ARTICLE

Vav3-induced cytoskeletal dynamics contribute to heterotypic properties of endothelial barriers

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Through multiple cell–cell and cell–matrix interactions, epithelial and endothelial sheets form tight barriers. Modulators of the cytoskeleton contribute to barrier stability and act as rheostats of vascular permeability. In this study, we sought to identify cytoskeletal regulators that underlie barrier diversity across vessels. To achieve this, we correlated functional and structural barrier features to gene expression of endothelial cells (ECs) derived from different vascular beds. Within a subset of identified candidates, we found that the guanosine nucleotide exchange factor Vav3 was exclusively expressed by microvascular ECs and was closely associated with a high-resistance barrier phenotype. Ectopic expression of Vav3 in large artery and brain ECs significantly enhanced barrier resistance and cortical rearrangement of the actin cytoskeleton. Mechanistically, we found that the barrier effect of Vav3 is dependent on its Dbl homology domain and downstream molecular interactions with the basement membrane through integrins (Zaidel-Bar and Geiger, 2010; Oldenburg and de Rooij, 2014). Finally, a third component, the cytoskeleton, has gained attention as a critical regulator of barrier function. As a dynamic intracellular network of actin fibers, microtubules, and intermediate filaments (Ingber, 2002), the cytoskeleton links junctional complexes and focal adhesions, coordinating tension forces that affect both cell shape and intercellular contacts (Fanning et al., 1998; Giannotta et al., 2013). Adhesive molecules of tight junctions directly interact with zonula occludin proteins (ZO-1, ZO-2, and ZO-3), which anchor the actin cytoskeleton to these junctional complexes (Itoh et al., 1999a, b). Similarly, the cytoplasmic tail of VE-cadherin is connected to the actin bundles via α- and β-catenin proteins (Dejana, 2004). This association to the actin cytoskeleton is essential for junction assembly, strength, and maintenance (Nelson et al., 2004; Huveneers et al., 2012; Hong et al., 2013). In this manner, the cytoskeleton has the capacity to quickly alter both cell–cell and cell–matrix interactions.

Cytoskeletal organization and dynamics are regulated by Rho GTPases such as RhoA, Rac1, and Cdc42. In turn, these GTPases have major effects on endothelial barrier regulation and permeability (Wojciak-Stothard and Ridley, 2002; Dejana, 2004). Importantly, inactivation of Vav3 in vivo resulted in increased vascular leakage, highlighting its function as a key regulator of barrier stability.

Introduction

The vascular endothelium functions as a dynamic barrier that regulates selective exchange of gases, solutes, proteins, and immune cells between the vessel lumen and the interstitial space (Dejana, 2004; Pries and Kuebler, 2006). Dysregulation of endothelial permeability is a hallmark of several inflammatory and vascular diseases and can result in uncontrolled vascular leakage leading to severe fluid loss and organ dysfunction (Mehta and Malik, 2006; Bakker et al., 2009; Lee and Slutsky, 2010). Paracellular permeability of the endothelium can be altered by soluble factors such as thrombin, bradykinin, TNF-α, histamine, and vascular endothelial (VE) growth factor (VEGF; Mehta and Malik, 2006) through a mechanism that relies on the discrete widening and tightening of endothelial cell (EC)–cell junctions (Giannotta et al., 2013). Two types of intercellular junctions, namely adherens junctions and tight junctions, are most crucial in regulating the barrier properties of the endothelium. The main molecular component of endothelial adherens junctions is VE-cadherin (Navarro et al., 1998; Dejana, 2004; Giannotta et al., 2013), whereas tight junctions rely on clusters of claudins, occludins, and junction adhesion molecules (Furuse et al., 1993, 1998; Martin-Padura et al., 1998). In addition to cell–cell contacts, the endothelial barrier is also influenced by molecular interactions with the basement membrane through integrins (Zaidel-Bar and Geiger, 2010; Oldenburg and de Rooij, 2014). Finally, a third component, the cytoskeleton, has gained attention as a critical regulator of barrier function. As a dynamic intracellular network of actin fibers, microtubules, and intermediate filaments (Ingber, 2002), the cytoskeleton links junctional complexes and focal adhesions, coordinating tension forces that affect both cell shape and intercellular contacts (Fanning et al., 1998; Giannotta et al., 2013). Adhesive molecules of tight junctions directly interact with zonula occludin proteins (ZO-1, ZO-2, and ZO-3), which anchor the actin cytoskeleton to these junctional complexes (Itoh et al., 1999a, b). Similarly, the cytoplasmic tail of VE-cadherin is connected to the actin bundles via α- and β-catenin proteins (Dejana, 2004). This association to the actin cytoskeleton is essential for junction assembly, strength, and maintenance (Nelson et al., 2004; Huveneers et al., 2012; Hong et al., 2013). In this manner, the cytoskeleton has the capacity to quickly alter both cell–cell and cell–matrix interactions.

Cytoskeletal organization and dynamics are regulated by Rho GTPases such as RhoA, Rac1, and Cdc42. In turn, these GTPases have major effects on endothelial barrier regulation and permeability (Wojciak-Stothard and Ridley, 2002; Dejana, 2004). Importantly, inactivation of Vav3 in vivo resulted in increased vascular leakage, highlighting its function as a key regulator of barrier stability.

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Table 1. Endothelial cell types used in the study

| Vascular bed | EC type                  | Abbreviation | Passage no. obtained | Passage no. used for experiments |
|--------------|--------------------------|--------------|----------------------|---------------------------------|
| Arterial     | Human aortic EC          | HAEC         | P1/P2                | P4–5                            |
|              | Human iliac artery EC    | HIAEC        | P3/P3                | P4–6                            |
| Venous       | Human saphenous vein EC  | HSaVEC       | P2/P3                | P4–6                            |
|              | Human umbilical venous EC| HUVEC        | P1/P1                | P4–5                            |
| Microvascular| Human brain microvascular EC | HBMVEC | P2/P3                | P4–6                            |
|              | Human adipose microvascular EC | HAMVEC | P2/P2                | P4–6                            |
|              | Human uterine microvascular EC | HUMVEC | P1/P2                | P4–5                            |
|              | Human lung microvascular EC | HLMVEC | P2/P2                | P4–6                            |
|              | Human dermal microvascular EC | HDMEC | P1/P2                | P4–6                            |

ECs for each tissue type were obtained from two different sources. Initial passage numbers of obtained cell samples per source and passage numbers used for final experiments are shown on the right.

Results

Human ECs from distinct vascular beds exhibit unique barrier properties

Transendothelial barrier experiments were conducted on confluent endothelial monolayers from nine distinct human tissues obtained from two different vendors, totaling 18 individual samples. This panel included human ECs of arterial (aorta and iliac artery), venous (saphenous vein and umbilical vein), and microvascular (brain, adipose tissue, uterus, lung, and dermis) origin (Table 1; Fig. 1 A). Brightfield images of the nine EC types are shown in Fig. S1. Barrier establishment and stabilization was monitored by using continuous electric cell–substrate impedance sensing (ECIS) for 48 h (Fig. 1 B). Different rates of barrier establishment were observed, although most EC types reached their maximum barrier plateau within 24–48 h (Fig. 1 C; see Fig. S1 [A and B] for measurements of individual EC sources). When absolute levels of barrier resistance were quantified at 48 h, human dermal microvascular ECs (HDMECs) and human lung microvascular ECs (HLMVECs) displayed the highest levels of resistance (2,513 ± 245 and 1,677 ± 311 Ω; Fig. 1 D). Lowest resistance levels were recorded for human aortic ECs (HAECs) and human saphenous vein ECs (HSaVECs) and HAMVECs) isolated from human cerebral cortex exhibited lower barrier resistance compared with HLMVECs and HDMECs (1,049 ± 64 Ω; Fig. 1, C and D). The overall subgroup of microvascular ECs exhibited higher levels of barrier resistance on average compared with the group of macrovascular ECs isolated from arterial vascular beds (1,687 ± 177 and 769 ± 321 Ω; Figs. 1 E and S1 C). Although it is well accepted that permeability is regulated at the capillary level, the lack of a supportive vascular wall other than arteries and veins demands microvascular ECs to both form at tight barrier on their own and also enable permeability depending on tissue needs (Fig. 1 A).

By simultaneous recording of multiple frequencies and deploying further data modeling, we determined individual impedance contributions of different compartments of the endothelial barrier. This ECIS modeling approach is based on a mathematical transfer function developed by Giaever and Keese (1991) that determines three model parameters that characterize...
specific properties of the cell monolayer: $\alpha$, $R_b$, and $C_m$ (Fig. 1B). Parameter values of $R_b$, $\alpha$, and $C_m$ at 48 h are shown in Fig. 1 F (entire data curves of $R_b$ values are presented in Fig. S1 D). HDM ECs and HLMVECs exhibit high $R_b$ values, indicating tight cell–cell junctions compared with ECs from the cerebral cortex (HBM VECs), venous system (HSAVECs and human umbilical ECs [HUVECs]), and aorta (HAECs).

High levels of endothelial barrier resistance correlate with continuous intercellular junctions and cortical arrangement of the actin cytoskeleton

To reveal the underlying structural characteristics that correlate with the cell-type–specific trends in barrier resistance observed, we evaluated junctional proteins (VE-cadherin, claudin-5, and ZO-1), cytoskeleton-related molecules (F-actin, cortactin, and...
Figure 2. High levels of barrier resistance correlate with continuous intercellular junctions and cortical arrangement of the actin cytoskeleton. 

(A) Confocal images of confluent endothelial monolayers displayed in order of low (HAEC, HBMVEC, and HUVEC) and high levels of barrier resistance (HLMVEC and HDMEC) left to right (bar, 20 µm; representative of n = 3). HLMVEC and HDMEC exhibit a linear and organized junctional staining of VE-cadherin, claudin-5, and ZO-1 compared with an irregular junctional pattern in cell types with lower levels of resistance. Phalloidin staining reveals a strict cortical arrangement of the actin cytoskeleton in HLMVEC and HDMEC, whereas cells with low resistance exhibit more stress fibers. Localization of cortactin is more peripheral and less cytoplasmic in HLMVECs and HDMECs. F-actin fibers aligned along the cell periphery, minimizing radial tension forces at cell junctions, indicated by linear junctional pattern of VE-cadherin/phalloidin overlay images (arrows) and lower presence of pMLC2 in HLMVECs and HDMECs compared with cell types with low barrier resistance. 

(B–F) Graphs presenting mean values of fluorescence intensity across multiple cell–cell junctions per cell type (Fig. S2 B) for each barrier protein shown in A. Junctional components as well as phalloidin and cortactin are concentrated along the cell borders in HLMVECs and HDMECs compared with cell types with low barrier resistance.
Figure 3. Correlation of RNA expression to barrier resistance associates VAV3 with a tight barrier phenotype, and single-cell sequencing confirms microvascular expression of Vav3 in vivo. (A) Gene expression levels (NanoString) of the microvascular (MV) EC panel shown in order of the strongest correlation to barrier resistance values. Display of genes is based on the Spearman’s rank correlation coefficient (rs) within a panel of 262 code sets (measurement in triplicates). Cytoskeletal regulators such as BAIAp2, VAV3, and SOR652, show a strong correlation of expression to barrier resistance (rs = 0.952, 0.903, and 0.891, respectively).
phosphorylated myosin light chain 2 [pMLC2]), and focal adhesions (vinculin) in high- (HDMCs and HLMVECs) and low- (HAECs, HUVECs, and HBMEVCs) resistance monolayers. HDMCs and HLMVECs exhibit junctional localization of VE-cadherin that appears both abundant and continuous, whereas HAECs, HUVECs, and HBMEVCs exhibited less VE-cadherin staining, which when present displayed a serrated pattern (Fig. 2, A–F; lower-magnification images shown in Fig. S2 A). Comparable findings were obtained for claudin-5 and ZO-1. Strikingly, the lower-magnification images shown in Fig. S2 A). Comparable findings were obtained for claudin-5 and ZO-1. Strikingly, the lower-magnification images shown in Fig. S2 A). Comparable findings were obtained for claudin-5 and ZO-1. Strikingly, the lower-magnification images shown in Fig. S2 A). Comparable findings were obtained for claudin-5 and ZO-1. Strikingly, the lower-magnification images shown in Fig. S2 A). Comparable findings were obtained for claudin-5 and ZO-1. Strikingly, the lower-magnification images shown in Fig. S2 A). Comparable findings were obtained for claudin-5 and ZO-1. Strikingly, the lower-magnification images shown in Fig. S2 A). Comparable findings were obtained for claudin-5 and ZO-1. Strikingly, the lower-magnification images shown in Fig. S2 A). Comparable findings were obtained for claudin-5 and ZO-1. Strikingly, the lower-magnification images shown in Fig. S2 A). Comparable findings were obtained for claudin-5 and ZO-1. Strikingly, the lower-magnification images shown in Fig. S2 A). Comparable findings were obtained for claudin-5 and ZO-1. Strikingly, the lower-magnification images shown in Fig. S2 A). Comparable findings were obtained for claudin-5 and ZO-1. Strikingly, the lower-magnification images shown in Fig. S2 A). Comparable findings were obtained for claudin-5 and ZO-1. Strikingly, the lower-magnification images shown in Fig. S2 A).

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Vav3 expression and barrier heterogeneity

0.891, respectively). (B) Genes that show a strong inverse correlation of RNA expression to barrier resistance such as SRC, TPM2, and RND3 (r_s = −0.939, P < 0.001, and −0.721, respectively). P < 0.05. (C) Single-cell sequencing analysis of 5,000 cells freshly isolated from both pulmonary lobes of a C57BL/6 mouse. Shown are all identified pulmonary cell-type populations visualized by t-SNE. (D) Endothelial identity is defined by simultaneous expression of Pecam1 (CD31) and Cdhl5 (VE-cadherin). (E) Shown is the population of Vav3-positive pulmonary cells (upper left graph), which is by a large fraction composed of ECs and by a subpopulation of immune cells, defined by coexpression of either Pecam1 and Cdhl5 or Pr ptrc (CD45), respectively. Although there is a large overlap of expression of Vav3 with Baiap2, this is not the case for Sorbs2 and Angpt2. Some Vav3-positive ECs also express Vav2, whereas Vav2 is expressed in CD45-positive cells only.
Silencing VAV3 reduces barrier resistance and cortical distribution of F-actin

To test the barrier regulatory role of the genes identified in the foregoing expression analyses, we used an siRNA-based knockdown strategy to silence the expression of BAIA2P, VAV3, and SORBS2. In ECs with a tight barrier phenotype (HDMECs), reduction of VAV3 but not BAIA2P or SORBS2 significantly reduced barrier resistance (2398 ± 6 vs. 3011 ± 56 Ω at the 48-h time point; Fig. 4, A and B). Given the presence of low VAV2 expression levels, we performed a dual siRNA knockdown to eliminate both VAV2 and VAV3 transcripts in HDMECs and HLMVECs. The reduction observed was similar to the one found with single elimination of VAV3 mRNA (Fig. 4, C–I), ruling out potential compensatory effects of VAV2. As positive control and for purpose of comparison, we also silenced CLND5 (claudin-5), a critical structural barrier protein (Fig. 4, C–I).

As observed earlier, EC types with high VAV3 expression levels such as HDMECs and HLMVECs displayed strongly defined junctional complexes and a cortical distribution of F-actin. Expression of VAV3 was closely linked to these cellular features because the knockdown in HDMECs resulted in substantial loss of cortical actin fibers, an increase in stress fibers, and a reduction of cortactin at the cell periphery (Fig. 4, J and K). These findings were also reproduced in HLMVECs (Fig. S4). Concurrent attenuation of VAV2 and VAV3 expression in HDMECs (Fig. 4, J and K) and HLMVECs (Fig. S4) was not additive to the phenotype seen by knockdown of VAV3 alone.

Overexpression of Vav3 converts low barrier resistance of HAEC and HBMVEC toward a high–barrier resistance phenotype

The lowest protein levels of endogenous Vav3 were observed for HAECs and HBMVECs (Fig. 5 A), which corresponds with their low levels of barrier resistance. We used lentiviral constructs to ectopically express HA-tagged Vav3 WT (VAV3WT) and Myc-tagged Vav3 mutant (VAV3N269A) in both of these EC types (Fig. 5, B–D). Although vector control-infected cells exhibited low levels of barrier resistance, overexpression of VAV3WT significantly elevated barrier resistance in both HAECs and HBMVECs (Fig. 5, E–I). This was not the case for overexpression of

Table 2. DAVID analysis of genes that correlate with high Vav3 expression

| Category | Count | P |
|----------|-------|---|
| Annotation cluster 1 | Enrichment score: 4.33 |
| Focal adhesion (KEGG_PATHWAY) | 18 | 5.1E−6 |
| Regulation of actin cytoskeleton (KEGG_PATHWAY) | 18 | 8.0E−6 |
| Proteoglycans in cancer (KEGG_PATHWAY) | 13 | 2.5E−3 |
| Annotation cluster 2 | Enrichment score: 3.42 |
| Cadherin binding involved in cell–cell adhesion (GOTERM_MF) | 18 | 8.1E−5 |
| Cell–cell adherens junction (GOTERM_CC) | 19 | 1.1E−4 |
| Cell–cell adhesion (GOTERM_BP) | 11 | 6.3E−3 |
| Annotation cluster 3 | Enrichment score: 2.58 |
| Outflow tract morphogenesis (GOTERM_BP) | 9 | 1.5E−5 |
| Palate development (GOTERM_BP) | 6 | 3.2E−2 |
| Heart looping (GOTERM_BP) | 5 | 4.0E−2 |
| Annotation cluster 4 | Enrichment score: 2.05 |
| Differentiation (UP_KEYWORDS) | 24 | 2.1E−3 |
| Neurogenesis (UP_KEYWORDS) | 12 | 6.4E−3 |
| Nervous system development (GOTERM_BP) | 14 | 5.2E−2 |
| Annotation cluster 5 | Enrichment score: 1.90 |
| GTP-binding (UP_KEYWORDS) | 15 | 3.6E−3 |
| GTPase activity (GOTERM_MF) | 12 | 4.4E−3 |
| Small GTPase mediated signal transduction (GOTERM_BP) | 12 | 1.1E−2 |
| Nucleotide-phosphate-binding region GTPase (UP_SEQ_FEATURE) | 15 | 1.2E−2 |
| GTP binding (GOTERM_MF) | 16 | 1.5E−2 |
| Small GTP-binding protein domain (INTERPRO) | 9 | 1.5E−2 |
| P-loop containing nucleoside triphosphate hydrolase (INTERPRO) | 24 | 1.1E−1 |
| Annotation cluster 6 | Enrichment score: 1.81 |
| Actin-binding (UP_KEYWORDS) | 13 | 2.6E−3 |
| Actin filament binding (GOTERM_MF) | 8 | 2.1E−2 |
| Brush border (GOTERM_CC) | 6 | 2.2E−2 |
| Actin binding (GOTERM_MF) | 13 | 5.1E−2 |
| Annotation cluster 7 | Enrichment score: 1.76 |
| Tight junction (KEGG_PATHWAY) | 9 | 1.4E−3 |
| Bicellular tight junction (GOTERM_CC) | 6 | 1.9E−2 |
| Tight junction (UP_KEYWORDS) | 5 | 2.0E−1 |

Annotation cluster defines a group of terms having similar biological functions. The enrichment score is the rank of the annotation cluster based on its significance within the dataset tested. The count column refers to number of genes involved in the same category.

*Annotation cluster categories related to barrier function.
Figure 4. Knockdown of VAV3 reduces barrier strength and alters cytoskeletal arrangement in HDMECs and HLMVECs. (A) siRNA knockdown of three candidate genes with a strong correlation of expression to barrier resistance (BAIAP2, VAV3, and SORBS2) validates the effect of VAV3 as an important regulator. (B) Efficiency of siRNA knockdown for BAIAP2, VAV3, and SORBS2. (C and E) Effect of VAV3 silencing on barrier resistance compared with combined siRNA.
VAV3N369A, which contains a loss-of-function mutation in the Dbl homology (DH) domain responsible for the GEF function of Vav3. Importantly, overexpression of VAV3WT enhanced cortical presence of actin fibers and junctional localization of cortactin compared with vector control and VAV3N369A (Fig. 5 J). Furthermore, Vav3 also increased the overall number of cross-linked F-actin and focal adhesions.

**Downstream activation of Rap1 is required for the barrier-enhancing effect of Vav3**

Our previous results demonstrated that the barrier-enhancing properties of Vav3 require its GEF-related DH domain. Thus, we sought to investigate activation levels of GTPases to seek further insight into its mechanism of action. Using GTP-bound GT-Pase pulldown assays, we detected significantly elevated levels of activated Rac1 at 24 h and activated Rap1 at multiple time points after plating of confluent monolayers of VAV3WT expressing HBMECs compared with vector control cells (Fig. 6, A, B, E, and F). This was not the case for RhoA, which is associated with barrier-disruptive rather than barrier-stabilizing effects (Fig. 6, C and D). To further investigate the relevance of Rap1 downstream of Vav3 given its prominent response to Vav3, we coexpressed a dominant-negative Rap1 construct (Rap1S17N) in VAV3WT expressing HBMECs and HBMECs (Fig. 6 G). Noticeably, expression of Rap1S17N abolished the barrier-enhancing effect of VAV3WT for both HBMECs and HBMECs and lowered barrier resistance levels in control cells by affecting Rap1 baseline activity (Fig. 6, H–J). Likewise, coexpression of Rap1S17N also disturbed the cortical localization of actin fibers, pMLC2, and cortactin (Figs. 6 K and S1). To examine potential upstream pathways, we also stimulated HBMECs with reduced levels of Vav3 with forskolin. Forskolin, a known activator of cAMP and Epac1, has been previously linked to endothelial barrier stabilization (Fukuhara et al., 2009b). Though maximum resistance levels remained lower in VAV3-silenced cells, VAV3 knockdown did not diminish the absolute change of resistance upon forskolin stimulation compared with control siRNA (Fig. S5, C–E).

**Loss of Vav3 promotes microvascular leakage in vivo**

To validate the biological significance of these findings in vivo, we assessed barrier function in the microvasculature of both Vav3−/− and Vav2−/−; Vav3−/− knockout mice. Double-null mice were initially selected to eliminate any compensatory effect of Vav2 in this in vivo context. Tail-vein injections of fluorescent microspheres in combination with VEGF resulted in greater microsphere accumulation in the microvascular wall of trachea capillaries for both Vav3−/− and Vav2−/−; Vav3−/− animals (Fig. 7, A and B; and Fig. S6 A). This phenotype was associated with a loss of barrier tightness, as inferred from the discontinuous pattern of CD31-positive microvascular junctions in the areas where the microspheres were trapped. Furthermore, we found perivascular deposition of fibrinogen in capillaries of VEGF-treated Vav2−/−; Vav3−/− animals, whereas no fibrinogen was noted in control littermates (Fig. S6 B). The deposition of fibrinogen/fibrin further supports the concept that Vav proteins are required for the physiological control of barrier function in microvessels. We systemically administered Evans blue to double-null mice and upon a small intradermal injection of either VEGF or histamine in the dorsal skin, endothelial permeability was evaluated. When compared with controls, we found that Vav2−/−; Vav3−/− mice exhibited a more pronounced permeability response to both VEGF and histamine (227 ± 47% and 296 ± 96% vs. 100%, respectively; Fig. S6, C and D).

Based on our findings and the recognized effects of Rac1 and Rap1 on actin fibers, we propose the following model: Vav3 promotes activation of Rap1 either downstream of Rac1 or through alternative mechanisms such as activation of Rap GEFs, autocrine loops, or other second messengers. In turn, activation of Rac1 and Rap1 promotes localization of cortactin to the cell periphery and cortical arrangement of actin fibers. The shift from RhoA-associated stress fibers with radial tension to actin fibers aligned along the cell perimeter with cortical tension stabilizes intercellular junctions and promotes barrier stability (Fig. 7 C). Hence, EC types with high endogenous levels of Vav3 exhibit cortical F-actin, continuous cell–cell junctions, and high levels of barrier resistance.

**Discussion**

It has been recognized that the endothelium, specific to distinct tissues and organs, exhibits unique morphological, functional, and molecular features (Aird, 2007; Nolan et al., 2013). In this study, we compared the tightness of endothelial barriers in confluent monolayers from a panel of 18 EC samples isolated from nine different vascular beds. An initial and important finding was that these cells retained a functional “memory” for barrier integrity as they segregated according to the hierarchy of the vessel type, with the single exception of brain microvascular ECs. In this manner, ECs isolated from vessels with robust mural layers (large arteries) had the lowest transendothelial resistance, whereas microvascular ECs exhibited the highest levels of barrier resistance. These results are consistent with the functional role of microvessels in fluid exchange. In the absence of continuous mural cells, ECs from capillaries must independently build tight barriers that are also quickly responsive to physiological needs. Thus, microvascular ECs are most likely to hold the

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**Table presenting ECIS data modeling values of Rb, α, and Cm at 48 h for HDMCs and HLMCs.** (D and F) Efficiency of siRNA knockdown for VAV2, VAV3, and CLDN5 (ECIS dataset C and E) in HDMCs and HLMCs versus control siRNA. (G and H) Bar graphs of barrier resistance levels at 48 h for HDMCs and HLMCs (ECIS dataset C and E). Error bars show mean ± SEM; *, P < 0.05; n = 3. (J) Immunofluorescence staining of VE-cadherin, ZO-1, F-actin (phalloidin), and cortactin in HDMCs monolayers subjected to either siRNA knockdown of VAV3 and VAV2/3 or siRNA control (bars, 20 μm). Magnification of phalloidin staining highlights loss of cortical actin and gain of stress fibers upon VAV3 and VAV2/3 knockdown. Translocation of cortactin to the cell periphery (filled arrows) is reduced in monolayers with siRNA knockdown of either VAV3 alone or VAV2/3 (open arrows). (K) Fluorescence intensity across cell–cell junctions (mean of n = 6) in cells exposed to siRNA control, siRNA VAV3, and siRNA VAV2/3 (for VE-cadherin, ZO-1, phalloidin, and cortactin, as shown in J).
Figure 5. **Overexpression of VAV3WT increases barrier resistance and promotes structural integrity of endothelial monolayers.**

(A) Protein levels of endogenous Vav3 in the panel of EC types evaluated by Western blotting. γ-Tubulin was used as loading control.

(B) Illustration of the lentiviral constructs used to overexpress VAV3 WT (VAV3WT) and VAV3 mutant (VAV3N369A).

(C) Lentiviral overexpression of HA-tagged VAV3WT and Myc-tagged VAV3N369A results in consistent protein expression in HAECs and HBMVECs as verified by Western blot. γ-Tubulin was used as the loading control.

(D) Immunofluorescence staining of HA-tagged VAV3WT shows uniform cytoplasmic distribution.

(E and F) ECIS measurements of barrier resistance by using HAECs and HBMVECs overexpressing vector control, VAV3WT, and VAV3N369A, respectively. (G) Table presenting ECIS data modeling values of the cell–cell junctional component Rb as well as values for α, and Cm at 48 h for HAECs and HBMVECs transfected with vector control, VAV3WT, and VAV3N369A expression constructs, respectively (mean ± SEM; n = 3).

(H and I) Bar graphs of ECIS resistance levels of HAECs and HBMVECs at 48 h (as shown in D and E; *, P < 0.05 vs. vector control; #, P < 0.05 vs. VAV3WT; mean ± SEM; n = 3).

(J) Immunofluorescence images of HBMVECs transduced
highest degree of regulatory control over vascular permeability, enabling quick opening and closing of the barrier in response to specific stimuli. Interestingly, the brain microvascular sub-group revealed low barrier resistance levels despite their role in supporting a highly impermeable blood–brain barrier. However, the establishment and maintenance of a tight blood–brain barrier strongly relies on the presence of and close interaction with pericytes and astrocytes (Janzer and Raff, 1987; Wolburg et al., 1994; Willis et al., 2004; Armulik et al., 2010; Daneman et al., 2010; Chang et al., 2015), cells that were absent from our monoculture approach. In terms of differences between permeability of macro- and microvascular ECs, it has been previously noted that ECs from microvessels have better developed junctional complexes than those in large vessels, a fact also supported by molecular tracer analysis in vivo (Simionescu et al., 1976, 1978a, b; Schnitzer et al., 1994).

Molecular heterogeneity of the endothelium has been explored by using in vitro approaches and thus met with some skepticism. Would removal of ECs from their native environment normalize their molecular profile despite their origin, resulting in a phenotypic drift? Although it is likely that some degree of drift occurs, there is experimental evidence to indicate that EC heterogeneity is epigenetically programmed (Børsum et al., 1982; Kelly et al., 1998; Chi et al., 2003; Aird, 2007). Chi et al. (2003) generated gene expression array data of a large panel of human EC types from in vitro culture. By using unsupervised hierarchical clustering, they found a striking order and consistency in the expression patterns based on the sites of endothelial origin (Chi et al., 2003). For microvascular ECs, elevated expression of cytoskeleton-associated genes was observed, including actin-binding LIM protein 1, actinin-associated LIM protein, Arg-binding protein 2, slingshot, Vav3, myosin IB, myosin 5C, myosin 7A, and myosin light chain kinase.

This focus to characterize molecular heterogeneity across vascular beds has been important, but it contrasts the limited efforts to functionally clarify endothelial heterogeneity in relation to barrier properties, with few exceptions. To date, levels of claudin-5 have been linked to differences in barrier strength among different vascular beds. It has been shown that HDMECs are more dependent on claudin-5, whereas VE-cadherin compensates for the lack of claudin-5 in HUVECs (Kluger et al., 2013). The tissue-specific relevance of claudin-5 was further revealed by the phenotype of claudin-5 knockout mice, which die of cerebroedema shortly after birth because of selective impairment in blood–brain barrier function (Nitta et al., 2003). In contrast, systemic administration of anti-VE-cadherin antibodies results in vascular leakage that predominantly affects the lung and heart (Corada et al., 1999). These findings clearly speak for distinctions in barrier function that are organ specific.

In seeking to identify additional signatures responsible for the establishment of tight EC barriers, we correlated gene expression with functional barrier resistance and identified several possible candidates. Interestingly, the number of genes with predicted regulatory effects on the cytoskeleton or cytoskeletal components themselves was enriched within the group of identified candidates. Hence, these findings support the concept that the cytoskeleton is a major determinant of barrier heterogeneity and permeability plasticity. This notion is also supported by the arrangements of actin filaments in the distinct EC monolayers. A disorganized and stress fiber–like phenotype was noted in EC types with low levels of barrier resistance, whereas ECs with tight barrier properties showed a highly cortical organization of actin fibers. The importance of cortical actin fibers for junctional stabilization and tight barrier properties has been previously acknowledged (Millán et al., 2010; Hoelzle and Svitkina, 2012; Oldenburg and de Rooij, 2014). In contrast, radial stress fiber–like actin bundles lead to discontinuous intracellular junctions imposed by their traction forces (Oldenburg and de Rooij, 2014).

Correlation analysis between barrier function and molecular profile highlighted several candidates, and among those, VAV3 emerged as an interesting candidate for further investigation given its expression pattern in human microvascular EC types, which was further validated by single-cell sequencing data. Vav3 belongs to the Vav protein family of Rho GEFs that, in mammals, encompasses two other members, Vav1 and Vav2 (Movilla and Bustelo, 1999; Bustelo, 2014). These GEFs have been linked to cellular signaling events associated with cytoskeletal organization, cell transformation, and oncogenesis (Movilla and Bustelo, 1999; Bustelo, 2014; Robles-Valero et al., 2018). In ECs Vav2 was shown to be a downstream target of VEGFR2 signaling and responsible for the activation of RhoA and Rac1 (Gavard and Gutkind, 2006; Garrett et al., 2007). Moreover, research using Vav2<sup>−/−</sup>; Vav3<sup>−/−</sup> mutant mice revealed that these GEFs are mediators of ephrin-induced Rac1 activation, EC migration, and angiogenesis (Hunter et al., 2006). As of yet, Vav3 has not been linked to endothelial barrier organization nor barrier heterogeneity. Gain- and loss-of-function experiments confirmed the critical role of Vav3 in the establishment and maintenance of the endothelial barrier. Evaluation of actin fiber formation provided further insight into the possible mechanism associated with Vav3 function. In particular, cortical actin accumulation, and cortactin localization were both highly responsive to the levels of Vav3.

In contrast with VAV3, levels of VAV2 exhibited no correlation to barrier resistance throughout the panel of EC types evaluated. Although baseline levels of Vav2 possibly add to barrier stabilizing effects of Vav3 or could compensate for loss of Vav3, in fact there is evidence that all Vav proteins perform overlapping, albeit not identical, functions (Bustelo, 2014). For example, Vav1 but not Vav2 or Vav3 can stimulate the nuclear factor of activated T cells in T-lymphocytes (Doody et al., 2000). Likewise, microarray experiments have shown that Vav2 and Vav3 use isoform-specific, redundant, and synergistic pathways to promote changes in the transcriptional landscape of breast cancer cells (Citterio et al., 2012). Perhaps pertinent to our study, early work has shown...
that Vav3 induces changes in the cytoskeleton that are distinct from those elicited by Vav1 or Vav2 (Movilla and Bustelo, 1999). Whether this isoform specificity is a result of subtle catalytic differences in GTPase substrates or possible subcellular localizations remains to be investigated.

Using a DH domain–mutated form of Vav3, we demonstrated that the barrier-related effect of Vav3 requires its GEF function. Vav3 can stimulate nucleotide exchange in a wide spectrum of GTPases, including Rho (RhoA, RhoB, and RhoC) and Rac (Rac1, Rac2, and RhoG) subfamily members (Movilla and Bustelo, 1999; Bustelo, 2014). We favor the implication of Rac-associated barrier stability given the negative correlation of RhoA-triggered stress fibers and barrier disruption noted in our panel of ECs. Upon overexpression of Vav3, we observed elevated activation levels of both Rac1 and Rap1, whereas concurrent expression of dominant-negative Rap1S17N abolished the barrier-enhancing effect of Vav3WT. (A–F) Levels of activated Rac1, RhoA, and Rap1 were determined in confluent monolayers of vector control and VAV3WT overexpressing HBMVECs at 6, 24, and 48 h after plating by using GTP-bound pulldown assays. Levels of GTP-Rac1, GTP-RhoA, and GTP-Rap1 (activated state) are compared with total levels of Rac1, RhoA, and Rap1 as well as γ-tubulin, respectively. Bar graphs show quantification of activation levels as the percentage of vector control per time point (controls set to 100% *, P < 0.05; n = 3–4). VAV3WT promotes activation of Rac1 (at 24 h), and Rap1 (all time points), whereas no effect is observed for RhoA. (G) Immunoblot of HAECs and HBMVECs overexpressing HA-tagged VAV3WT, Rap1S17N, and a combination of both. γ-Tubulin was used for loading control. (H and I) ECIS measurements of HAECs and HBMVECs overexpressing vector control, VAV3WT, Rap1S17N, and VAV3WT with Rap1S17N in combination. (J) Bar graphs of ECIS resistance levels of HAECs and HBMVECs at 48 h (as shown in H and I; *, P < 0.05 vs. vector control, #, P < 0.05 vs. VAV3WT; n = 3). Error bars show means ± SEM. (K) Immunofluorescence images of HBMVECs overexpressing vector control, VAV3WT, Rap1S17N, and VAV3WT with Rap1S17N in combination, respectively (bar, 20 µm). Overexpression of VAV3WT promotes cortical organization of actin fibers, pMLC2, and cortactin (as noted by arrows) in comparison with both vector control, and VAV3WT in combination with Rap1S17N. It also elicits an increase in small focal adhesions as shown by vinculin staining (arrows). ctrl, control.
Vav3. Whereas the direct interaction of Vav proteins with Rac1 is well established (Movilla and Bustelo, 1999; Bustelo, 2014), activation of Rap1 most likely occurs further downstream of the Vav3–Rac1 axis according to the catalytic specificity of the DH domain, which does not favor binding to Ras-like proteins. In this context, it is of interest that the Rap1 GDP/GTP exchange factor RasGRP2 has been shown to be activated via an F-actin–mediated translocation mechanism upon Rac1 signaling in COSI cells (Caloca et al., 2004). Arthur et al. (2004) observed binding of Rap1 to the DH–Pleckstrin homology module of Vav2 and revealed Rap1-mediated translocation of Vav2 toward the plasma membrane. Rap1 in particular has been associated with cortical formation of the F-actin cytoskeleton and therefore is thought to shift RhoA-mediated radial tension forces toward a cortical tension pattern running parallel to cell–cell contacts, thereby stabilizing junctions (Noda et al., 2010; Ando et al., 2013; Pannekoek et al., 2014). Hence, depletion of Rap1 decreased endothelial barrier function in vitro (Pannekoek et al., 2011), whereas activation of Epac1/Rap1 has been linked to barrier-protective effects (Fukuhara et al., 2005a; Adamson et al., 2008). Other possible actions include the indirect effect of Vav3 on the expression of proteins connected to this process. For example, we have found that Vav2 and Vav3 are important to block epithelial–mesenchymal transitions in breast cancer cells. This action, which is GTPase dependent, relies at least in part on the engagement of a distal transcriptional program that regulates the abundance of critical proteins involved in cell–cell contacts such as E-cadherin, plackoglobulins, connexins, and Claudins (unpublished data). Whether these Vav3-mediated downstream effects are of direct or indirect nature needs to be determined. In summary, our findings support a new barrier-enhancing role of the cytoskeletal regulatory molecule Vav3 in the endothelium and highlight its contribution to barrier heterogeneity across different vascular beds.

Materials and methods

Mice

Vav3−/− and Vav2−/−;Vav3−/− mice in the C57BL/10 genetic background were generated by standard crosses from the single knockout mice (Doody et al., 2001; Sauzeau et al., 2006) as described previously (Sauzeau et al., 2007). All animals were housed in a pathogen-free environment, and experiments were conducted in accordance with University of California, Los Angeles, Department of Laboratory Animal Medicine’s Animal Research Committee guidelines.

Cell culture

Human ECs were purchased from different vendors to capture a broad spectrum of sampling. Specifically, we obtained HUVECs (C2519A; Lonza; C-12203; PromoCell), HSaVECs (HSVEC/A; VEC-Technologies; CAP-0019; Angio-Proteomie), HAECs (PCS-100-011; ATCC; 6100; SciencCell), HIAECs (CC-2545; Lonza; CAP-0020; Angio-Proteomie), HBMVECs (ACBRI 376 V; Cell-Systems; CAP-0002; Angio-Proteomie), HUMVECs (C-12295; PromoCell; 7000; SciencCell), HLMVECs (3000; SciencCell; C-12281; PromoCell), HAMVECs (7200; SciencCell), and HDMECs (C-12212; PromoCell; 2000; SciencCell). All types of ECs were cultured in EGM-2 endothelial growth medium (CC-3162; Lonza) supplemented with 5% FBS (Omega Scientific) and were maintained at 37°C with 5% CO2. Similar EC passages 4–6 were used for final experiments. Human dermal fibroblasts were a gift from W. Lowry (University of California, Los Angeles, Los Angeles, CA), expanded in DMEM supplemented with 10% FBS, and cultured in EMG-2 medium over 48 h before RNA expression experiments. Brightfield images were acquired by using an Axiovert 200M inverted microscope (Zeiss).

Lentiviral vector, particle production, and transduction

pRRL-HA-VAV3WT was constructed by cloning VAV3 from pC.HA-VAV3 (4555, Addgene) into the lentiviral vector pRRL-IRES-RFP (University of California, Los Angeles, Vector Core) by using Gibson Assembly. For the Vav3 DH domain mutated form, pLVX-Myc-VAV3S369A was used. The sequence of Rap1S17N was provided by X. Zhang (Southwestern University, Dallas, TX) as part of pcDNA3-HA-Rap1-S17N. Lentiviral particles were generated by transfecting Lenti-X HEK293 cells (Takara Bio Inc.) with target, VSV-G pseudotype, and delta8.2 packaging plasmids by using Lipofectamine 2000 (Invitrogen). Conditioned medium was collected after 48 h and passed through 0.45-µm filters. ECs were transduced at 50% confluence overnight in EGM-2 medium containing protamine 4 µg/ml sulfate. Empty pRRL-IRES-RFP was used as control vector.

Quantitative real-time PCR

Total RNA was purified by using the RNaseasy mini kit (QIAGEN). Complementary DNA synthesis was performed with iScript cDNA Synthesis kit (Bio-Rad Laboratories) by using OligodT primers.
Quantitative real-time PCR was performed on CFX Connect Real Time Cycler PCR detection system (Bio-Rad Laboratories) by using SoSo Advanced SYBR green (Bio-Rad Laboratories). The following primers were used: BAIA2, 5′-GAAACAGACCTG CCTGCGCT-3′ (forward) and 5′-CTTCAAGGATCAAGGCTG-3′ (reverse); CLDN5, 5′-CTGTTCATAGGCAGAGCG-3′ (forward) and 5′-AGGACAGGTCTCCAGGCTT-3′ (reverse); PARDEG, 5′-GTC AAGAGCAAGTGGGCCC-3′ (forward) and 5′-AGAAACAGCCTTCC-3′ (reverse); SOX10, 5′-GGGCACTGACTCCTACTTC CC-3′ (forward) and 5′-GGTGACTGAGAATCAGCCC-3′ (reverse); VAV2, 5′-GAATCACTTACGCTGCTCC-3′ (forward) and 5′-TGC ACCTCCACCTTGATATG-3′ (reverse); VAV3, 5′-GGAGCCAAATG GACTGGGAAGAA-3′ (forward) and 5′-CGGAGCTGTAAAAGGGG TC-3′ (reverse); TJP1, 5′-TCACCTACCACTCTGCTG-3′ (forward) and 5′-CTAGGCCCTCAGATGACCA-3′ (reverse); CDH5, 5′-ATG AGATGCTGTGTAAG-3′ (forward) and 5′-TGTGACTTGG TCTGGGTA-3′ (reverse); CTTN, 5′-TTTTGCGGCAAGTATGCC-3′ (forward) and 5′-AGCGCCTCTTTACCTCATT-3′ (reverse); HPRT, 5′-GCCCTGGGCTGTTAGTATG-3′ (forward) and 5′-AGCAAGGAGG TTAGCCCTGTC-3′ (reverse). Each reaction was run in duplicates; results are reported as ΔCt and are normalized to HPRT.

NanoString measurement and data analysis
For RNA expression analysis, equal numbers of ECs (500,000 per 35-mm dish) were plated, and monolayers were cultured over 24 h in EMG-2 media with 5% FBS. Cell lysates were harvested at 24 h, and RNA extraction was performed as described above. Before running the NanoString array, RNA sample quality had been assessed using the BioAnalyzer (Agilent Technologies). Probe sequences were custom designed and manufactured by NanoString, including sequences of eight housekeeping genes for data normalization as well as sequences of negative and positive controls. Samples were measured in triplicate per EC type, loading 100 ng mRNA per well. RNA samples were processed according to the standard nCounter instructions (NanoString). Expression data were collected as absolute counts; detectable levels of nonspecific binding (background) were measured by six negative controls and the means plus two SDs were subtracted from each mRNA count. Expression levels were further normalized to the panel of eight housekeeping genes (CNOT10, DHX16, FCFI, HPRT1, TLK2, TUBG1, USP39, and ZKSCAN5). RNA samples of human dermal fibroblasts served as biological comparison and control for a vessel-specific endothelial expression profile. For correlation analysis of RNA expression to resistance levels, genes were ranked in order of their individual Spearman’s rank correlation coefficient (rs), and a heat map was generated based on the absolute RNA expression counts per gene.

Lung tissue dissociation and single-cell sequencing
Before dissection, lungs were perfused with sterile saline via the left ventricle, and afterward, large vessel branches removed from the pulmonary lobes. Tissue dissociation was performed by using a lung dissociation kit (130-095-927; Miltenyi Biotec) according to the manufacturer’s protocol. Red blood cells were eliminated by incubating the cell suspension with a red blood cell lysis buffer (eBioscience) for 1 min. To keep the processing time between tissue harvesting and single-cell lysis at a minimum, no further cell type enrichment step was performed.

For the generation of single-cell gel beads in emulsion, a suspension of 8,700 cells was loaded on a Chromium single cell instrument (10x Genomics) with an estimated targeted cell recovery of ~5,000 cells. Single-cell RNAseq libraries were prepared by using the Chromium single cell 3’ library and gel bead kit v2 (10x Genomics). Sequencing was performed on Illumina HiSeq2500, and the digital expression matrix was generated by demultiplexing, barcode processing, and gene unique molecular index counting by using the Cell Ranger v2.0 pipeline (10x Genomics). To identify different cell types and find signature genes for each cell type, the R package Seurat was used to analyze the digital expression matrix. Specifically, cells that express <200 genes and genes detected in less than three cells were filtered out. Second, 2,112 variable genes were selected by Seurat for further analysis. The data were then regressed by sequencing depth to remove this unwanted source of variation. Principal component analysis and t-distributed stochastic neighbor embedding (t-SNE) were used to reduce the dimensionality of the data, and the data were plotted on a two-dimensional graph. A graph-based clustering approach was later used to cluster the cells; then signature genes were found and used to define cell types for each cluster. ECs were selected based on the presence of at least three reads of the Pemai1 gene and three reads of the Cdhs5 gene. To identify Vav3-positive cell population, cells that contained one or more reads of Vav3 were selected. After this, Seurat was used to cluster only the endothelial and Vav3-expressing cells.

Immunocytochemistry and immunohistochemistry
For immunocytochemistry of cultured human EC types, cells were seeded onto Lab-Tek II eight-well uncoated glass slides (80,000 per well; Thermo Fisher Scientific), and confluent monolayers were cultured over 48 h. Subsequently, cells were fixed for 10 min with 2% PFA and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) or fixed with methanol for 15 min at −20°C (claudin-5 staining). After a blocking step of 1 h with 5% donkey serum (017–000–121; Jackson ImmunoResearch Laboratories, Inc.), primary antibodies were incubated overnight in 5% serum and included VE-cadherin (1:200; sc-6458; Santa Cruz Biotechnology, Inc.), claudin-5 (1:200; ab15106; Abcam), ZO-1 (1:200; 40–2200; Zymed), cortactin (1:100; clone 4F11; EMD Millipore), vinculin (1:200; V4505; Sigma-Aldrich), and pMLC2 (1:200; 3675; Cell Signaling Technology). Alexa Fluor secondary antibodies (1:400; Invitrogen), Texas red phalloidin (1:50; Thermo Fisher Scientific), and DAPI (1:1,000; Thermo Fisher Scientific) were incubated for 1 h at room temperature.

For immunohistochemistry, mice were perfused with 2% PFA (AC41678-5000; Acros Organics), and tissue was isolated and fixed with 2% PFA overnight and embedded in paraffin blocks. Slides were hydrated, and 10 mM citrate 0.05% TWEEN-20 was used for heat-mediated antigen retrieval. 5% donkey serum was used to block tissue and incubate primary antibody overnight. Primary antibodies included CD31 (1:50, clone S23; Dianova), and fibrinogen (1:200, ab18533; Abcam). Alexa Fluor (Invitrogen) secondaries were used at 1:400. For whomemounted stainings, the trachea was dissected and separated into two parts by a
longitudinal incision. All slides were mounted in Mowiol 4-88. A Zeiss LSM880 confocal microscope was used for fluorescence imaging, and ZEN software was used for image processing.

**Western blotting and GTPase activation assay**
Protein lysates for Western blot analysis were generated by using modified radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 0.15 NaCl, 1 mM sodium orthovanadate, and 10 mM β-glycerophosphate), containing protease inhibitor cocktail (11873580010; Roche) and 200 µM sodium orthovanadate. The following primary antibodies were used: VE-cadherin (1:1,000; sc-6458; Santa Cruz Biotechnology, Inc.), claudin-5 (1:1,000; ab15106; Abcam), ZO-1 (1:1,000; 40-2200; Thermo Fisher Scientific), β-actin (1:2,000; ab8227; Abcam), cortactin (1:1,000; 05-180; EMD Millipore), vinculin (1:1,000; V4505; Sigma-Aldrich), Vav3 (1:500; homemade rabbit polyclonal antibody), HA epitope tag (1:1,000; 901501; BioLegend), and γ-tubulin (1:2,000; ab1322; Abcam). For detection of activated Rap1, Rac1, and Rho, a GTP-bound GTPase pulldown assay was used (8818, 8815, and 8820; Cell Signaling Technology) and conducted according to the manufacturer’s instructions. Immunocomplexes were detected by enhanced chemiluminescence (Thermo Fisher Scientific) by using the ChemiDoc XR+ Molecular Imager and ImageLab Software (Bio-Rad Laboratories). Quantification of bands by densitometry analysis was performed by using ImageJ software (National Institutes of Health).

**Microsphere extravasation assay**
A combination of VEGF-A165 (3 µg/mouse; 450-32; PeproTech) with 100 µl of 30-nm red fluorescent microspheres (Fluoro-Max–dyed red aqueous fluorescent particles; 0.03 µm; Thermo Fisher Scientific) was administered via tail-vein injection in knockout mice and littermates. After 30 min, intravascular microspheres were removed by perfusion with 2% FFA in sterile saline via the left ventricle. Tracheas were dissected and fixed in 2% PFA over 2 h after a permeabilization and blocking step with 0.3% Triton X-100 and 3% donkey serum in PBS for 1 h. Tissue samples were incubated with rat anti–mouse CD31 (1:50; clone SJ31; Dianova) overnight and with Alexa Fluor secondary antibodies (1:100; Invitrogen), and DAPI (1:1,000) over 2 h the next day. Trachea samples were mounted in Mowiol 4-88, and the amount of extravasated microspheres was assessed by using confocal imaging (z stack) and 3D image reconstruction (Imaris; Bitplane). Microvascular leakage was quantified by determining the area of microsphere extravasation versus the total vessel area.

**Miles assay**
100 µl Evans blue (0.5% in sterile saline; E2129; Sigma-Aldrich) was injected in the tail vein of Vav2−/−;Vav3−/− and control mice followed by intradermal administration of VEGF (150 ng in 30 µl/site), histamine (50 ng in 30 µl/site), or sterile saline at the dorsal skin. 20 min after intradermal injection of VEGF, histamine, and control saline, the dorsal skin sites were excised, images taken, and Evans blue extracted by immersion in formamide at 55°C over 48 h. The levels of extravasated Evans blue per sample was determined by spectrometry at 620 nm and quantified after normalization with control samples (SpectraMaxPlus microplate reader; Molecular Devices).

**Statistical analyses**
Differences between groups were evaluated by using one-way ANOVA followed with Tukey’s post hoc multiple-comparison test. Data pairs were compared by using a Student’s unpaired two-tailed t test and Mann-Whitney test. For correlation analyses, the Spearman correlation coefficient (r_s) was determined. To reduce dimensionality and visualize the single-cell RNA sequencing dataset, principal component analysis and t-SNE were used. All analyses were performed by using Prism (v4.0c; GraphPad Software) unless otherwise indicated. P values of <0.05 were considered significant. Data are presented as means ± SEM. Functional annotation analysis and clustering were performed in DAVID v6.8 (Huang et al., 2009a,b).

**Online supplemental material**
Fig. S1 shows the levels of barrier resistance and Rb values in each of the EC subtypes as well as their morphology under phase contrast. Fig. S2 provides distribution and abundance of barrier-related proteins in the EC subtypes by immunocytochemistry and total levels by Western blotting. Fig. S3 compares levels of barrier-associated transcripts across all nine VE subtypes and compares lung and aorta endothelium by single-cell sequencing focusing on expression of Vav3, Baiap2, and Sorbs2. Fig. S4 demonstrates that silencing of VAV3 affects cortical deposition of actin and cortactin in HLMVECs. Fig. S5 includes the quantification of the confocal images shown in Figs. 5 and 6 and demonstrates the effects of cAMP activation in the presence and absence of VAV3. Fig. S6 shows the effects of combined loss of Vav2 and Vav3 in vascular permeability in vivo. Table S1 provides the raw (mean of n = 3) expression data from the NanoString array.

**Acknowledgments**
The authors thank Michelle Steel for technical assistance, Christian Renken (Applied BioPhysics) for support regarding the ECIS system, Dr. Xuewu Zhang for sharing the pcDNA3-HA-Rapi-S17N plasmid, and the Tissue Procurement Core Laboratory Shared Resource at the University of California, Los Angeles, for its contribution. Lentiviral particle production (University of California, Los Angeles, Vector Core) and NanoString array measurements were performed at the University of California, Los Angeles, Integrated Molecular Technologies Core, which is supported by CURE/P30 DK041301.

This study was supported by the Deutsche Forschungsgemeinschaft (scholarship HI1727/1-1) to G. Hilfenhaus. M.L. Iruela-Arispe is supported by grants 1R01CA197943 and 1R01HL140014. X.R. Bustelo is supported by grants from the Castilla-León Government (BIO/SA01/15 and CSIO49U16), the Ministry of Economy and Competitiveness (SAF2015-64586-R, RD12/0036/0002, and CB16/12/00351), Worldwide Cancer Research (14-1248), the Fundación Ramón Areces, and the Spanish Association Against Cancer (GC16173472GARC).

The authors declare no competing financial interests.

Author contributions: G. Hilfenhaus designed and performed experiments, analyzed the data, and wrote the manuscript. D. Nguyen, J. Freshman, D. Prajapati, and D. Song performed the experiments and analyzed the data. F. Ma and M. Pellegrini
performed the single-cell sequencing data analysis. S. Ziyad assisted in the plasmid cloning and revised the manuscript. M. Cuadrado generated and provided critical reagents. X.R. Bustelo provided critical reagents and revised the manuscript. M.L. Irueła-Arispe designed the study, provided guidance, and revised the manuscript.

Submitted: 14 June 2017
Revised: 12 April 2018
Accepted: 8 May 2018

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