Cellular Biology

The Polarity Protein Scrib Is Essential for Directed Endothelial Cell Migration

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Rationale: Polarity proteins are involved in the apico-basal orientation of epithelial cells, but relatively little is known regarding their function in mesenchymal cells.

Objective: We hypothesized that polarity proteins also contribute to endothelial processes like angiogenesis.

Methods and Results: Screening of endothelial cells revealed high expression of the polarity protein Scribble (Scrib). On fibronectin-coated carriers Scrib siRNA (siScrib) blocked directed but not random migration of human umbilical vein endothelial cells and led to an increased number and disturbed orientation of cellular lamellipodia. Comunoprecipitation/mass spectrometry and glutathione S-transferase (GST) pulldown assays identified integrin α5 as a novel Scrib interacting protein. By total internal reflection fluorescence (TIRF) microscopy, Scrib and integrin α5 colocalize at the basal plasma membrane of endothelial cells. Western blot and fluorescence activated cell sorting (FACS) analysis revealed that silencing of Scrib reduced the protein amount and surface expression of integrin α5 whereas surface expression of integrin αV was unaffected. Moreover, in contrast to fibronectin, the ligand of integrin α5, directional migration on collagen mediated by collagen-binding integrins was unaffected by siScrib. Mechanistically, Scrib supported integrin α5 recycling and protein stability by blocking its interaction with Rab7a, its translocation into lysosomes, and its subsequent degradation by pepstatin-sensitive proteases. In siScrib-treated cells, reinduction of the wild-type protein but not of PSD95, Dlg, ZO-1 (PDZ), or leucine rich repeat domain mutants restored integrin α5 abundance and directional cell migration. The downregulation of Scrib function in Tg(kdrl:EGFP)−/− transgenic zebrafish embryos delayed the angiogenesis of intersegmental vessels.

Conclusions: Scrib is a novel regulator of integrin α5 turnover and sorting, which is required for oriented cell migration and sprouting angiogenesis. (Circ Res. 2013;112:924-934.)

Key Words: angiogenesis ■ cell migration ■ cell polarity ■ endothelial cells ■ fibronectins ■ integrin α5

Cell polarity is essential for the function of all eukaryotic cells because it is involved in processes like cell differentiation, cell division, and morphogenesis. Especially in epithelial cells, apico-basal polarity accounts for the asymmetrical distribution of cell organelles, junctions, and proteins within the cell. Planar cell polarity is the organization of epithelial cells in the plane of a tissue that is perpendicular to the apico-basal axis and a prerequisite for oriented cell division and convergent extension.1

So far, 3 different evolutionarily conserved polarity protein complexes have been identified, the Par, the Crumbs, and the Scrib complex, which are all highly expressed in mammalian epithelial cells. In renal epithelial cells the Crumbs complex regulates the formation of the apical surface, and the Par complex modulates the balance between apical and lateral surfaces via multiple interactions.2 In Drosophila, the Scrib complex contributes to the formation of the baso-lateral surface and regulates apico-basal polarity of epithelial cells.3 The protein complex consists of Scribble (Scrib), Lethal giant larvae (Lgl), and Discs large (Dlg), and Discs large (Dlg), which interact genetically,
whereas the exact nature of physical interaction of the proteins is less clearly defined. Scrib acts as a scaffold protein, and its deficiency results in the loss of epithelial apico-basal polarity manifested in the misdistribution of apical proteins and adherens junctions to the basolateral cell surface and abnormal cell growth. Scrib also regulates planar cell polarity of epithelial cells because the lack of Scrib causes a defect in the planar polarization of the inner ear. Mammalian Scrib may also regulate epithelial cell proliferation. In the colon, Scrib is downregulated during malignant progression, and its expression pattern correlates with loss of cell polarity. The functions of Scrib in epithelial cell polarization are mediated in part by the leucine rich repeat (LRR) domain, which tethers Scrib to the plasma membrane. Via its PDZ domains Scrib interacts with ZO-2, zyxin, adenomatous polyposis coli, and the guanine nucleotide exchange factor SPIX. The latter has been implicated in the effect of Scrib on cell migration of epithelial cells and fibroblasts by scaffolding p21-activated protein kinase and Scrib in a protein complex.

Different to the majority of epithelial cells, the vasculature is of mesodermal origin. Although the peculiarities of the mesenchyme render these cells less polarized, a certain polarization is present in particular in the endothelium: Similar to epithelial cells, the endothelium orients toward a lumen, is firmly attached to a basal lamina, and complex intercellular junctions facilitate monolayer formation and growth control. During processes like cell division and migration, endothelial cells also exhibit planar polarity and this feature might be important during angiogenic formation of new blood vessels. Nevertheless, little is known about a possible expression of the Scrib complex in endothelial cells and the function of Scrib in the vasculature has not been studied, yet.

We hypothesize that polarity proteins of the Scrib complex contribute to planar endothelial polarization. We found that Scrib is essential for endothelial cell polarization and orientation during directed migration by controlling Rac localization and integrin α5 turnover. By this mechanism, Scrib controls endothelial cell tube formation and sprouting angiogenesis in vitro and in vivo.

### Methods

See Online Materials and Methods for standard experimental methods for human cell culture, siRNA and plasmid transfection, isolation of RNA and RT-PCR, protein isolation and Western blot analysis, immunoprecipitation and mass spectrometry, glutathione S-transferase (GST) pulldown, fluorescence activated cell sorting (FACS) analysis, analysis of integrin α5 recycling by antibody feeding and cell surface biotinylation, immunofluorescence, and total internal reflection fluorescence (TIRF) microscopy. DuoLink analysis, cell migration and in vitro angiogenesis assays, zebrafish studies, retinal angiogenesis, and mouse wholemount staining.

### Results

**Scrib Is Required for Endothelial Cell Polarization**

Comparison of endothelial and epithelial cells revealed a similar mRNA expression of major members (Scrib, Dlg5, Lgl1, and Lgl2) of the Scrib complex among the different cells. Given its prominent function in the polarity complex we focused our further study on Scrib. Scrib protein expression in human umbilical vein endothelial cells (HUVEC) and the human microvascular endothelial cells-1 (HMEC-1) cell line was comparable with that of different epithelial cell lines (Online Figure IA). To assess the role of Scrib for endothelial cell polarization, we investigated the effect of Scrib siRNA on the localization of the nucleus in HUVEC. During epithelial cell migration, the relative position of the nucleus, which is typically positioned in the rear part of the cell, can be used as an indicator of the cell polarity axis. In the scratch wound assay, this characteristic of planar polarization was also observed in HUVEC (Online Figure IB). Downregulation of Scrib by siRNA (siScrib) prevented the effect and resulted in an almost central localization of the nucleus (Online Figure IB). Moreover, also the localization of the golgi apparatus changed from a positioning in front of the nucleus to a random localization in response to Scrib siRNA (Online Figure IC). These observations establish that Scrib controls planar cell polarity in endothelial cells.

**Silencing of Scrib Enhances Tube Formation but Attenuates Sprouting**

To assess the effect of Scrib on in vitro angiogenesis, tube formation assays on Matrigel and spheroid outgrowth assays were performed in endothelial cells. Downregulation of Scrib significantly increased tube formation, whereas sprouting out of endothelial spheroids in response to basic fibroblast growth factor was significantly attenuated by Scrib siRNA (HUVEC, Figure 1; HMEC-1, Online Figure IIA). The latter effect was predominantly a consequence of a reduction in the number of tubes formed (Online Figure IIB). This may suggest that Scrib regulates endothelial tip cell formation. Indeed, in a compensation assay in which control and Scrib siRNA–treated, labeled HUVEC were mixed in 1 spheroid, Scrib downregulation was associated with a decrease in the frequency of cells in the tip position (Online Figure IIC).

**Scrib Is Required for Directional Endothelial Cell Migration**

To address a possible role for Scrib in migration, we performed scratch wound and chemotaxis assays. Downregulation of Scrib had no effect on migration in the
Scratch wound assay suggesting that random endothelial cell movement is Scrib-independent (Figure 2A). In contrast, Scrib was required for the chemotactic response. In a transwell assay, Scrib siRNA reduced migration in response to basic fibroblast growth factor and fetal calf serum by ≈50% (Online Figure IIIA). To address the basis of this inhibition, we used chemotaxis chambers coated with fibronectin and live cell tracking. Downregulation of Scrib had no effect on the totally migrated distance but led to a loss of the cellular ability to migrate into the direction of the gradient. After Scrib siRNA, their migrated path was more tortuous and the distance migrated toward the chemotactic gradient was reduced by ≈50% (Figure 2B and 2C, Online Figure IIIB).

Scrib Regulates Lamellipodia Localization and Number
During directed migration, cells form a stabilized lamellipodium at their leading front, which is characterized by Rac1 accumulation. Rac1 staining confirmed that usually, each migrating endothelial cell has only one defined lamellipodium, which is predominantly oriented in the direction of the chemoattractant. In contrast, silencing of Scrib with siRNA increased the mean number of lamellipodia per cell to 2 and the orientation of these protrusions toward the chemotactic gradient was lost (Figure 2D and 2E).

Integrin α5 Is an Interaction Partner of Scrib
It is generally believed that epithelial Scrib mediates its effect by an adaptor function through targeting of signaling molecules into defined cellular compartments. To identify the proteins involved in the Scrib-dependent effects on directional endothelial cell migration, we performed Scrib immunoprecipitations and identified coprecipitating proteins by mass spectrometry. By this method, we could confirm several known interactors of Scrib, like βPIX and G protein–coupled receptor kinase interacting ArfGAP1 (GIT1). Importantly, as a so far unreported interactor we coprecipitated integrin α5. Two to 3 peptides corresponding to the integrin α5 sequence were identified by mass spec analysis in three independent experiments (Online Table I). Importantly, the interaction of Scrib and integrin α5 was also evident by coimmunoprecipitation followed by Western blot (Figure 3A). To determine the domains of Scrib required for the interaction with integrin α5, GST pulldown assays were performed with the intracellular domain of integrin α5 and lysates of cells over-expressing either wild-type or mutant Scrib missing the LRR and the PDZ domains, respectively. This assay demonstrated that only wild-type but not mutant Scrib interacts with the integrin α5 intracellular domain (Figure 3B). Duolink proximity ligation assay analysis, which selectively stains sites of protein–protein interactions revealed that Scrib and integrin α5 form complexes in paxillin-containing submembrane regions like focal adhesions in the intact cell (Online Figure IVA). Indeed, by TIRF microscopy exclusively visualizing the basal plasma cell membrane, distinct colocalization of Scrib and integrin α5 was observed in focal adhesions (Figure 3C).

Scrib Controls Integrin α5 Surface Expression
Because integrin α5 is involved in cell migration, we determined whether Scrib downregulation affects this adhesion molecule. Whereas Scrib did not control integrin α5 mRNA expression (Online Figure IVB), its downregulation reduced the integrin α5 protein amount by ≈70% (Figure 3D). By confocal microscopy it was obvious that particularly the integrin α5 abundance in focal adhesions was reduced by Scrib siRNA (Figure 3E). Therefore, also integrin α5 surface expression was reduced by silencing Scrib. Integrin α5 binds to RGD-peptides, and the binding capacity of cells to FITC-labeled RGD-peptides as determined by FACS was significantly reduced after Scrib downregulation (Figure 3F). In line with this, Scrib siRNA reduced the surface expression of integrin α5 and also that of integrin β1, its interacting β subunit. Importantly, surface expression of integrin alpha V was not affected by Scrib siRNA, suggesting that Scrib is a specific regulator for integrin α5 (Figure 3G). To obtain confirmation for the specificity of the interaction at the functional level, we studied directed migration on chemotaxis chambers coated with collagen I, in which binding is mediated by a different set of integrins, such as α2β1 and α1β1. Importantly, directional migration on collagen was not affected by Scrib downregulation (Figure 3H and Online Figure IIIIC and IIIID). To vice versa establish integrin α5 as a functional effector of Scrib, we determined the effect of integrin α5 knockdown by siRNA on directed migration. Similar as in response to Scrib siRNA, integrin α5 siRNA inhibited directed migration on fibronectin but not collagen matrix (Online Figure V through VC). Silencing of integrin...
Figure 2. Role of Scrib for endothelial cell migration and lamellipodia formation. A, Effect of Scrib siRNAs (siScrib1&3) and scrambled control (siScr) on migration in the scratch wound assay. B and C, Representative tracings (B) and statistical analysis (C) of the effect of siRNA on the migration of human umbilical vein endothelial cells (HUVEC) in fibronectin-coated chemotaxis chambers in response to a gradient of fetal calf serum (0% to 20%). Bar graphs show means of totally migrated distance and direction relative to the gradient. Representative photographs (D) and statistical analysis (E) of lamellipodia localization in the aforementioned assay. Cells were stained with anti–Rac-1 antibody (green) and phalloidin for F-actin (red) and nuclei were counterstained with DAPI (white). Lamellipodia orientation was determined from the site of Rac1 accumulation relative to the nucleus and the direction toward the chemotactic gradient. Upper bar graph shows lamellipodia localization determined by overlay of a coordinate system and quantification of the lamellipodia in each direction, lower bar graph depicts the average number of lamellipodia per cell. The scale bar represents 20 μm. n=4 to 5 independent experiments, 15 to 36 cells per experiments were analyzed. *P<0.05, **P<0.01 vs scrambled (siScr).
Figure 3. Scrib and integrin α5 interaction and effect of Scrib on integrin α5β1 surface localization in human umbilical vein endothelial cells (HUVEC). **A**, Western blot for integrin α5 and Scrib after immunoprecipitation of Scrib. **B**, Western blots of a pulldown assay of a GST construct of the intracellular domain of integrin α5. HEK 293 cells were transfected with GFP, wild-type Scrib, a mutant missing the PDZ domains (ΔPDZ) and a mutant missing the LRR (ΔLRR), respectively, lysed and incubated with the intracellular domain of integrin α5 coupled to GST-beads. GFP indicates green fluorescent protein; GST, glutathione S-transferase; Ini, initial input; LRR, Leucine rich repeat; PD, elution of the GST-pull down; and PDZ, PSD95, Dlg, ZO-1. **C**, Representative total internal reflection fluorescence (TIRF) microscopic images of Scrib (green) and integrin α5 (red) in the basal plasma membrane of HUVEC. The scale bar represents 4 μm. **D**, Effect of scrambled (siScr) or Scrib (siScrib) siRNA on integrin α5 protein expression. **E**, Exemplary confocal microscopy images for the effect of Scrib downregulation on integrin α5 staining in HUVEC subjected to a chemotactic gradient (fetal calf serum, 6 hours). The scale bar represents 20 μm. **F** and **G**, Representative FACS recordings for the effect of Scrib downregulation on binding of FITC-RGD-peptides and of antibodies directed against integrin α5, integrin β1 (F) and integrin αV (G). **H**, Quantification of chemotaxis assays performed on type I collagen in response to fetal calf serum. The total migration distance and the direction relative to the gradient are shown. n=4 to 6.
α5 did, however, not affect endothelial cell planar polarity as determined by nucleus localization (data not shown). Furthermore, integrin α5 overexpression partially restored directed cell migration of cells missing Scrib (Online Figure VD). These data illustrate that Scrib specifically facilitates integrin α5 surface expression and that this mechanism is required for directional migration on matrices containing integrin α5β1 binding partners.

The Effect of Scrib on Migration and Integrin α5 Requires the PDZ and the LRR Domain of the Protein

To identify the domains of Scrib involved in integrin α5 expression and migration, HUVEC treated with and without Scrib siRNA were transfected with siRNA-resistant expression plasmids of wild-type Scrib and of Scrib mutants either missing the PDZ or the LRR domain. Whereas this approach had no effect on cells treated with control siRNA, wild-type Scrib restored integrin α5 expression (Figure 4A) and migration in cells pretreated with Scrib siRNA (Figure 4B). Similar as for the interaction observed in the GST pulldown, neither of the Scrib mutants was effective in restoring the normal phenotype although the ΔPDZ mutant appeared to have a small, yet insignificant, positive effect. These observations suggest that integrin α5 surface expression as well as oriented migration requires a full-length functionally intact Scrib.

Scrib Protects Integrin α5 From Lysosomal Degradation

As Scrib siRNA treatment had no effect on integrin α5 mRNA, posttranslational effects probably account for the Scrib-dependent maintenance of integrin α5 protein. Indeed, when translation was inhibited with cycloheximide, Scrib siRNA decreased the half life of integrin α5 protein from ≈30 to 15 minutes (Online Figure V1A). As these data suggest that Scrib prevents integrin α5 degradation, we studied a panel of inhibitors affecting different protein degradation pathways. Whereas the effect of most inhibitors was identical between cells treated with scrambled or Scrib siRNA, pepstatin, an inhibitor of aspartyl proteases including cathepsin D selectively increased integrin α5 protein levels in the siRNA-treated group (Online Figure V1B). To address the role of Scrib for a potential lysosomal degradation of integrin α5, we studied the intracellular localization of green fluorescent protein (GFP)-tagged integrin α5 in the presence of pepstatin to allow its lysosomal accumulation. Importantly, Scrib siRNA induced a massive increase in the abundance of integrin α5-GFP in lysosomes (Figure 4C). This could also be demonstrated biometrically as Scrib siRNA significantly increased the correlation coefficient of the fluorescence signal of integrin α5-GFP and LysoTracker-Red (siScr+Pep R= -0.039±0.14; siScrib+Pep R=0.475±0.07; n=11–12; P<0.0001, Figure 4C). In the absence of pepstatin, integrin α5-GFP could not be detected in lysosomes under any conditions illustrating the efficiency of the degradation process. Importantly, the pepstatin-dependent increase in integrin α5 protein amount cannot be the consequence of a potential stabilization of the Scrib protein, as Scrib expression remained unchanged during the 2 hours of the experiment (siScr+Pep 85.7% ± 25.8 compared with siScr; siScrib+Pep 114.6% ± 21.6 compared with siScrib). These data suggest that Scrib prevents degradation of integrin α5 and therefore either stabilizes the protein at the plasma membrane, facilitates its recycling after internalization in response to ligand binding, or protects integrin α5 from sorting into lysosomes. Antibody feeding studies and analysis of internalization and recycling after cell surface biotinylation demonstrated that after Scrib siRNA treatment, internalization remained unchanged but recycling was reduced by approximately 40% (Figure 4D and 4E, Online Figure V1C). To further characterize the involved mechanism, we performed a second proteomic screen, this time for the effect of Scrib on the integrin α5 interactome. Mass spectrometry for integrin α5 coprecipitating proteins identified a selective interaction of the protein with Rab7a after Scrib knock-down. These data could also be confirmed by proximity ligation assay (Online Figure 7A). For other proteins, GTPase Rab7a has been suggested to control their sorting from late endosomes into lysosomes. To determine whether something similar occurs in response to Scrib siRNA, integrin α5 expression was studied after treatment with Rab7 siRNA. Indeed, silencing Rab7a rescued integrin α5 protein amount after Scrib downregulation (Online Figure VIIIB). Thus, Scrib is required to maintain normal integrin α5 recycling and prevents its sorting into a lysosomal degradation pathway mediated by Rab7a.

Scrib Contributes to Developmental Angiogenesis In Vivo

Because directional migration is an essential part of angiogenesis, we studied retinal angiogenesis in heterozygous circle tail (crc) mice. These animals carry a frame shift mutant of Scrib and homozygosity of the mutation is embryonically lethal because of failure to initiate neural tube closure and cardiac malformation. Also, severe vascular malformations are present in homozygous crc embryos (Online Figure VIII). Wholemount staining of homozygous crc mice at day E11 show that especially the vessels in the dorsal region of the embryo are disorganized and vascular development in total is delayed, but it is unclear whether this is secondary to the previously observed alterations. The vascular phenotype of heterozygous crc mice in contrast was minor: The mutation led to a minimal, yet significant, reduction in the width of the retinal vascularized area (Online Figure VIII). Given that homozygosity should result in a 50% reduction in functional protein, we studied Scrib expression in crc mice. Surprisingly, heterozygous crc mice had comparable level of Scrib protein as wt mice, suggesting that Scrib expression is largely controlled on the posttranslational level (Online Figure VIIIIB). Indeed, inhibitors of proteasomal as well as of lysosomal degradation increased Scrib level in HUVEC by 2- to 3-fold after 8 hours of treatment (Online Figure VIIIC). As an approach to confirm the delayed retinal angiogenesis induced by Scrib deficiency, we studied vascular development in Tg(kdrl:EGFP)M22 zebrafish embryos, in which the endothelium is labeled by kdrl-promoter–driven GFP. Scrib expression was detectable in GFP-positive endothelial cells as well as GFP-negative remaining cells of fish.
as determined by FACS followed by Western blot as well as RT-PCR (Online Figure IXA). Two different morpholinos were injected, which both attenuated Scrib expression in the zebrafish embryos (Online Figure IXB). Targeting Scrib caused a clear delay in the formation of the intersegmental vessels (Figure 5A and 5B). Furthermore, zebrafish treated with scrib morpholinos showed severe malformation of the vessels in the brain with spontaneous hemorrhages (Figure 5C). Importantly, also integrin α5 morphants delayed the formation of intersegmental vessels, which may suggest that also in the zebrafish, integrin α5 among other important proteins is an effector of Scrib (Figure 5B). These data could be...
strengthened by the accumulative effect of Scrib and integrin α5 morpholinos. When both morpholinos were coinjected in concentrations which alone do not affect vessel formation, the double knock down resulted in a clear delay of intersegmental vessel formation (Online Figure IXC and IXD). These observations demonstrate that also in vivo Scrib contributes to angiogenic processes involving directed endothelial cell migration and integrin α5 signaling.

**Discussion**

In this study we demonstrate that Scrib is essential for directional endothelial cell migration toward a chemotactic stimulus and that Scrib controls endothelial tube formation in vitro and angiogenesis in vivo. In this regard, Scrib specifically regulates integrin α5 abundance by preventing integrin α5 interaction with Rab 7a and subsequent lysosomal degradation. By this mechanism, Scrib facilitates integrin α5 recycling to the plasma membrane.

Migration is a complex cyclic cellular process, which involves a dynamic elongation and retraction of parts of the cell so that eventually, as consequence of a random process, the cell moves away from its original spot. Directionality of the process is the result of chemotaxis or in the case of the monolayer wounding model (scratch wound assay) of an inhibition of backwards migration to areas covered by cells. The latter can either be a consequence of simple physical hindering factors (similar to diffusion) or result from inhibitory signaling through cellular junctions and cell–cell contacts. Although the distinctions on the nature of directionality are often overlooked, we here demonstrate that Scrib in endothelial cells is specifically required for chemotactic directional migration but not the migration in the monolayer wounding assay.

Although initially identified as a protein involved in apico-basal polarity, it is progressively becoming clear that the basolateral localization predisposes Scrib to also contribute to planar and front-rear polarity. Indeed, several studies linked Scrib to migratory processes in epithelial cells, fibroblasts, and lymphocytes.20 There is, however, little consensus on the specific role and functions of Scrib during migration, and its localization reportedly varies between cells and assay systems used. In epithelial Madin Darby canine kidney (MDCK) cells, Scrib downregulation induces cell migration in Boyden chamber experiments,16 whereas in breast carcinoma cells (T47D, MCF10A) and fibroblasts Scrib downregulation reduced cell migration.14,21

Little is known regarding the role of Scrib in endothelial cells. Although it was reported that the protein can interact with vimentin,22 the functional implications of this interaction are unclear. There is consensus that Scrib acts as an adaptor protein and by its PDZ domain positions the Rac/Cdc42 guanine nucleotide exchange factor βPIX in the baso-lateral compartment of cell–cell contacts. In confluent endothelial cells, prominent Scrib staining is observed in cell–cell contacts. However, we failed to observe any specific translocation of Scrib to the leading edge of migrating cells, the site of Rac1 accumulation during oriented migration, which is in contrast to a previous report in epithelial cells.14 Nevertheless, Scrib seems to regulate Rac localization in endothelial cells. During chemotaxis, directionality is a consequence of an imbalance in the localized activation of GTPases mediating retraction and extension of the cell.23 Indeed, our observation that deletion of Scrib results in the formation of multiple lamellipodia together with loss of directionality implies that Scrib acts by limiting excessive activity toward lamellipodia formation, a process which requires the small GTPases Rac1 and Cdc42.24 Via its PDZ
domain Scrib recruits guanine nucleotide exchange factors (GEFs) like βPIX, which are involved in Rac1 and Cdc42 activation and therefore might regulate the availability of GEFs, Cdc42, and Rac during the formation of new protrusions and thus suppress excessive lamellipodia formation.

Although Scrib was not detected in focal adhesions in astrocytes by epifluorescence, screening of the proteome of focal adhesions recovered Scrib as one of many proteins enriched in these complexes. This finding suggests that specific interaction partner in endothelial cells locate Scrib to different sites than those described for other cell types. Searching for such new interactors we identified integrin α5 to coprecipitate with Scrib. This finding implies that Scrib is not only localized in lateral cell–cell contacts but also in focal adhesions. Indeed, TIRF microscopy revealed a clear colocalization of Scrib and integrin α5 in these basal cell contacts in migrating cells during chemotaxis. Importantly, this interaction was not detectable by conventional confocal microscopy because the accumulation of Scrib in lateral cell contacts dominates the picture. Moreover, our data demonstrate that this interaction is functionally relevant, because Scrib stabilized integrin α5 abundance in focal adhesions.

It is well established that integrin α5 is involved in migration per se, but not only the presence on the cell surface but also the recycling of integrins is necessary for regulated cell migration. This process is regulated at several steps, including the internalization of the protein, the return to the plasma membrane, and the transport to degrading compartments. By analyzing internalization and recycling we could show that internalization of integrin α5 is not changed by silencing Scrib but less integrin α5 returns to the plasma membrane. Thus, Scrib does not stabilize integrin α5 at the plasma membrane but interferes with the recycling or the degradation of the protein. Several studies investigated the recycling process of integrin α5 and showed the involvement of the small GTPases Rab11a and Rab25 and Rab coupling protein (RCP) in mediating integrin α5 recycling from perinuclear recycling compartments. Interestingly, also βPIX and GIT1, which we both confirmed as Scrib interacting proteins in endothelial cells in the present study, and ARF6 were previously recognized to control integrin recycling. We investigated the role of these known regulators in the Scrib-mediated effect on integrin α5 recycling by the use of different siRNAs and co-immunoprecipitation, but we could not find evidence for the involvement of these pathways. Therefore, Scrib has to affect a novel, unreported pathway, which could only be uncovered by a proteomics approach. By mass spec analysis we determined the effect of Scrib siRNA on the integrin α5-interacting proteome. We found that the interactions of integrin α5 with several proteins are changed after silencing of Scrib. One of the proteins interacting with integrin α5 to a greater extent after downregulation of Scrib is Rab7a. This small GTPase has been described to regulate the sorting from late endosomes to lysosomes. We could confirm the mass spec data by Duolink proximity ligation assay analysis. Importantly, downregulation of Rab7a indeed rescued integrin α5 protein amount after downregulation of Scrib. These data reveal a new small GTPase regulating integrin α5 sorting and demonstrate that Scrib inhibits the interaction of integrin α5 and Rab7a and therefore protects it from lysosomal degradation.

It is generally believed that the LRR domains of Scrib facilitate correct localization of Scrib at the membrane and that the PDZ domains mediate protein–protein interactions. In line with this observation both domains of Scrib were required in the present study to maintain integrin α5 expression, interaction, and oriented migration. The function of Scrib in controlling integrin α5 sorting appears to be relatively specific toward a single, Rab7a-dependent degradation pathway. This observation was confirmed by the use of inhibitors of different degradation pathways: Integrin α5 is degraded in the proteasome or in lysosomes. Additionally, extracellular release of integrin α5 fragments has been described for monocytes. The integrin α5 degradation mediated by the knock down of Scrib was selectively abolished by pepstatin, an inhibitor of lysosomal aspartatic protease cathepsin D, whereas pepstatin had little effect on integrin α5 in cells containing Scrib. Inhibitors of cysteine cathepsins did not affect integrin α5 degradation (chloroquine, leupeptin). Moreover, inhibition of integrin shedding by the serine protease inhibitor PMSF or inhibition of cytosolic integrin α5 degradation by the calpain inhibitor E64d all increased the protein abundance of integrin α5, but this effect was unrelated to Scrib. Proteasomal degradation of integrin α5 seems to be of little relevance in endothelial cells, as MG132 massively decreased integrin α5 level in a Scrib-independent manner. These findings further confirm that Scrib is required to prevent degradation of integrin α5 in lysosomes.

Several lines of evidence of the present study support the physiological relevance of the present observations: Tube formation in the Matrigel assay, which reflects random formation of cell–cell contacts, potentially is a result of increased lamellipodia formation by Scrib downregulation. Similarly, attenuation of Scrib expression increased branching complexity of the basal dendrites of pyramidal neurons in the brain. In accordance with the loss of oriented migration, sprouting out of a spheroid was attenuated by downregulation of Scrib. This finding could be recapitulated in developmental angiogenesis in the Tg(kdr:EGFP) zebrafish model using previously developed Scrib morpholinos. In addition to the delay in angiogenesis we also observed cerebral vascular malformations after Scrib morpholino treatment. However, Scrib morphants also show defects in the migration of the nVII motor neurons, and it is unclear whether the defects in cerebral vessel formation are primary or consequence of the malformation of the brain.

In summary, with the present work, we have identified Scrib as a novel factor controlling endothelial cell planar polarity. Scrib protects integrin α5 from Rab 7a-mediated sorting into lysosomes. By this mechanism, Scrib facilitates directional endothelial cell migration in a chemotactic gradient and controls developmental angiogenesis.
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Disclosures

None.

References

What Is Known?

• Cell polarity is a fundamental characteristic particularly of epithelial cells and is essential for their function.
• The polarity protein Scrib regulates apico-basal as well as planar cell polarity of epithelial cell and neuronal cells.
• Some polarization is also present in the vascular intima, but a contribution of Scrib to endothelial cell polarization has not been reported.

What New Information Does This Article Contribute?

• Scrib regulates endothelial cell planar polarity, lamellipodia localization, directed cell migration, and angiogenesis in vitro.
• Scrib interacts in endothelial cells with integrin α5, a previously unknown interaction, and protects it from Rab7a-dependent lysosomal degradation.
• The interaction of Scrib and integrin α5 is essential for directed cell migration and angiogenesis in vitro and in vivo. We showed that Scrib interacts with integrin α5 and protects this adhesion molecule from Rab7a-dependent lysosomal degradation. This interaction with integrin α5 is essential for the Scrib-mediated regulation of directed cell migration and angiogenesis in vitro and in vivo. Therefore, our study provides new insights into the regulation of endothelial cell polarity, affecting oriented cell migration and angiogenesis. Additionally, we identified a so far unreported mechanism regulating lysosomal degradation of integrin α5. As a scaffolding protein, Scrib takes a central place in polarity signaling and may regulate additional functions of endothelial cells. Scrib might therefore be an attractive target for pro- and antiangiogenic therapies. This appears to be particularly relevant for tumor angiogenesis, because Scrib also acts as a tumor suppressor in epithelial cells.

Novelty and Significance

Movement and orientation of cells are polarized processes, but the regulation of endothelial cell polarity is incompletely understood.

In epithelial cells Scrib is one of several proteins regulating polarization, and loss of epithelial polarity is a frequent feature of malignancy in cancer. Here, we identified Scrib as a novel regulator of endothelial cell planar polarity, lamellipodia localization, directed cell migration, and angiogenesis in vitro. We showed that Scrib interacts with integrin α5 and protects this adhesion molecule from Rab7a-dependent lysosomal degradation. This interaction with integrin α5 is essential for the Scrib-mediated regulation of directed cell migration and angiogenesis in vitro and in vivo. Therefore, our study provides new insights into the regulation of endothelial cell polarity, affecting oriented cell migration and angiogenesis. Additionally, we identified a so far unreported mechanism regulating lysosomal degradation of integrin α5. As a scaffolding protein, Scrib takes a central place in polarity signaling and may regulate additional functions of endothelial cells. Scrib might therefore be an attractive target for pro- and antiangiogenic therapies. This appears to be particularly relevant for tumor angiogenesis, because Scrib also acts as a tumor suppressor in epithelial cells.


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Supplemental Material

The polarity protein Scrib is essential for directed endothelial cell migration

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Materials and Methods

Materials

Human fibronectin and type I collagen were purchased from BD Biosciences (Heidelberg, Germany), DAPI and chloroquin from Sigma-Aldrich (München, Germany), MG132 from Merck (Darmstadt, Germany), Leupeptin, Pepstatin and phenylmethylsulfonylfluorid (PMSF) from Applichem (Darmstadt, Germany). E64d was a gift from the Institute of Biochemistry II, Goethe-University, Frankfurt. The integrin α5-GFP construct was a generous gift from Dr. Jim C. Norman (Beatson Institute for Cancer Research, Glasgow, U.K).

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Walkersville, MD). Cells were cultured in EBM (Lonza, Walkersville, MD) supplemented with 10% FCS, bovine brain supplement and human recombinant EGF, penicillin (50 U/ml) and streptomycin (50 µg/ml). First to third passage endothelial cells were used throughout.

siRNA and plasmid transfection

For siRNA treatment, endothelial cells (80-90 % confluent) were transfected with siRNA using GeneTrans II according to the instructions provided by MoBiTec (Göttingen, Germany). Two different scrambled siRNAs were used, one general siRNA negative control and one specific scrambled for the Scrib siRNAs (Invitrogen, Darmstadt, Germany). Four different Scrib siRNAs (Invitrogen) and two different integrin α5 siRNAs (Qiagen) were used for the experiments. Plasmid overexpression was achieved with the Neon electroporation system (Invitrogen, Darmstadt, Germany). When no specific siRNA is named, the results of at least two different siRNAs are summarized.

siRNA-resistant expression plasmids were generated by introduction of still mutations at the siRNA binding sites of different Scrib-1 expression plasmids (after removal of the GFP-tag by blunt end cloning) described previously 1 with the aid of the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Böblingen, Germany).

Isolation of RNA and RT-PCR

RNA was isolated with the RNA Miniprep Kit (Stratagene, La Jolla, CA, USA). cDNA synthesis was performed with SuperScript III Reverse Transcriptase (Invitrogen, Darmstadt, Germany) and random hexamer primers. For semiquantitative real-time PCR Absolute QPCR SYBR Green Mix was used with appropriate primers and ROX as a reference dye (Thermo Scientific) in a Mx4000 qPCR cycler. Relative expressions of target genes were normalized to eukaryotic translation elongation factor 2 (EEF2) or RNA polymerase II, analyzed by delta-delta-Ct method and given as percentage compared to control experiments. Primer and probe sequences: Scrib forward: TGGCTCACCTGGCCCTGAATGATG; reverse: TCCAGATCGTTGCCTCCCAGATCC. Integrin α5 forward: ATACCAGCCAGCGGAGTG; reverse: GCCATGGGCTCGAACTGTTG.

Protein isolation and Western blot analysis

After treatment as described in the “results” section, cells were lysed with Triton X-100 lysis buffer and subjected to SDS-PAGE as described.2 Western blot analyses were performed with an infrared-based laser scanning detection system (Odyssey, Licor, Bad Homburg, Germany). Proteins were detected by antibodies against Scrib (Millipore, Schwalbach, Germany), Integrin α5 (Millipore) and β-actin (Sigma-Aldrich, München, Germany). The infrared-fluorescent-dye-conjugated secondary antibodies were purchased from Licor (Bad Homburg, Germany).

IP and LTQ Orbitrap mass spectrometry


Scrib immunoprecipitation was performed after cross linking the Scrib antibody (C-20, Santa Cruz Biotechnologies, Heidelberg, Germany) or the control antibody (unspecific goat IgG) to magnetic beads (Dynabeads Protein G, Invitrogen) with dimethyl pimelimidate. Cells were lysed with Triton X-100 lysis buffer and incubated with the antibody at 4°C over night. After washing, bound proteins were eluted with citrate buffer (pH 2.8) and separated by SDS-PAGE. The gels were stained with coomassie blue. The corresponding lanes were cut in several gel pieces (approx. 149) and in gel digested by trypsin as essentially described by Collins et al. 3

After washing, bound proteins were eluted with 10mM glutathione. The eluates were subjected to Western blot. A GST construct was used as a control and did not bind Scrib.

**FACS**

For FACS analysis the cells were detached with Versene and incubated with FITC-conjugated integrin α5 antibodies (clone SAM-1, Millipore), FITC-labeled integrin β1 antibodies (4B4-FITC, Beckman Coulter, Fullerton, CA), PE-conjugated integrin αV antibodies (clone 13C2, Millipore) and FITC-RGD peptides (FITC-LC-GRGDSP, Anaspec, Fremont, CA), respectively. Subsequently, the cells were analysed by FACS analysis in a FACS Calibur machine (BD Biosciences, Heidelberg, Germany).

**Analyzing integrin α5 recycling**

**Antibody feeding.** Integrin α5-specific antibody (5µg/mL in EGM buffered with Hepes; clone HA5, Millipore) was bound to the surface of serum-starved cells at 4°C and after washing the cells were warmed so that internalization of the antibody-bound integrin occurred. Residual antibodies remaining on the surface were removed with an acidic washing step (0.5% acetic acid, 0.5M NaCl in PBS with Ca/Mg, pH3.0) on ice and subsequently, warm (37°C) medium containing 10% FCS was added to initiate integrin α5 recycling. After 5 to 15 minutes the cells were fixed and stained with AlexaFluor 488-conjugated goat anti-mouse antibody without permeabilization. By this only the integrin α5 which reappeared on the surface was labelled. Thereafter, the cells were permeabilized staining with AlexaFluor 647-conjugated goat anti-mouse for the total antibody-marked integrin α5. Cells were analyzed by laser scanning microscopy (LSM 510 meta, Zeiss). During each experiment, identical microscope and computer settings were used. For quantification the ratio of surface to total integrin α5 was determined in 5 fields of vision per slide and averaged.

**Surface biotinylation.** Integrin α5 internalization and recycling was measured as described in 4. Briefly, following surface labelling using NHS-SS-biotin, cells were transfected to serum-free EBM for 30 min at 37°C to allow internalization. On ice, biotin was removed from proteins remaining at the cell surface by reduction with MesNa. Recycling was allowed by returning the cells to 37°C for the times indicated and following, the biotin was removed from recycled proteins by a second reduction with MesNa on ice. Biotinylated integrin α5 was determined in

**GST pulldown**

The GST construct of the intracellular domain of integrin α5 was a generous gift from Dr. Jim C. Norman (Beatson Institute for Cancer Research, Glasgow, U.K.). HEK cells were transfected with GFP, wildtype Scrib and mutants either missing the PDZ or LRR domains, respectively using lipofectamine 2000 (Invitrogen) with dimethyl pimelimidate. Cells were lysed with a Triton-X100 lysis buffer and incubated with a GST construct of the intracellular domain of integrin α5 over night at 4°C. The beads were washed, centrifuged and the bound proteins were eluted with 10mM glutathione. The eluates were subjected to Western blot. A GST construct was used as a control and did not bind Scrib.
the different fractions by capture-ELISA using an integrin α5 antibody.

**Immunofluorescence/TIRF microscopy**

Cells were treated as described in the “results” section, fixed in phosphate-buffered formaldehyde solution (4%), permeabilized with Triton X-100 (0.2%) and blocked with bovine serum albumin solution (3%) in phosphate-buffered saline. Scrib was detected with a specific polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), integrin α5 by a monoclonal antibody (Millipore, Schwalbach, Germany), Rac-1 by a monoclonal antibody (Millipore) and F-actin by Alexa Fluor 647 labelled phalloidin (Invitrogen). The cells were then washed and incubated with fluorescent secondary antibodies (Alexa), mounted and images were acquired either by confocal microscopy (LSM 510, Zeiss) or by TIRF microscopy (Olympus). Lysosomes were stained with Lyso Tracker red DND99 (Molecular Probes).

**Duolink analysis**

Duolink analysis was performed as described in the manufacturer’s protocol (Duolink II Fluorescence, Olink, Upsalla, Sweden). Briefly, HUVEC were treated as described in the “results” section, fixed in phosphate buffered formaldehyde solution (4%), permeabilized with Triton X-100 (0.2%), blocked with serum albumin solution (3%) in phosphate buffered saline and incubated over night with antibodies against Scrib (Santa Cruz Biotechnology, Santa Cruz, CA), integrin α5 (Millipore, Schwalbach, Germany), integrin β1 (provided by Johannes A. Eble) and cyclooxygenase-2 (BD Bioscience, Heidelberg, Germany), respectively. After washing, samples were incubated with the respective PLA-probes for one hour (37°C), washed and ligated for 30min (37°C). After an additional washing, amplification with polymerase was allowed for 100min (37°C). To show the specificity of the Scrib antibody, a blocking peptide was used (Scrib C-20 P, Santa Cruz). The basal plasma membrane was shown by paxillin (BD Bioscience) and the nuclei were stained using DAPI. Images were acquired by confocal microscope (LSM 510, Zeiss).

**Cell migration assays**

**Scratched wound assay.** Random cell migration was analyzed in a scratched wound assay. Cells were transfected with scrambled or Scrib siRNA and 72 hours after transfection the monolayer was wounded with the aid of a sterile pipette tip and cells were allowed to migrate in the presence of 8% FCS for the times indicated.

**Transwell assay.** Directed endothelial cell migration was investigated in a modified Transwell chamber system as previously described. Briefly, after transfection, endothelial cells were counted and seeded on membrane inserts (FluoroBlok, 3 µm pore size, BD Bioscience, Heidelberg, Germany) in the presence of EBM supplemented with 0.1% BSA. The lower chamber contained EBM supplemented with 4% FCS. After 20 hours, the cells on the upper surface of the filter were removed mechanically and cells that had migrated into the lower compartment were fixed (4% paraformaldehyde in PBS), stained with DAPI and counted (8 images per well, x200 magnification).

**Chemotaxis assay.** Endothelial cells were transfected, seeded in chemotaxis chambers (Ibidi, München, Germany) and a gradient of FCS (0-20%) was applied to the cells as suggested by the manufacturers protocol. Cell migration was studied over a period of 20 hours in a Zeiss Z1 Cell Observer equipped for automated live cell imaging at 37°C. The Zeiss AxioVision software was used for quantification.

**In vitro angiogenesis assays**

**Tube formation.** For the Matrigel assay, HUVEC were transfected with scrambled or Scrib siRNA and seeded onto Matrigel (1x10⁴ cells/cm²) 72 hours after transfection. Tube formation was assessed after 4 hours and quantified by counting the number of branch points.

**Endothelial cell spheroids.** HUVEC transfected either with scrambled or Scrib siRNA for 48 hours were used to generate spheroids containing 400 cells as described. After 24 hours in the collagen gel, angiogenic sprouting was quantified by measuring mean tube number, mean tube length and cumulative tube length of all capillary like sprouts originating from the central plain of an individual spheroid by computer assisted microscope. At least five spheroids per data point and experiment were analyzed. In a variation of this assay (compensation assay), spheroids were generated from two differently treated groups of cells labelled with genetically coded fluorescence probes. For this, cells were transduced with lentivirus to express YFP and CFP, respectively. One of the cell populations served as control cells transfected with scrambled siRNA, whereas the other was transfected with Scrib siRNA. The cells were mixed (1:1) and the spheroid assay was performed as described above.
Zebrafish studies

Zebrafish strains and lines and morpholino injection. Zebrafish were raised under standard laboratory conditions at 28° C. The Tg(fli1:EGFP)y1 and Tg(kdrl:EGFP)s847 transgenic lines were used. Embryos were injected with 8ng of previously reported morpholinos: scribMo1 designed against the start codon of scribble: 5'-CCACAGCGGGATAACACTTCAGCATG-3', or scribMo2: 5'-ACAAAAGTTTGCATACCATTTCTAG-3' at the 1 to 4-cell stage. Both morpholinos blocked scribble expression as demonstrated by Western blot. To block integrin α5 expression in the zebrafish, embryos were injected with 8ng of a previously described itga5 morpholino (itga5Mo): 5'-AATCAACAACGAAAATGGCCATTGGA-3'.

Imaging of live zebrafish embryos. One day old live embryos were embedded in 1.5% low melting agarose (Roth) in embryo medium (E3) in glass bottom dishes (MatTek). Development of the embryos was either observed over a period of 24 hours with a Zeiss Z1 Cell Observer equipped for automated live cell imagine or single photos were taken at 30 and 48 hours post fertilization. Stacks of optical sections of GFP fluorescent blood vessels were acquired with a Zeiss 510 meta confocal microscope and processed in Zeiss Image Browser and Adobe Photoshop.

Scrib expression in zebrafish. After mRNA isolation of approx. 20 zebrafish embryos (control and Scrib morpholino treated, respectively, 48 hours post fertilization) with the RNA Minikit (Bio&Sell, Feucht, Germany), cDNA synthesis was performed with SuperScript III Reverse Transcriptase (Invitrogen, Darmstadt, Germany) and random hexamer primers. For semiquantitative real-time PCR Fast Plus EvaGreen MasterMix qPCR w/low Rox (Biotium, Hayward, California, USA) was used with appropriate primers in a Mx3005 qPCR cycler (Stratagene). The PCR products were separated in an agarose gel.

For determination of Scrib protein expression in zebrafish, for each sample two zebrafish were homogenized in Laemli buffer and subjected to SDS-PAGE. Western blot analyses were performed with an infrared-based laser scanning detection system (Odyssey, Llicor, Bad Homburg, Germany). Scrib was detected by a polyclonal antibody raised in rabbit (Millipore, Schwalbach, Germany).

Scrib expression in endothelial cells of the zebrafish was performed as previously described. Briefly, zebrafish embryos (48 hours post fertilization) were digested with trypsin for 1 hour at 28 °C during which they were triturred with a 200 µL pipette tip every 10 min. Digestion was stopped by fetal calf serum to a final concentration of 10%. Cells were centrifuged for 3 min at 3000 rpm, rinsed once with PBS and resuspended with phosphate buffer saline containing CaCl2, MgCl2 and fetal calf serum (1%). FACS of single cell suspensions was performed at room temperature using a FACSaria (BD Biosciences). The resulting GFP+ and GFP- cells were subjected to PCR or Western blot as described above.

Mouse embryo wholemount staining

Mouse embryos were fixed in phosphate-buffered formaldehyde solution (4%), washed with phosphate buffered saline containing 0.1% Triton X-100 and blocked with horse serum solution (10%) in phosphate-buffered saline. Embryos were incubated with an endomucin antibody (eBioscience) over night (4°C) and following washed with phosphate buffered saline containing Triton (0.1%) and horse serum (10%). After washing and incubation with a secondary antibody (Alexafluor 546), embryos were analyzed by confocal microscopy (LSM 510, Zeiss).

Retinal angiogenesis

The experiments were performed in accordance with the National Institutes of Health Guidelines on the Use of Laboratory Animals. Both the University Animal Care Committee and the Federal Authorities for Animal Research of the Regierungspräsidium Darmstadt (Hessen, Germany) approved the study protocol. Eyes from postnatal day 5.5 C57Bl/6 (wt) and heterozygous crc (crc/+) mice (N=6 of each group) were fixed for 2 hours in 4% paraformaldehyde. Retinas were permeabilized in 3% BSA and 0.5% Triton X- 100 then incubated overnight with FITC-conjugated isolecitin-B4 (Sigma). After washing, retinas were mounted on slides for visualization with a Zeiss LSM meta 510 confocal microscope.

Statistics

Data are expressed as the mean ± SEM. Statistical analyses were performed by student’s t-test or one-way ANOVA followed by Bonferroni’s multiple comparison test, where appropriate. Values of P<0.05 were considered statistically significant.
References


Online Figure I. Scrib mRNA and protein expression in different cell types and role of Scrib for endothelial cell polarization. (A) Representative mRNA and protein expression of Scrib as determined by qRT-PCR relative to EEF2 and Western blot in neuroblastoma and epithelial tumour cell lines and human umbilical vein endothelial cells (HUVEC) as well as the human microvascular endothelial cell line HMEC-1. (B&C) Role of Scrib in planar polarity: HUVEC transfected with either scrambled (siScr) or Scrib (siScrib) siRNA were seeded in ibidi inserts to obtain a cell free border of the cells. One hour after removing the insert, cells were fixed. (B) After staining the cells for VE-cadherin (green) and the nuclei (DAPI, white), the relative position of the nucleus in the cell was determined. Representative photographs and bar graph showing the quantification of the localization of the nucleus relative to the rear end of the cells close to neighbouring cells are depicted. Western blot showing Scrib expression after treatment with scrambled or Scrib siRNA, β-actin served as a loading control. (C) Golgin 97 (green), phalloidin (red) and DAPI (white) were stained to determine golgi localization relative to the nucleus. Results of three to four independent experiments are summarized; The scale bars represent 20µm. **P<0.01; ***P<0.001 vs. scrambled (siScr).
Online Figure II. Effect of Scrib downregulation on in vitro angiogenesis. (A&B) Effect of Scrib siRNA treatment (siScrib) or control siRNA (siScr) on tube formation of human microvascular endothelial cells (HMEC-1). (A) Representative microscopic image and statistical analysis of sprouting from spheroids. The scale bar represents 100 µm. (B) Representative image and branching point analysis of the tube formation assay on Matrigel. The scale bar represents 200 µm. (C) Statistical analysis of sprouting from spheroids after transfection of HUVEC with scrambled (siScr) and Scrib (siScrib) siRNA. Endothelial sprouting was determined 24 hours after seeding into a collagen matrix by a computer assisted microscope. (D) Compensation assay: Exemplary confocal microscopy image and statistical analysis of spheroids generated from cells treated with scrambled siRNA (labelled by lentiviral transduction of CFP shown in green) and Scrib siRNA (labelled by lentiviral transduction of YFP shown in red) -treated HUVEC. Statistical analysis for the distribution of siScr and siScrib treated cells identified by their colour. Data shown as mean number of sprouted cells per spheroid as well as in tip and stalk cell position. The scale bar represents 50µm. N=4-5, *P<0.05 ; **P<0.001 vs. scrambled (siScr).
Online Figure III. Effect of Scrib downregulation on directional cell migration. (A) Transwell assay: Relative effect of Scrib siRNA as compared to scrambled siRNA on the migration of HUVEC in response to FCS (4%) and bFGF (30 ng/mL). (B,C&D) Chemotaxis assay in chambers coated with (B) fibronectin and (C&D) collagen: Effect of scrambled (siScr) and Scrib (siScrib) siRNA on migration of HUVEC in a gradient of FCS (0-20%). The cells were allowed to migrate for 20 hours. Straight distance and the tortuosity of migration were determined by computer aided analysis. n=4-5, *P<0.05, **P<0.01 vs. scrambled (siScr)
Online Figure IV. Interaction of Scrib and integrin α5. (A) Representative images of Duolink analyses of the interaction of Scrib and integrin α5 and the respective controls as indicated. Positive signals demonstrating an interaction of the indicated proteins are shown as red dots. Co-staining with paxillin (green) and DAPI (white) to show the basal plasma membrane and the nucleus, respectively. α5: integrin α5; β1: integrin β1; blocking peptide: blocking peptide for the Scrib antibody; COX-2: cyclooxygenase 2. The scale bars represent 20µm. (B) Bar graph showing the effect of Scrib siRNA treatment on integrin α5 mRNA expression determined by qRT-PCR. n=3.
Online Figure V. Effect of silencing and overexpression of integrin α5 after Scrib siRNA treatment on directed cell migration. (A,B&C) Tracings (A) and statistical analysis of the effect of integrin α5 siRNA on the migration of HUVEC in fibronectin (B) or collagen (C) coated chemotaxis chambers in response to a gradient of FCS (0-20%). (D) HUVEC were transfected with Scrib siRNA followed by integrin α5 expression plasmid and migration was determined in a fibronectin coated chemotaxis chambers in response to a gradient of FCS (0-20%). Bar graphs show mean of totally migrated distance, direction relative to the gradient, straight distance and tortuosity which were determined by computer aided analysis. n=3-4, *P<0.05, **P<0.01, ***P<0.001 vs. scrambled (siScr) and siScr+GFP, respectively.
Online Figure VI. Effect of Scrib downregulation on integrin α5 protein stability, internalization and recycling. (A) Determination of integrin α5 protein stability. Scrambled or Scrib siRNA treated HUVEC were incubated with cycloheximide (15µg/ml) for the times indicated, and integrin α5 expression was determine by Western blot. The time course shows the results of three independent experiments. (B) Effect of MG132 (MG, 10µmol/L), leupeptin (Leu, 30µmol/L), chloroquine (Chloro, 200µmol/L), pepstatin (Pep, 2µmol/L), E64d (20µmol/L) and PMSF (200µmol/L) treatment for 2 hours on integrin α5 protein expression as determined by Western blot in HUVEC treated with scrambled (siScr) or Scrib siRNA (siScrib). Data show the relative change in protein abundance compared to solvent treated cells. N=4, *p<0.05 siScr vs siScrib. (C) HUVEC were transfected with either scrambled (siScr) or Scrib (siScrib) siRNA and cell surface proteins marked by sulfo-NHS-SS-biotin. Thereafter, the cells were allowed to internalize and/or recycle the cell surface proteins and the protein amounts of total, internalized and recycled biotinylated integrin α5 were determined by ELISA. N=4 independent experiments, *P<0.05 vs. scrambled (siScr)
Online Figure VII. Scrib regulates integrin α5 and Rab7a interaction and silencing Rab7a rescues integrin α5 protein amount. (A) Representative images and quantification of Duolink analyses of the interaction of integrin α5 and Rab7a and the respective secondary antibodies alone as controls. Positive signals demonstrating an interaction of the indicated proteins are shown as red dots and DAPI (white) shows the nuclei. The scale bars represent 20µm. For the quantification, the positive signals were counted automatically by the microscope software (Axiovision) and related to the number of cells and the integrin α5 protein amount present in the cells determined by Western blot. (B) HUVEC were transfected with scrambled (siScr) or Scrib (siScrib) siRNA with or without Rab7a siRNA (siRab7a) and integrin α5, Scrib and Rab7a were detected by Western blot. Representative Western blot are depicted. N=3-4, ***p<0.001 vs. siScr
Online Figure VIII. Role of Scrib during developmental angiogenesis and posttranscriptional regulation of Scrib expression. (A) Representative microscopic images of wholemount stainings of wild type (wt) and homozygous crc (crc/crc) mouse embryos. Endothelial cells were stained by endomucin. (B) Representative microscopic images and statistics for width of vascularisation and vascularised area relative to retina size of the retina in wild type (wt) and heterozygous crc mice (crc/+) on day P5.5 as stained with FITC-labelled lectin. The scale bar represents 500µm. N=6, **P<0.01. (B) Effect of an 8 hours treatment with MG132 (MG, 10µmol/L), leupeptin (Leu 10µmol/L) and chloroquine (Chloro, 100µmol/L µM) and solvent (Sol) on Scrib protein abundance. Data shown are relative to the solvent control, n=5, *P<0.05 **P<0.01 vs. solvent.
Online Figure IX. Expression of Scrib in Tg(fli1:EGFP)y1 zebrafish and endothelial cells of Tg(fli1:EGFP)y1 zebrafish and contribution of Scrib and integrin α5 to developmental angiogenesis in the zebrafish. (A) Scrib protein and mRNA expression in GFP⁺ (endothelial cells) and GFP⁻ cells, respectively, sorted from Tg(fli1:EGFP)y1 zebrafish by FACS. (B) Western blot showing the expression of Scrib in control zebrafish and after injection of Scrib morpholino 1 (Mo1) and Scrib morpholino 2 (Mo2), respectively. (C) Bar graph showing the quantification of the mean length of the intersegmental vessels at 30 hours post fertilization in embryos after injection of 4ng of control (CtlMo), Scrib (ScribMo1), integrin α5 (itga5Mo) or a combination of Scrib and integrin α5 morpholinos. (D) Representative fluorescence microscopic images showing the effect of a combined injection of Scrib morpholinos (ScribMo1) and integrin α5 morpholinos (itga5Mo) on vessel development 53 hours post fertilization. N=6-8; **P<0.01, ***P<0.001 vs. scrambled (CtlMo)
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**Online Table I.** Table showing Integrin α5 peptide sequences indentified after immunoprecipitation of Scrib by LC-MS/MS analysis: observed mass over charge ratio of precursor ion, measured and calculated mass of peptide in Dalton, mass deviation in ppm and peptide Mascot score.