimentin peripheral membrane staining (Figure 1D, a–c, yellow inset, and d–f, arrowheads). Short vimentin fragments were also observed in proximity of this vimentin staining. These fragments may represent vimentin squiggles that move along the cell periphery and are intermediates of vimentin filament turnover and rearrangement (Martys *et al.*, 1999).

To further ascertain the cytoskeletal localization of Scrib, colocalization studies of exogenously expressed epitope-tagged human (EGFP-hScrib) or mouse (HA-mScrib) Scrib with proteins of the major cellular cytoskeletal networks were performed in MDCK or COS-1 cells. EGFP-hScrib or HA-mScrib was observed in a filamentous network when expressed in MDCK epithelial (Figure 1E, a and d) or in monkey kidney fibroblast COS-1 (Supplemental Figure 1Ba) cells, respectively. In MDCK cells, these filaments were particularly prominent in sparse cell cultures during cell spreading and the establishment of cell–cell contact. EGFP-hScrib-containing filaments emanated from a perinuclear region and extended toward the cell periphery. These filaments extensively overlapped with vimentin and keratin 18 of IFs (Figure 1E, a–f). Likewise, HA-tagged mScrib expressed in COS-1 cells occurred as filaments that aligned with vimentin, showing that colocalization with IFs was not restricted to a single cell line and was independent of the

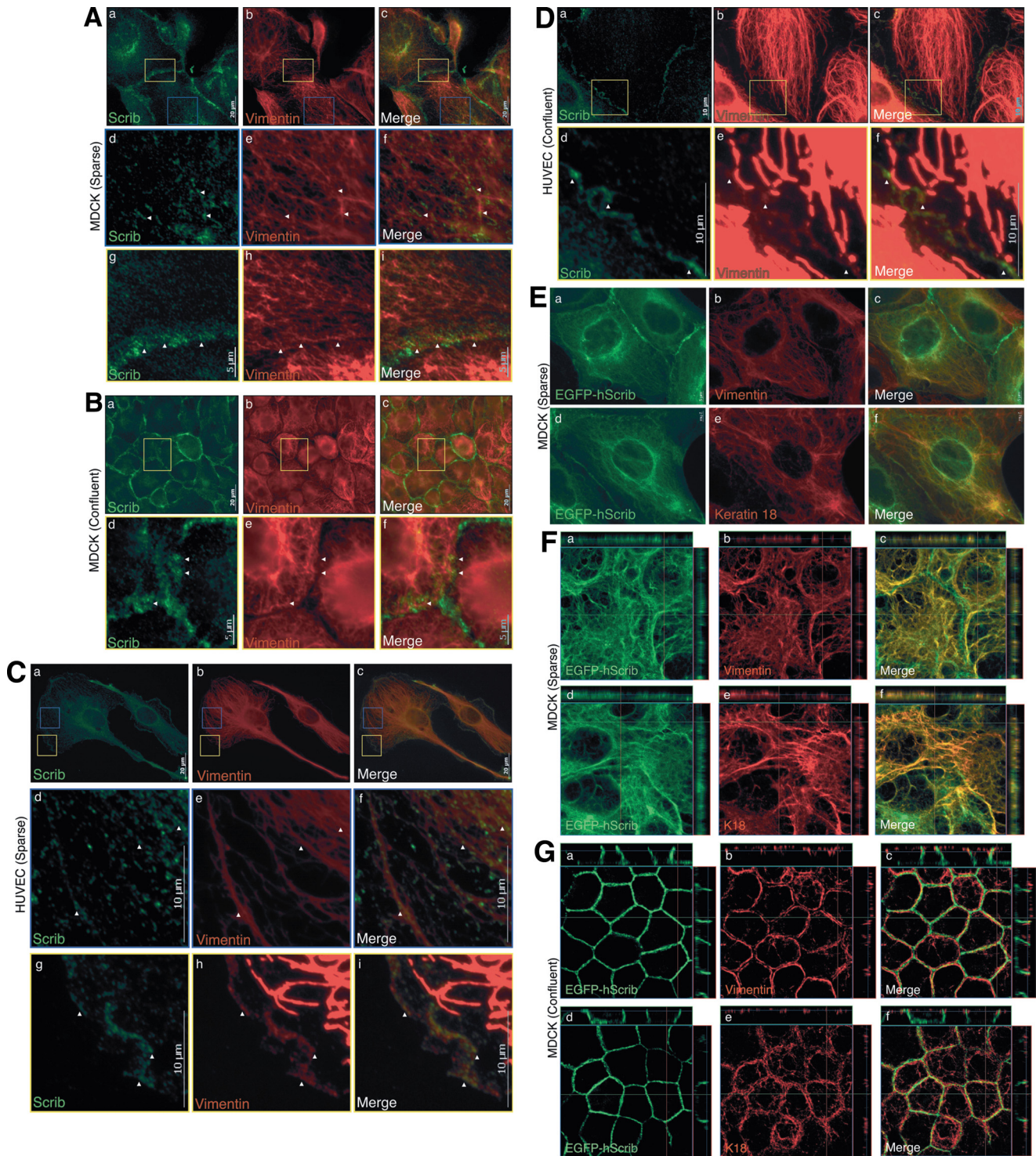


Figure 1. Scrib localizes to intermediate filaments. (A–D) Localization of endogenous Scrib to vimentin IFs in MDCK cells and HUVECs. A few coverslips were placed into a 10-cm dish and MDCK cells (A and B) were seeded at 1×10^6 (A; Sparse) or 1×10^7 (B; Confluent) cells and grown for 2 d. HUVECs (C and D) were seeded at 1.6×10^5 (C; Sparse) or 6.5×10^5 (D; Confluent) cells per 10-cm dish and grown for 4 d. Coverslips were then removed and used for immunofluorescence microscopy; the remaining culture in the dish was imaged by phase contrast to assess cell confluence and then processed for biochemical analysis (see Figure 2A, a and c). Scrib (A–D, a, green) and vimentin (A–D, b, red) were immunostained. Yellow in the merged images (A–D, c) indicates colocalization. In sparse cultures of both cell types, punctate staining of endogenous Scrib can be observed along vimentin filaments emanating from the perinuclear region toward the cell periphery (arrowheads in magnification of blue inset in A and C, d–f). In addition, in MDCK cells, Scrib can also be detected on vimentin filaments peripheral to the plasma membrane (arrowheads in magnification of yellow inset in A, g–i). In HUVECs, both Scrib and nonfilamentous vimentin colocalize at the plasma membrane as punctate staining in what seem to be membrane protrusions (arrowheads in magnification of yellow inset in C, g–i). In confluent cultures, Scrib is concentrated at contact sites and present on vimentin filaments in the cell periphery of MDCK cells (arrowheads in magnification of yellow inset in B, d–f). In HUVECs, the Scrib positive contact sites colocalize

particular tag (Supplemental Figure 1B, a–c). Furthermore, no significant EGFP-hScrib colocalization was observed with either the microtubule (Supplemental Figure 2A, a–c) or the actin (Supplemental Figure 2B, a–c) networks in MDCK cells. Treatment of cells with nocodazole or cytochalasin D to disrupt the microtubule (Supplemental Figure 2A, d–f) or actin (Supplemental Figure 2B, d–f) cytoskeleton, respectively, neither affected the filamentous appearance of EGFP-hScrib (Supplemental Figure 2, A and B, d–f) nor its colocalization with vimentin (Supplemental Figure 2, A and B, g–i). This thus confirms the absence of Scrib from either actin microfilaments or microtubules.

The colocalization of Scrib with IFs was further corroborated in MDCK cell monolayers cultured on permeable polycarbonate filters (Figure 1, F and G). Under sparse culture conditions when cell–cell contact was being established, EGFP-hScrib showed extensive colocalization with vimentin (Figure 1F, a–c) and keratin 18 (Figure 1F, d–f) based IFs and the lateral membrane. In confluent MDCK cell monolayers, EGFP-hScrib localized along the length of the lateral plasma membrane and the IFs had acquired an apical localization (Figure 1G, a–f), apparently lining the apical pole of the lateral plasma membrane and only showing partial overlap with hScrib at the apical pole of the lateral membrane (Figure 1G, c and f).

In conclusion, in HUVECs and MDCK cell cultures, endogenous Scrib partially colocalized with vimentin at cell–cell contacts and along perinuclearly emanating filaments. In addition, colocalization was also observed at membrane protrusions of sparsely cultured HUVECs. In Scrib-overexpressing COS-1 or sparse MDCK cell cultures, Scrib showed extensive decoration of vimentin and keratin IFs. In MDCK cells, Scrib redistributed to the lateral plasma membrane during the establishment and maturation of cell–cell adhesion, in which it showed limited overlap with IFs lining the apical pole.

Figure 1 (cont). with nonfilamentous vimentin or short filament staining (arrowheads in magnification of yellow inset in D, d–f). Note the presence of short vimentin filaments in the cell periphery. (E) Epitope-tagged hScrib colocalizes with vimentin and keratin 18. A few coverslips were placed into a 10-cm dish and MDCK cells expressing EGFP-hScrib were seeded at 1×10^6 cells per 10-cm dish and grown for 2 d to obtain sparse cultures. Coverslips were then removed and used for immunofluorescence microscopy; the remaining culture in the dish was imaged by phase contrast to assess cell confluence and then processed for biochemical analysis (see Figure 2Ab). EGFP-hScrib (a and d, green) and vimentin (b, red) or keratin 18 (e, red) were visualized in sparse MDCK cells expressing EGFP-hScrib. Yellow in the merged images (c and f) indicates colocalization of hScrib with the IF. (F and G) Relocalization of EGFP-hScrib, vimentin, and keratins during formation of polarized MDCK cell monolayers grown on permeable supports. EGFP-hScrib expressing MDCK cells were seeded at 3×10^5 cells per 12-mm-diameter permeable support insert and grown for 1 d (Sparse) or 7 d (Confluent). EGFP-hScrib (F and G, a and d, green) and vimentin (F and G, b, red color) or keratin 18 (F and G, e, red) were visualized by confocal fluorescence microscopy in sparse (F) or polarized (G) MDCK cell cultures. Blue lines indicate the location of the horizontal confocal section, and red and green lines indicate the site of vertical confocal sections along the apical–basal axis of the monolayer. Yellow in the merged images indicate colocalization (F and G, c and f). Note the extensive colocalization of EGFP-hScrib with vimentin and keratins in nonpolarized cells. In polarized cells, vimentin and keratin 18 accumulate at the apical pole, whereas EGFP-hScrib is present on the lateral membrane and only shows minimal overlap with IFs at the apical end of the lateral membrane.

Scrib Directly Associates with Intermediate Filaments

Next, we biochemically corroborated the colocalization of Scrib with IFs by using MDCK, MCF10A, HeLa, and COS-1 cells, which all express endogenous Scrib (Supplemental Figure 3a). Endogenous vimentin was immunoprecipitated from cell lysates and probed for the coprecipitation of endogenous Scrib. As shown in Supplemental Figure 3b, Scrib specifically coprecipitated with vimentin in the cell lines tested. We further tested this interaction in MDCK cells and HUVEC of varying cell densities. Endogenous vimentin was immunoprecipitated from cell lysates harvested from MDCK cell cultures of increasing confluence and probed for the coprecipitation of endogenous Scrib. As shown in Figure 2Aa, Scrib specifically coprecipitated with vimentin over the range of cell confluence tested. This is consistent with the colocalization of Scrib and vimentin in both sparse and confluent cultures (Figure 1). Moreover, the amount of Scrib that coprecipitated with vimentin proportionally reflected the increasing amounts of Scrib expression observed with increasing cell confluence (Figure 2Aa, Input). In MDCK cells, protein levels of overexpressed EGFP-hScrib remained similar with increasing cell confluence, unlike those of endogenous Scrib (Figure 2Ab, Input). However, more EGFP-hScrib was coprecipitated with vimentin in sparse compared with confluent culture conditions (Figure 2Ab). This disproportionate increase in interaction is consistent with the extensive colocalization of EGFP-hScrib with filamentous vimentin in sparse cell cultures, which was no longer observed once high cell density was reached and may thus reflect a phenomenon of overexpression. A similar immunoprecipitation assay was done on both sparse and confluent HUVEC cultures (Figure 2Ac). Consistent with the result obtained for MDCK cells, endogenous Scrib showed an increase in expression levels with confluence, whereas vimentin levels remained unchanged (Figure 2Ac, Input). Vimentin coprecipitated with Scrib under both culture conditions, corroborating the colocalization observed for the two proteins in HUVECs.

To determine whether the association of Scrib with IFs reflects a direct binding to vimentin, we carried out cosedimentation assays between purified nonpolymerized or in vitro polymerized hamster vimentin and in vitro translated N-terminally HA-tagged mScrib (Figure 2B). Although purified nonpolymerized vimentin remained in the supernatant after ultracentrifugation, in vitro-polymerized vimentin sedimented to the pellet (Figure 2Ba). In vitro-translated HA-mScrib was recovered in the pellet, but only in the presence of polymerized vimentin (Figure 2Bb). Furthermore, both polymerized (Figure 2Bc) and nonpolymerized (Figure 2Bd) vimentin coimmunoprecipitated with in vitro-translated HA-mScrib. These experiments thus confirm the association between Scrib and vimentin observed in vivo and show that the interaction is direct and, at least in vitro, occurs with both nonpolymerized vimentin and vimentin filaments.

Scrib Associates with Intermediate Filaments via Its PDZ Domain-containing Region

To map the domain in Scrib responsible for the interaction with IFs, two N-terminally EGFP-tagged hScrib constructs encompassing either of the two major domains, namely, the LRR and the PDZ regions, were generated (Figure 2C) and tested for their association with IFs. Confirming the data above (Figure 2Ab), vimentin, but also keratin 18, coimmunoprecipitated with full-length EGFP-hScrib WT (Figure 2D, a and b). Intermediate filaments predominantly bound to

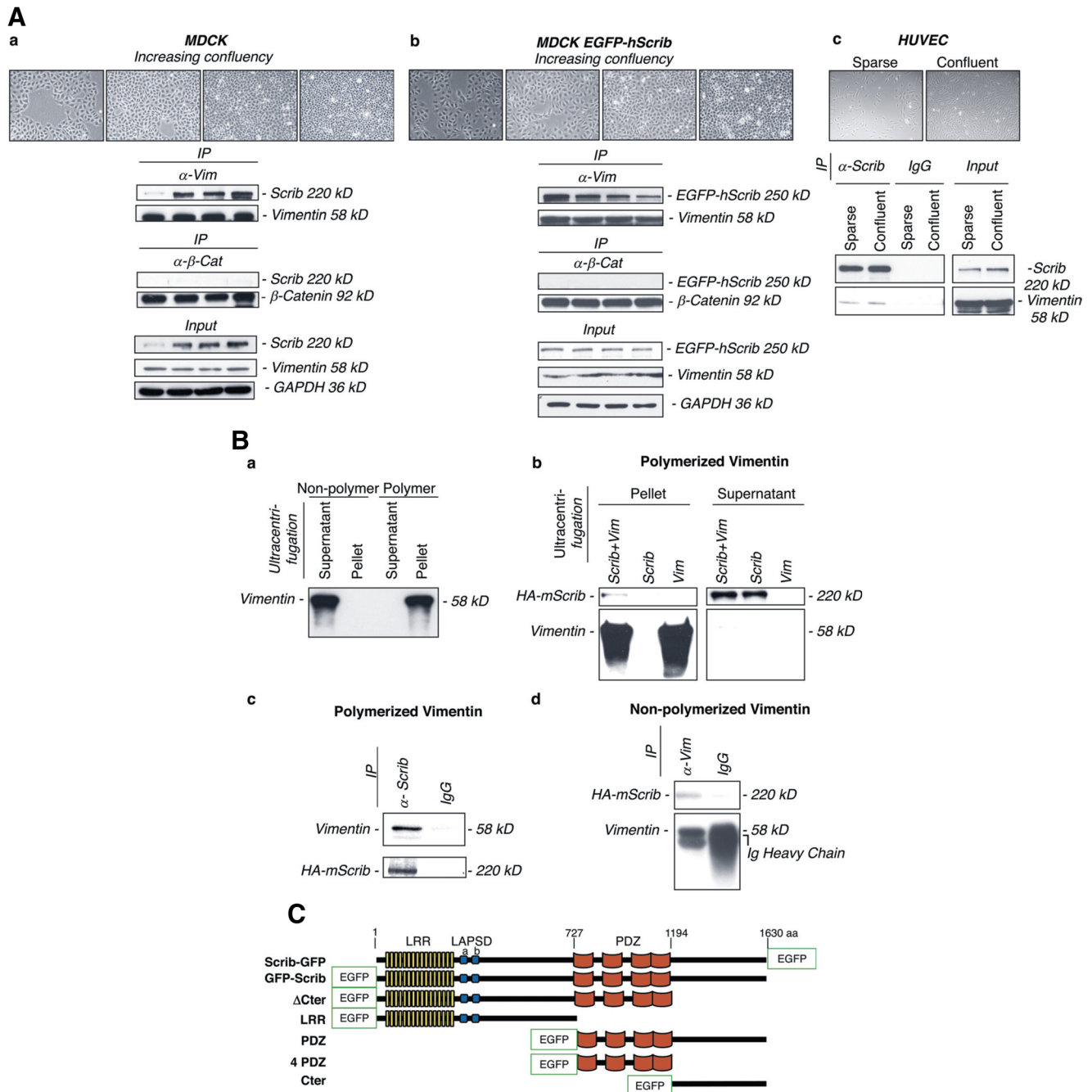


Figure 2. Scrib associates with intermediate filaments via its PDZ domains. (A) Scrib interacts with vimentin in MDCK cells and HUVECs at various culture confluences. Control MDCK cells (a) or cells expressing EGFP-hScrib (b) were seeded at 1×10^6 , 2×10^6 , 5×10^6 , or 1×10^7 cells per 10-cm dish and grown for 2 d to reach different confluences (top, phase contrast images). Vimentin was immunoprecipitated from harvested lysates. Associated endogenous (a) or exogenous (b) Scrib was detected by Western blot. Precipitation with an antibody to β -catenin served as a negative control. An aliquot of the cell lysate (5%) was directly analyzed by Western blot to monitor Scrib and vimentin expression levels (Input). GAPDH was detected to monitor for equal cell lysate loading. HUVECs were seeded at 1.6×10^5 (Sparse) or 6.5×10^5 (Confluent) cells per 10-cm dish and grown for 4 d (top, phase contrast images). Scrib was immunoprecipitated from harvested lysates. Associated vimentin was detected by Western blot. Precipitation with normal IgG served as a negative control (c). An aliquot of the cell lysate (5%) was directly analyzed by Western blot to monitor Scrib and vimentin expression levels (Input). (B) Scrib associates directly with both nonpolymerized or polymerized vimentin in vitro. Vimentin assembly was monitored through fractionation by ultracentrifugation. Fractions were subjected to Western blot and vimentin visualized as nonpolymerized (NonPolymer) or polymerized (Polymer) forms in the supernatant or pellet, respectively (a). In vitro-translated HA-mScrib cosedimented with vimentin only when applied to polymerized vimentin but not alone (b). HA-mScrib also associated with both polymerized (c) and nonpolymerized (d) vimentin in coimmunoprecipitation assays. Note that even in the presence of a large excess of normal IgG (d), little if any Scrib is found in the precipitate. (C) Schematic diagram of EGFP-hScrib deletion mutants. Full-length human Scrib was either tagged at the N terminus (GFP-Scrib) or C terminus (Scrib-GFP) with EGFP. Δ Cter lacks the region C-terminal to the PDZ domains, which is contained in the Cter construct. LRR and PDZ encode the N-terminal LRR-LAPSD or the C-terminal four PDZ domains, respectively. 4PDZ contains the PDZ domains only. aa, amino acids.

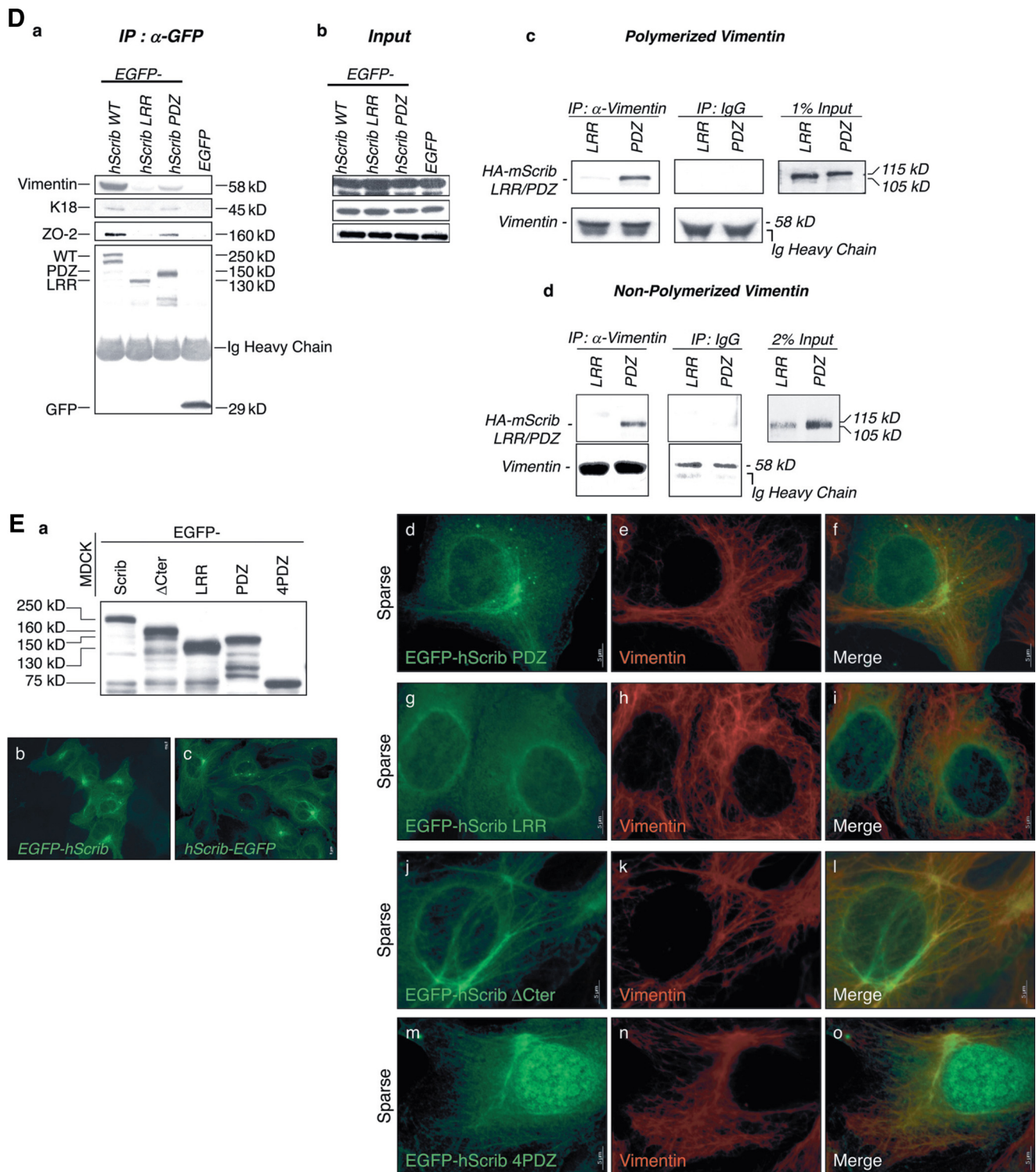


Figure 2 (cont). (D) Scrib associates with IFs via the region containing the four PDZ domains. EGFP-hScrib WT, LRR, and PDZ were immunoprecipitated from sparse MDCK cells and associated vimentin and keratin 18 as detected by Western blot analysis (a). The detection of ZO-2 served as a positive control for the interaction with hScrib WT or PDZ and as a negative control for the interaction with LRR (Métais *et al.*, 2005). An aliquot (5%) of the cell lysate was directly subjected to Western blot analysis to monitor the expression levels of vimentin, keratin 18, and ZO-2 (Input) (b). Scrib directly binds to vimentin via its PDZ domains. Purified in vitro-polymerized (c) or nonpolymerized (d) vimentin and in vitro-translated HA-mScrib LRR or HA-mScrib PDZ were combined and incubated. Vimentin was then immunoprecipitated and vimentin or HA-mScrib detected by Western blot. Normal IgG served as a negative control. (E) hScrib PDZ localizes to vimentin filaments in sparse MDCK cells. The different hScrib constructs tagged with EGFP were expressed in MDCK cells and visualized by Western blot analysis (a) or fluorescence microscopy (b–o). Note that both N- and C-terminally tagged Scrib (b and c) and only constructs containing the PDZ domains (d–f, j–l, and m–o) show extensive filamentous localization.

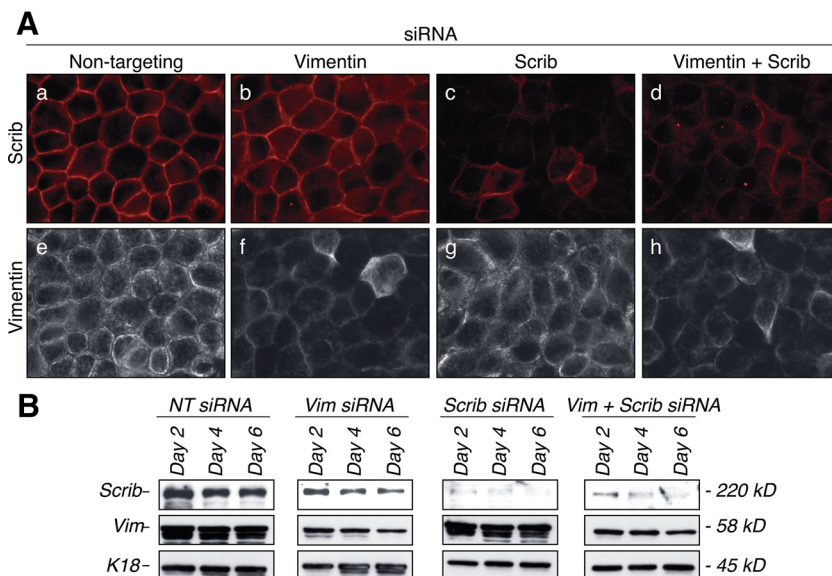


Figure 3. siRNA-mediated depletion of endogenous vimentin and Scrib in MDCK cells. (A) Silencing of Scrib and vimentin monitored by immunofluorescence microscopy. Scrib (a–d, red) and vimentin (e–h, white) were visualized in MDCK cells treated for 3 d with a nontargeting siRNA (a and e) or siRNAs to vimentin (b and f), Scrib (c and g), or both Scrib and vimentin (d and h). (B) Silencing of Scrib and vimentin monitored by Western blot analysis. Scrib and vimentin protein levels in lysates of cells treated with siRNA over a 6-d period were monitored by Western blot on days 2, 4, and 6. Keratin 18 was detected to monitor for equal cell lysate loading.

hScrib PDZ, with only a comparatively weak interaction with hScrib LRR observed. As a positive control, zona occludens (ZO)-2 bound both hScrib WT and hScrib PDZ, but not hScrib LRR, consistent with a previous report (Métais *et al.*, 2005). As a negative control, EGFP did not associate with the IF proteins (Figure 2D, a and b). Furthermore, in vitro-translated HA-mScrib PDZ domains, but not the HA-mScrib LRR domain, coimmunoprecipitated with purified in vitro-polymerized (Figure 2Dc) or nonpolymerized (Figure 2Dd) vimentin. These data thus implicate the regions containing the four PDZ domains in mediating the association of Scrib with IFs.

The interaction of the Scrib PDZ domain with vimentin was corroborated by expressing the different EGFP-hScrib deletion constructs (Figure 2Ea) in both sparse (Figure 2E, b–o) and confluent (Supplemental Figure 4, a–l) MDCK cells and analyzing their localization. Similarly to the EGFP-hScrib, a C-terminally tagged protein (hScrib-EGFP) was present on filaments (Figure 2Ec), excluding the possibility that filamentous localization was due to the location of the tag or to truncated Scrib molecules arising from either premature translational arrest or C-terminal degradation. Consistent with the binding data, hScrib PDZ (Figure 2E, d–f) but not hScrib LRR (Figure 2E, g–i) displayed a filamentous localization. Deletion of the region C-terminal to the PDZ domains (hScrib Δ Cter) did not affect filamentous localization (Figure 2E, j–l). Furthermore, the four PDZ domains themselves were sufficient for colocalization with vimentin (Figure 2E, m–o), whereas the C-terminal fragment downstream of the PDZ domains (hScrib-Cter) was absent from filaments (data not shown).

To determine whether a particular PDZ domain is responsible for the interaction with IFs, each of the four PDZ domains of hScrib were expressed as GST-fusion proteins and tested in binding assays. PDZ3 efficiently interacted with both vimentin and keratin 18 (Supplemental Figure 5A). Less efficient associations were also observed for PDZ1 and PDZ2, whereas no binding to vimentin or keratin 18 could be detected for PDZ4. To further corroborate these results, GST-hScrib PDZ3 was incubated with in vitro translated FLAG-tagged full-length vimentin (Supplemental Figure 5B). Expectedly, PDZ3 associated with vimentin but not the negative control FLAG-ZO2 PBM, which lacked the PDZ

binding motif essential for interaction with hScrib PDZ3 (Métais *et al.*, 2005). Thus, Scrib directly binds via its PDZ domains to the IF components vimentin and keratin 18.

Silencing of Either Scrib or Vimentin Leads to Similar Effects on Cell Motility and Morphology

To explore the functional relationship of the interaction between Scrib and vimentin, their protein levels were reduced in MDCK cells by using pools of four siRNAs targeting specifically either canine Scrib or vimentin. Immunofluorescence (Figure 3A) and Western blot (Figure 3B) analysis confirmed the gradual decrease of Scrib and/or vimentin protein levels after treatment with the respective siRNAs. We then analyzed the effect of the siRNAs on several cellular processes that have been linked to Scrib function, including cell morphology, migration, and polarity. All subsequent assays were carried out on the fourth day after addition of the siRNA, unless indicated otherwise.

Directed migration of cells in which Scrib and/or vimentin expression had been silenced was studied using wound healing assays. Depletion of either vimentin or Scrib alone or in combination resulted in an aberrant cell morphology and orientation at the migration front (Figure 4A). Whereas control cells migrated as an organized sheet with their long axis perpendicular to the migration front, silencing of Scrib and/or vimentin lead to a randomized cell orientation and a disorganized appearance of the cell sheet at the wound edge. At the leading edge, cells normally polarize their Golgi complex toward the direction of migration (Kupfer *et al.*, 1982), and this polarization is affected upon depletion of Scrib (Osmani *et al.*, 2006; Dow *et al.*, 2007). In agreement with these findings, Scrib knockdown cells failed to polarize their Golgi complex in our assay (Figure 4B, a–f). Interestingly, a randomized Golgi orientation was also observed in vimentin siRNA-treated cells, either alone or in combination with Scrib siRNA. In conclusion, cell morphology and Golgi polarization were similarly affected in migrating MDCK cells upon silencing of either Scrib, vimentin, or both.

Silencing of Scrib or Vimentin Affects Wound Closure Rates Due to Randomized Cell Migration

In light of the cellular abnormalities observed in migrating Scrib and vimentin knockdown cells, we analyzed in more

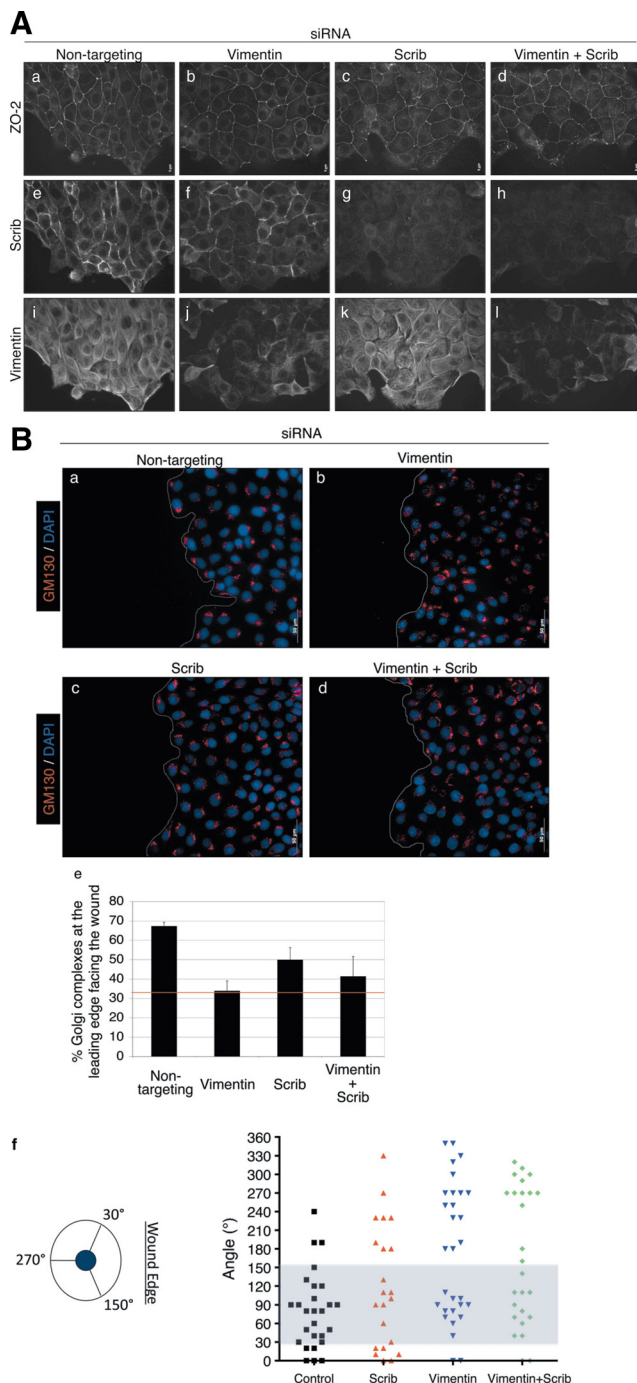


Figure 4. Silencing of Scrib or vimentin expression in MDCK cells leads to defects in cell morphology and Golgi complex orientation during directed cell migration. (A) Aberrant morphology. Monolayers of cells treated with nontargeting (a, e, and i), vimentin (b, f, and j), Scrib (c, g, and k), or Scrib and vimentin (d, h, and l) siRNA were wounded and stained with an antibody to ZO-2 (a–d) to visualize the cell outline. Scrib (e–h) and vimentin (i–l) were stained to monitor the effectiveness of the siRNA treatment. Note how in control cells the long axis of the cells is directed toward the wound edge (bottom of the images), whereas it is random in cells treated with the specific siRNAs. (B) Monolayers of cells treated with nontargeting (a), vimentin (b), Scrib (c), or Scrib and vimentin (d) siRNA were wounded and stained with an antibody to the *cis*-Golgi marker GM130 (red) and DAPI (blue) to label nuclei. The wound edge is demarcated with a white line. (e) Golgi complex orientation relative to the nucleus and the migration front was quantified as described

detail migration parameters, such as rate of wound closure, migration speed, and tortuosity. Whereas wounds in control cell monolayers closed over a 16-h period, cells treated with Scrib and/or vimentin siRNA showed significantly slower wound closure rates (Figure 5A), although this was less pronounced for cells treated with vimentin siRNA alone. To explore the parameters responsible for this reduced wound closure rate, we monitored velocity and tortuosity of cell movement at the wound edge by using time-lapse microscopy and cell tracking. In contrast to control cells, which migrated unidirectionally toward the wound as a cohesive sheet, knockdown cells displayed a random and uncoordinated movement (Supplemental Movies 1–4). No significant differences in velocity of migration could be established for the different knockdown cells (data not shown). We therefore analyzed the straightness of migration, which can be quantitatively assessed in terms of tortuosity, with a straight track having a value of 1 and a more tortuous or twisted route a value >1 . Control cells migrated with a mean tortuosity of 1.1, whereas cells treated with siRNAs to Scrib, vimentin, or both recorded significantly increased tortuosities of 2.1, 1.5, and 2.2, respectively (Figure 5B). The differences in tortuosity thus correlate well with the reduced wound closure rates for cells depleted of Scrib and Scrib/vimentin and the less pronounced effect in cells treated with vimentin siRNA only (see above). Together, these data thus demonstrate similar requirements for Scrib and vimentin in directed migration of MDCK cells, consistent with the two proteins acting in a common regulatory pathway.

Scribble and Vimentin Are Required for Efficient Cell Aggregation

Altered cell morphology and directed migration of Scrib and vimentin knockdown cells suggested defects in cell–cell adhesion. This possibility was explored using a hanging drop cell aggregation assay (Redfield *et al.*, 1997; Kim *et al.*, 2000). Control cells suspended in a hanging drop for 24 h formed dense cell aggregates (Figure 6A). In contrast, cells treated with Scrib or vimentin siRNA, or both, only showed sparse aggregates. If aggregates from the hanging drop were transferred onto coverslips, control cells adhered to the substratum, spread, and after 24 h formed cobblestone-like cell islands typical for MDCK cells (Figure 6B). Knockdown cells, in contrast, failed to remain tightly organized after attachment to the coverslip and spreading. Thus, Scrib and vimentin are required for normal cell–cell adhesion and reduced levels in these proteins could explain the loss of directional migration in knockdown cell monolayers.

in *Materials and Methods*. Shown is the fraction of leading edge cells with correctly polarized Golgi complexes that position in front of the nucleus, facing the wound. Results represent the means of three independent experiments, in which at least 400 cells were scored for each condition. Error bars, SD of the mean. A red line indicates basal levels for a random orientation of 33%. (f) Schematic representation of Golgi complex orientation. The position of Golgi complex relative to the nucleus (blue) and wound edge was determined for ~30 individual cells for each siRNA treatment and plotted. The shaded sector from 30° to 150° faces the wound edge and is bisected perpendicular to this edge. Note how the positioning of the Golgi complex of most control siRNA-treated cells falls within this sector, whereas that of cells where vimentin, Scrib, or both had been silenced is randomized.

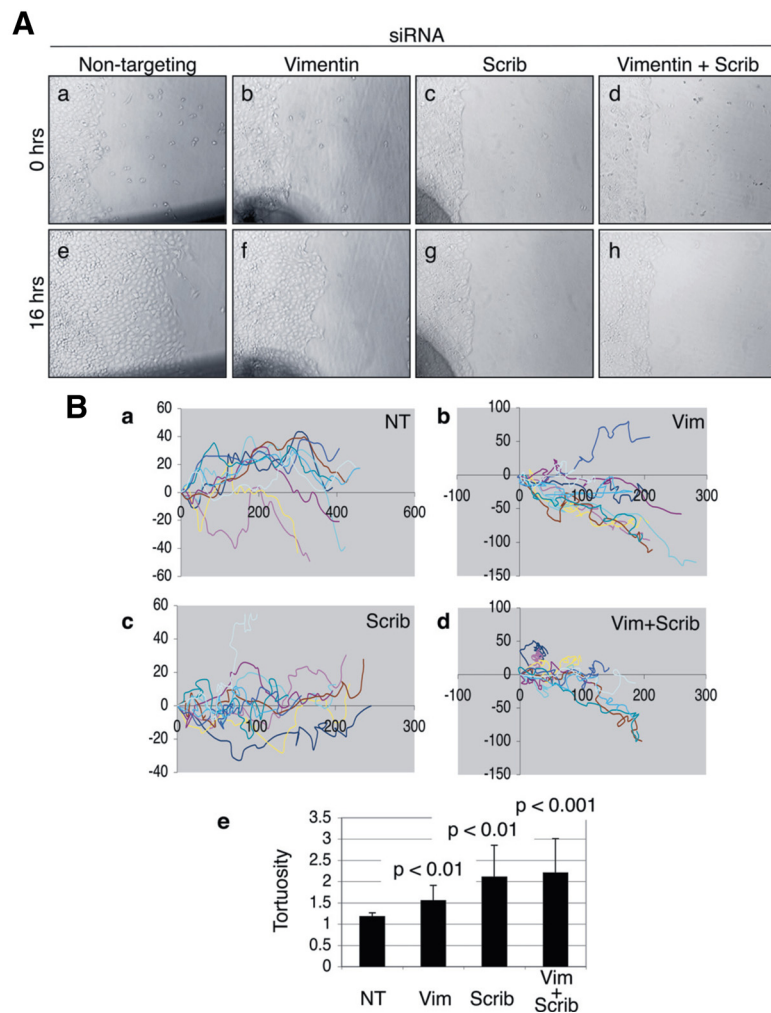


Figure 5. Slower wound closure rates due to a less directed migration of MDCK cells treated with Scrib or vimentin siRNA. (A) Wound closure. Monolayers of cells treated with nontargeting (a and e), vimentin (b and f), Scrib (c and g), or Scrib and vimentin (d and h) siRNA were wounded and allowed to migrate for 16 h. Images were taken after wounding (0 h; s a–d) or 16 h of migration (e–h). The black marks at the bottom of the dishes allow alignment of the wounds. Panels shown are representative of at least three independent experiments. (B) Quantification of cell migration directionality using live cell tracking. The X-Y graphs represent migration coordinates of 10 different cells at the wound edge treated with nontargeting (a), vimentin (b), Scrib (c), or vimentin and Scrib (d) siRNA, tracked over time 4 d after siRNA transfection. Start points for the different cells were adjusted to (0,0) coordinates. Results are representative of at least three independent experiments. (e) Tortuosity was scored for at least 30 individual cells for each siRNA treatment ($n = 3$; $p < 0.01$ – 0.001 , Student's t test). A value of 1 indicates linear movement. Error bars represent SD.

Vimentin Stabilizes Scrib by Protecting It from Proteasomal Degradation

The phenotypic convergence of silencing Scrib and vimentin inferred a functional relationship for the interaction of the two proteins. To gain insight into possible mechanisms underlying this function, we examined possible effects of Scrib or vimentin depletion on vimentin or Scrib protein levels,

respectively. Silencing of Scrib did not alter vimentin or keratin 18 expression levels (Figure 3B) or IF organization (data not shown). Interestingly, however, endogenous Scrib levels were significantly reduced after knockdown of vimentin in both sparse and confluent MDCK cell cultures (Figures 3B and 7A). Quantification of three independent experiments showed a correlation between the extent of vimentin

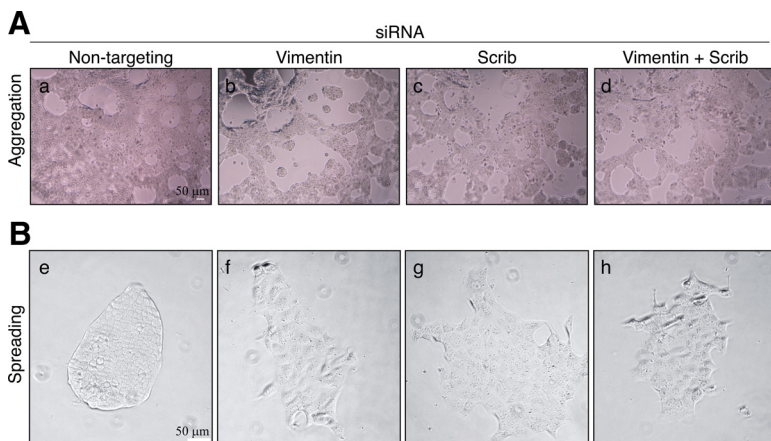


Figure 6. Silencing of Scrib and vimentin expression affects cell–cell aggregation and spreading. (A) Cell aggregation. MDCK cells treated with nontargeting (a and e), vimentin (b and f), Scrib (c and g), or Scrib and vimentin (d and h) siRNA were allowed to aggregate in a hanging drop and photographed (a–d). (B) Cell spreading. Cell aggregates were transferred from the hanging drop onto coverslips and allowed to adhere and spread (e–h). Note how cells treated with specific siRNAs form less compact aggregates (b–d) and show enhanced spreading (f–h) compared with control cells (a and e, respectively). Assays were carried out 4 d after siRNA transfection.

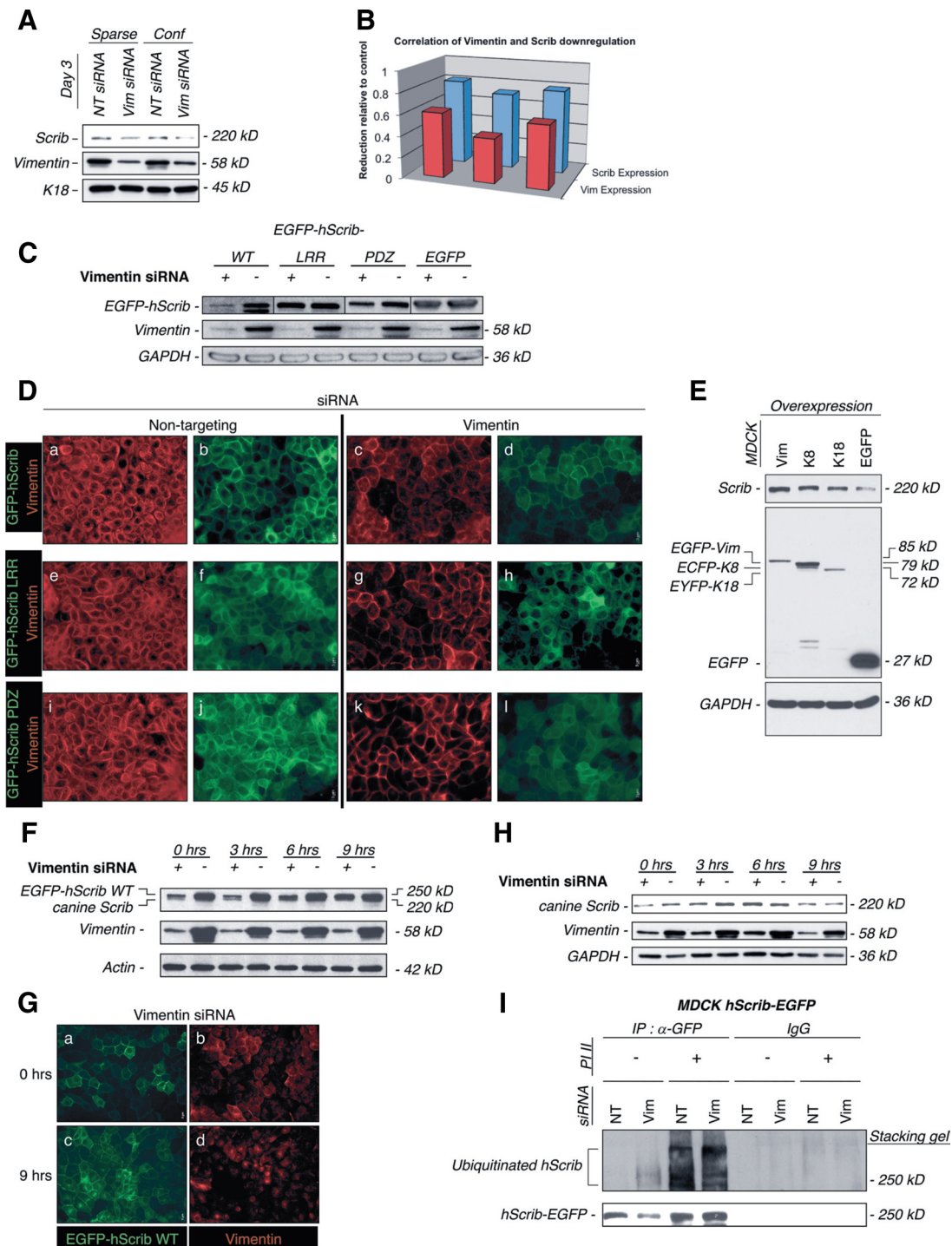


Figure 7. Proteasome-dependent degradation of Scrib is inhibited by its interaction with vimentin. (A) Vimentin expression in MDCK cells was silenced using siRNA over 3 d. Cells were subsequently reseeded to sparse and confluent cultures, and Scrib protein levels were monitored by Western blot analysis on day 4. K18 was detected to check for equal cell lysate loading. (B) Quantitative representation of Scrib down-regulation relative to levels of vimentin silencing in MDCK cells. (C) MDCK cells expressing EGFP-hScrib WT (~250 kDa), LRR (~130 kDa), PDZ (~150 kDa), or, as a negative control, EGFP alone, were treated with vimentin (+) or nontargeting (-) siRNA. hScrib expression was analyzed by Western blot using antibodies to GFP. GAPDH served as a control for equal lysate input. (D) MDCK cells expressing EGFP-hScrib WT (a-d), LRR (e-h), or PDZ (i-l) were treated with nontargeting (a, b, e, f, i, and j) or vimentin (c, d, g, h, k, and l) siRNA and EGFP-hScrib (b, f, j, d, h, and l; green) and vimentin (a, e, i, c, g, and k; red) expression was visualized by fluorescence microscopy. (E) MDCK cells exogenously expressing EGFP vimentin, ECFP-K8, EYFP-K18, or EGFP alone were analyzed by Western blot for expression of Scrib. GAPDH served as a control for equal lysate input. (F-H) Effect of a proteasome inhibitor on Scrib turnover. (F) Western blot. MDCK cells expressing EGFP-hScrib WT were treated with vimentin (+) or nontargeting (-) siRNA for 3 d and subsequently in the presence of a proteasome inhibitor for 0, 3, 6, or 9 h. Scrib levels and vimentin expression levels were then analyzed by Western blot. Note how in vimentin depleted cells, EGFP-hScrib (250 kDa) as well as endogenous Scrib (220 kDa) degradation is blocked by the proteasome inhibitor (also see H). Actin served as a control for equal lysate input. (G) Immunofluorescence microscopy. MDCK cells expressing EGFP-hScrib WT were

silencing and the degree of endogenous Scrib protein decrease (Figure 7B). Silencing of keratin 18 also resulted in reduced Scrib protein levels, and similar effects were observed in other cell lines, including HeLa and MCF10A (Supplemental Figure 6A). Interestingly, Erbin, a protein related to Scrib (Borg *et al.*, 2000; Santoni *et al.*, 2002), was up-regulated in vimentin siRNA-treated cells, suggesting a possible compensatory mechanism (Supplemental Figure 6B).

A similar but more dramatic effect was observed for cells overexpressing Scrib. Both EGFP-hScrib and EGFP-hScrib PDZ, but not EGFP-hScrib LRR, which does not associate with vimentin, were significantly reduced in vimentin siRNA treated MDCK cells as evidenced by Western blot (Figure 7C) or immunofluorescence microscopy (Figure 7D). Conversely, overexpression of vimentin, keratin 8, or keratin 18 in MDCK cells resulted in a significant increase in endogenous Scrib protein levels (Figure 7E).

Scrib has been reported to undergo E6AP ubiquitin ligase-mediated ubiquitinylation and proteasomal degradation in high-risk human papilloma virus (HPV)-infected epithelial cells (Nakagawa and Huibregtse, 2000; Massimi *et al.*, 2007). We therefore tested whether proteasomal degradation accounted for the reduction of Scrib protein levels in vimentin knockdown cells. Indeed, in vimentin knockdown cells treated with a proteasome inhibitor, EGFP-hScrib protein remained at similar levels as in control siRNA-treated cells (Figure 7, F and G). Furthermore, although less pronounced, endogenous Scrib protein levels were also reduced in vimentin deficient MDCK cells, and this decrease was blocked in the presence of the proteasome inhibitor (Figure 7H).

To further corroborate the role of proteasomal degradation of Scrib in vimentin knockdown cells, we immunoprecipitated hScrib-EGFP from control or vimentin knockdown MDCK cells and analyzed whether it was ubiquitinylated. Indeed, ubiquitinylated hScrib was readily detected in vimentin siRNA-treated cells (Figure 7I). Furthermore, in the presence of proteasome inhibitor, ubiquitinylation of hScrib was also observed in control cells and this was enhanced in vimentin siRNA-treated cells. Together, these data therefore reveal a role for vimentin in stabilizing Scrib protein by protecting it from proteasomal degradation.

DISCUSSION

Since its discovery as a tumor suppressor in *Drosophila* (Bildler and Perrimon, 2000), Scrib has been attributed roles in an array of polarity-related processes in morphologically and functionally different cells, such as epithelial, neuronal, and T cells (Murdoch *et al.*, 2003; Ludford-Menting *et al.*, 2005; Wada *et al.*, 2005). Recently, mammalian Scrib has been implicated in cell–cell adhesion and polarized migration in

various cell types (Qin *et al.*, 2005; Osmani *et al.*, 2006; Dow *et al.*, 2007; Nola *et al.*, 2008). Similarly, IFs such as vimentin and keratins are known to play nonmechanical roles in protein trafficking and signaling (reviewed in Ivaska *et al.*, 2007; Magin *et al.*, 2007; Oriolo *et al.*, 2007), which in turn influence cellular processes such as cell adhesion and polarization. Here, we report the identification and functional characterization of an interaction between Scrib and IFs. We show that Scrib can colocalize with IFs and bind the IF components vimentin and keratin 18. Silencing of Scrib, vimentin, or both affects different cellular functions associated with epithelial polarization, including anterior–posterior cell polarization, wound healing, directed migration, and cell aggregation. In the absence of vimentin, Scrib is subject to increased proteasomal degradation, implicating that the interaction with the IF cytoskeleton is important to stabilize Scrib protein levels required for directed migration and cell–cell adhesion. Although we provide evidence based on colocalization and coprecipitation experiments to support an interaction of Scrib with keratin 18, the biochemical and functional details of this association with keratin IFs will require additional work. The discussion will thus focus on the role of vimentin in Scrib function.

Endogenous Scrib localizes as punctate structures that line up with vimentin-positive filaments emanating from the perinuclear region and with vimentin bundles spanning the cytoplasm in both sparse HUVECs and MDCK cell cultures. In MDCK cells forming nascent cell–cell contacts, Scrib membrane staining colocalizes with vimentin filaments at the cell periphery. In spreading HUVECs, both Scrib and vimentin coincide at membrane protrusions. Confluent cultures of both cell lines exhibit vimentin colocalization with Scrib at the plasma membrane periphery. In overexpressing MDCK cells, the filamentous localization of exogenous EGFP-hScrib is most prominent in sparse cell cultures, where hScrib shows an extensive filamentous localization. The filamentous labeling of hScrib partially colocalizes with vimentin of IFs, but not with the actin or microtubule cytoskeleton. Interestingly, during establishment of cell–cell contacts and apical–basal cell polarization, exogenous EGFP-hScrib redistributes from a predominant filamentous to a mainly plasma membrane localization. This redistribution is observed both in cells grown on glass coverslips and polarized monolayers grown on permeable supports. On coverslips, hScrib is often concentrated in the vicinity of the plasma membrane, where there is partial overlap with IFs. In fully polarized MDCK cell monolayers, hScrib is found along the length of the lateral membrane, whereas vimentin accumulates on the apical-most end of the lateral membrane (Oriolo *et al.*, 2007), in which it shows limited overlap with hScrib. The mechanism by which hScrib redistributes during establishment of cell–cell contact is not known, but live cell videomicroscopy provided no evidence for motility of EGFP-hScrib along IFs (data not shown).

Both exogenously expressed and endogenous Scrib binds to vimentin, both in sparse and confluent MDCK cell cultures. This association between endogenous Scrib and vimentin was also observed in HUVEC cultures and in several other cell lines, including MCF10A, HeLa, and COS-1. Because *in vitro*-translated Scrib specifically binds purified vimentin, the interaction is likely direct. Scrib was found to associate with both nonpolymerized and polymerized vimentin. However, it is unclear whether Scrib interacts with vimentin monomers, because purified nonpolymerized vimentin generally contains low molecular oligomers (Herrmann and Aeby, 2004). The N-terminal part of Scrib containing the LRR domain shows little if any binding to vimentin.

Figure 7 (cont). treated with vimentin siRNA and subsequently, a proteasome inhibitor for 0 h (a and b) or 9 h (c and d) and EGFP-hScrib (a and c; green) and vimentin (b and d; red) expression was visualized by fluorescence microscopy. (H) Western blot for endogenous Scrib. MDCK cells were treated with vimentin (+) or nontargeting (–) siRNA and subsequently in the presence of a proteasome inhibitor for 0, 3, 6, or 9 h. Endogenous levels of canine Scrib and vimentin were then analyzed by Western blot. GAPDH served as a control for equal lysate input. (I) hScrib-EGFP of nontargeting or vimentin siRNA-treated MDCK cells in the 9 h presence (+) or absence (–) of proteasome inhibitor was immunoprecipitated and ubiquitinylated hScrib detected by Western blot. Normal IgG served as a negative control.

In contrast, the C-terminal region containing the four PDZ domains binds vimentin, albeit less efficiently than full-length Scrib. A similar behavior was observed for ZO-2, which also binds to the PDZ domains of Scrib (Métais *et al.*, 2005), and may reflect a role for the N terminus of Scrib on the conformation or accessibility of the PDZ domains. In accordance with the binding data, only constructs containing the PDZ domains showed filamentous localization. Analysis of individual PDZ domains revealed an efficient binding of vimentin to PDZ3, less efficient associations with PDZ1 and PDZ2, and no detectable interaction with PDZ4, consistent with an interaction with the PDZ domains themselves as opposed to intervening sequences. The interaction of *in vitro* translated vimentin with PDZ3 also corroborates the interaction data for the *in vitro* translated Scrib and purified vimentin, indicating that this association is direct. Because vimentin does not encode a typical C-terminal PDZ-binding motif, the association could be mediated by an internal loop in vimentin. Such a mode of interaction is not uncommon and has been established for several PDZ-domain proteins, including ZO-1 (Harris and Lim, 2001; Utepergenov *et al.*, 2006). Moreover, TBEV NS5 has been reported to bind PDZ4 of Scrib via an internal binding site (Werme *et al.*, 2008).

Several lines of evidence support the notion that the interaction with IFs stabilizes Scrib by protecting it from proteasomal degradation. First, silencing of vimentin expression leads to reduced Scrib protein levels. Second, the extent of the decrease in Scrib protein levels correlates with the extent of vimentin knock-down. This stabilizing function of vimentin is observed both in sparse and confluent MDCK cells, as well as in other cell lines. Third, only Scrib constructs that contain the PDZ domains are affected if vimentin is depleted, showing that the interaction with vimentin is important for protection from degradation. Fourth, Scrib protein levels remain high in vimentin siRNA treated cells in the presence of a proteasome inhibitor. Fifth, Scrib proteins levels are increased in cells overexpressing vimentin. Finally, in vimentin siRNA-treated cells, ubiquitinated Scrib can be detected, and its levels are increased in control and vimentin siRNA-treated cells in the presence of a proteasome inhibitor. Importantly, both overexpressed as well as endogenous Scrib show an enhanced turnover if vimentin expression is silenced. In MDCK, HeLa, and MCF10A cells, the simultaneous silencing of vimentin and keratin 18 lead to a larger reduction in Scrib levels, consistent with a contribution of both types of IFs in stabilizing Scrib.

Reduction of endogenous Scrib protein levels due to the treatment of cells with vimentin siRNA results in a similar phenotype as observed in Scrib knockdown cells. Indeed, several well-established effects linked to reduced Scrib protein levels were phenocopied in cells where vimentin was silenced. The role of Scrib in anterior-posterior cell polarization during migration has been extensively characterized. In wound healing assays, MDCK cells migrate as a sheet to close the wound. Cells at the leading edge polarize their MTOC and Golgi apparatus in the plane of migration (Kupfer *et al.*, 1982). As reported previously (Osmani *et al.*, 2006; Dow *et al.*, 2007), silencing of Scrib abolished the reorientation of the Golgi complex, and this was also observed in cells treated with vimentin siRNA. Furthermore, wound closure was slower in both Scrib and vimentin knockdown cells. Live imaging and computation of tortuosity indexes showed a more randomized migration for both knockdown cells. Loss of directionality likely accounts for the slower wound closure because velocity of migration was not significantly affected (data not shown). The slower clo-

sure of wounded Scrib knockdown cell monolayers in our study contrasts with a previous report also using MDCK cells (Qin *et al.*, 2005), but it is consistent with the delayed migration of MCF10A cells upon Scrib silencing and the delayed wound closure in mice lacking Scrib (Dow *et al.*, 2007). In agreement with (Qin *et al.*, 2005), we observed a defect in cell-cell aggregation of Scrib knockdown cells, and this was also the case for cells exposed to vimentin siRNA. The concomitant silencing of both Scrib and vimentin showed no synergistic effect on polarization, directionality of migration or aggregation, consistent with the notion that the effect of suppressing vimentin expression reflects to a significant extent the concomitant reduction in Scrib protein levels below a critical threshold.

The phenotypic convergence of Scrib and vimentin silencing in MDCK is not surprising considering the role of vimentin on Scrib protein stability. This relationship with Scrib is also reflected in the similar functions of vimentin in cell migration and adhesion reported previously. In the event of epithelial-mesenchymal transitions (Thiery and Sleeman, 2006), epithelial cells acquire mesenchymal characteristics, including increased cell motility, that are correlated with the upregulation of vimentin expression (Lee *et al.*, 2006). This correlation has been demonstrated in migrating epithelial MCF10A cells, where vimentin is transiently and exclusively expressed in actively migrating cells at the wound edge, where it positively regulates migration (Gilles *et al.*, 1999). Furthermore, fibroblasts of vimentin null mice exhibited defective wound healing due to reduced cell migration (Eckes *et al.*, 1998, 2000) and in a pathological context, expression of vimentin promoted cell migration and invasion in breast, colon and prostate carcinomas (McInroy and Määttä, 2007; Zhao *et al.*, 2008). The function of vimentin in cell migration and adhesion has also been reported in the transendothelial adhesion and extravasation of leukocytes. Here, the reorganization and polarization of vimentin in both the receiving endothelial sheets and the migrating lymphocytes positively regulates the protein levels and defined surface expression of cell adhesion molecules and integrins on the respective cell types (Nieminen *et al.*, 2006). Incidentally, vimentin is polarized in the uropod of lymphocytes similarly to Scrib in T cells (Ludford-Menting *et al.*, 2005; Nieminen *et al.*, 2006).

Our findings that Scrib associates with IFs and that this association stabilizes Scrib by sequestering it from proteasomal degradation expands the list of proteins whose stability, localization or function are regulated by IFs. Vimentin sequesters soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAP) 23 and creates a mobilizable reservoir of SNAP23 for target membrane-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor function (Faigle *et al.*, 2000). 14-3-3 and tumor necrosis factor receptor death domain-associated proteins are sequestered by either vimentin and/or keratins to regulate cellular processes like cell growth and apoptosis (Kim and Coulombe, 2007). Activated mitogen-activated protein kinase is protected from phosphatases and transported by vimentin during nerve injury (Perlson *et al.*, 2005). Brush-border localization of ezrin in intestinal epithelial cells is dependent on a transient interaction with keratins (Wald *et al.*, 2005). More recently, the stability and localization of Albatross, a polarity protein that in conjunction with Par3 regulates cell adhesion complexes, was shown to depend on its interaction with keratin IFs (Sugimoto *et al.*, 2008). Interestingly, Scrib is also sequestered in HTLV-1 virus infected T cells through an interaction with the viral Tax protein (Arpin-Andre and Mesnard, 2007).

In conclusion, we propose a working model in which the interaction of Scrib with vimentin IFs occurs during remodeling of the plasma membrane during EMT, cell migration and cell-cell contact maturation, as well as in confluent epithelial cell monolayers. Although it is well established that junctional and polarity proteins are in a dynamic flux during cell migration (Matsuda *et al.*, 2004; Drees *et al.*, 2005; Thiery and Sleeman, 2006), only recent evidence suggests that even in confluent epithelial cell monolayers, these proteins may also be in a dynamic equilibrium between cytoplasmic and membrane-associated pools (Drees *et al.*, 2005; Shen *et al.*, 2008). In high-risk HPV E6-containing epithelial cells, soluble Scrib is degraded via the proteasome, whereas the insoluble pool remains largely protected (Massimi *et al.*, 2004). We hypothesize that Scrib present in such a soluble pool interacts with the IF networks, preventing its degradation as part of the machinery that regulates the natural homeostatic turnover of Scrib. In addition, however, IFs may also provide a reservoir for dynamic exchange of Scrib with the plasma membrane in polarized cells, or to enlarge the nonmembrane bound fraction of Scrib during directed migration. As discussed above, similar roles for IFs are not unprecedented and have been postulated for the interaction of SNAP23 with vimentin (Faigle *et al.*, 2000) or ezrin and Albatross with keratins (Wald *et al.*, 2005; Sugimoto *et al.*, 2008), respectively. This interpretation would be consistent with the observation that silencing of vimentin affects the known functions of Scrib in cell polarization, directed migration, and cell-cell adhesion. It will now be of interest to elucidate the molecular mechanisms that regulate Scrib turnover and homeostasis.

ACKNOWLEDGMENTS

We thank Ronald Liem, Rudolf Leube, Sachdev Sidhu, and Birgit Lane for kindly providing reagents and Lynette Chen for help with cell sorting. This work was supported by the Agency for Science, Technology and Research (A*STAR), Singapore. W. H. is an adjunct faculty member at the Department of Physiology, National University of Singapore, and P.O.H. is an RD Wright Research Fellow of the Australian National Health and Medical Research Council.

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