The ability of blood vessels to sense and respond to stimuli such as fluid flow, shear stress, and trafficking of immune cells is critical to the proper function of the vascular system. Endothelial cells constantly remodel their cell–cell junctions and the underlying cytoskeletal network in response to these exogenous signals. This remodeling, which depends on regulation of the linkage between actin and integral junction proteins, is controlled by a complex signaling network consisting of small G proteins and their various downstream effectors. In this commentary, we summarize recent developments in understanding the small G protein RAP1 and its effector RASIP1 as critical mediators of endothelial junction stabilization, and the relationship between RAP1 effectors and modulation of different subsets of endothelial junctions.

The vasculature is a dynamic organ that is constantly exposed to a variety of signaling stimuli and mechanical stresses. In embryogenesis, nascent blood vessels form via a process termed vasculogenesis, wherein mesodermally derived endothelial precursor cells aggregate into cords, which subsequently form a lumen that permits trafficking of plasma and erythrocytes. Angiogenesis occurs after establishment of this primitive vascular network, where new vessels sprout from existing vessels, migrate into newly expanded tissues, and anastomose to form a functional and complex circulatory network. In the mouse, this process occurs through the second half of embryogenesis and into postnatal development in some tissues, such as the developing retinal vasculature. Further, angiogenesis occurs in a variety of pathological conditions, such as diabetic retinopathy, age-related macular degeneration, inflammatory diseases such as rheumatoid arthritis, wound healing, and tumor growth. Both vasculogenesis and angiogenesis are driven through signaling by vascular endothelial growth factor (VEGF), and therapeutic agents targeting this pathway have shown efficacy in a number of diseases.

Blood vessels must have a sufficient degree of integrity so as to not allow indiscriminate leak of plasma proteins and blood cells into the underlying tissue. However, vessels must be able to sense their environment, respond to local conditions, and mediate the regulated passage of protein, fluid, and cells. For example, endothelial cells are the primary point of attachment for immune cells leaving the bloodstream and entering tissue, and leukocytes subsequently migrate either through the endothelial cell body itself (the transcellular route), or through transient disassembly of cell–cell junctions (the paracellular route). Precise regulation of endothelial junctions is critical to the proper maintenance of vascular integrity and related processes, and disruption of vascular cell–cell contacts is an underlying cause or contributor to numerous pathologies such as cerebral cavernous malformations (CCM) and hereditary hemorrhagic telangiectasia (HHT). Understanding the basic mechanisms of endothelial junction formation and maintenance will therefore lead to a greater chance of success of therapeutic intervention in these pathologic conditions, especially in instances where targeting of
VEGF signaling is insufficient to resolve vascular abnormalities.

**Endothelial Junctions: Their Composition and Regulation**

Similar to epithelial cells, endothelial cell–cell junctions are primarily comprised of two types, tight junctions (TJs) and adherens junctions (AJs), which form through homotypic trans-interaction of molecules on adjacent cells, as well as cis-interactions on the same cell. The major constituents of TJs are the transmembrane proteins claudin-5, occludin, nectins, and junctional adhesion molecules (JAMs), whereas AJs are largely composed of calcium-dependent vascular endothelial cadherin (VE-cadherin). Other molecules, such as nectins, participate in the establishment of initial cell–cell contacts and organize both TJs and AJs. In both cases, these classes of junctional proteins are linked to the underlying actin cytoskeleton through various adaptor proteins, such as zonula occludens 1 (ZO1) in the case of TJs and the catenins in the case of AJs. β-catenin interacts directly with the C-terminal tail of VE-cadherin, and α-catenin binds both to actin and to β-catenin, although there is still controversy as to whether ternary or higher-order complexes assemble in vitro and in vivo. Additional catenins, such as p120-catenin, also bind to the juxtamembrane region of VE-cadherin and modulate adhesion strength, largely through prevention of cadherin endocytosis. AJ integrity is regulated by a number of factors, including linkage of AJs to the actin and microtubule cytoskeleton, which can prevent lateral diffusion and internalization of integral AJ components.

Much recent work has demonstrated that interactions between adjacent cells mediated by junction proteins, as well as between junctions and the actin cytoskeleton, play a key role in control of barrier function and leukocyte diapedesis. Antibodies directed against the extracellular domain of VE-cadherin have been demonstrated to block VE-cadherin-dependent adhesion, and thus reduce barrier function, as assessed by measurement of transendothelial electrical resistance and passage of FITC-dextran across endothelial monolayers. Injection of VE-cadherin blocking antibodies in vivo results in increased vascular permeability and neutrophil infiltration. Conversely, stabilization of the linkage between adherens junctions and the actin cytoskeleton, achieved through fusion of VE-cadherin with α-catenin, reduced permeability and leukocyte extravasation both in vitro and in vivo. Taken together, the data indicate that modulation of AJs, either through disruption of homophilic interactions or control of actin–juncion linkages, may have a profound impact on endothelial barrier function. In addition to the role in barrier function, stable VE-cadherin-dependent cell–cell linkages are important for establishment of endothelial apical-basolateral polarity in major arteries.

Recently, interest has grown concerning the precise composition and morphology of distinct subsets of endothelial junctions. In contrast to epithelial junctions, where tight junctions are located at the apical surface of cells and adherens junctions are found at the baso-lateral segment, endothelial AJs and TJs are typically not stratified in most tissues, but instead, intermingle in many junctional contacts. Further, endothelial junctions display several distinctive morphologies, at least when examined in confluent cultures of primary endothelial cells from various vascular beds. Linear junctions, which are thought to represent stable cell–cell contacts, display close association with cortical actin (also described as circumferential actin bundles), as shown in numerous immunofluorescence microscopy experiments. Reticular junctions, which exhibit a honeycomb-like staining pattern of AJ markers, may represent a specialized type of junction formed by sliding contacts between two or more adjacent cells. These contacts were reported to be devoid of actin and associated mechanotransducing proteins, such as myosin, within the reticular network. In addition, reticular structures were not rich in TJ markers such as ZO-1. This, combined with the fact that PECAM-1 was found within the reticular junctions, yet was interspersed between cadherin–catenin regions, supports the hypothesis that these regions may be a specialized area of junction that facilitates leukocyte extravasation and specific aspects of barrier function under distinct conditions.

A third type of junctional morphology, variously referred to as “discontinuous” “punctate,” or “focal” adherens junctions (FAJs), is associated both with initial AJ formation and remodeling of AJs. When two endothelial cells initiate contact with one another, VE-cadherin-containing filopodia form initial adhesions, called focal or spot adherens junctions, which are linked to radial actin stress fibers. These adhesions subsequently expand laterally to broaden the contact interface, and form linear junctions with associated bundled linear actin. Contraction of endothelial cells, induced by stimuli such as VEGF, thrombin, or TNFα, is able to promote formation of nascent FAJs from linear junctions. Interestingly, FAJs contain vinculin, which is recruited to focal adhesions to facilitate integrin clustering and linkage to the actin cytoskeleton. Recent data indicate that vinculin is recruited to the remodeling FAJs, and may act to maintain cell–cell contacts in the presence of an orthogonal mechanical force. α-catenin function is controlled in part by mechanically induced conformational changes, and this may facilitate vinculin recruitment to specific junctional regions that sense local strain. Intriguingly, vinculin also binds to talin, a focal adhesion protein that also undergoes force-induced remodeling, and tension is required to maintain vinculin at focal adhesions. This suggests that vinculin may be a general means to link the actin cytoskeleton to areas of the cell that are encountering mechanical stress, either at sites of matrix attachment or linkage to other cells.

**RAP1 as a Central Mediator of Endothelial Cell–Cell and Cell–Matrix Adhesions**

RAP proteins are small monomeric guanosine triphosphatases (GTPases) that are members of the RAS GTPase superfamily. GTPases function as switches in numerous cellular signaling and trafficking processes.
Figure 1. Signaling by RAP1 effectors in endothelial barrier control. Upstream signaling at the cell membrane, potentially mediated by G-protein coupled receptors (GPCRs), triggers activation of adenyl cyclase via Go, resulting in formation of cyclic AMP (cAMP). cAMP binds to EPAC1, a guanine nucleotide exchange factor, which catalyzes exchange of GDP on RAP1A/B for GtP, activating the small G protein. Active RAP1A/B may bind to effectors such as RASIP1, RADIL, AF6, and KRIT1, in many cases triggering their movement to the cell cortex (not shown). RASIP1 promotes bundling of cortical actin, cross-linked by non-muscle myosin heavy chain II (nmMHCII). The cortical actin bundles are linked to transmembrane VE-cadherin molecules through α-catenin and β-catenin, cytosolic adaptor proteins. Assembly of this actin network may inhibit the formation of focal adherens junctions, comprised of cadherin–catenin complexes linked to longitudinal stress fibers. AF6 (also known as afadin) promotes accumulation of junctional components, and binds directly to β-catenin, as well as KRIT1. AF6 and KRIT1 suppress RHOD activation in certain cell types (e.g., AF6 in lymphatic endothelial cells). Inhibition of RHOD reduces phosphorylation of myosin light chain (MLC) by Rho kinase (ROCK), reducing contraction and potentially FAJ formation. The role of RADIL is less clear, but it may partner with RASIP1 and also inhibit RHOD signaling in some contexts.
switching behavior is controlled by cycling between GDP (inactive) and GTP-bound (active) forms, processes that are catalyzed by specific exchange factors. \(^{35,36}\) RAP1 is typically bound to GDP, but guanine nucleotide exchange factors (GEFs), such as PDZ-GEF, RAPGEF3/EPAC1, and C3G, promote exchange of GDP for GTP (Fig. 1). \(^{36}\) The GTP-bound form of RAP1 is then primed to specifically interact with its downstream effectors, by binding to their RAS-association (RA) domains. Inactivation of the cycle is achieved through action of GTPase-activating proteins (GAPs), which promote hydrolysis of bound GTP to GDP. \(^{35}\) Although RAS and RAP proteins share significant amino acid sequence identity, RAS signaling functions primarily in the regulation of growth, differentiation, and apoptosis, whereas RAP signaling regulates adhesion of cells to extracellular matrix, as well as formation and possibly stabilization of cell–cell junctions. \(^{36}\)

Two isoforms of RAP1, RAP1A and RAP1B, have been identified, which appear to have overlapping and distinct functions. \(^{37}\) Targeted systemic knockout of murine \(Rap1a\) results in defective leukocyte adhesion, altered myeloid cell function, and partially penetrant embryonic and peri-natal lethality resulting from hemorrhage (Table 1), although the severity of these effects is dependent on the genetic background utilized. \(^{38-40}\) \(Rap1a^{−/−}\) mice also display reduced neovascularization in hindlimb ischemia models. \(^{35}\) \(Rap1b\) knockouts have 50% lethality after E12.5, and also a mild deficiency in platelet function (Table 1). \(^{41}\) Closer examination of postnatal retinas in \(Rap1b^{−/−}\) mice indicated a delay in angiogenesis at day 7 (P7), although this delay resolved by P14. \(^{42}\) Ex vivo aortic ring sprouting assays also indicated a reduction in angiogenic sprout outgrowth in response to basic FGF (bFGF) and VEGF. \(^{42}\) Vascular defects and hemorrhage are present in each knockout, and the endothelial cell-autonomous requirements for each \(Rap1\) gene have yet to be clearly defined. Conditional deletion of \(Rap1b\) and one allele of \(Rap1a\) from endothelial and a small subset of hematopoietic cells using a \(Tie2-Cre\) driver causes retinal angiogenesis defects similar to those observed in the single \(Rap1b\) knockout. \(^{43,44}\) The primary data describing any embryonic phenotypes of these animals, as well as the EC-specific double homozygous knockout of \(Rap1a\) and \(Rap1b\) have not yet been published. \(^{43,44}\) These experiments would shed light on potential redundancies and unique functions of \(Rap1a\) and \(Rap1b\) in the endothelium.

The roles of RAP1A and RAP1B in endothelial cells have been best studied in vitro, primarily through overexpression and gene knockdown studies examining the RAPI isoforms themselves, or various GAPs and GEFs that regulate RAPI function. Knockdown of either isoform leads to disruption of integrin-mediated adhesion, with consequences such as

Table 1. In vitro and in vivo phenotypes of EPAC1-RAP1-effector genes

| Gene name       | Loss-of-function endothelial junction phenotype in vitro | Loss-of-function phenotype in vivo | Endothelial junction morphology in vivo | Key references          |
|-----------------|--------------------------------------------------------|-----------------------------------|---------------------------------------|--------------------------|
| EPAC1/RAPGEF3   | Increased permeability, disorganized junctions, loss of cortical actin | Systemic KO not lethal, EC-specific KO not reported | Not reported | 48,73:75 |
| RAP1A           | Increased permeability, disorganized junctions, loss of cortical actin, increase in gaps between cells, apparent increase in FAJs | Systemic KO displays partially penetrant embryonic/perinatal lethality, hemorrhage, edema | Not reported | 51,54 |
| RAP1B           | Increased permeability, disorganized junctions, loss of cortical actin, apparent increase in FAJs | Systemic KO shows 50% hemorrhage, lethality after E12.5, transient delay in angiogenesis in neonatal retina | Not reported | 41,51,54 |
| Afadin/AF6      | Reduction of junctional marker staining at cell periphery, increased actin stress fibers in lymphatic ECs | Systemic KO embryonic lethal by E10.5, EC-specific KO shows mostly penetrant edema and lethality by E16.5 | Punctate VE-cadherin staining in lymphatic ECs | 57-60 |
| KRIT1/CCM1      | Increased P-MLC and stress fibers, disrupted α-catenin localization | Systemic KO is embryonic lethal between E10–11; EC-specific neonatal KO displays hemorrhage, vessel dilation | Disorganized VE-cadherin in neonatal cranial vessels | 12,63,66 |
| RASIP1          | Increased permeability, disorganized junctions, loss of cortical actin, increase in FAJs, compromised barrier function | Embryonic lethality between E9.5–10.5 in systemic knockout, abnormal blood vessel development, hemorrhage | Increase in FAJ proportion in yolk sac | 53,67,69,70 |
| RADIL           | Loss of cortical actin, increase in FAJs, compromised barrier function in combination with RASIP1 knockdown | Altered neural crest migration (zebrafish), systemic murine knockout not reported | Not reported | 70,72 |
impaired VEGF-dependent migration; overexpression of constitutively active RAP1 also inhibits migration, resulting from increased integrin-dependent adhesion.\textsuperscript{38,45,46} RAP1 and its upstream GEF EPAC1 have been implicated in endothelial barrier control, as activation of RAP1 by EPAC1 impairs induction of permeability by thrombin or VEGF.\textsuperscript{47-50} Further, loss-of-function studies of RAP1 in vitro result in VE-cadherin disorganization, a reduction in linear junction-associated actin, and an increase in FAJs (Table 1).\textsuperscript{31-54} There are discrepancies in the literature as to whether RAP1A or RAP1B is the primary contributor to the formation and stabilization of endothelial junctions, as RNAi-mediated knockdown of either isoform leads to disruptions of VE-cadherin staining, increased formation of FAJs and/or gaps between cells, and reduction in transendothelial resistance (Table 1).\textsuperscript{54} These inconsistencies may be a result of utilization of endothelial cells from different vascular beds, different siRNA sequences with possible off-target effects, or different culture conditions. Nonetheless, recent work indicates that differential localization of RAP1 isoforms may be a contributor to distinct isoform-dependent functions, as RAP1A localizes more strongly to junctions than RAP1B.\textsuperscript{54}

### RAP1 Effectors Regulate Actin Linkage and Remodeling at Endothelial Junctions

RAP1 exerts its effects on junctions through control of effectors that impact cytoskeletal and junctional actin organization. The effects of RAP1 on actin remodeling and barrier stabilization may be partially through GEFs for the small G protein RAC1, as RAP1 has been shown to interact with VAV1 and TIAM1, which activate RAC1 and promote actin bundling at the leading edge of migrating cells, and thus, stabilize nascent or remodeling contacts.\textsuperscript{56} Recent data have implicated RAP1 in direct regulation of actin bundling in epithelial cells, through signaling to non-muscle myosin heavy chain II isoform B (nmMHCIIIB), which promotes formation of linear junctions with circumferential actin bundles (Fig. 1).\textsuperscript{55} In addition, RAP1-specific effectors such as afadin and KRIT1/CCM1 carry out distinct functions at junctions.

Afadin/AF6 is a broadly expressed cytosolic protein that localizes to junctions and interacts with nectins and the actin cytoskeleton, as well as a number of other cytoskeletal regulators.\textsuperscript{56} The N terminus of afadin contains two Ras superfamily-domain (RA) domains, which mediate interaction of afadin with active RAP1.\textsuperscript{56} RAP1 signaling drives afadin localization, as overexpression of constitutively active RAP1 increases afadin localization at the cell periphery, whereas inhibition of RAP1 signaling through overexpression of RAP1GAP redistributes afadin to the perinuclear region of the cytosol.\textsuperscript{57} Knockdown of afadin in endothelial cells results in decreased staining of junctional components at cell–cell contacts, although it is unclear if this reflects a reduction in total levels or a reorganization of junctional structures (Fig. 1, Table 1).\textsuperscript{57} Knockout of afadin is embryonic lethal but endothelial-specific knockout of afadin results in partially penetrant embryonic lethality, resulting from subcutaneous lymphedema (Table 1).\textsuperscript{58-60} Animals surviving embryogenesis exhibit aberrant retinal angiogenesis and mislocalized junctional VE-cadherin, as well as defective angiogenic sprouting in Matrigel or hindlimb ischemia assays in vivo.\textsuperscript{56} Thus, afadin appears to be critical for proper vascular function, at different stages of angiogenesis, both in blood vessels and lymphatic vessels.

KRIT1/CCM1 was identified in a yeast two-hybrid screen for Rap1 interactors, and contains a FERM domain responsible for interactions with the actin cytoskeleton and integral membrane proteins.\textsuperscript{61} Activation of Rap1 signaling stimulates release of KRIT1 from microtubules and promotes its translocation to cell–cell junctions, where it interacts with CCM2, CCM3, and other components of junctions, including afadin and β-catenin, to promote barrier function and endothelial polarity.\textsuperscript{62-64} KRIT1 is thought to act by suppressing RhoA activation that leads to phosphorylation of myosin light chain (MLC), activating myosin-based contraction and leading to the formation of stress fibers and barrier disruption (Table 1).\textsuperscript{64} KRIT1 also plays a role in activating integrin-based focal adhesions, as binding of KRIT1 to ICAP-1, a negative regulator of β1 integrin, permits talin binding to integrin and subsequent activation (Figure 1).\textsuperscript{65} Mouse Krit1 knockouts are embryonic lethal by E11.0, with dilation of both the dorsal aorta and cranial vessels.\textsuperscript{66} Inducible endothelial-specific knockout of Krit1 in neonatal mice also results in cranial vessel dilation, with accompanied leak, hemorrhage, and mislocalization of VE-cadherin (Table 1).\textsuperscript{12} The distinct roles of KRIT1 and afadin in promoting vascular barrier function through disparate mechanisms suggests that Rap1 effectors may cooperate, either additively or through coordination, of different signaling cascades.

### A Role for the RAP1 Effector RASIP1 in Junctional Actin Assembly and Stabilization

Ras-interacting protein 1 (RASIP1) was also identified through a yeast two-hybrid screen as a protein that preferentially interacts with H-RAS, K-RAS, as well as RAP1.\textsuperscript{67} It was predicted to be a RAS/RAP effector and member of the afadin protein family on the basis of an identified N-terminal RA domain, as well as forkhead-associated and dilute domains also shared with afadin.\textsuperscript{67} Subsequent work demonstrated that Rasip1 is highly expressed in embryonic and adult vasculature and is below detectable levels in non-vascular tissue in vertebrates.\textsuperscript{53,68,69} We and others have shown that knockout of Rasip1 in mice results in pericardial edema, multifocal hemorrhage, and mid-gestational embryonic lethality, resulting from malformation of vasculogenic blood vessels and disruption of circulation (Table 1).\textsuperscript{53,69} In addition, morpholino-mediated knockdown of rasip1 expression in the developing zebrafish embryo causes blood vessel defects, including irregularly shaped axial vessels and hemorrhage around cranial and intersomitic vessels.\textsuperscript{53} Taken together, the data from multiple vertebrate species support a critical role of Rasip1 in embryonic blood vessel development.\textsuperscript{53,68,69}

Biochemical and fluorescent resonance energy transfer studies demonstrated that RASIP1 preferentially interacts
with GTP-bound RAP1. Knockdown of RASIP1 in human umbilical vein endothelial cells (HUVEC) results in increased permeability of cell monolayers to FITC-dextran, indicating functional disruption of the endothelial barrier. These effects have also been observed with knockdown of RAP1, and support the hypothesis that RASIP1 is a downstream RAP1 effector that promotes endothelial barrier function. Loss of RASIP1 further increased monolayer permeability when endothelial cells were exposed to thrombin, which induces cell contraction, and counteracted the effect of Angiopoietin 1, which signals through Tie2 to strengthen barrier function. Physical evidence of disrupted cell–cell contacts was shown, as nascent junctions in RASIP1-deficient ECs displayed a greater distance between adjacent membranes when examined via electron microscopy. The compromised barrier function in response to physiological stimuli in RASIP1 knockout HUVEC suggests that RASIP1 may play a key role in controlling how vessels behave in pathological angiogenesis and in response to inflammatory stimuli, but this hypothesis awaits examination in preclinical in vivo disease models.

The reduction in barrier function in RASIP1-deficient cells is likely a consequence of a loss of linear junctions and associated circumferential actin bundles, as actin no longer efficiently assembles at endothelial junctions, but rather is found shifted away from cell-cell contacts at endothelial junctions, but rather is actin no longer efficiently assembles

preclinical in vivo disease models. This hypothesis awaits examination in preclinical in vivo disease models.

Many intriguing questions remain concerning RASIP1 function, possible interplay with other RAPI effectors, and upstream and downstream binding partners. Upstream activators remain somewhat elusive, although the involvement of cAMP suggests a role for Gαi coupled GPCRs. The RAPI effectors RASIP1, afadin, KRIT1, and RADIL promote circumferential actin bundling to some extent (Fig. 1, Table 1), and their loss disrupts function of the endothelial barrier. However, comparison of RASIP1 and KRIT1 knockdown HUVEC showed a subtle yet distinct change in actin organization, and a difference in activation of RHOA signaling. The relationship of RASIP1 to RHOA signaling is still unclear, as RASIP1 is reported to signal to ARHGAP29 and inhibit RHOA-mediated activation of myosin light chain phosphorylation (and thus actin contraction), yet there are conflicting data as to whether loss of RASIP1 results in global RHOA activation and MLC2 phosphorylation. Perhaps, RAPI effectors act at distinct stages of circumferential actin bundling, and provide specific signals to activate or suppress regulators of actin remodeling such as RHOA, RAC1, and CDC42. Recent data indicates that RAPI suppresses RHOA-dependent activation of nmMHCII, yet promotes circumferential actin bundling through activation of myotonic dystrophy kinase-related CDC42-binding kinase, but it is unknown which, if any RAPI effector functions in this pathway. Finally, the differing effects of afadin, RASIP1, and RADIL, which is a third member of the afadin protein family, on vascular development in vivo remain to be fully understood. As mentioned, loss of RASIP1 in mice and fish causes early defects in embryonic blood vessels, whereas afadin knockout only affects embryonic lymphatic vessels. Radil-knockout mice have not yet been described, but knockdown of radil in the zebrafish has no overt defect in vascular development (Table 1). We hypothesize that these three family members may share some redundant functions, and gene duplication followed by subsequent subfunctionalization and/or localization has contributed to the distinct phenotypes reported. Further in vivo examination is required, especially EC-specific combinatorial knockouts, to fully address this question.

Disclosure of Potential Conflicts of Interest

The authors are employees of Genentech, Inc., a member of the Roche Group.

Acknowledgments

The authors thank J Burton and P Vitorino for comments and suggestions regarding the manuscript.

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