The Polarity Protein Scrib is Essential for Directed Endothelial Cell Migration

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ABSTRACT

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 Scrib. On fibronectin-coated carriers Scrib siRNA (siScrib) blocked directed but not random migration of human umbilical vein endothelial cells (HUVECs) and lead to an increased number and disturbed orientation of cellular lamellipodia. Co-immunoprecipitation/mass spectrometry and GST pulldown assays identified integrin α5 as a novel Scrib interacting protein. By TIRF microscopy, Scrib and integrin α5 colocalize at the basal plasma membrane of HUVEC. Western blot and FACS analysis revealed that silencing of Scrib reduced the protein amount and surface expression of integrin α5 subunit whereas surface expression of integrin αV subunit 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, translocation into lysosomes and its subsequent degradation by pepstatin-sensitive proteases. In siScrib-treated cells, reinduction of the wildtype protein but not of PDZ- or LRR-domain mutants restored integrin α5 abundance and directional cell migration. The downregulation of Scrib function in Tg(kdrl:EGFP)s843 transgenic zebrafish embryos delayed the angiogenesis of intersegmental vessels.

Conclusion: Scrib is a novel regulator of integrin α5 turnover and sorting, which is required for oriented cell migration and sprouting angiogenesis.

Keywords: Angiogenesis, polarity proteins, integrin α5, endothelial cell, migration, fibronectin

Nonstandard Abbreviations:

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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>Scrib</td>
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<tr>
<td>Lgl</td>
<td>Lethal giant larvae</td>
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<td>Dlg</td>
<td>Discs large</td>
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<tr>
<td>LRR</td>
<td>Leucine rich repeat</td>
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<td>PDZ</td>
<td>PSD95, Dlg, ZO-1</td>
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<tr>
<td>ZO-2</td>
<td>Zona occludens-2</td>
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<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
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<td>PAK</td>
<td>p21-activated protein kinase</td>
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<td>GST</td>
<td>glutathione S-transferase</td>
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<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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<td>TIRF</td>
<td>total internal reflection fluorescence</td>
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<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
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<td>HMEC-1</td>
<td>human microvascular endothelial cells-1</td>
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<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>GIT1</td>
<td>G-protein-coupled receptor kinase interacting ArfGAP1</td>
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<tr>
<td>RGD</td>
<td>L-ArginyGlycyl-L-Aspartic acid</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>crc</td>
<td>circle tail</td>
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<td>wt</td>
<td>wild type</td>
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<td>MDCK</td>
<td>Madin Darby canine kidney</td>
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INTRODUCTION

Cell polarity is essential for the function of all eukaryotic cells since it is involved in processes like cell differentiation, cell division and morphogenesis. Especially in epithelial cells, apico-basal polarity accounts for the asymmetric 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, three 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), 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.4 Scrib also regulates planar cell polarity of epithelial cells since the lack of Scrib causes a defect in the planar polarization of the inner ear.5 Mammalian Scrib may also regulate epithelial cell proliferation,6, 7 In the colon Scrib is downregulated during malignant progression and its expression pattern correlates with loss of cell polarity.8 The functions of Scrib in epithelial cell polarization are mediated in part by the LRR domain, which tethers Scrib to the plasma membrane. Via its PDZ domains Scrib interacts with ZO-2 10, zyxin 11, APC 12 and the guanine nucleotide exchange factor βPIX.13 The latter has been implicated in the effect of Scrib on cell migration of epithelial cells and fibroblasts by scaffolding PAK and Scrib in a protein complex.14

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 towards 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 Supplementary 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 LTQ Orbitrap mass spectrometry, GST pulldown, FACS analysis, analysis of integrin α5 recycling by antibody feeding and cell surface biotinylation, immunofluorescence and 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 similar mRNA expression of all members (Scrib, Dlg5, Lgl1 and Lgl2) of the Scrib complex. 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 HMEC-1 endothelial cell line was comparable to that of different epithelial cell lines (Online Fig. 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.\(^1\) In the scratch wound assay, this characteristic of planar polarization was also observed in HUVEC (Online Fig. IB). Down-regulation of Scrib by siRNA (siScrib) prevented the effect and resulted in an almost central localization of the nucleus (Fig. S1B). 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 Fig. 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 bFGF was significantly attenuated by Scrib siRNA (HUVEC: Fig. 1, HMEC-1: Online Fig IIA). The latter effect was predominantly a consequence of a reduction in the number of tubes formed (Online Fig. IIB). This may suggest that Scrib regulates endothelial tip cell formation. Indeed, in a compensation assay in which control and Scrib siRNA treated, labelled HUVEC were mixed in one spheroid, Scrib downregulation was associated with a decrease in the frequency of cells in the tip position (Online Fig. 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 (Fig. 2A). In contrast, Scrib was required for the chemotactic response. In a transwell assay, Scrib siRNA reduced migration in response to bFGF and FCS by approx. 50% (Online Fig. 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 siRNA their migrated path was more tortuous and the distance migrated towards the chemotactic gradient was reduced by approx. 50% (Fig. 2B&C, Online Fig. IIIB).

Scrib regulates lamellipodia localization and number.

During directed migration, cells form a stabilized lamellipodium at their leading front, which is characterized by Rac-1 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 two and the orientation of these protrusions towards the chemotactic gradient was lost (Fig. 2D & E).

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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 co-precipitating proteins by LTQ orbitrap mass spectrometry. By this method, we could confirm several known interactors of Scrib, like βPIX and GIT1. Importantly, as a so far unreported interactor we co-precipitated integrin α5. Two to three peptides corresponding to the integrin α5 sequence were identified by mass spect analysis in three independent experiments (Online Table I). Importantly, the interaction of Scrib and integrin α5 is also evident by co-immunoprecipitation followed by Western blot (Fig. 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 overexpressing 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 (Fig. 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 sub-membrane regions like focal adhesions in the intact cell (Online Fig. IVA). Indeed, by TIRF microscopy exclusively visualizing the basal plasma cell membrane, distinct colocalization of Scrib and integrin α5 was observed in focal adhesions (Fig. 3C).

Scrib controls integrin α5 surface expression.

As 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 Fig. IVB), its down-regulation reduced the integrin α5 protein amount by approx. 70 % (Fig. 3D). By confocal microscopy it was obvious, that particularly the integrin α5 abundance in focal adhesions was reduced by Scrib siRNA (Fig. 3E). Therefore, also integrin α5 surface expression was reduced by silencing Scrib. Integrin α5 binds to RGD-containing peptides, and the binding capacity of cells to FITC-labelled RGD-containing peptides as determined by FACS was significantly reduced after Scrib down-regulation (Fig. 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 (Fig. 3G). In order 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 (Fig. 3H and Online Fig. IIIC&D). 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 Fig. VA, B & C). Silencing of integrin 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 Fig. VD). These data illustrated 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

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effect on cells treated with control siRNA, wild-type Scrib restored integrin α5 expression (Fig. 4A) and migration in cells pre-treated with Scrib siRNA (Fig. 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, post-translational 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 approximately 30 to 15 min (Online Fig. VIA). 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 Fig. VIB). To address the role of Scrib for a potential lysosomal degradation of integrin α5, we studied the intracellular localization of 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 (Fig. 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, Fig 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 to siScr; siScrib+Pep 114.6 % ± 21.6 compared to 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% (Fig. 4D&E, Online Fig. VIC). To further characterize the involved mechanism, we performed a second proteomic screen, this time for the effect of Scrib on the integrin α5 interactome. LTQ Orbitrap mass spectrometry for integrin α5 co-precipitating 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 Fig. VIIA). 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 Fig. VIIB). 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.

As 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 Fig. VIII A). 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 published 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 vascularised area (Online Fig. VIII A). Given that heterozygosity 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 Fig. VIII B). 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 Fig. VII C). As an approach to confirm the delayed retinal angiogenesis induced by Scrib deficiency, we studied vascular development in $Tg(kdrl:EGFP)^{3843}$ zebrafish embryos, in which the endothelium is labelled by kdr1-promoter-driven green fluorescent protein (GFP). Scrib expression was detectable in GFP-positive endothelial cells as well as GFP-negative remaining cells of fish, as determined by FACS sorting followed by Western blot as well as RT-PCR (Online Fig. IX A). Two different morpholinos were injected which both attenuated Scrib expression in the zebrafish embryos (Online Fig. IX B). Targeting Scrib caused a clear delay in the formation of the intersegmental vessels (Fig. 5 A & B). Furthermore, zebrafish treated with scrib morpholinos showed severe malformation of the vessels in the brain with spontaneous haemorrhages (Fig. 5 C). Importantly, also integrin $\alpha_5$ morphants delayed the formation of intersegmental vessels, which may suggest that also in the zebrafish, integrin $\alpha_5$ among other important proteins is an effector of Scrib (Fig. 5 B). These data could be strengthened by the accumulative effect of Scrib and integrin $\alpha_5$ 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 Fig. IX C & D). These observations demonstrate that also in vivo Scrib contributes to angiogenic processes involving directed endothelial cell migration and integrin $\alpha_5$ signaling.

DISCUSSION

In this manuscript we demonstrate that Scrib is essential for directional endothelial cell migration towards a chemotactic stimulus and that Scrib regulates endothelial tube formation in vitro and angiogenesis in vivo. In this regards, Scrib specifically regulates integrin $\alpha_5$ abundance by preventing integrin $\alpha_5$ interaction with Rab 7a and subsequent lysosomal degradation. By this mechanism, Scrib facilitates integrin $\alpha_5$ 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 signalling 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 baso-lateral 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 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, 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 GEF 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. 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. 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. Via its PDZ domain Scrib recruits GEFs like βPIX which are involved in Rac1 and Cdc42 activation and therefore might regulate the availability of GEFs, Cdc42 and Rac for 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 other sites than those described for other cell types. Searching for such new interactors we identified integrin α5 to co-precipitate 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 co-localization 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 as the accumulation of Scrib in lateral cell contacts dominates the picture. Moreover, our data demonstrate that this interaction is functionally relevant, as Scrib stabilizes 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 the recycling of integrins is necessary for regulated cell migration. This process can be 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 the 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 coimmunoprecipitation, 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 down-regulation of Scrib is Rab7a. This small GTPase has been described to regulate the sorting from late endosomes to lysosomes. These data could be confirmed by Duolink proximity ligation assay analysis. Importantly, downregulation of Rab7a indeed rescued integrin α5 protein amount after down-regulation 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 towards a single, Rab7a-dependent degradation pathway. This observation was
confirmed by the use of inhibitors of different degradation pathways: Inteegrin 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 sphere was attenuated by downregulation of Scrib. This finding could be recapitulated in developmental angiogenesis in the Tg(kdrl: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
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FIGURE LEGENDS

Figure 1. Role of Scrib for in vitro angiogenesis: Effect of Scrib downregulation (siScrib) or control siRNA (siScr) on human umbilical vein endothelial cells (HUVEC). (A) Representative microscopic image and branching point analysis of the tube formation assay on Matrigel. The scale bar represents 200 µm N=4, ***p<0.001 (B) Representative image of endothelial cell sprouting from spheroids 24 hours after seeding into a collagen matrix. The scale bar represents 100µm.

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&C) Representative tracings (B) and statistical analysis (C) of the effect of siRNA on the migration of HUVEC in fibronectin coated chemotaxis chambers in response to a gradient of FCS (0-20 %). Bar graphs show mean of totally migrated distance and direction relative to the gradient. (D) Representative photographs and (E) statistical analysis of lamellipodia localization in the afore mentioned 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 Rac-1 accumulation relative to the nucleus and the direction towards 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=4-5 independent experiments, 15-36 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 HUVEC. (A) Western blot for integrin α5 and Scrib after immunoprecipitation of Scrib. (B) Western blots of a pull down 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. Ini: initial input; PD: Elution of the GST-pull down. (C) Representative 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 (FCS, 6 hours). The scale bar represents 20 µm. (F &G) Representative FACS recordings for the effect of Scrib downregulation on binding of FITC-RGD-containing 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 FCS. The total migration distance and the direction relative to the gradient are shown. N=4-6.

Figure 4. Role of Scrib for integrin α5 recycling and degradation. (A&B) Effect of transfection of Scrib wild-type and mutant plasmids on (A) integrin α5, β-actin and Scrib expression and (B) directed cell migration in a transwell assay in HUVEC treated with scrambled (siScr) or Scrib siRNA (siScrib). The plasmids wild type Scrib (wt) and Scrib deletion mutants of the PDZ domain (ΔPDZ) or the LRR domain (ΔLRR) were modified by introducing still mutations to render them siRNA resistant. Green fluorescent protein (GFP) was used as control plasmid. Numbers below the integrin α5 Western blot indicate the results of the densitometry relative to the housekeeping gene β-actin, N=3 separate experiments. *p<0.05. (C) Effect of Scrib siRNA treatment on lysosomal accumulation of integrin α5-GFP. HUVEC were transfected with scrambled (siScr) or Scrib siRNA (siScrib) and integrin α5-GFP, treated with pepstatin for 2 hours and incubated with Lyso Tracker red DND99. Representative images and graphs showing the correlation of the relative signal intensity of the integrin α5-GFP and Lyso Tracker signal along a line in one representative cell. R= mean correlation coefficient of 11 to 12 cells. The scale bar represents 10 µm. (D) Representative images and (E) statistical analysis of integrin α5 recycling as determined by antibody feeding in HUVEC. Quantification depicts the ratio of surface to total integrin α5 staining. The scale bar represents 20µm. N=3, p<0.05 vs. scrambled (siScr).
**Figure 5. Contribution of Scrib and integrin α5 to developmental angiogenesis in the flkGFP zebrafish mutant.** Representative fluorescence microscopic images showing the effect of Scrib morpholinos (ScribMo) on vessel development 48 hours post fertilization (A) of the intersegmental vessels and (C) of vessels in the brain. (B) mean length of the intersegmental vessels at 30 hours post fertilization in Scrib (ScribMo1 & 2) and integrin α5 (itga5Mo) morphants. BA, basilar artery; the scale bar represents 200µm. N=8; ***P<0.001 vs. scrambled (CtlMo)

**Novelty and Significance**

**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 endothelial 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 vivo in the mouse and in the zebrafish.

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 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 anti-angiogenic therapies. This appears to be particularly relevant for tumor angiogenesis as Scrib also acts as a tumor suppressor in epithelial cells.
Figure 2

A) Bar graph showing the migrated distance (% SiScr) for Scr, SiScrib1, and SiScrib3 over 3h, 6h, and 24h.

B) Diagram illustrating the gradient and cell movement.

C) Bar graph showing the total distance and directed migration for SiScr and SiScrib.

D) Images of cell morphology with SiScr and SiScrib.

E) Graphs showing lamellipodia localization and lamellipodia per cell for different conditions.
Figure 5
The Polarity Protein Scrib is Essential for Directed Endothelial Cell Migration
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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 silent 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 (Stratagene). 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: ATACCAGCCAGGAGTG; reverse: GCCATGGGCTCGAATGCCATACCTCATATGGAGTG; reverse: GCCATGGCGCTGAACTGTTG.

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. and analyzed by mass spectrometry. Briefly, the tryptic peptides obtained from each gel slice were subjected to LC-MS/MS analysis on an Orbitrap XL mass spectrometer (Thermo) with a nano-HPLC (Agilent) on the front end. Peptides were separated on a C18-reversed phase silica filled in a 75 µm ID PicoTip emitter (New Objectives) in 60 min HPLC runs using gradients with 0.1% formic acid from 5% to 50% acetonitrile following by 90% wash and 5% re-equilibration steps. Eluted peptides were analyzed by MS/MS method in positive mode programmed to fragment top ten most abundant ions using dynamic exclusion with a resolution of 30000 at 400Th. Single charged precursor ions were rejected, the fragmentation of peptide ions occurred in the linear ion trap by CID at 35% collision energy. Fragmentation spectra were extracted from RAW spectra using extract_msn (Thermo) and matched against the human database (Uniprot) containing 20366 sequences (Thermo) and matched against the human database (Uniprot) containing 20366 sequences. The search parameters were set as following: 15 ppm deviation on the precursor and 0.6 Da on fragment masses, fixed carbamidomethylation of cysteine, variable oxidation of methionine and one missed cleavage. Only peptides with Mascot scores above the significance threshold of 0.05 were taken into account.

**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) as described by the manufacturer. 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.

**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 Heps; 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 transferred 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
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 (1x10^4 cells/cm^2) 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'-CCACAGCGGGATACACTTCAGCATG-3’ or scribMo2: 5'-ACAAAAGTTTGCATACCATTTCTAG-3’ at the 1 to 4-cell stage. Both morpholinos blocked scribble expression as demonstrated by Western blot.

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 (Olympus, Licor, 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 triturated 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 isoelectin-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 200µm. (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 Srcib 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)\textsuperscript{y/l} zebrafish and endothelial cells of Tg(fli1:EGFP)\textsuperscript{y/l} zebrafish and contribution of Scrib and integrin α5 to developmental angiogenesis in the zebrafish. (A) Scrib protein and mRNA expression in GFP\textsuperscript{+} (endothelial cells) and GFP\textsuperscript{-} cells, respectively, sorted from Tg(fli1:EGFP)\textsuperscript{y/l} 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)
**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.

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