The mammalian Scribble polarity protein regulates epithelial cell adhesion and migration through E-cadherin

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Scribble (Scrib) is a conserved polarity protein required in *Drosophila melanogaster* for synaptic function, neuroblast differentiation, and epithelial polarization. It is also a tumor suppressor. In rodents, Scrib has been implicated in receptor recycling and planar polarity but not in apical/basal polarity. We now show that knockdown of Scrib disrupts adhesion between Madin–Darby canine kidney epithelial cells. As a consequence, the cells acquire a mesenchymal appearance, migrate more rapidly, and lose directionality. Although tight junction assembly is delayed, confluent monolayers remain polarized. These effects are independent of Rac activation or Scrib binding to βPIX. Rather, Scrib depletion disrupts E-cadherin–mediated cell–cell adhesion. The changes in morphology and migration are phenocopied by E-cadherin knockdown. Adhesion is partially rescued by expression of an E-cadherin–α-catenin fusion protein but not by E-cadherin–green fluorescent protein. These results suggest that Scrib stabilizes the coupling between E-cadherin and the catenins and are consistent with the idea that mammalian Scrib could behave as a tumor suppressor by regulating epithelial cell adhesion and migration.

Introduction

The development of epithelial sheets, which was one of the earliest steps in the evolution of the metazoa, is of fundamental importance in animal development (Schock and Perrimon, 2002; Nelson, 2003; Zegers et al., 2003). Apical/basal polarization of epithelial cells is essential to their function, and the loss of polarity, as occurs during epithelial–mesenchymal transitions (EMTs), has been implicated in tumor progression and metastasis (Thiery, 2003). Genetic screens in model organisms have uncovered several conserved proteins that are required for cell polarization in many different contexts (Kemphues, 2000; Tepass et al., 2001; Macara, 2004).

One group of three such proteins—Scribble (Scrib), Discs large (Dlg), and Lethal giant larvae (Lgl)—identified in *Drosophila melanogaster*, also behave as tumor suppressors (Jacob et al., 1987; Woods and Bryant, 1991; Bilder et al., 2000; Bilder, 2004), and mutations in any of the genes for these proteins cause overgrowth of embryonic tissue, particularly the imaginal disc and brain cells, forming large amorphous masses. Additionally, *Scrib*, *Lgl*, and *Dlg* mutants cooperate with oncogenic *Ras* in the transformation of *D. melanogaster* eye disc cells (Brumby and Richardson, 2003; Pagliarini and Xu, 2003). Expression of activated Ras causes overproliferation, but the cells remain in the epithelial layer. However, in the context of a *Scrib* mutant, the Ras cells become metastatic. They degrade the basement membrane, migrate, and invade neighboring wild-type tissues. The key mechanism underlying this transition is the loss of E-cadherin, a transmembrane protein that forms the adherens junction between epithelial cells and is essential for apical/basal polarization. Forced coexpression of E-cadherin inhibits invasion (Pagliarini and Xu, 2003).

Scrib is required for maintenance of apical/basal polarity in *D. melanogaster* epithelial cells (Bilder et al., 2000) but is also important in synaptic function (Peng et al., 2000) and in neuroblast asymmetric cell divisions (Albertson and Doe, 2003). The molecular basis for loss of polarity in *D. melanogaster* embryos lacking Scrib is not yet entirely understood. However, elegant genetic analyses revealed that in embryonic epithelial cells they are part of a complex network involving multiple polarity proteins (Bilder et al., 2003; Tanentzapf and Tepass, 2003). The Par-3 polarity complex functions in the initial specification of the apical domain, and Scrib apparently helps specify the basolateral surface by repressing the activity of Par-3. The Crumbs polarity complex is recruited to the apical

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Abbreviations used in this paper: EMT, epithelial–mesenchymal transition; ERK, extracellular signal–regulated kinase; HGF, hepatocyte growth factor; KD, knockdown; PDZ, PSD-95, ZO-1, and Discs-large; RNAi, RNA interference; shRNA, small hairpin RNA.

The online version of this article contains supplemental material.

The Journal of Cell Biology, Vol. 171, No. 6, December 19, 2005 1061–1071
http://www.jcb.org/cgi/doi/10.1083/jcb.200506094

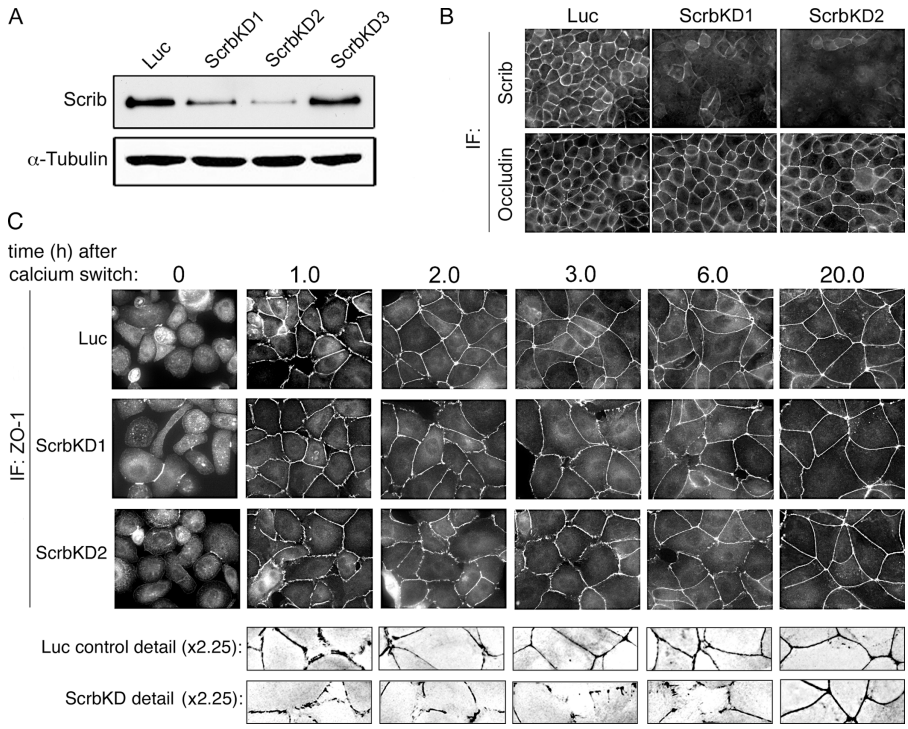

Figure 1. **Suppression of Scrib expression in MDCK cells causes a delay in tight junction assembly.** (A) MDCK II cells were transiently transfected with either pS-Luciferase vector (Luc) as a control or three pSUPER constructs targeting different sequences of canine Scrib mRNA (ScrbKD). Equal amounts of proteins were analyzed by SDS-PAGE and immunoblot. (B) Control and ScrbKD MDCK cells grown at high density were fixed and stained for Scrib and occludin. (C) Control and ScrbKD cells were subjected to a calcium switch and then fixed and stained for ZO-1 at the indicated times after readdition of calcium. Magnified details of the junctions are shown in inverted black-and-white scale to highlight the defects caused by loss of Scrib.

surface by Par-3 and somehow represses Scrib activity. Thus, the balance between these three groups of polarity proteins limits the extent of the apical and basolateral membranes, but the molecular mechanisms by which they do this are still unknown.

The mammalian orthologue of Scrib has not yet been implicated in apical/basal polarization or as a tumor suppressor. Intriguingly, however, it is targeted for destruction by the E6 oncoprotein of human papillomavirus, the major cause of cervical cancer (Nakagawa and Huibregtse, 2000). Moreover, progression of uterine cervical carcinomas from precursor lesions to invasive cancers correlates with a dramatic decrease in Scrib expression (Nakagawa et al., 2004). Unexpectedly, murine Scrib appears to be involved in planar polarity because a mutation that introduces a premature stop codon in the protein causes a defect in the planar polarization of the inner ear epithelium (Montcouquiol et al., 2003; Murdoch et al., 2003). Nonetheless, Scrib is widely expressed and associates with the lateral membranes in epithelial cells through a mechanism that appears to involve E-cadherin (Navarro et al., 2005).

Scrib is a large multidomain protein that contains 16 NH_2-terminal leucine-rich repeats, four PSD-95, ZO-1, and Discs-large (PDZ) domains, and an uncharacterized COOH-terminal region (Humbert et al., 2003; Bilder, 2004). It belongs to a family of so-called LAP (leucine-rich repeats and PDZ) proteins, which includes Erbin and Densin-180, although it remains unclear whether any of these proteins possess related functions. Recently, mammalian Scrib was found to bind through its PDZ domains to the COOH terminus of βPIX, a guanine nucleotide exchange factor for Rac (Audebert et al., 2004). This interaction has been implicated in thyrotropin receptor endocytosis and recycling, but whether it is involved in cell polarity is not known (Lahuna et al., 2005). In *D. melanogaster* it is unlikely that a homologous interaction is important because the PDZ domains of Scrib are dispensable for epithelial polarization and for control of cell proliferation (Albertson et al., 2004; Zeitler et al., 2004). Other binding partners for mammalian Scrib have been reported recently, but their physiological significance remains unclear (Metais et al., 2005; Petit et al., 2005).

Given the paucity of data on the functions of mammalian Scrib and the potential importance of the protein in embryonic development and tumor progression, we investigated the role of Scrib in MDCK epithelial cells by RNA interference (RNAi). Cells lacking Scrib appear relatively normal at high density, when they have formed polarized monolayers, but they exhibit profound defects at lower cell densities. The cells appear to undergo a morphological EMT, migrate more rapidly, and lose directionality during migration. A substantial loss of cell–cell adhesion occurs as a result of reduced E-cadherin activity. Therefore, mammalian Scrib plays a key role in regulating E-cadherin activity, and its loss is predicted to enhance tumor migration and invasion.

Results

Gene silencing of Scrib in MDCK cells

Several target sequences were selected from the partial canine *Scrib* gene and used to construct pSUPER vectors for the expression of small hairpin RNAs (shRNAs). Of the three sequences tested, two efficiently knocked down Scrib expression when expressed by transient transfection in MDCK II cells (Fig. 1 A). Immunofluorescence microscopy revealed Scrib to be associated with the lateral membranes in mammalian epithelial cells, and staining was substantially reduced by transfection with the ScrbKD1 or 2 vectors (Fig. 1 B).

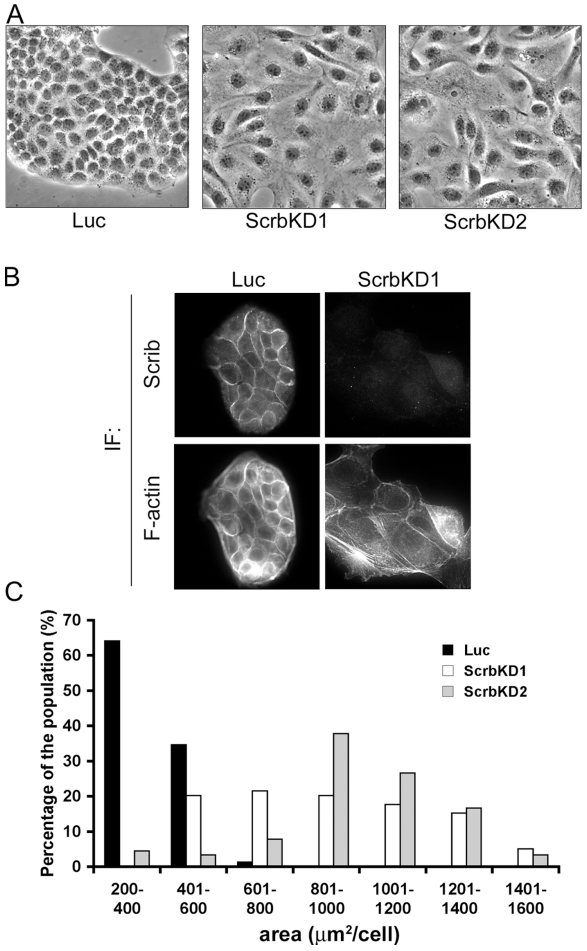

Figure 2. **Cells lacking Scrib lose their epithelial morphology at low density.** (A) Control (Luc) and ScrbKD cells were grown on 6-well plates at low density for 3 d. Images were obtained by phase-contrast microscopy using a 10× objective. (B) Control and ScrbKD cells were fixed and stained with phalloidin to visualize F-actin. Typical colonies are shown. (C) Surface areas per cell (μm^2) were measured for 80–90 frames (as shown in A) and sorted into bins 200 μm^2 wide. The percentage of cells in each bin is shown.

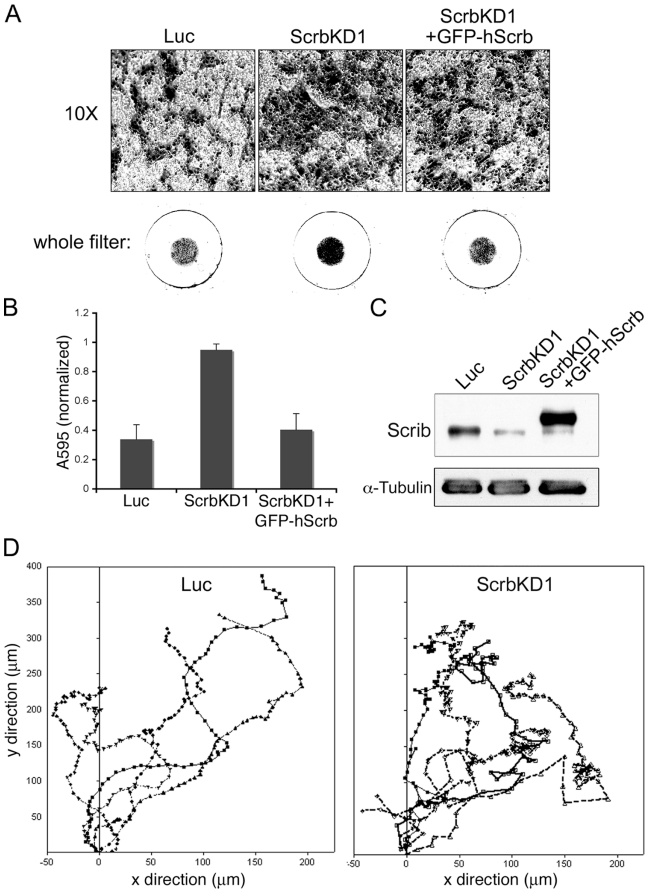

Figure 3. **Suppression of Scrib expression increases cell motility and attenuates orientated migration.** (A) Motility of cells transfected with Luc, ScrbKD1 shRNA, or ScrbKD1 plus GFP-tagged human Scrib was measured using a Boyden chamber assay. Images were captured after crystal violet staining of the cells at the bottom side of the filter. (B) Quantification of cell migration by measuring A595 of eluted crystal violet. Error bars represent mean ± SD. (C) Immunoblot analysis of transfected cells with anti-Scrib antibody. Equal protein concentrations were loaded and normalized using anti-tubulin. (D) Rose plot of individual cell tracks from time-lapse movies of the wounding assay.

Surprisingly, however, the tight junctions appeared to be intact in these cells, as assessed by occludin staining (Fig. 1 B) or ZO-1 staining (not depicted). Moreover, confocal sections of cells stained for the apical marker gp135 revealed no loss of apical/basal polarization in cells expressing reduced Scrib levels (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200506094/DC1), and cysts grown in Matrigel appeared to be polarized normally (not depicted). These results suggest that depletion of Scrib does not disrupt tight junction assembly. However, when the transfected cells were subjected to a calcium switch and stained for ZO-1, a short delay in junction assembly was observed (Fig. 1 C). ZO-1 accumulated rapidly at the cell–cell contacts in both the control and Scrib knockdown (KD) cells but, in the absence of Scrib, the fusion of the ZO-1 lines into a continuous band encircling each cell was delayed. The defect was particularly noticeable at vertices where several cell boundaries meet. By 20 h after calcium switch, however, the ZO-1 staining in the cells lacking Scrib was indistinguishable from that in the control cells.

Scrib is required for maintenance of an epithelial phenotype at low cell densities

When cells were transfected with pS-ScrbKD vectors and plated at low densities, they consistently displayed a mesenchymal phenotype. Normal MDCK cells organize into discrete, tight islands with smooth boundaries, but cells lacking Scrib appeared more fibroblastic (Fig. 2, A and B). They spread over a much larger surface area (approximately three to five times larger; Fig. 2 C), and the edges of the cell clusters were disorganized as though the cells were moving apart from one another. When stained with phalloidin, the normal cortical actin rings were absent in cells lacking SCRIB and were replaced by stress fibers often oriented along the long axis of the cells (Fig. 2 B). These observations suggested that Scrib might regulate epithelial cell adhesion and/or migration.

Scrib inhibits cell motility and is required for oriented migration

To determine whether Scrib regulates MDCK cell motility, transfected cells were plated onto 8-μm filters in Boyden

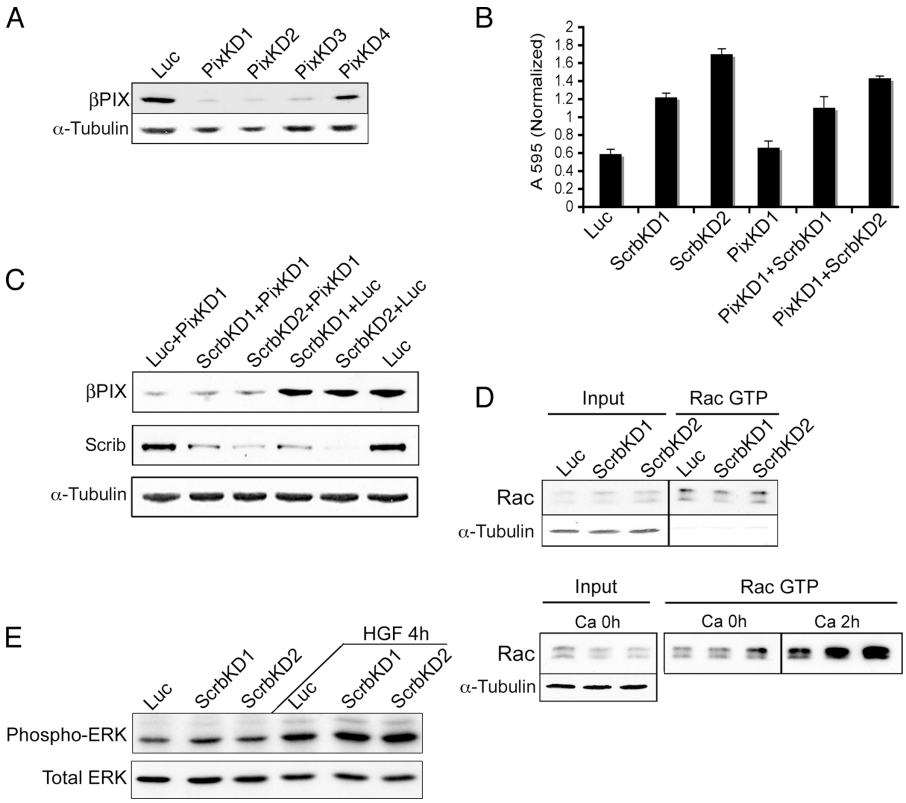

Figure 4. **βPIX is not involved in Scrib function at adherens junctions.** (A) MDCK II cells were transiently transfected with either pS-Luciferase control vector (Luc) or three pSUPER constructs targeting different sequences of canine βPIX mRNA (PixKD). Proteins were analyzed by SDS-PAGE and immunoblot. (B) Quantification of Boyden chamber migration assay with Luc, ScrbKD, and ScrbKD plus PixKD cells. Error bars represent mean ± SD. (C) Immunoblot showing Scrib single KD and Scrib and βPIX double KD. (D) Rac pull-down assays with control and ScrbKD cells in both normal medium (top) and low-calcium medium (bottom). (E) Immunoblot analysis of phospho-ERK in control and ScrbKD cells with and without stimulation by 15 ng/ml HGF.

chambers and incubated in normal medium containing 10% serum (both above and below the filter). The same number of cells was plated onto each filter. After 16–20 h, cells that had migrated through the pores to the bottom surface of the filters were stained with crystal violet. Loss of Scrib substantially increased the number of cells that had migrated through the filter (Fig. 3, A and B). Importantly, when we expressed a GFP fusion of a human Scrib in the cells transfected with the pS-ScrbKD1 vector, the number of cells migrating through the filter was reduced to control levels (Fig. 3, A and B). It was conceivable that the reversion to a wild-type phenotype was caused by reexpression of the endogenous protein. To test for this possibility, we blotted cell lysates for Scrib (Fig. 3 C). The endogenous Scrib protein level was reduced upon expression of the ScrbKD1 shRNA and did not respond to coexpression of the human GFP–Scrib fusion. The GFP–Scrib fusion can be distinguished by its lower mobility on SDS-PAGE and was expressed at approximately two to three times the level of the endogenous protein in control cells (Fig. 3 C). These data prove that the effects of Scrib RNAi on motility are indeed caused by loss of the Scrib protein rather than by off-target effects of the shRNA.

The filter assay depends not only on cell motility but also on the rate of cell attachment to and spreading on the filter surface. Therefore, as an alternative approach to measuring cell motility, we performed wounding assays on MDCK monolayers and tracked the movement of individual cells within the population at the wound edge by time-lapse microscopy. The overall rate of wound closure was similar for both the control cells and those lacking Scrib (0.35 vs. 0.32 μm/min). However, the behavior of cells lacking Scrib was remarkably different from that of the control (Videos 1 and 2, available at http://www.jcb.org/cgi/content/full/jcb.200506094/DC1). At the edges of the wound, control cells extruded lamellipodia and moved forward as an organized sheet, but the KD cells were less organized. Some of the KD cells lost their attachment to the sheet, pulled away from the leading edge, and moved in random directions. This difference can be seen in the Rose plots of cells tracked over the period of the assay (Fig. 3 D). Calculation of directionality parameters confirmed that the cells lacking Scrib move at a significantly higher speed than control cells (2.54 ± 0.19 vs. 1.73 ± 0.41 μm/min; $P = 0.026$) and with a lower persistence coefficient (4.3 vs. 11.6 min; $P = 0.02$). The reduced persistence accounts for the similarity in the overall rate of wound closure. Interestingly, cells further back from the wound exhibited a continual jiggling motion, as if they had lost adhesion to their neighbors and were trying to move away from one another, and transient gaps appeared in the monolayer (Video 2). These results support the data shown in Fig. 2, suggesting that loss of Scrib causes a defect in cell–cell adhesion.

Scrib effects are not mediated by βPIX binding

Cell movement is regulated by Rac, and this GTPase has also been implicated in controlling adhesion between epithelial cells (Ehrlich et al., 2002; Van Aelst and Symons, 2002; Chu et al., 2004). Interestingly, Scrib has been reported to bind, via its PDZ domains, to the COOH terminus of the Rac guanine nucleotide exchange factor, βPIX (Audebert et al., 2004). To determine whether the SCRIB–βPIX interaction is important in

mediating the regulation of cell migration and adhesion, we first confirmed that in MDCK cells we could detect this interaction (unpublished data) and then assessed the role of βPIX by silencing expression of the canine protein in MDCK cells.

Of four pSUPER constructs tested, three efficiently suppressed βPIX expression (Fig. 4 A). In particular, the PixKD1 shRNA reduced expression of the protein by >90%. However, loss of βPIX had no detectable effect on cell migration as measured using the Boyden chamber assay (Fig. 4 B). Moreover, in our hands, overexpression of βPIX did not increase cell migration in the filter assay (unpublished data). We then performed double KD experiments in which the expression of both Scrib and βPIX was suppressed (Fig. 4 C). We reasoned that one function of Scrib might be to sequester and inactivate βPIX. In this case, loss of Scrib would release the βPIX, leading to inappropriate activation of Rac and increased migration. If this hypothesis were correct, a double KD would reverse the migration phenotype by removing the excess free βPIX from the cell. As depicted in Fig. 3 (A and B), migration through filters was increased by Scrib KD. However, the coordinate loss of βPIX did not significantly perturb this effect (Fig. 4 B). Note that cotransfection of the PixKD shRNA did not interfere with gene silencing of Scrib (Fig. 4 C). We therefore conclude that Scrib function in cell adhesion and migration is independent of βPIX binding.

Because βPIX is a guanine nucleotide exchange factor for Rac, we also asked whether loss of Scrib would alter Rac activity. Rac-GTP was detected by pull-down assays using a GST fusion of the Rac binding domain of PAK. No consistent differences in Rac-GTP were detected, however, in control cells versus those lacking Scrib (Fig. 4 D). When the cells were subjected to a calcium switch, Rac was activated within 2 h of calcium addition in both the control and Scrib KD cells (Fig. 4 E). We therefore conclude that the polarity defects associated with suppression of Scrib expression are independent of βPIX and are not mediated through the Rac GTPase. Finally, we asked whether Scrib might regulate the extracellular signal–regulated kinase (ERK) signaling pathway, which is activated by scatter factor (hepatocyte growth factor [HGF]; Tanimura et al., 1998). HGF induces an EMT in which cells lose adhesiveness and become more migratory, a phenotype similar to that observed in cells lacking SCRIB. However, no significant differences in phospho-ERK were detected when Scrib expression was knocked down either before or after addition of HGF (Fig. 4 E). These data suggest that Scrib does not function to regulate the HGF signaling pathway.

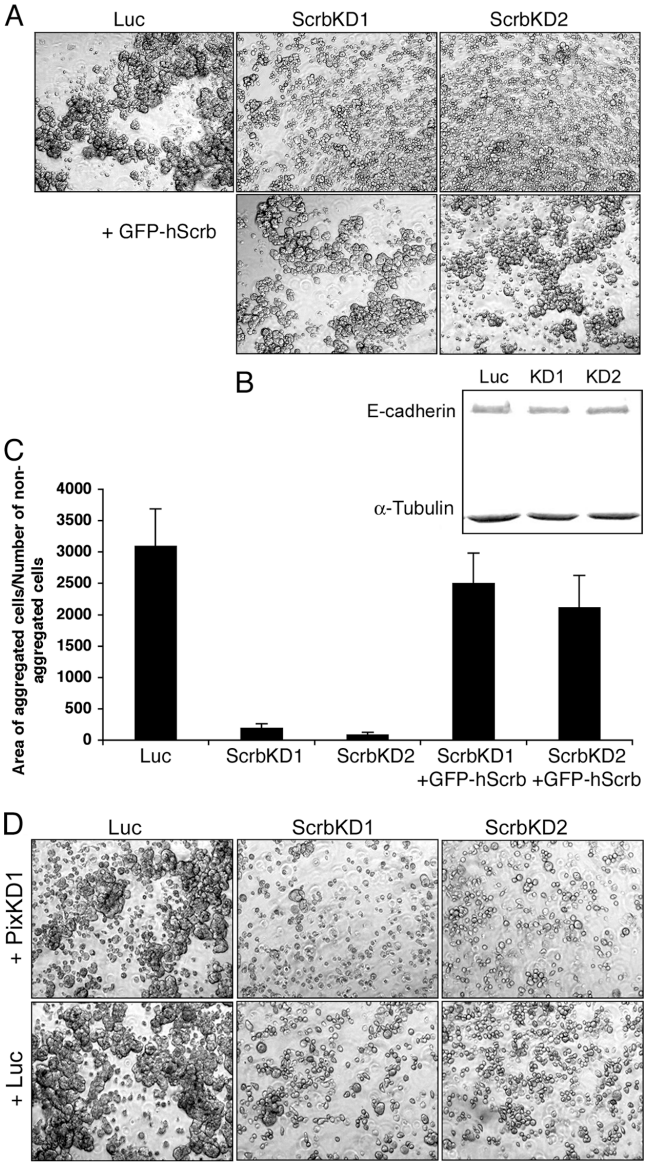

Figure 5. **Decreased adhesiveness of cells lacking Scrib.** (A) 3×10^4 control and ScrbKD cells were seeded into hanging drop cultures and allowed to aggregate overnight. After trituration by passing the cell cluster 10 times through a 200-μl pipette tip, images were captured by phase-contrast microscopy using a 10× objective. (B) E-cadherin is not preferentially lost from the ScrbKD cells in suspension culture. (C) Quantification of the degree of aggregation shown in A. Data are presented as the area of the aggregated cells/number of individual nonaggregated cells and represent means of 10–12 images from triplicates of each sample ± SD. (D) Effect of βPIX silencing on cell aggregation.

Scrib is required for E-cadherin–mediated adhesion

To determine whether cell–cell adhesion is compromised in the absence of Scrib, we first used an aggregation assay. Cells were trypsinized, triturated to break up clumps into individual cells, resuspended in fresh medium in a hanging drop beneath the lid of a tissue culture plate, and incubated for 18–20 h. Cell aggregation was then assessed microscopically. A dramatic loss of aggregation was apparent in cells expressing either pS-ScrbKD1 or 2 vectors, as compared with the control cells that were transfected with pS-Luc (Fig. 5, A and C). This effect was not a result of differential loss of E-cadherin in the cell suspensions as assessed by immunoblotting lysates from the suspended cell cultures (Fig. 5 B). Importantly, coexpression of human GFP-Scrib reversed the adhesion defect caused by the loss of endogenous SCRIB, proving that the effect of the shRNAs on adhesion is specifically mediated through destruction of the Scrib mRNA rather than through off-target effects (Fig. 5, A and C). We also tested for a possible role for βPIX on aggregation, using an shRNA directed against the canine gene (Fig. 4, A and B). However, loss of βPIX from the cells had no effect on

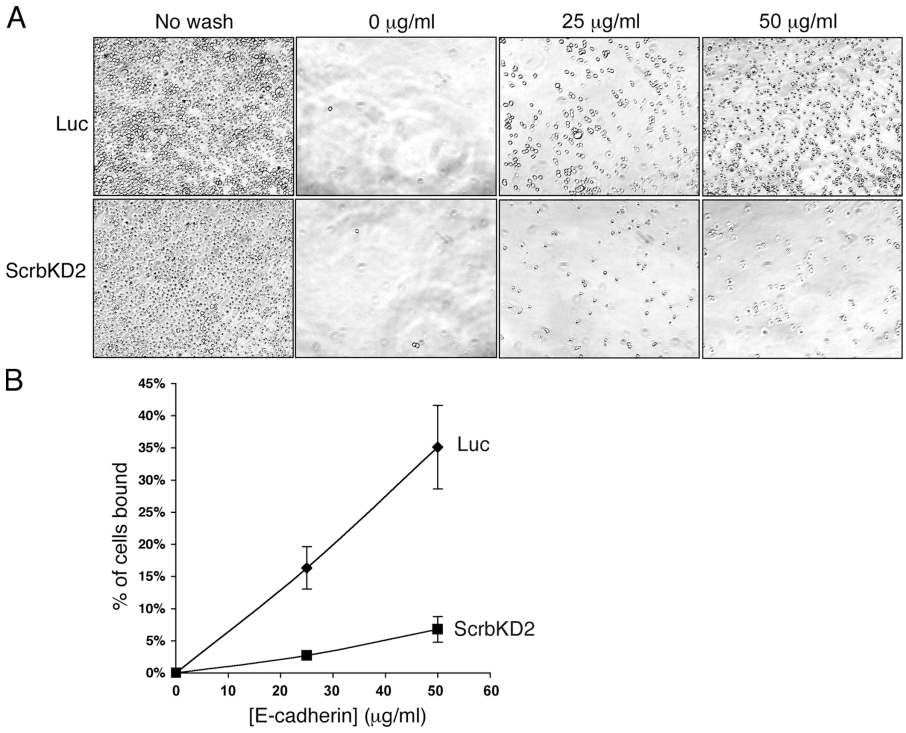

Figure 6. **Suppression of Scrib expression reduces E-cadherin–mediated adhesion.** (A) In vitro E-cadherin binding assay. Different concentrations of recombinant E-cadherin extracellular domain were coated on plates. Control (Luc) and ScrbKD cells were collected in suspension and allowed to adhere to the plates for 60 min. Images were captured for nonwashed plates (to give total cell numbers), and plates were subjected to washing (to determine attached cell numbers). (B) Quantification of cell attachment to E-cadherin ectodomain. Data represent means of 10–12 images from triplicates of each condition ± SD.

the aggregation of control cells and did not reverse the loss of aggregation observed in the absence of Scrib (Fig. 5 D).

To determine whether the aggregation defect is mediated through E-cadherin or some other cell adhesion protein, we assayed the ability of the MDCK cells to attach to a surface coated with the extracellular domain of E-cadherin. Cells were disassociated using an EGTA solution (with no trypsin), centrifuged and resuspended in fresh medium, and added to 96-well plates coated with the ectodomain of E-cadherin. After 60 min, the plates were washed and remaining attached cells were counted. Results are shown in Fig. 6. Almost no cells attached to the plates in the absence of the E-cadherin ectodomain, demonstrating that during the 60-min incubation period integrin-mediated attachment is negligible. Control cells attached efficiently, and attachment was proportional to the amount of E-cadherin ectodomain on the plate (Fig. 6 B). Importantly, loss of Scrib caused a substantial drop (approximately threefold) in cell attachment, demonstrating that E-cadherin homophilic adhesion is compromised in the absence of Scrib. Addition of an arginine–glycine–aspartic acid peptide to block integrin-mediated adhesion had no significant effect (unpublished data).

Depletion of Scrib causes a defect in cell–cell adhesive junctions

These data suggest that Scrib is required for normal E-cadherin function at cell–cell junctions. We therefore examined the distribution of E-cadherin and of the Na/K-ATPase, which is a marker for the basolateral membrane. In control cells, these proteins colocalize along the lateral cell boundaries. Scrib KD caused a distinctive phenotype in which the lateral membranes of the cells became disorganized. The membranes appeared less vertical and had convoluted edges (Fig. 7 A). A similar phenotype was observed for β-catenin distribution (Fig. 7 B). However, the total amounts of E-cadherin, β-catenin, and α-catenin expressed in cells depleted of Scrib were the same as the amounts in control cells (Fig. 7 C). Scrib does not, therefore, regulate the expression of these junctional proteins. Moreover, when surface proteins were biotinylated, captured on streptavidin beads, and blotted for E-cadherin, no reproducible difference was observed between the control and Scrib KD cells (Fig. 7 D). These data demonstrate that there is no change in the amount of E-cadherin on the cell surface, and we conclude that Scrib is not involved in controlling the exocytosis or endocytosis of E-cadherin.

When adherens junctions form, a fraction of the α- and β-catenin becomes stabilized at the cell cortex, either through clustering of the E-cadherin or perhaps through attachment to actin, and is detergent insoluble. We measured the detergent-insoluble fraction in control and Scrib KD cells and found that in the absence of Scrib the amounts of both α- and β-catenin in this fraction were substantially reduced (Fig. 7, E and F). Together, these results suggest that Scrib is required for the normal stabilization of α- and β-catenin at the cell cortex.

Depletion of E-cadherin phenocopies the effects of Scrib KD

If both the adhesion defect and the increased motility observed in response to Scrib silencing are caused by decreased E-cadherin activity, one would predict that depletion of E-cadherin would produce the same phenotype. We therefore expressed a shRNA targeted against the canine E-cadherin in MDCK cells and achieved a >50% reduction in E-cadherin expression (Fig. 8 A). Interestingly, cells depleted of E-cadherin migrated through filters significantly faster than the control (Fig. 8 B). Moreover, these cells were larger and more fibroblastic in appearance than control cells when plated at low densities (Fig. 8 C),

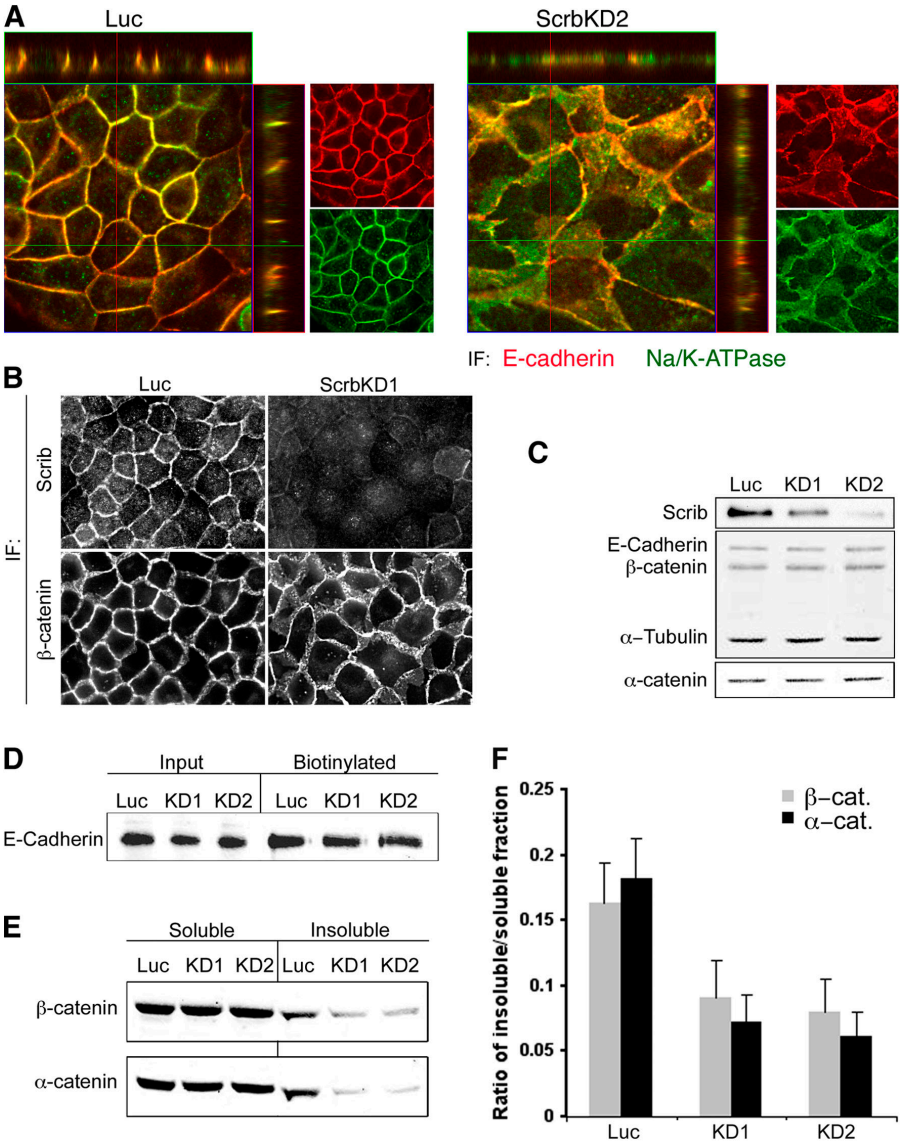

Figure 7. **Loss of Scrib causes a defect in adherens junction structure.** (A) Control and Scrib-depleted cells were plated at subconfluence and allowed to form islands of cells. They were then fixed and stained for E-cadherin (red) and Na/K-ATPase (green). Image stacks were collected using confocal microscopy with 0.95-μm z steps. (B) Control and ScrbKD cells were fixed and stained for Scrib and β-catenin. (C) Immunoblot of junctional proteins in control and ScrbKD cells. Whole cell lysates were blotted for E-cadherin, α- and β-catenin, and α-tubulin. (D) Cells were treated with a nonpermeable biotin linker, sulfo-NHS-SS-biotin, and biotinylated proteins were captured onto streptavidin-agarose and detected by immunoblot. (E) Control and ScrbKD cells plated at low density were lysed with buffer containing 0.5% Triton X-100. After centrifugation, supernatants and pellets were resolved by SDS-PAGE. Distributions of α- and β-catenin were detected with immunoblot. 25% of the soluble and 50% of the insoluble fractions are shown. (F) Quantification by Odyssey image system. Data are presented as the ratio of insoluble to soluble fractions. Error bars represent the SD from four independent experiments.

just as observed for the Scrib KD cells. Based on these data, we conclude that both the morphological changes and increased motility in cells depleted of Scrib can be ascribed to a failure of the E-cadherin to form normal trans-adhesive interactions.

An E-cadherin–α-catenin fusion protein can reverse the effects of silencing Scrib expression

To determine the locus of action of Scrib, we attempted to reverse the effects of Scrib depletion by the ectopic expression either of a cadherin–GFP fusion (Ecad–GFP) or of a cadherin–α-catenin fusion protein (Ecad–αcat). This latter construct lacks β-catenin binding sites but can connect to the actin cytoskeleton through the COOH-terminal domain of the α-catenin and can promote homophilic adhesion (Nagafuchi et al., 1994; Gottardi et al., 2001). The Ecad–GFP has been shown previously to be fully functional (Adams et al., 1998).

Both constructs were expressed only at very low levels compared with the level of endogenous E-cadherin (Fig. 8 D). Nonetheless, the Ecad–αcat fusion was partially able to reverse the increase in migratory behavior of the cells depleted of Scrib (Fig. 8 E). Importantly, however, a similar level of Ecad–GFP was unable to reduce migration of these cells. Next, using an aggregation assay, we asked whether the fusion proteins could also reverse the adhesion defect in the Scrib KD cells. Again, the Ecad–αcat provided a partial restoration of cell–cell adhesion, whereas the Ecad–GFP fusion did not (Fig. 8, F and G). The Ecad–αcat fusion did not appear to increase aggregation of control cells, although a small effect would not have been detectable in this assay. Therefore, the forced, constitutive linkage of E-cadherin to α-catenin can restore normal adhesive and migratory behavior on cells in which Scrib expression has been reduced, suggesting that Scrib acts to modulate this linkage.

Discussion

Polarization is a fundamental aspect of metazoan development, and a core set of proteins is required for the polarization of cells in many different developmental contexts (Macara, 2004). These proteins appear to execute conserved functions during

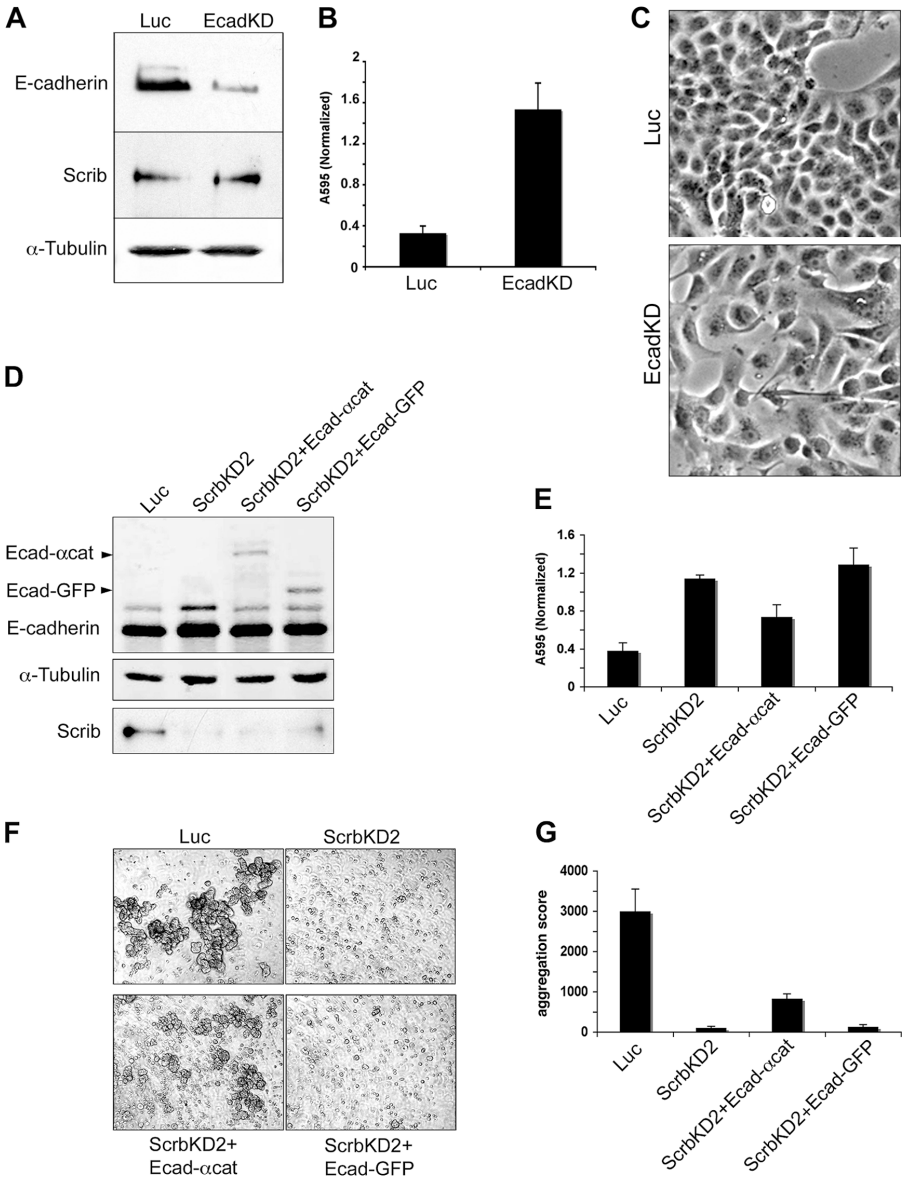

Figure 8. **Effects of E-cadherin depletion and expression of an E-cadherin–α-catenin fusion protein.** (A) Cells were transfected with an shRNA targeted against canine E-cadherin. Lysates were immunoblotted for E-cadherin and Scrib. Equal protein concentrations were loaded and normalized using anti-tubulin. (B) Quantification of the motility of cells transfected with Luc or EcadKD shRNAs using Boyden chamber assay as described in Fig. 3. (C) Morphology of cells depleted of E-cadherin. Control and EcadKD cells were grown on six-well plates at low density for 3 d. Images were obtained by phase-contrast microscopy using a 10× objective. (D) Expression of an Ecad–αcat and Ecad–GFP fusion in MDCK cells. The fusion proteins and endogenous E-cadherin were detected by immunoblot with anti–E-cadherin antibodies. (E) Effects of the Ecad–αcat and Ecad–GFP fusions on motility of cells depleted of Scrib. Migration through filters was quantified as in Fig. 3. (F) Aggregation assay. 3×10^4 cells were seeded into hanging drop cultures and allowed to aggregate for overnight. After trituration with a 200-μl pipet tip, images were captured by phase-contrast microscopy using a 10× objective. (G) Quantification of the aggregation assay. Data are presented as the area of the aggregated cells/number of individual nonaggregated cells per field and represent means of 10–12 fields from triplicates of each sample ± SD.

polarization, but they also possess tissue- and organism-specific functions. For example, the Par-3 polarity protein interacts with Par-6 in worms, flies, and vertebrates, but its association with the Rac exchange factor Tiam1 might be vertebrate specific (Chen and Macara, 2005). In *D. melanogaster*, Scrib is a tumor suppressor that is essential for apical/basal polarization of epithelial cells and neuroblasts, but in mice it has been implicated in planar polarity of the inner ear epithelium and has not so far been linked to epithelial cell or neuroblast apical/basal differentiation or to neoplastic transformation (Humbert et al., 2003; Bilder, 2004).

We have now found that mammalian Scrib is a key regulator of E-cadherin adhesive activity in MDCK epithelial cells. E-cadherin is an essential component of the adherens junction and is required both for adhesive contacts between epithelial cells and, in vertebrates, for the assembly of tight junctions (Gumbiner, 2000, 2005). Initial interactions between E-cadherin on adjacent cells form rapidly and are independent both of the cytoplasmic domain of E-cadherin and of the actin cytoskeleton (Chu et al., 2004). Over time, however, the adhesive force between cells increases as the cytoplasmic domain of E-cadherin clusters and attaches to the actin cytoskeleton via α- and β-catenin (Yap et al., 1998; Chu et al., 2004).

E-cadherin also possesses the characteristics of a tumor suppressor. For example, it is down-regulated in many carcinomas, heterozygosity in the *E-cadherin* gene increases the risk for diffuse gastric cancer, and mutations in *E-cadherin* are present in many types of epithelial cancer (Cavallaro and Christofori, 2004). In *D. melanogaster*, mutation of *Scrib* in the context of Ras-transformed eye disc cells suppresses E-cadherin expression, which induces invasion of the basement membrane and metastasis (Brumby and Richardson, 2003; Pagliarini and Xu, 2003). We found that KD of E-cadherin in MDCK cells phenocopied the effects of Scrib KD on migration and adhesion, confirming that Scrib acts on E-cadherin function and that the changes in migratory behavior and cell–cell adhesion are causally related to one another. However, unlike in *D. melano-*

gaster, reduction of Scrib levels in MDCK cells had no effect on E-cadherin expression, and it did not alter either delivery to the plasma membrane or endocytosis because the surface expression of the protein was unchanged after Scrib depletion. But the amounts of detergent-insoluble α- and β-catenin were increased, suggesting that Scrib acts downstream of E-cadherin.

How does Scrib operate? We were unable to detect any association of Scrib with E-cadherin, α-catenin, or β-catenin by coimmunoprecipitations (unpublished data), suggesting that the mechanism does not involve direct binding. Rac and Cdc42 are activated during the formation of cell–cell adhesions through E-cadherin, and expression of dominant-interfering Rac and Cdc42 mutants can block this formation (Chu et al., 2004). Therefore, we initially assumed that Scrib might act by promoting the activation of Rac through association with the guanine nucleotide exchange factor βPIX. However, suppression of Scrib expression did not detectably alter E-cadherin trafficking, and its role in cell–cell adhesion and migration appears to be independent of βPIX expression. Moreover, although Rac has been shown to be important in epithelial cell–cell adhesion (Hordijk et al., 1997), Rac activity was not altered by suppression of Scrib expression. These data therefore identify a new, βPIX-independent function for Scrib in mammalian epithelial cells.

To identify the step at which Scrib acts, we tested the effects of a fusion between E-cadherin and α-catenin, which cannot bind to β-catenin. Remarkably, even very low amounts of this construct could partially rescue aggregation and reduce migration of Scrib KD cells, whereas expression of an Ecad–GFP fusion had no effect. Therefore, we propose that the lesion in the Scrib KD cells is confined to the coupling between the cadherin and α-catenin. The data also suggest that the coupling of E-cadherin to α-catenin is normally dynamic and that Scrib is required to stabilize the linkage so as to permit adhesive junctions to form. The covalent attachment of E-cadherin to α-catenin would eliminate the need for this stabilization, thus permitting adhesions to form even when Scrib levels are reduced. If Scrib acted at another point, for instance to stabilize the coupling of α-catenin to the actin cytoskeleton, one would not expect the expression of an Ecad–αcat fusion to reverse the effects of Scrib depletion. We speculate that Scrib reduces the mobility of the catenins so as to encourage the formation of connections to the actin cytoskeleton, which in turn will reduce the lateral mobility of E-cadherin in the plasma membrane and facilitate clustering at nascent junctions.

It is important to note that Scrib might possess other functions in mammalian epithelial polarization that were not revealed by partial silencing of its expression in MDCK cells. For example, it is unclear at present whether the minor defect in tight junction assembly would be exaggerated in a Scrib knockout. However, we note that shRNA-mediated reduction of ZO-1 expression by 80–90% in MDCK cells (unpublished data) closely phenocopies a ZO-1 knockout, suggesting that in some cases at least the principal biological functions of proteins are uncovered even by incomplete gene silencing. Future studies will focus on the nature of the link between Scrib and the cadherin–catenin complex and on the mechanism by which the dynamics of the complex regulate adhesiveness.

Materials and methods

Constructs

A partial cDNA to human Scrib was provided by J. Huibregtse (University of Texas, Austin, TX). The 5′ and 3′ ends of the open reading frame were obtained by PCR from human kidney cDNA and subcloned into the fragment to recover full-length hScrb cDNA. GFP-hScrb was constructed by cloning full-length human Scrb into the HindIII and EcoRI sites of the pEGFPC1 (CLONTECH Laboratories, Inc.) vector. GFP–E-cadherin was a gift from J. Nelson (Stanford University, Stanford, CA).

To generate shRNAs against the canine Scrib, partial sequences were obtained by RT-PCR from MDCK II cells and screened for candidate small interfering RNA primers using rational design criteria (Reynolds et al., 2004). Target sequences ScrbKD1 and 2 gave efficient suppression of Scrib expression. Sequences of the ScrbKD sense oligonucleotides are as follows: ScrbKD1 (5′-GATCCCCCAGATGGTCCTCAGCAAGTTTCAAGAGAACTTGCTGAGGACCATCTGTTTTTTGGAAA-3′) and ScrbKD2 (5′-GATCCCCGAGGTGACACTGTGCAGCATTCAAGAGATGCTGCACAGTGTCACCTCTTTTTTGGAAA-3′). Partial canine βPIX and E-cadherin sequences were obtained from the boxer genome project (GenBank, National Center for Biotechnology Information, National Institutes of Health). ShRNA sequences are as follows: PixKD1 (5′-GATCCCCCGAGCTCTCCTTTACGAAATTCAAGAGATTTCGTAAAGGAGAGCTCGTTTTTTGGAAA-3′), PixKD2 (5′-GATCCCCCACGCACAATGGCAAGACTTTCAAGAGAAGTCTTGCCATTGTGCGTGTTTTTTGGAAA-3′), PixKD3 (5′-GATCCCCCATCCAGCAAGCATGCAGATTCAAGAGATCTGCATGCTTGCTGGATGTTTTTTGGAAA-3′), and EcadKD (5′-GATCCCCGTCTAACAGGGACAAAGAATTCAAGAGATTCTTTGTCCCTGTTAGACTTTTTGGAAA-3′).

Sense and antisense oligonucleotides for shRNAs were annealed, phosphorylated, and ligated into the BglII and HindIII sites of pSUPER. As a negative control we used pS-Luc, which targets a sequence within the luciferase gene that is not present in the canine genome (Chen and Macara, 2005).

Cell culture, transfection, and calcium switch

Cell culture, transfection, and calcium switch of MDCK II cells were performed as described previously (Chen and Macara, 2005). Cells (2×10^6) were transiently transfected in suspension by electroporation, using 2.5–18 μg DNA (Amaxa, Inc.). Transfection efficiency was generally >70%. For calcium switch experiments, 3×10^4 MDCK II cells were plated into 8-well Lab-Tek II chambers (Nunc) with normal growth medium. After 40–44 h, the medium was replaced with MEM lacking calcium and supplemented with 2% dialyzed calf serum. After 16–20 h, cells were switched back to normal growth medium.

Immunological methods

For analysis of total cell extracts by immunoblot, cells were scraped directly into Laemmli sample buffer. After SDS-PAGE, proteins were transferred to nitrocellulose and detected by chemiluminescence (Kirkegaard and Perry Laboratories) or, for quantification of the proteins, with the Odyssey Infrared Imaging System (LI-COR Corp.). For immunoblots of the soluble fraction, cells were washed with cold PBS and lysed in lysis buffer (25 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 1% Triton X-100, 1 mM DTT, and 1 mM PMSF). After centrifugation (10 min at 14,000 rpm), supernatants were assayed for protein concentration using Bradford reagent and then boiled in Laemmli sample buffer.

Antibodies used were as follows: anti-Scrib (1:150; Santa Cruz Biotechnology, Inc.), anti–β-catenin (1:3,000); anti-βPIX (1:500), anti–E-cadherin (1:3,000), anti-ERK (1:5,000), and anti–α-catenin (1:2,000; BD Biosciences); anti–α-tubulin (1:5,000; Sigma-Aldrich); anti-Rac (1:500, Upstate Biotechnology); anti-ERK and phospho-ERK antibodies (provided by D. Lannigan, University of Virginia, Charlotteseville, VA); and 1 μg/ml anti-myc 9E10 and anti-HA 12CA5. HRP-conjugated goat anti–mouse, mouse anti–goat, or goat anti–rabbit secondary antibodies were used at a dilution of 1:5,000–1:10,000 (Jackson ImmunoResearch Laboratories). For Odyssey detection, Alexa Fluor 680–conjugated goat anti–mouse, mouse anti–goat (Invitrogen), or IRDye 800–conjugated goat anti–rabbit (Rockland, Inc.) secondary antibodies were used. For immunofluorescence, cells were usually fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 in PBS, and blocked with 5% BSA in PBS for 1 h before incubation with antibodies. For detection of Na/K-ATPase, cells were fixed in methanol/acetone (1:1) at −20°C for 10 min. Primary antibodies were anti-Scrib (1:100) or anti–ZO-1 (1:500), anti-occludin (1:500; Zymed Laboratories), anti–β-catenin, anti–E-cadherin (1:500), and

anti–Na/K-ATPase (1:250; Abcam). Anti-gp35 (1:300) was a gift from G. Ojakian (State University of New York Downstate Medical Center, Brooklyn, NY). Alexa Fluor–conjugated secondary antibodies (Invitrogen) were used at a dilution of 1:1:500–1:1,000. Alexa 594–conjugated phalloidin was used at a dilution of 1:50. Cells were mounted in Slowfade (Invitrogen).

Imaging

Epifluorescence images were collected using an inverted microscope (T200; Nikon) with a 60× water-immersion lens (Plan Achromatic, NA 1.2) coupled to a charge-coupled device camera (Orca; Hamamatsu), controlled by Openlab 4.0 software (Improvision). Images were collected at 12-bit depth, 1,024 × 1,280 pixels resolution, and converted to TIFF files. Images were postprocessed in Photoshop 7.0 (Adobe) to increase the grayscale range and to reduce haze using an unsharp mask and were converted to 8-bit depth. In some cases (Fig. 1 C), the black and white values were inverted, and the contrast was further enhanced to emphasize cell junctions. Movies were converted to QuickTime format. Confocal imaging was performed using a microscope (LSM510; Carl Zeiss MicroImaging, Inc.) with a 100× oil-immersion objective (Plan Achromatic, NA 1.3).

Triton X-100 solubility

The Triton X-100 solubility assay was performed as previously described (Tsukamoto and Nigam, 1999; Palacios et al., 2002). After transfection, 1.2×10^5 cells were plated on 6-well plates. Cells were lysed after 3 d in 100 μl CSK-A buffer containing 0.5% Triton X-100 for 15 min on ice. Cell lysates were collected and centrifuged at 14,000 rpm for 10 min. Supernatants and pellets were boiled with sample buffer, and equal volumes were resolved by SDS-PAGE. Proteins were visualized by immunoblotting and quantified with the Odyssey Infrared Imaging System.

Cell surface biotinylation

Cell surface biotinylation was performed as described previously (Le et al., 1999). MDCK cells were grown on filters and incubated with 1.0 mg/ml sulfosuccinimidyl 2-(biotinamido) ethyl-dithioproprionate (sulfo-NHS-SS-biotin) (Pierce Chemical Co.) on both sides of the filter for 1 h. Cells were then washed with quenching reagent (50 mM NH_4Cl in PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$) for 10 min, followed by further washes in PBS. Cells were then scraped off the filters and lysed in RIPA buffer, and cell lysates were incubated with streptavidin beads (Pierce Chemical Co.) to collect biotinylated proteins. Biotinylated E-cadherin was detected by immunoblot.

Rac activity assays

Assays were performed as described previously using a GST fusion of the Rac binding domain of PAK1 to capture Rac-GTP (Ren et al., 1999; Chen and Macara, 2005).

Aggregation assay

The hanging drop aggregation assay was performed essentially as described previously (Thoreson et al., 2000). Cells were trypsinized in the presence of EDTA, washed twice in PBS, and resuspended at 10^6 cells per milliliter in normal growth medium. 3×10^4 cells were then suspended as hanging drops from the lid of a 24-well culture dish and allowed to aggregate overnight. The cells were subjected to shear force by passage 10 times through a 200-μl filter pipet tip (Continental Laboratory). Cells were then imaged using a 10× phase-contrast objective. For quantification, in each field, the degree of aggregation was measured as the ratio of the area of the aggregates to the number of individual nonaggregated cells using Openlab software (Improvision).

E-cadherin binding assay

The E-cadherin binding assay was a modified version of one described previously (Yap et al., 1998). Wells in a 96-well ELISA high-binding plate (Corning) were coated with 0–50 μg/ml of E-cadherin extracellular domain–Fc fusion protein (provided by B. Gumbiner, University of Virginia, Charlottesville, VA) in coating buffer (100 mM NaCl, 20 mM Hepes, and 1 mM $CaCl_2$, pH 7.2) overnight at 4°C. Wells were then blocked with 3% BSA for 2 h, followed by three washes in HBSS+ (containing 1.2 mM Ca; Invitrogen). MDCK cells were dispersed using a cell disassociation solution (Sigma-Aldrich) at 37°C for 30 min and washed twice with normal medium. Cells were then resuspended in HBSS+ with 1 mM additional Ca. About 10^5 cells were added to wells in the presence of 0.2 mg/ml GRGDTP peptide (Sigma-Aldrich) and allowed to incubate for 60 min. After gentle washing with HBSS+, cells bound to plates were imaged. The number of cells in unwashed wells was counted, and data are presented as the percentage of cells remaining in each washed well, compared with the unwashed control.

Boyden chamber cell migration and wound-healing assays

Modified Boyden chamber assays were performed essentially as described previously (Sander et al., 1998). Approximately 10^5 cells in DME with 10% serum were seeded in the upper compartment of cell culture–treated Transwell filters (6.5-mm diam and 8-μm pores; Corning). The lower compartment contained DME with 10% serum. After 16–20 h at 37°C, nonmigrating cells in the top chamber were removed with a cotton swab and cells that had migrated to the underside of the filter were fixed with 4% paraformaldehyde and stained with 0.4% crystal violet. For quantification, crystal violet was eluted with 10% acetic acid and the absorbance at 595 nm of eluant was measured. To normalize for variability in cell numbers, an identical volume of cells was seeded into another well, fixed, stained, and eluted, without the cells from the upper well being removed.

For the wounding assay, confluent monolayers grown on Delta T dishes (Fisher Scientific) in DME + 10% serum + 20 mM Hepes, pH 7.4, were scraped with the tip of a microinjection pipette to form a linear wound. After 4 h of recovery, wound closure was recorded using a phase-contrast 20× objective lens at 5-min intervals for 16 h at 37°C. Individual cells were tracked over the course of the movie using Openlab software, transposed to the same initial x,y coordinates (0,0), and displayed as Rose plots. The mean square displacement, $d^2(t)$, was calculated for each cell. Migration parameters were then estimated, assuming the cells migrate as persistent random walkers: $d^2(t) = 2S^2P[t - P(1 - e^{-t/P})]$, where S is the cell speed and P is the directional persistence time. An Excel macro for calculating the correlation functions was provided by R. Horwitz (University of Virginia, Charlottesville, VA).

Online supplemental material

Fig. S1 shows confocal images of control and Scrib KD cells stained for Scrib and for the apical marker gp135. Videos 1 and 2 show cell migration in response to wounding of MDCK monolayers. Video 1 (control) shows the migration of wild-type cells; Video 2 (ScrbKD) shows the migration of cells depleted of Scrib by RNAi. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200506094/DC1.

We thank James Nelson for Ecad–GFP, Rick Horwitz for the cell migration analysis macro, Deborah Lannigan for the ERK antibodies, George Ojakian for anti-gp135, members of the Macara group for helpful suggestions, and the anonymous reviewers for insightful comments. We also thank Kathleen Schwartz, Xiao Chen, and Xuejun Chen from the Gumbiner Laboratory for invaluable help with the E-cadherin binding assay. We thank Anne Spang for critical reading of the manuscript.

This work was supported by grants GM070902 and CA040042 from the National Institutes of Health, Department of Health and Human Services.

Submitted: 16 June 2005
Accepted: 8 November 2005

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