Polarity Protein Scrib Facilitates Endothelial Inflammatory Signaling

Christoph Kruse, Angela R.M. Kurz, Katalin Pálfí, Patrick O. Humbert, Markus Sperandio, Ralf P. Brandes, Christian Fork,* U. Ruth Michaelis*

Objective—The polarity protein Scrib is highly expressed in endothelial cells and is required for planar cell polarity. Scrib also facilitates recycling of integrin α5 to the plasma membrane. Because integrin α5 signals the presence of the inflammatory matrix protein fibronectin, we hypothesized that Scrib contributes to endothelial inflammatory signaling.

Approach and Results—Cytokine treatment of human umbilical vein endothelial cells induced an inflammatory response as evident by the induction of vascular cell adhesion molecule-1 (VCAM-1). Downregulation of Scrib greatly attenuated this effect. In endothelial-specific conditional Scrib knockout mice, in vivo lipopolysaccharide treatment resulted in an impaired VCAM-1 induction. These effects were functionally relevant because Scrib small interfering RNA of human umbilical vein endothelial cells attenuated the VCAM-1–mediated leukocyte adhesion in response to tumor necrosis factor-α. In vivo, tamoxifen-induced endothelial-specific deletion of Scrib resulted in a reduced VCAM-1–mediated leukocyte adhesion in response to tumor necrosis factor-α in the mouse cremaster model. This effect was specific for Scrib and not mediated by other polarity proteins. Moreover, it did not involve integrin α5 or classic pathways supporting inflammatory signaling, such as nuclear factor κ light chain enhancer of activated B-cells or MAP kinases. Co-immunoprecipitation/mass spectrometry identified the zinc finger transcription factor GATA-like protein-1 as a novel Scrib interacting protein. Small interfering RNA depletion of GATA-like protein-1 decreased the tumor necrosis factor-α–stimulated VCAM-1 induction to a similar extent as loss of Scrib did. Silencing of Scrib reduced GATA-like protein-1, but not mRNA abundance.

Conclusions—Scrib is a novel proinflammatory regulator in endothelial cells, which maintains the protein expression of GATA-like protein-1. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBAHA.115.305678.)

Key Words: cell polarity ■ endothelial cells ■ GATA-like protein-1 ■ inflammation ■ VCAM-1

Cell polarity is a fundamental characteristic of various cell types. Polarity is maintained by establishing an asymmetrical distribution of proteins at the plasma membrane. Cellular and tissue polarization is essential for processes, such as differentiation, cell division, junction formation, and migration. Three conserved multi-protein complexes have been identified to act as key polarity regulators in vertebrates and invertebrates: the Par complex, the Crumbs complex, and the Scribble complex.

The proteins are best studied in epithelial cell, where specific functions have been attributed to the distinct polarity complexes. The Par (Par3/Par6/aPKC) and Crumbs (Crumbs/Pals1/Patj) complexes are localized at the apical region of the cell and facilitate apical membrane identity.2,3 The Scribble module consists of Scribble (Scrib), lethal giant larvae, and discs large and is localized at the basolateral region. This complex promotes basal membrane identity, and thus, it regulates apico-basal polarity of epithelial cells.2

The Scrib protein is a highly conserved scaffold protein, containing 16 leucine-rich repeats,4 which restrict Scrib to the plasma membrane,2 and 4 PDZ domains.4 Through these, Scrib interacts with junctional adhesion molecules,5 adhesion molecules,6 and the guanine nucleotide exchange factor β-PIX.5 In epithelial cells, Scrib regulates cell adhesion and migration by stabilizing the coupling between E-cadherin and catenins.10 Analysis of homozygous mutant mice of Scrib demonstrated that Scrib is essential for planar cell polarity11 and for maintaining epithelial cohesion during lung development.12 Although the function of Scrib in epithelial cells is well studied, its role in endothelial cells remains unclear. We have...
previously reported that Scrib is involved in endothelial-directed cell migration and is required for angiogenesis. In particular, Scrib was involved in matrix signaling because it facilitates integrin α5 recycling to the plasma membrane and prevents its lysosomal degradation.

Integrins mediate cell–cell and cell–matrix interaction by binding ligands, such as fibronectin, vitronectin, collagen, and laminin. Depending on the matrix type, the endothelial activity state is altered. Although collagen is associated with endothelial quiescence, fibronectin is typical for an active endothelium and is associated with inflammatory activation. These matrix constituents facilitate inflammatory signaling, and integrin–ligand crosstalk support endothelial response to cytokines.

Based on our previous observation of an important role for Scrib in integrin α5 signaling, we hypothesize that the Scrib polarity protein promotes endothelial inflammatory signaling. To test this directly, we have examined cultured human endothelial cells and the systemic and local response of conditional tamoxifen-inducible endothelial-specific Scrib knockout mice.

**Material and Methods**

**Results**

Scrib Mediates Cytokine-Induced Vascular Cell Adhesion Molecule-1 Expression in Endothelial Cells

To assess the role of endothelial Scrib in inflammation processes, human umbilical vein endothelial cells (HUVEC) and human microvascular endothelial cells were transfected with small interfering RNAs (siRNAs) directed to Scrib or a control Scramble siRNA followed by stimulation with tumor necrosis factor (TNF)-α. Downregulation of Scrib led to a strong attenuation of the TNFα-stimulated vascular cell adhesion molecule-1 (VCAM-1) protein expression, but did not influence the intercellular adhesion molecule-1 (ICAM-1) protein abundance (Figure 1A). As inflammatory signaling is often nonspecifically disturbed by siRNA and transfection, knockdown experiments were performed with small hairpin RNA (shRNA) directed against Scrib. VCAM-1 induction in response to TNFα but also interleukin (IL)-1β and lipopolysaccharide was similarly attenuated by Scrib shRNA, suggesting that the inhibitory effect of Scrib downregulation is independent of the type of inflammatory stimulus (Figure 1B). Similar effects were observed on VCAM-1 mRNA expression, indicating that the effects of Scrib were likely through transcriptional regulation of VCAM-1 rather than the level of VCAM-1 protein translation, trafficking, or stability (Figure 1C). Rescue experiments were performed with a Scrib-expression plasmid containing silent mutations rendering it resistant to the Scrib shRNA. Scrib overexpression had no effect of TNFα-induced VCAM-1 expression in control cells, but restored VCAM-1 expression in cells

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>GLP-1</td>
<td>GATA-like protein-1</td>
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<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
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<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule-1</td>
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<tr>
<td>shRNA</td>
<td>small hairpin RNA</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<tr>
<td>TNFα</td>
<td>tumor necrosis factor α</td>
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<td>VCAM-1</td>
<td>vascular cell adhesion molecule 1</td>
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**Figure 1.** Effect of Scrib downregulation on vascular cell adhesion molecule-1 (VCAM-1) in human umbilical vein endothelial cells (HUVEC). A, Representative Western blot and densitometry for VCAM-1 and intercellular adhesion molecule-1 (ICAM-1) in HUVEC after treatment with Scrib siRNA (siScrib1, siScrib3) or Ctl (siScr, H2O) and stimulation with and without tumor necrosis factor (TNF)-α (1 ng/mL, 3 h), n=4. B, Representative Western blot and densitometry of VCAM-1 protein expression in Scrib shRNAs (shScrib)– or control shRNAs (Ctl)–transduced HUVEC stimulated with TNFα (1 ng/mL, 3 h), interleukin (IL)-1β (1 ng/mL, 3 h), or lipopolysaccharide (LPS; 10 μg/mL, 3 h), n≥4. C, Normalized VCAM-1 mRNA expression in Scrib shRNAs (shScrib)– or control shRNAs (Ctl)–transduced HUVEC stimulated with TNFα (1 ng/mL, 2 h), IL1β (1 ng/mL, 2 h), or LPS (10 μg/mL, 2 h), n≥4. *P<0.05 relative to the corresponding control. shRNA indicates small hairpin RNA; and siRNA, small interfering RNA.
treated with Scrib shRNA (Figure IB in the online-only Data Supplement). As we previously observed that integrin α5 is stabilized by Scrib13 and as matrix impacts on endothelial inflammatory signaling,19 we examined the relevance of these on the Scrib-dependent regulation of VCAM-1. Importantly, the anti-inflammatory effect of Scrib siRNA was matrix-independent, and downregulation of integrin α5 had no influence on TNFα-, IL1β- or lipopolysaccharide-induced VCAM-1 expression in HUVEC (Figure IC and ID in the online-only Data Supplement). Thus, the effect of Scrib in cytokine-induced VCAM-1 expression is independent of the previously reported role of Scrib in integrin α5 sorting.

VCAM-1 Expression Is not Attenuated by Other Polarity Proteins

As Scrib is involved in endothelial planar cell polarity and apico-basal polarity of epithelial cells, the impact of other polarity proteins on the TNFα-induced VCAM-1 expression was examined. siRNA against lethal giant larvae homolog 2 did not influence the responses, whereas siRNA against discs large homolog 1 and Pals1-associated tight junction protein actually potentiated the TNF-stimulated VCAM-1 expression (Figure 2). Thus, the effect of Scrib in maintaining endothelial inflammatory response is specific for this particular polarity protein and suggests that it may not be related to Scrib’s function in cellular polarity.

Scrib Is not Involved in Classic Inflammatory Signaling

Because nuclear factor κ light chain enhancer of activated B-cells (NF-κB) is the main driver of VCAM-1 expression in endothelial cells, the impact of Scrib on this transcription factor was studied. Unexpectedly, Scrib shRNA had no effect on NF-κB expression, phosphorylation, or translocation, nor was the IκB level affected by downregulation of Scrib (Figure 3A; Figure II in the online-only Data Supplement). Similarly, TNFα-induced VCAM-1 expression was not affected by Scrib downregulation (Figure 3B). Therefore, the NF-κB pathway is not involved in Scrib-mediated VCAM-1 expression.

As Scrib is involved in endothelial planar cell polarity and apico-basal polarity of epithelial cells, the impact of other polarity proteins on the TNFα-induced VCAM-1 expression was examined. siRNA against lethal giant larvae homolog 2 did not influence the responses, whereas siRNA against discs large homolog 1 and Pals1-associated tight junction protein actually potentiated the TNF-stimulated VCAM-1 expression (Figure 2). Thus, the effect of Scrib in maintaining endothelial inflammatory response is specific for this particular polarity protein and suggests that it may not be related to Scrib’s function in cellular polarity.

Figure 2. Effect of downregulation of polarity proteins on vascular cell adhesion molecule-1 (VCAM-1) in human umbilical vein endothelial cells (HUVEC). Normalized polarity protein mRNA (left) and VCAM-1 mRNA expression (middle) in lethal giant larvae homolog 2 (LLGL2) siRNA (siLLGL2), discs large homolog 1 (DLG1) siRNA (siDLG1), Pals1-associated tight junction protein (Patj) siRNA (siPatj), or GFP siRNA (siGFP)-transfected HUVEC stimulated with tumor necrosis factor (TNF)-α (1 ng/mL, 2 h). Right, Representative Western blots for VCAM-1 in HUVEC treated with siLLGL2, siDLG1, siPatj, or siGFP and stimulated with TNFα (1 ng/mL, 3 h). n=3. *P<0.05 relative to the corresponding control. siRNA indicates small interfering RNA.

GATA-Like Protein-1 Is an Interacting Partner of Scrib

In the search of potential other mediators of the effect of Scrib knockdown, we analyzed the VCAM-1 promoter. The promoter contains many GATA transcription factors–binding sites, and GATAs have previously been suggested to contribute to endothelial inflammatory signaling.21 Because we recovered the similar nuclear zinc finger protein GATA-like protein-1 (GLP-1) in our previous interaction search (Table I in the online-only Data Supplement),13 we focused on this protein. Little is known about GLP-1, although it was reported to act as transcriptional repressor of GATA factor function in somatic cells of the gonads.22 Its function in endothelial cells, however, is completely elusive. Importantly, we readily detected GLP-1 protein in HUVEC, human fibroblasts, human dermal microvascular endothelial cells, and human aortic smooth muscle cells (Figure IVA in the online-only Data Supplement). Immunoprecipitation from the cytosolic fraction of HUVEC clearly identifies Scrib as an interactor of GLP-1 (Figure 4A), and this interaction was also seen by proximity ligation assay (Figure 4B).

In the few reported on GLP-1, the protein is considered a transcriptional repressor,23 and thus, depletion of Scrib should result in higher GLP-1 level in the nucleus. In HUVEC, however, just the opposite was true: siRNA against...
GLP-1 attenuated the TNFα-stimulated VCAM-1 protein and mRNA expression to a similar extent as Scrib siRNA did (Figure 4C; Figure IVB and IVC in the online-only Data Supplement). Thus, Scrib controls the nuclear abundance and thus potential binding of GLP-1 to the VCAM-1 promoter. To test this aspect, chromatin immunoprecipitation experiments were performed. Indeed, it was possible to specifically recover elements of the VCAM-1 promoter by chromatin immunoprecipitation with an anti–GLP-1 antibody. Importantly, downregulation of Scrib reduced the recovered signal to the same extent as did downregulation of GLP-1. Thus Scrib, by limiting GLP-1 abundance, reduces the amount of this transcription factor binding to the VCAM-1 promoter (Figure 4F).

Scrib Mediates Leukocyte Adhesion In Vitro and In Vivo

To address the physiological relevance of the present findings, adhesion experiments were performed. First, the adhesion of freshly isolated human leukocytes to TNFα-stimulated HUVEC was studied. Downregulation of Scrib alone did not affect TNFα-induced leukocyte adhesion. As this might be a reflection of the fact that leukocytes can adhere to VCAM-1 and to ICAM-1 and as ICAM-1 expression was not controlled by Scrib, the experiments were repeated in the presence of an ICAM-1 blocking peptide. Importantly, in the presence of the peptide, leukocyte adhesion to Scrib-deficient endothelial cells was significantly attenuated (Figure 5A). To investigate whether these observations are also relevant in vivo, tamoxifen-induced endothelial-specific Scrib knockout mice were generated by crossing previously reported Scrib1-flox/flox mice24 with Cdh5-CreERT2 mice.25 By comparing intima and the remaining part of the carotid artery, effective tamoxifen-inducible selective Scrib knockout in the endothelium could be proven on mRNA level (Figure 5B). Moreover, similar to the cultured HUVEC, GLP-1 protein was also significantly reduced in the intima of the Scrib-deficient mouse aortas (Figure 5C).

Next, in vivo lipopolysaccharide, as a global strong inflammatory stimulus, was used to induce vascular VCAM-1 and ICAM-1 systemically. Similarly as in cultured HUVEC, VCAM-1 mRNA induction was significantly attenuated in the intima of endothelial-specific Scrib knockout mice as compared with wild-type animals, whereas Scrib did not affect ICAM-1 expression (Figure 5D). As an approach to directly visualize leukocyte adhesion to the endothelium in vivo, we used intravital microscopy of the mouse cremaster muscle. Microvascular and hemodynamic parameters were similar between endothelial-specific Scrib knockout mice and wild-type controls (Table). In addition, systemic leukocyte counts were not different between both groups (Table). Similar to the in vitro observations, our in vivo study revealed that TNFα-stimulated leukocyte adhesion in the cremaster muscle post-capillary venules of endothelial-specific Scrib-knockout mice was similar to that of the wild-type mice. However, after administration of the ICAM-1-blocking antibody YN1, leukocyte adhesion to Scrib-deficient endothelium was significantly reduced as compared with the endothelium of
wild-type control mice (Figure 5E). These data identify Scrib as a facilitator of VCAM-1 induction in vivo and, therefore, as an important regulator of leukocyte adhesion.

Discussion
In the present study, we demonstrated that Scrib promotes cytokine-stimulated expression of VCAM-1. Loss of Scrib attenuated VCAM-1–dependent leukocyte adhesion ex vivo and in the mouse cremaster model. The effect of Scrib was mediated through changes in VCAM-1 mRNA because Scrib maintained the cellular level of GLP-1, a novel inducer of VCAM-1 expression.

Our previous studies revealed that Scrib is essential for recycling integrin α5 to the plasma membrane. As fibronectin, the ligand of integrin α5, is a relatively proinflammatory matrix, we speculated that Scrib may indirectly regulate the inflammatory response of endothelial cells. Although we found evidence in favor for this theory, the impact of Scrib on inflammatory signaling was different from our initial concept. Scrib does not seem to be involved in cytokine or matrix signaling...
of inflammation per se, but rather specifically contributed to VCAM-1 induction. In fact, the inflammatory responses to the classical stimuli TNF-α, IL-1β, and lipopolysaccharide were not differentially affected by Scrib knockdown in cultured cells or in vivo. Rather, only the induction of VCAM-1 but not of ICAM-1 and E-selectin was affected by Scrib downregulation. Moreover, typical inflammatory pathways were totally independent of Scrib: cytokine-stimulated activation of NF-κB, the main driver for VCAM-1 and ICAM-1 expression, was not affected by Scrib knockdown. Also protein kinase B and MAP kinase, such as p38, c-Jun N-terminal kinases, and extracellular signal-regulated kinase 1/2 activation, which all are involved in VCAM-1 and ICAM-1 induction, were independent of Scrib.

These findings were unexpected as was our observation that integrin α5 did not influence the endothelial response to cytokines. Scrib is a known interactor of the Rac GEF betaPIX. Rac not only elicits effects on the cytoskeleton, but is also a main activator of NADPH oxidases of the Nox family. In fact,
inflammation is a main stimulus of Rac1 and of the NADPH oxidase, and Nox activation has been shown to contribute to inflammatory signaling. Despite this, we found no impact of Scrib on Nox activity (K.P., unpublished observations), and although we previously reported a role of Scrib on Rac1 localization within the cell, this function seems to occur in a compartment unrelated to inflammatory signaling.

Based on this, it is tempting to speculate that the effect of Scrib is not a specific function of this polarity protein, but common to the whole family of polarity complex proteins. Unexpectedly, of all the polarity proteins we knocked down in the present study, only loss of Scrib elicited an anti-inflammatory effect, suggesting that Scrib is special as a polarity protein. Structurally, Scrib is mainly an adaptor protein carrying leucine-rich repeats and PDZ domains. Both domains are frequent in cells, and other proteins harboring them contribute to inflammatory signaling. For example, PDZD12, a PDZ-domain-containing nuclear protein, acts as an ubiquitin E3 ligase for the NF-kB subunit p65, and the leucine-rich repeats protein LRRC33 inhibits NF-kB and AP-1 activation and cytokine production. Pals1-associated tight junction protein of the Crumbs polarity complex, however, is also a PDZ protein and did not promote inflammatory signaling, suggesting that the underlying mechanism is more complex.

In the present study, we observed that Scrib affects cytokine-stimulated VCAM-1 mRNA expression similarly as it changed protein abundance. This excludes that Scrib contributes to VCAM-1 protein stability, trafficking, or sorting and leaves mRNA transcription and thus the promoter as the most probable targets of Scrib. Our further analyses were focused on Fox protein transcription factors, which are present in the VCAM-1 promoter, and also previous studies have identified FoxO1 as a VCAM-1 promoter–binding protein. Indeed, Scrib was able to reduce FoxO1 in the nucleus of HUVEC, but against all odds, FoxO1 overexpression failed to rescue this effect. Moreover, inhibition of FoxO1 by an inhibitor did not alter VCAM-1 protein abundance in HUVEC.

This left GATA transcription factors as the most probable targets. The GATA family of transcription factors has 6 members as categorized by a DNA consensus sequence of 2 characteristic zinc-finger motifs. Interestingly, many pathways controlling inflammation alter GATA factors like MAP kinases or protein kinase B and the PTEN system.

By reevaluation of our mass spectrometry data from Scrib immunoprecipitation, we found GLP-1 to be a Scrib-interacting protein. GLP-1 is reported as a nuclear protein containing 2 zinc fingers, one of which is highly homologous to the conserved GATA zinc finger DNA-binding motif. Forcing expression of GLP-1 represses GATA in the activation of target promoters in Leydig cells of the testis and granulosa cells in the ovary. GLP-1lacZ null mice are infertile, caused by a marked reduction in oocyte development and severe sperm differentiation defects. Based on the similarity of GLP-1 and GATAs and on the fact that we recovered GLP-1 as Scrib-interacting protein, we focused on this factor. Scrib siRNA lowered the GLP-1 protein expression in the cytoplasm and in the nucleus. GLP-1 mRNA, in contrast, was not affected by Scrib knockdown, suggesting altered translation efficiency or protein stability. Using different inhibitors, we failed to obtain support for the latter aspect, but the experiments were technically difficult because low protein abundance and limited antibody quality. Additional experiments will be required to ultimately clarify this point. Because Scrib seems to maintain GLP-1 protein expression, it should allow sufficient GLP-1 to enter the nucleus and to promote VCAM-1 gene expression. Indeed, chromatin immunoprecipitation experiments demonstrated that GLP-1 binds the VCAM-1 promoter, and this effect was not only reduced by siRNA against GLP-1 but also against Scrib. Although silencing of Scrib limited VCAM-1 expression to a physiologically important extent, the contribution of Scrib to VCAM-1 expression was somewhat modest. Given that classic inflammatory signaling through NF-kB was Scrib-independent, this was expected. Interestingly, however, downregulation of GLP-1 and of Scrib yielded a similar reduction of VCAM-1 expression. This might suggest that the effect of Scrib specifically involves GLP-1.

ICAM-1 and VCAM-1 promote the adhesion of lymphocytes, monocytes, and granulocytes to the activated endothelium by establishing strong bonds with integrins. Inhibition or loss of one or more of the members of the immunoglobulin superfamily decreases leukocyte adhesion and migration. In our hands, adhesion experiments of leukocytes to inflamed HUVEC showed similar effects in Scrib-deficient cells, however, only

**Table. Hemodynamic and Microvascular Parameters in TNFα-Induced Inflammation**

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<th>Venules, n</th>
<th>Diameter, μm</th>
<th>Centerline Velocity, μm/s</th>
<th>Wall Shear Rate, per second</th>
<th>Systemic Leukocyte Count, cells/μL</th>
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after blocking of ICAM-1, suggesting that ICAM-1 functionally compensates the lack of VCAM-1 expression. Consistent with our in vitro data, inducible endothelial-specific Scrib knockout mice exhibit a reduced VCAM-1 mRNA expression in the endothelium of carotid arteries. Furthermore, in vivo microscopy experiments clearly demonstrated that the leukocyte adhesion after ICAM-1 inhibition is attenuated. Despite the fact that adhesion of leukocytes is mediated by ICAM-1 and VCAM-1, the latter adhesion molecule is not dispensable: Genetic deletion of VCAM-1 results in embryonic lethality as a result of placenta malformation. Also immune responses are dependent on VCAM-1 because, for example, infection with toxoplasmas cannot be controlled after VCAM-1 deletion in mice.

Importantly, VCAM-1 has a central role in early atherosclerosis development and in the inflammatory response after myocardial infarction. VCAM-1 but not ICAM-1 deficiency reduces atherosclerotic development in Ldlr–/– mice.49 apoE(−/−) knockout mice, lacking the VCAM-1 D4D allele, have clearly reduced arterial VCAM-1 expression, monocyte adherence in the aortic root, and fatty streak formation.50 A recent study also demonstrated that an anti–VCAM-1 antibody markedly reduced adhesion of inflammatory cells in ApoE(−/−) mice with a subsequent attenuation of atherosclerosis development.51 Thus, although ICAM-1 and VCAM-1 are both increased in inflammation and promote adhesion of white blood cell, they are not merely redundant in their function. In fact, VCAM-1 is an attractive mediator of inflammation-driven atherosclerosis. Thus, considering that Scrib selectively interferes with VCAM-1, inhibition at Scrib might be a strategy to attenuate atherosclerosis development.

In summary, with this study, we identified GLP-1 as a novel interaction partner of Scrib controlling inflammatory response. Scrib indirectly reduces VCAM-1 expression, and by this it alters the cytokine-induced leukocyte adhesion to the endothelium.

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Disclosures
None.

Reference


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Data Supplement (unedited) at:
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Supplemental Material

The polarity protein Scrib facilitates endothelial inflammatory signaling
Christoph Kruse\textsuperscript{1,4}, Angela R.M. Kurr\textsuperscript{2,4}, Katalin Pálfi\textsuperscript{1,4}, Patrick O. Humbert\textsuperscript{1}, Markus Sperrando\textsuperscript{2,4}, Ralf P. Brundel\textsuperscript{3,4}, Christian Fork\textsuperscript{1,4,8}, U. Ruth Michaelis\textsuperscript{1,4,8}

Supplemental Materials and Methods

Materials
Hank’s buffer (\#A3140.5000) was purchased from Applichem (Darmstadt, Germany). Human recombinant TNF\textalpha\textsuperscript{a} (#300-01A) and IL-1\textbeta\textsuperscript{a} (#200-01B) were purchased from PeproTech (Rocky Hill, NY, USA), Lipopolysaccharid (LPS) from Escherichia coli (#L3129) from Sigma-Aldrich (München, Germany), and Collagen Type I Rat Tail (#354236) from Corning Incorporated (Tewksbury, MA, USA). Anti-Scrib (C-20) (#sc-11049), anti-Scrib (K-21) (#sc-11048), anti-Scrib (H300) (#sc-28379), anti-NFkB p65 (#sc-109), anti-p50 (#sc-33022), anti-IkB-alpha (#sc-371), anti-VCAM-1 (#sc-8304), anti-JNK1 (F-3) (#sc-1648), anti-GLP1 (#sc-167994), anti-Topoisomerase 1 (#sc5342), and anti-Tubulin beta (sc-9104) antibodies were acquired from Santa Cruz (Heidelberg, Germany). Anti-Integrin alpha5 (#AB1949), and anti-GLP-1 (#ABE421) antibodies were supplied by Millipore (Darmstadt, Germany). Anti-Akt, phospho-Ser473- (#4058S), anti-AKT (#2920), anti-phospho- Thr180/Tyr182- (#9211), anti-p44/42, phospho- (#9101), anti-p44/42 (#4696), anti-JNK, phospho- (#4668), anti-p65, phospho- (#3033) and anti-FoxO1 (#2880) were acquired from Cell Signaling (Danvers, MA, USA). Anti-COX2 antibody (#610204) was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Anti-p38 (#ab31828) was acquired from Abcam (Cambridge, UK). Trypsin (#T3924) and anti-beta-Actin (#A1978) were purchased from Sigma-Aldrich (München, Germany).

Cell Culture
Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (#CC-2519, Lot No.186864; 191772; 192485; 76524; 76921; 7F3111, Walkersville, MD, USA) and PELOBiotech (#PB-CH190-8013, Lot No. QC-18P13F11, Planegg, Germany). Cells were cultured on fibronectin-coated (356009, Corning Incorporated, Tewksbury, MA, USA) dishes in endothelial growth medium (EGM), consisting of endothelial basal medium (EBM) supplemented with human recombinant epidermal growth factor (EGF), EndoCGS-Heparin, (PELOBiotech, Planegg, Germany), 8% fetal calf serum (FCS) (356009, Biochrom, Berlin, Germany), penicillin (50 U/ml) and streptomycin (50 µg/ml) (15140-122, Gibco (lifeotechnologies, Carlsbad, CA, USA) in a humidified atmosphere of 5% CO\textsubscript{2} at 37 °C. For each experiment at least three different batches of HUVEC from passage 3 were used.

Immortalized human microvascular endothelial cells (HMEC-1) (#98247) were requested from CDC (Atlanta, GA, USA). Cells were cultured on fibronectin-coated dishes in endothelial growth medium (EGM), consisting of endothelial basal medium (EBM) containing human recombinant epidermal growth factor (EGF) and EndoCGS-Heparin, (PELOBiotech, Planegg, Germany), supplemented with 8% fetal calf serum (FCS), penicillin (50 U/ml), and streptomycin (50 µg/ml) in a humidified atmosphere of 5% CO\textsubscript{2} at 37 °C.

Human embryonic kidney (HEK) 293T/17 cells (#CRL-11268) were purchased from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), high glucose, GlutaMAX from Gibco, lifeotechnologies (Carlsbad, CA, USA), supplemented with 8% fetal calf serum (FCS), penicillin (50 U/ml), and streptomycin (50 µg/ml) in a humidified atmosphere of 5% CO\textsubscript{2} at 37 °C.

Human Aortic Smooth Muscle Cells (H\textalpha\textomega\textomega\textomegaSMC) (#354-05a) were requested from PELOBiotech (Planegg, Germany). Cells were cultured in Smooth Muscle Cell Medium (#PB-MH-200-2190) supplemented with with 8% fetal calf serum (FCS), penicillin (50 U/ml), streptomycin (50 µg/ml), EGf, FGF, glutamin, and insulin from singlequots (PELOBiotech, Planegg, Germany). Cells were cultured in humidified atmosphere of 5% CO\textsubscript{2} at 37 °C. For experiments cells from passage 7 to 9 were used.

Self-isolated human fibroblasts were cultured in DMEM/F12 (11039-021) from Gibco (lifeotechnologies, Carlsbad, CA, USA) supplemented with 8% fetal calf serum (FCS), penicillin (50 U/ml), and streptomycin (50 µg/ml) in a humidified atmosphere of 5% CO\textsubscript{2} at 37 °C.

siRNA and plasmid transfection, shRNA transduction
Endothelial cells were cultured to 80% confluence and transfected with siRNA using GeneTrans II from MoBiTec (Göttingen, Germany) according to the manufacturer’s instructions. After 4 h and 16 h medium was replaced with fresh EGM. Cells were
used for experiments 72 h after transfection. Two different control siRNAs were used, one general scrambled Stealth RNAi™ siRNA Negative Control, Med GC (#12935-300) from lifetechologies (Carlsbad, CA, USA) and one specific GFP siRNA (#SR-CL020-005) from Eurogentec (Köln, Germany). Two different Scrib siRNAs (#HSS146451, #HSS177461) from lifetechologies (Carlsbad, CA, USA), three different GLP-1 siRNAs (#HSS170027, #HSS170028, #HSS170029), and one Integrin α5 siRNA (#8SIO2654841) from QIAGEN (Hilden, Germany) were used for experiments. When no specific siRNA is named, the results of two different siRNAs were summarized and indicated as control (Ctl).

siRNA-resistant expression plasmids were generated by introduction of at least five silent mutations at the siRNA binding sites of Scrib expression plasmids (after removal of the GFP-tag by blunt end cloning) described previously\(^1\) with the aid of the QuikChange\(^\text{TM}\) II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Böblingen, Germany).

Plasmid overexpression was achieved with the NEON electroporation system (Invitrogen, Darmstadt, Germany) according to the manufacturer’s instructions.

For lentiviral shRNA transduction of HUVEC, two different Scrib shRNA constructs were designed and cloned into an empty lentiviral backbone with a puromycin resistance gene for selection (plasmid 8453: pLKO1 puro). Two different control shRNA constructs were used, one general scrambled shRNA (Plasmid 1864: scramble shRNA) and one specific GFP shRNA (Plasmid 30323: pLKO.1 GFP shRNA). All pLKO1 plasmids as well as packing plasmid (Plasmid 12260: psPAX2) and envelope plasmid (Plasmid 12259: pMD2.G) were purchased from Addgene (Cambridge, MA, USA). Cloning, transfection and transduction were performed as described in the pLKO.1 protocol on http://www.addgene.org/. HUVEC were selected with a puromycin concentration of 0.4 µg/ml. Cells were used for experiments 7 days after infection. When no specific shRNA is named, the results of two different shRNAs were summarized.

**Isolation of mRNA and RT-qPCR**

Total mRNA was isolated with the Bio&Sell RNA-MiniKit (Bio&Sell, Feucht, Germany) according to the manufacturer’s instructions. cDNA synthesis was performed with Superscript III Reverse Transcriptase (Invitrogen, Darmstadt, Germany) and random hexamer primers as described in the manufacturer’s protocol. For semi quantitative real-time PCR EvaGreen qPCR Mix with ROX (#76.580.5000) from Bio&Sell (Feucht, Germany,) was used with appropriate primers in a Mx3000P qPCR cycler (Agilent Technologies, Santa Clara, CA, USA). Relative expression of target genes were normalized to eukaryotic translation elongation factor 2 (EEF2) or RNA polymerase II, analyzed by delta-delta Ct method with the MxPro software (Agilent Technologies, Santa Clara, CA, USA) and given as relative values compared to control experiments.

**Protein isolation and Western blot analysis**

Cells were lysed with Triton X-100 lysis buffer (20 mM TRIS/Cl pH 7.5, 150 mM NaCl, 10 mM NaPP\(_{3}\), 20 mM NaF, 1% Triton, 2 mM Orthovanadat (OV), 10 mM Okadaic Acid, protein-inhibitor mix (PIM), 40 µg/ml Phenylmethylsulfonylfluorid (PMSF)) or RIPA lysis buffer (20 mM TRIS/Cl pH 7.5, 150 mM NaCl, 10 mM NaPP\(_{3}\), 20 mM NaF, 1% Deoxycholat, 1% Triton, 0.1% SDS, 2 mM Orthovanadat (OV), 10 mM Okadaic Acid, PIM, 40 µg/ml PMSF). For nucleus protein extraction cells were lysed in buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM DTT, PIM, 40 µg/ml PMSF). After incubation on ice for 15 min Nonidet was added (0.75%) and cells were centrifuged for 1 min at 16,000 g. Pellet was lysed in buffer B (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM DTT, PIM, 40 µg/ml PMSF). Protein amount was determined with Bradford protein assay and subjected to SDS-PAGE followed by Western blot as described.\(^2\) Infrared-fluorescent-dye-conjugated secondary antibodies were purchased from Licor (Bad Homburg, Germany) and detected with an infrared-based laser scanning detection system (Odyssey Classic, Licor, Bad Homburg, Germany).

**Immunoprecipitation (IP)**

For GLP-1 immunoprecipitation, HUVEC were lysed in buffer A and Nonidet as previously described. Cytoplasm (supernatant) was incubated with GLP-1 antibody for 90 min followed by incubation with magnetic beads for an additional 90 min. Beads were washed with buffer A 3 times and subsequently incubated with sample buffer (8.5% glycerin, 2% SDS, 6.25% Tris/HCl pH 6.8, 20 mM DTT, 0.013% bromphenol blue) for 5 min at 95 °C. Protein was subjected to SDS-PAGE followed by Western blot.

**Chromatin immuno-precipitation (ChIP)**

Cell preparation, crosslinking and nuclei isolation were performed with the truCHIP™ Chromatin Shearing Kit (Covaris, Woburn, USA) according to the manufacturers protocol. Afterwards, the lysates were sonified with the Bioruptur Plus (9 cycles, 30 seconds on, 90 seconds off; Diagenode, Seraing, Belgium) at 4°C. Cell debris was removed by centrifugation and the lysates were diluted 1:3 in dilution buffer (20 mM Tris/HCl pH 7.4, 100 mM NaCl, 2 mM EDTA, 0.5% Triton X-100 and protease inhibitors). After preclearing with 20µL DiaMag protein A coated magnetic beads (Diagenode, Seraing, Belgium) for 30 minutes at 4°C, samples were incubated overnight at 4°C with 3-5 µg of anti-H3 (#pAb-003-050 #C15310135, Diagenode, Seraing, Belgium), anti-GLP-1 (#ABE421, Millipore, Darmstadt, Germany) and IgG from rabbit (#C15410206, Diagenode, Seraing, Belgium) antibodies. 5% of the samples served as input. The
antibody complexes were collected with 35 µl DiaMag protein A coated magnetic beads (Diagenode, Seraing, Belgium) for 3 hours at 4 °C, subsequently washed twice for 5 minutes with each of the wash buffers 1-3 (Wash Buffer 1: 20 mM Tris/HCl pH 7.4, 150 mM NaCl, 0.1% SDS, 2 mM EDTA, 1% Triton X-100; Wash Buffer 2: 20 mM Tris/HCl pH 7.4, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100; Wash Buffer 3: 10 mM Tris/HCl pH 7.4, 250 mM lithium chloride, 1% Nonidet, 1% sodium deoxycholate, 1 mM EDTA) and finally washed with TE-buffer pH 8.0. Elution of the beads was done with elution buffer (0.1 M NaHCO3, 1% SDS) containing 1x proteinase K (Diagenode, Seraing, Belgium) and shaking at 600 rpm for 1 hour at 55°C, 1 hour at 62°C and 10 minutes at 95 °C. After removal of the beads, the eluate was purified with the QiaQuick PCR purification kit (Qiagen, Hilden, Germany) and subjected to qPCR analysis.

Proximity ligation analysis
Proximity ligation 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 0.9% (0.2%), incubated with the respective PLA-probes for one hour (37°C), washed and ligated for 30 min (37°C). After an additional washing, amplification with polymerase was allowed for 100 min (37°C). To show the specificity of the Scrib antibody, a blocking peptide was used (Scrib-C-20 P, SantaCruz, Heidelberg, Germany, #sc-11049 P). The nuclei were stained using DAPI. Images were acquired by confocal microscopy (LSM 510, Zeiss).

Cell adhesion assay
The second day after transfection with siRNA, HUVEC were detached by Trypsin and seeded on 6 channel µ-Slide (#80606) from ibidi (München, Germany) at a concentration of 2x10^4 cells/ml. Next day cells were stimulated with TNFα (10 ng/ml) for 3 h followed by incubation with and without ICAM-1 blocking antibody (10 µg/ml) (R&D systems, Minneapolis, MN, USA, #BBA3) for 30 min. Leukocytes were separated from human blood by Ficoll purification. 100 µl of leukocytes (1.2x10^6 cells/1.2 ml) were added to each channel of the slide and incubated for 3 min. The channels were subsequently connected to the syringe pump (KDScientific, Holliston, MA, USA, #78-8210INT) in a humidified atmosphere of 5% CO2 at 37 °C and flushed with EGM and stepwise increased flow speed. Images were taken at 0.35 dyn/cm² and 15 dyn/cm² with Observer Z1 and AxioCam MRm from Zeiss (Jena, Germany). ImageJ software was used for quantification.

Experimental animals and animal procedures
All experimental procedures were approved by the local governmental authorities (approval numbers: FU1035) and were performed in accordance with the animal protection guidelines exclusively in male mice. C57BL/6 mice were purchased from Charles Rivers (Deisenhofen, Germany). The generation and general characterization of Scrib1flox/flox has been described previously and were kindly provided by one of the authors. Cdh5-CreERT2 mice were kindly provided by Ralf Adams, MPI Münster. Activation of CreERT2 was achieved by oral tamoxifen administration in the chow (LASCRdiet CreActive TAM400, LASvendi, Soest, Germany) on 10 consecutive days followed by a “wash-out” phase of 2 weeks. In all experiments Cre positive (denoted as Cre+/0) as well as the Cre negative (denoted as Cre0/0) control animals received tamoxifen to exclude direct effects of this anti-estrogen. Breeding of the Cre-lines was carried out by crossing Cre +/0 and Cre 0/0 animals so that Cre +/0 and Cre 0/0 littermates could always be compared side by side. Mice were housed in a specified pathogen-free facility with 12/12 hours day/night cycle and free access to chow and water.

Intimal RNA isolation from mouse carotid arteries
RNA isolation from carotid endothelium has been described previously. Lipopolysaccharides (LPS) from Escherichia coli 0111:B4 were dissolved in saline 0.9% saline and injected intraperitoneally (i.p.) in tamoxifen-administrated Scrib1flox/flox_Cdh5-CreERT2 mice (8 mg LPS/kg mouse). After 2 h mice were killed by exsanguination in deep isofluraneesthesia and the remaining blood was then removed by transcardiac perfusion with Hank’s buffer. Subsequently, the carotid arteries were isolated and cleaned of adhering tissue, followed by flushing them with 100 µl QIAzol Lysis Reagent (# 79306, Qiagen, Hilden, Germany) in order to lyse the carotid intima. The lysates of both carotids were pooled and RNA was isolated with the help of miRNAeasy MiniKit from Qiagen (#217004, Hilden, Germany).

Intimal protein isolation from mouse aorta
Aortas were isolated and cleaned of adhering tissue, followed by flushing them with RIPA buffer (20 mM TRIS/Ci pH 7.5, 150 mM NaCl, 10 mM NaPP, 20 mM NaF, 1% Desoxycholat, 1% Triton, 0.1% SDS, 2 mM Orthovanadat (OV), 10 mM Okadaic Acid, PIM, 40 µg/ml PMSF). Protein amount was determined with Bradford protein assay and subjected to SDS-PAGE followed by Western blot as described. Infrared-fluorescent-dye-conjugated secondary antibodies were purchased from Licor (Bad Homburg, Germany) and detected with an infrared-based laser scanning detection system (Odyssey Classic, Licor, Bad Homburg, Germany).

TNFα-induced inflammation model of mouse cremaster muscle
The TNFα-induced inflammation model of the mouse cremaster muscle was performed in tamoxifen-
administrated Scrib1flox/flox_Cdh5-CreERT2 mice as previously described. Briefly, mice were pretreated intraperitoneally (i.p.) with and without rat anti-mouse ICAM-1 monoclonal antibody (YN1/1.7.4, 100 µg, LGC). 500 ng of mTNFα (#410-MT-10, R&D Systems, Minneapolis, MN, USA) was injected into the scrotum 1 h later and after additional 2 h the cremaster muscle was exteriorized for intravital imaging using an Olympus BX51WI microscope equipped with a water immersion objective (x20, 0.95NA, Olympus). During the entire observation, the cremaster muscle was superfused with thermo-controlled (35 °C) bicarbonate-buffered saline. Postcapillary venules were recorded using a CCD camera (model CF8/1, Kappa) and virtual dub software for later offline analysis. Microvascular parameters (venular diameter, venular vessel, segment length), the number of adherent cells/mm² and adhesion efficiency (adherent cells/mm² / systemic white blood cell count) were determined using Fiji software. Postcapillary venules under observation ranged from 20 µm to 40 µm in diameter.

Statistics
Unless otherwise indicated, data are given as means ± standard error of mean (SEM). Calculations were performed with BiAS.10.12 or Prims 3.02. In case of multiple testing, Bonferroni correction was applied. For multiple group comparisons ANOVA followed by post hoc testing (Bonferroni) was performed. Individual statistics of unpaired samples was performed by T-test and if not normal distributed by Mann-Whitney test. p-value of <0.05 was considered as significant. Unless otherwise indicated, N indicates the number of individual experiments.

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Supplemental Material

The polarity protein Scrib facilitates endothelial inflammatory signaling
Christoph Kruse1,4, Angela R.M. Kurz2,4, Katalin Pálfi1,4, Patrick O. Humbert1, Markus Sperandio2,4, Ralf P. Brandes1,4,*, Christian Fork1,4, U. Ruth Michaelis1,4,8

Supplemental Figures

Supplemental Figure I: Role of Scrib on VCAM-1 in human microvascular endothelial cell (HMEC-1), effect of Scrib overexpression in HUVEC and role of Scrib in cultured HUVEC on different matrices. A, Representative Western blot and densitometry of VCAM-1 protein expression in Scrib siRNA (siScrib) or control siRNA (Ctl) transfected HMEC-1 stimulated with TNFα (1 ng/ml, 3 h), n=4. B, Representative Western blot and densitometry of VCAM-1 protein expression in Scrib shRNA9 (shScrib9) or scrambled shRNA (shScr) transduced HUVEC overexpressed with Scrib or GFP followed by stimulation with TNFα (1 ng/ml, 3 h), n=6. C, Representative Western blots for Scrib, Integrin α5 and VCAM-1 in HUVEC treated with GFP siRNA (siGFP), Scrambled siRNA (siScr), Scrib siRNA (siScrib) and Integrin α5 siRNA (siα5), respectively and followed stimulation with and without TNFα (1 ng/ml, 3 h). HUVEC were cultured on different matrices (uncoated, fibronectin, collagen). D, Representative Western blot and densitometry of VCAM-1 protein expression in Scrib siRNA (siScrib), Integrin α5 siRNA (siα5) or control siRNA (Ctl) transfected HUVEC treated with and without IL1β (1 ng/ml, 3 h) and LPS (1 µg/ml, 3 h), n=3, p*<0.05 relative to the corresponding control.
Supplemental Figure II: Effect of Scrib for p50 expression. Representative Western blot and densitometry of p50 protein in the nucleus of Scrib siRNA (siScrib) and scrambled siRNA (siScr) transfected HUVEC treated with and without TNFα (10 ng/ml, 15 min), n=4. *p<0.05 relative to the corresponding control. *p<0.05 with vs. without TNFα.

Supplemental Figure III: Role of Scrib for FoxO1 expression. A, Representative Western Blots and densitometry of FoxO1 protein expression in the cytoplasm and nucleus in Scrib shRNAs (shScrib) or control shRNAs (Ctl) transduced HUVEC stimulated with and without TNFα (1 ng/ml, 3 h), n=3. B, Representative Western blot for VCAM-1 and FoxO1 in Scrib shRNA8 (shScrib8) and scrambled shRNA (shScr) transduced HUVEC, overexpressing GFP, FoxO1 WT and constitutive active (caFoxO1) followed by stimulation with TNFα (1 ng/ml, 3 h). C, Representative Western blot and densitometry for VCAM-1 in HUVEC treated with the FoxO1 inhibitor (AS1842856) for 2 h at the indicated concentrations followed by stimulation with and without TNFα (1 ng/ml, 3 h). *p<0.05 relative to the corresponding control.
Supplemental Figure IV: Role of GLP-1 for VCAM-1 expression. A, Representative Western blots of GLP-1 in the cytoplasm and nucleus of human fibroblasts, HMEC-1, HUVEC, HAOSMC and HEK. B, Representative Western blot and densitometry for GLP-1 in GLP-1 siRNA (siGLP-1) and control siRNA (Ctl) treated HUVEC, n=3. C, Normalized VCAM-1 mRNA expression in Scrib siRNA (siScrib), GLP-1 siRNAs (si1GLP1, si2GLP1, si3GLP1) and ctl siRNAs (Ctl) treated HUVEC after stimulation with TNFα (1 ng/ml, 2 h), n≥3. D, Normalized GLP-1 mRNA expression in Scrib siRNA (siScrib) and control siRNAs (Ctl) treated HUVEC after stimulation with TNFα (1 ng/ml, 2 h), n≥3. p<0.05 relative to the corresponding control.