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Distinct functions for Rap1 signaling in vascular morphogenesis and dysfunction

Magdalena Chrzanowska-Wodnicka
Blood Research Institute, Blood Center of Wisconsin, Milwaukee, USA

Abstract

Rap1 signaling is important for both major processes of vessel formation: vasculogenesis, or de novo vessel formation, and angiogenesis, sprouting of new vessels from pre-existing ones. We provide an overview of genetic studies in mice and zebrafish and discuss some of the proposed underlying mechanisms derived from cellular models, with particular emphasis on Rap1's role in angiogenesis, maintenance of endothelial barrier and connection with cerebral cavernous malformation (CCM), a neurological deficit that leads to seizures and lethal stroke. Lastly, we provide a brief summary of studies in cardiac and smooth muscle cells, where the Epac-Rap1 signaling axis is emerging as an important regulator of contractility.

Genetic models

Rap1 proteins are highly evolutionarily conserved, with 97% and 99% identity between zebrafish, mouse and human proteins. Genetic models of Rap1 implicate its involvement in multiple functions during development [1]. The role of two Rap1 proteins, Rap1A and Rap1B in vascular development has been studied in mouse and zebrafish models. Because signaling pathways governing vessel development are preserved across species, these models are likely to provide insight into Rap1 function in human vascular development and vascular dysfunction in disease.

Vasculogenesis

Both Rap1 isoforms are ubiquitously expressed, with Rap1B the predominant isoform in endothelial cells (ECs) [2]. Deletion of either Rap1 isoform leads to some degree of embryonic lethality, although adult homozygous animals can be obtained with low efficiency (Table) [3, 4]. While no lethality or hematopoietic or homing defects were described in Rap1A$^{-/-}$ mice on a mixed genetic background [5], deletion of Rap1A in mice in a C57Bl6 background leads to a partial embryonic lethal phenotype that includes bleeding and edema; however, specific vascular defects have not been described [6]. Rap1B$^{-/-}$ mice develop normally until embryonic day (E)12.5, following which up to 50% of embryos succumb to interspersed hemorrhage and hemorrhage on the side of the head [3] (and M.C.-W., unpublished data). Rap1A and Rap1B double knockout mice die due to major malformation between E8.5 and E10.5, consistent with Rap1's major role in promoting adhesion (M.C.-W. unpublished data). Interestingly, endothelial-lineage restricted knockouts of either Rap1B and Rap1A do not exhibit embryonic lethal phenotype observed in total knockouts of each isoform [7]. Deletion of both isoforms leads to lethality due to

hemorrhage at various times between E10.5 and E13.5, with 50% of the embryos having normal vasculature at E10.5 (M.C.-W. and Kevin Whitehead, University of Utah, unpublished data). It is not clear if death is due to a primary vascular problem or if it is secondary to a hemodynamic or physiologic abnormality. Interestingly, Tie2-Cre mediated deletion of all but one Rap1A allele phenocopies the embryonic pathology observed in total Rap1B$^{-/-}$ mice (M.C.-W., unpublished data). These observations suggest Rap1 in endothelium is not absolutely required for early vasculogenesis and that there is redundancy of functions between two Rap1 isoforms. However, at least one Rap1 allele is required for normal vascular development and function. Since Tie2 promoter is active in hematopoietic and ECs [8], the function of Rap1 in both these cellular compartments has to be considered as potentially critical for development.

In zebrafish, morpholino-mediated inhibition of Rap1B expression leads to vascular malformations [7, 9] (and M.C.-W., Sribalaji Lakshmikanthan and Ramani Ramchandran, unpublished data). We found that morpholino-mediated knockdown of Rap1B did not interfere with early development in the majority of embryos; however, it significantly impaired angiogenesis at 20-28 hours post fertilization (hpf), as described below. At 48 hpf head bleeding and heart defects were observed in Rap1B morphants [10, 11] (and M.C.-W., S.L. and R.R., unpublished data).

Several Rap1 GEFs regulate Rap1 function in vascular cells [12, 13], but only a few of them have been implicated in vasculogenesis in vivo: PDZ-GEF-1 and CalDAG-GEF1/RasGRP2 in endothelium and C3G in pericyte recruitment (Table). PDZ-GEF-1 is an adaptor protein with (PSD-95/DlgA/ZO-1) PDZ and Ras/Rap-association (RA) domains [14] and an evolutionarily conserved GEF specific for Rap1 [14, 15]. In mice, PDZ-GEF1 is transiently expressed at E8.5-E9.5 and PDZ-GEF-1$^{-/-}$ mouse embryos die shortly after that, most likely due to failure in yolk sac vasculature, as blood islands fail to form a primary vascular plexus [16]. Consistent with the yolk sac failure are extended range in the severity of vascular phenotypes and other observed defects: growth retardation, defective neural tube closure and incomplete embryonic turning. In 25% of PDZ-GEF-1$^{-/-}$ embryos allantois is not connected to the chorion, a developmental defect similar to that in mice lacking integrin α_4[17] or its receptor, VCAM-1 [18], both required for the formation of chorioallantois. Thus, Rap1, activated by PDZ-GEF-1, may be involved in integrin α4-VCAM-1-dependent chorioallantoic fusion. The in vitro allantois explant culture of PDZ-GEF1-deficiency led to reduced accumulation of VE-cadherin at cell-cell junctions and abnormal blood vessel formation, defects that were suppressed by ectopic expression of constitutively activated Rap1 [19].

In the Xenopus embryo, XRASGRP2 (an ortholog of human CalDAG-GEF1/RasGRP2), induced by VEGF, is required for endothelial differentiation of hemangioblast cells and its knockdown leads to delayed vascular development [20]. In human ECs, CalDAG-GEF1 has been implicated in both Ras and Rap1 pathways: its overexpression leads to increased expression of Ras, but increased activity only of Rap1, through which it promotes EC adhesion and migration [21].

Crk SH3-binding Guanine Nucleotide Release Protein (C3G), a ubiquitously expressed adaptor protein and a GEF for Rap1, Rap2 and R-Ras is essential for early embryonic development [22, 23]. Mice with hypomorphic C3G mutation die around E11.5 due to hemorrhage and vascular integrity defects resulting from defective pericyte development and abnormal PDGF response necessary for vascular myogenesis [24]. It remains to be determined if Rap1 is the C3G effector regulating this process.

Angiogenesis

During the second half of gestation, the vascular bed is formed through angiogenesis, a process of sprouting new capillaries from existing vasculature [25]. Prompted by the smaller size of surviving Rap1B$^{-/-}$ mice, suggestive of a cardiovascular developmental defect, we investigated the effect of Rap1B-deficiency on developmental angiogenesis and found that both retinal angiogenesis and neoangiogenesis were inhibited [26]. Similar studies in Rap1A$^{-/-}$ mice also demonstrated a defect in hindlimb ischemia and in a Matrigel plug neovascularization model, indicating that both Rap1 isoforms promote angiogenesis [6, 7, 26, 27]. Deletion of Rap1 in endothelium leads to similar angiogenesis defects, in a dose-dependent manner [7]. In zebrafish, Rap1B is essential for angiogenesis, as Rap1B loss-of-function (LOF) leads to a severe defect in intersomitic vessel (ISV) sprouting [7], a process dependent on Vascular Endothelial Growth Factor (VEGF) signaling [28]. Furthermore, a combination of genetic and pharmacological studies indicate that Rap1 and VEGF Receptor 2 (VEGFR2) act in the same pathway in ISV formation [7]. This result, combined with the observation that all responses to VEGF are inhibited in Rap1-deficient ECs [29], prompted us to hypothesize that Rap1 regulation of VEGF responses occurs at the level of its receptor, VEGFR2 (discussed in the next chapter).

Lymphatics

Deficiency of either Rap1 isoform leads to edema [3, 6] (and M.C.-W., unpublished data), however Rap1 function in the development of lymphatic vessels has not been studied. Although the function of Rap1A$^{-/-}$ myeloid cells is altered, with increased haptotaxis and decreased neutrophil adhesion and decreased superoxide production, the development of Rap1A$^{-/-}$ T-cells and B-cells is normal [4]. Nonetheless, Rap1B, which is the predominant isoform in B-cells, is critically required for the formation of lymphatic organs. Rap1B deficiency leads to markedly reduced marginal zone B-cells in the spleen and decreased numbers of mature B-cells in peripheral and mucosal lymph nodes, without affecting early B-cell development. Underlying these defects are decreased adhesion and chemotaxis and lessened homing to lymph nodes [30, 31].

Hemostasis

Platelet function—Rap1B promotes integrin $\alpha_{IIb}\beta_3$ activation and platelet function and its deficiency leads to a mild hemostatic defect in adult mice without spontaneous bleeding [3]. As discussed previously, hemorrhage has been observed in up to 50% of Rap1B$^{-/-}$ embryos and therefore Rap1B-deficiency in platelets might be considered as a cause of this phenotype. However, conditional deletion of Rap1B using a Tie2-driven Cre/flox system, which in addition to deleting Rap1B in endothelium also deletes Rap1B in hematopoietic cells and, specifically, in platelets [7], does not lead to embryonic bleeding. Therefore, defective hemostasis in Rap1B$^{-/-}$ embryos is likely caused by loss of Rap1B in additional tissues.

Vascular integrity & connection with cerebral cavernous malformations (CCM)—Data from Rap1-knockout mice suggest that Rap1 function in both endothelial and nonendothelial compartments is required for normal vasculogenesis. Of the two, the role of Rap1 in endothelium is better understood, and, as suggested by cellular models, involves stabilization of cell-cell junctions. Genetic models of two of the Rap1 effectors regulating this aspect of vascular function have been reported: Afadin and CCM1/KRIT1. Furthermore, connection with CCM implicates defective Rap1 signaling in vascular malformations.

Afadin (similar to AF6 gene product) is a scaffolding protein that links Ig-like adhesion molecules, nectins, with actin cytoskeleton, other adapter molecules, and, via its RA domain binds Rap1 and other small GTPases [32]. Afadin/AF6-Rap1 interactions play a well

documented role in facilitating adherens junction (AJ) formation, particularly in epithelial cells [13, 33]. Endothelial knockout of Afadin leads to decreased Matrigel plug neovascularization and decreased capillary density in a hindlimb ischemia model [34]. In the retina, retarded retinal angiogenesis at postnatal day (P)4 is accompanied by a decreased number of fine vascular meshes, and the discontinuous VE-cadherin localization in AJs, consistent with Afadin's role in promoting their formation. Postnatal viability of Afadin homozygous knockout mice is dramatically reduced, suggesting that Afadin is involved also in other aspects of vasculogenesis [34] (Table).

CCMs are vascular defects of the central nervous system consisting of abnormally enlarged capillary cavities without intervening brain parenchyma [35]. Susceptible to hemorrhaging, these brain angiomas result in focal neurological deficits that cause seizures and stroke. Three genetic loci have been implicated in autosomal forms of CCM: CCM1/KRIT1 [36, 37], CCM2/OSM/malcavernin [38] and CCM3/PDCD10 [39], and proteins encoding them form a functional complex. While other loci are likely to be involved, autosomal dominant LOF mutations in any of the three CCM proteins have a causative role in CCM.

KRIT1 (Krev/Rap1 Interaction Trapped 1) was originally identified as a Rap1-binding partner in a yeast two-hybrid screen [40]. In humans, KRIT is expressed in neuronal and vascular tissues [35]. In the endothelial compartment, KRIT1 is predominantly expressed in capillaries and arterioles, with higher expression levels in organs with specialized blood-organ barriers and lower expression in fenestrated capillaries in organs where ECs do not form cell-cell junctions [41]. CCM2 is a scaffolding protein whose binding partners include actin, Rac1 and p38 kinase activating MEKK3 and MKK3, with a function in osmoregulation [42].

CCM genes are evolutionarily conserved and several genetic models of CCM have been generated, some of which, particularly the zebrafish models, share similarity with Rap1-deficient phenotypes, suggesting functional interaction. Cardiac development in zebrafish embryos with the recessive lethal mutations of ccm1 (santa) and ccm2 (valentine) orthologs is abnormal, leading to enlarged, thin-wall hearts [43]. A similar phenotype was observed in the Rap1B morpholino LOF model [9]. Interestingly, the heart defect in CCM morphants could be rescued with exogenous expression of WT KRIT, but not a (R452E) KRIT1 mutant with decreased affinity to Rap1 and altered cellular localization [10].

In addition to its role in cardiac development, Rap1-KRIT1 interaction has been implicated in zebrafish vessel integrity. CCM1 and CCM2 LOF lead to severe and progressive dilation of major vessels in zebrafish embryos, with progressive vessel wall thinning, but structurally normal cell-cell junctions. This defect in endothelial morphogenesis is cell-autonomous, as demonstrated by transplantation studies [44]. Interestingly, at sub-effective doses, combined knockdown of CCM1 and Rap1B increases the occurrence of intracranial hemorrhage observed in Rap1B LOF morphants, suggesting Rap1 and CCM1 act in the same pathway promoting vascular integrity [9] (and M.C.-W., SL, RR, unpublished observations).

Mouse models of CCM—During early mouse development KRIT1 is ubiquitously expressed, including in the central nervous system, epithelia and endothelium [45-47]. With progressing development, endothelial expression is particularly pronounced in large vessels, a pattern different from that in human tissues [41]. Targeted mutation of CCM1 in mice failed to induce CCM pathology, however additional loss of the tumor suppressor p53, shown to increase the rate of somatic mutation, led to vascular lesions in the brains of 55% of the double-mutant animals [48] (Table). Consistent with the human phenotype, mice heterozygous for CCM2, predominantly expressed in neural parenchyma in normal brains, also develop brain lesions [47].

Complete ablation of CCM1 and CCM2 leads to early and severe vascular pathologies that result in embryo lethality. KRIT1$^{-/-}$ embryos develop dilatation of brain vessels and a defect in branchial arch artery formation [45]. In CCM2 total and endothelial-restricted knockout mice, vascular lumen formation and arteriogenesis are defective and the heart is malformed [49]. In comparison, mice deficient in both Rap1 isoforms in endothelium develop relatively normal vasculature, without visible defects in branchial arches or lumen but at E13.5 about 50% of them develop hemorrhage in the head, that is distinct from CCM lesions, and all embryos die before E15.5 (M.C.-W. and K.W., unpublished data). As mentioned before, most total double knockouts of Rap1A and Rap1B die before midgestation (M.C.-W., unpublished data), precluding the analysis of the vasculature.

In conclusion, CCM genes are critical for several aspects of cardio- and vasculogenesis, and functional interaction with Rap1 appears to be important for the first of these functions. Endothelial deletion of Rap1 leads to a phenotype that is distinct from CCM. However, additional cell types, such as astrocytic foot processes and pyramidal neurons in the cerebral cortex – structures integral to cerebral angiogenesis and formation of the blood-brain barrier - may contribute to the pathophysiology of CCM [41], and the role of the Rap1-KRIT1 signaling axis in these cells needs to be examined.

Molecular basis of Rap1 vascular phenotypes

Angiogenesis

Rap1 promotes angiogenic signaling by several factors, including Fibroblast Growth Factor 2 (FGF2), VEGF and a sphingolipid, Sphingosine-1-phosphate (S1P) (Figure 1). Activated downstream from their receptors, Rap1 promotes signaling pathways leading to activation of Erk, Akt, Rac1 and Afadin, thereby regulating endothelial cell proliferation, migration, and tubule formation [29, 34]. We have recently shown both Rap1 isoforms promote responses to VEGF by an additional mechanism that implies that Rap1 impact on VEGF signaling may not be limited to selective pathways downstream from VEGFR2 [7]. Because Rap1-deficiency in ECs attenuates all VEGF-induced responses [29], we examined the ability of VEGF to activate VEGFR2 in Rap1-deficient cells. Genetic knockout of Rap1B or siRNA knockdown of either Rap1A or Rap1B leads to decreased VEGFR2 activation, indicating that Rap1 is required for full activation of that receptor. Rap1 modulation of VEGFR2 activity is mediated in part by integrin $\alpha_v\beta_3$[7]. This integrin, together with integrin $\alpha_5\beta_1$, plays an important role in angiogenesis [50, 51]. Upregulated in angiogenic endothelium through function-blocking studies, integrin $\alpha_v\beta_3$ has been implicated in promoting angiogenesis and, specifically in VEGF-dependent angiogenesis through cooperation with VEGFR2 [52]. The structural basis of that cooperation has recently been revealed. Integrin $\alpha_v\beta_3$ and VEGFR2 form a physical complex via their cytoplasmic domains, an interaction that is promoted by phosphorylation of the Tyr747 of the integrin β_3 chain, which maintains the integrin in the activated state [53, 54]. However, under conditions when integrin $\alpha_v\beta_3$ is engaged and activated, we found that formation of the complex between VEGFR2 and the integrin is Rap1- and RIAM-independent, suggesting that Rap1 promotes integrin $\alpha_v\beta_3$-VEGFR2 cooperation via an additional signaling mechanism [7]. While both Rap1 isoforms promote integrin β1 activation and EC adhesion and migration [7, 27], Rap1 signaling to VEGFR2 is integrin β_1–independent [7]. The finding that Rap1 is a positive, upstream regulator of VEGFR2 signaling implicates a broader role for Rap1 in maintaining blood vessel homeostasis. VEGF-induced EC hyperpermeability is a critical aspect of the EC response to VEGF stimulation, yet the underlying cellular mechanisms are poorly understood [55]. Tissue-specific Rap1 knockout mice may provide a valuable tool to examine the role of Rap1 in that process.

Vascular integrity

Rap1 GEFs—A number of Rap1 GEFs and several Rap1 effectors regulate cell-cell junction formation in epithelial and ECs, and the mechanisms involved have been discussed in several comprehensive reviews [12, 13]. Of the Rap1 GEFs involved in this process, the function of Epac is probably best known. Activation of Rap1 in endothelium by cAMP-activated Epac promotes cell-cell junction formation and enhances endothelial barrier. First described in cultured cells [56-58], significance of Epac-Rap1 signaling pathway in barrier formation in vivo has been demonstrated in PAF-induced microvascular hyperpermeability of rat mesentery [59] and in protection of lung endothelium in a ventilator-induced lung injury [60]. Recently, targeting the Epac-Rap1 pathway has been validated as a potential therapeutic for endothelial hyperpermeability in vivo [61].

Rap1 effectors

Afadin: Afadin is known for regulating epithelial cell-cell junctions [32], but also acts as a Rap1 effector in promoting endothelial cell-cell junction formation and facilitates angiogenesis in vivo. Studies in model cell systems suggest that Afadin is required for the tubulogenesis step of angiogenesis. In HUVEC and HEK293 cells, Rap1 activated downstream from VEGFR2 and S-1-P receptors, promotes association of Afadin with PI3K and their translocation to AJs. Knockdown of Afadin blocks accumulation of AJ and tight junction (TJ) proteins in cell-cell contact sites and tubule formation. Once localized to junctions, Afadin further promotes Rap1 activation, providing a positive feedback [34].

In addition to its function during vasculogenesis, Afadin mediates barrier-protective effects in the murine model of ventilator induced lung injury. Cyclopenthenone-containing products resulting from oxidation of a natural phospholipid, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (OxPAPC), have protective effects in lung EC barrier dysfunction models and act by promoting Rac1-and cdc42-mediated peripheral actin assembly and accumulation of AJ and TJ proteins, a process dependent on Rap1 activity [62]. Recent in vivo studies demonstrate that Afadin mediates that effect as its depletion in vivo with Afadin-specific siRNA abolishes protective effects by OxPAPC [63].

KRIT1: Studies in animal models demonstrate a critical role for CCM proteins in endothelium in the regulation of blood vessel development. While it is likely that CCM proteins in other tissue compartments also contribute to the pathophysiology of CCM, studies in cultured ECs and analysis of human samples implicate Rap-KRIT1 functional interactions in the regulation of endothelial cell barrier.

Molecular interactions: CCM1/KRIT1 is a multidomain protein containing C-terminal band 4.1/ezrin/radixin/moesin (FERM) which mediates the linkage between cortical actin and integral membrane proteins, four ankyrin repeats involved in protein-protein interactions, and three NPXY motifs, the first two involved in binding CCM2 [64] and the most N-terminus proximal in binding integrin β_1-binding protein ICAP-1 [65]. The ICAP-1 binding site on KRIT1 is similar to the I-CAP1 binding site on integrin β_1 and therefore it was proposed that KRIT1 and integrin compete for binding to ICAP-1 [66]. Recent studies defined the structural basis of this interaction and revealed the role of ICAP-1 in modulation of integrin activation [67]. ICAP-1 bound to integrin β_1 acts as an antagonist of talin-mediated integrin β_1 activation; KRIT1 binding of ICAP-1 releases integrin β_1 from this inhibitory association, allowing binding of talin and subsequent steps of integrin activation.

First shown in a yeast two-hybrid system [40], direct interaction between KRIT1 and Rap1 was controversial for a time [66], likely because binding of KRIT1 RA domain to purified Rap1B is rather weak with a micromolar affinity, and requires neighboring ankyrin repeats

and FERM domain [68].Studies from Faurobert and Ginsberg labs shed more light on this interaction. [69] In BHK cells, exogenously expressed WT and, in particular, DA Rap1A interacts with KRIT1 and promotes KRIT1 interaction with PIP2 in liposomes. Rap1 interacts with the FERM domain of KRIT1 in vitro [70] and a point mutation in KRIT1 that significantly decreases KRIT1's affinity for Rap1 (R452E) alters KRIT1's cellular localization [10] (as described below). Recently, a co-crystal structure of KRIT1 in complex with Rap1 has been solved [71]. Rap1-KRIT1 interaction encompasses an extended surface, including Rap1 Switch I and II and KRIT1 FERM F1 and F2 subdomains.

Cellular localization and function: The FERM domain of KRIT1, like that of other FERM-containing proteins, exists in two conformations: open and closed, mediated by intramolecular interactions between the N- and C-terminus [69, 72]. Exogenously expressed KRIT1 localizes to the nucleus or the cytoplasm and the shuttling between the two compartments was proposed to depend on KRIT1's FERM domain open or closed conformation and interaction with CCM2 and ICAP-1, respectively [72]. In ECs [70, 73], KRIT1 localizes to microtubules [69, 73], with increased localization to microtubule plus ends during mitosis [73]. Overexpression of DA mutant of Rap1 induces release of KRIT1 from microtubules and its translocation to the membrane and, specifically, to cell-cell junctions [69]. In cell-cell junctions, KRIT1 binds Heart-of-glass (HEG1) receptor [74] and associates with junctional proteins, including β-catenin and Afadin [70]. Rap1 activity is required for KRIT1's interaction with junctional molecules, but not localization to junctions [71]. Interestingly, (R452E) KRIT1 mutant with decreased affinity for Rap1 does not localize to junctions (as it is sequestered by microtubules) and fails to support endothelial cell-cell junctions [71].

Knockdown of endogenous KRIT1 decreases β-catenin localization to junctions, increasing vascular permeability to thrombin in BAEC monolayers, and prevents Epac1- and Rap1-mediated stabilization of junctions [70]. Further, physical interaction of KRIT1 and CCM2 is required for endothelial cell-cell junctional localization, and lack of either protein destabilizes endothelial barrier by promoting RhoA-mediated contractility. Therefore, the KRIT1-CCM2 interaction promotes endothelial barrier by suppressing Rho/ROCK signaling. Inhibition of ROCK corrects the vascular leak in $KRIT1^{+/-}$ and $CCM2^{+/-}$ mice [75].

A model for regulation of endothelial barrier: Based on the above-described cellular studies, a couple of mechanisms have been proposed to explain the role of Rap1 in regulating KRIT1 function in cell-cell junctions (Figure 1). One proposes that KRIT1 is transported along microtubules by an unknown motor protein to the plasma membrane, where it encounters active Rap1, which releases it from microtubules. Next, KRIT1 sequesters ICAP-1, releasing it from integrin β_1 and thus facilitating integrin activation. It has been proposed that loss of KRIT1 may reduce integrin β_1 activation, contributing to increased vascular permeability. However, integrin expression pattern indicates that integrins $\alpha_v\beta$ and $\alpha_v\beta_5$ rather than $\alpha_v\beta_1$ are expressed more strongly in CCM endothelium, and therefore regulation of these integrins may be more significant in CCM pathology [76]. In an alternative model Rap1 plays an active role in releasing KRIT1 from microtubules allowing KRIT1 binding to HEG1 [74] and localization to cell-cell junctions, where it associates with junctional proteins, including β-catenin and VE-cadherin [10].

Additional function: Most KRIT1 LOF mutations described in patients occur within the FERM domain, leading to a truncated KRIT1 protein [77]. It has been suggested that the open conformation assumed by such mutants, blocks their ability to shuttle to the nucleus and that the loss of that function, mediated by interaction with ICAP-1, may contribute to CMM pathogenesis [72]. In the nucleus KRIT1 may modulate the expression levels of the

antioxidant protein SOD2, contributing to the maintenance of intracellular Reactive Oxygen Species homeostasis [78].

A study in HUVECs overexpressing KRIT1 demonstrated its anti-angiogenic functions, mediated by DLL4/Notch signaling, and suggested that during early stages of CCM development, loss of functional KRIT1 promotes endothelial proliferation and capillary sprouting by dysregulated Notch signaling [79]. Consistent with this, increased endothelial proliferation has been observed in KRIT1$^{-/-}$ embryos [45], a phenotype distinct from Rap1B$^{-/-}$ mice [26].

Lastly, studies using human and mouse ECs cultured in 3D implicate Rap1 as a downstream target of VE-cadherin and CCM1, as a component of a signaling complex in AJs that is required for establishment of endothelial polarity and formation of vascular lumen [80]. The role of CCM proteins in lumen formation has been demonstrated in a mouse knockout model [49].

Rap1 functions in other cells

Outside of endothelium, there is little information about Rap1 functions in pericytes and other cellular components contributing to vasculogenesis. Emerging reports, however, demonstrate that in cardiomyocytes and smooth muscle cells the Epac-Rap1 signaling axis, often in cooperation with PKA signaling, regulates several basic functions, including permeability and cellular contractility, disruption of which would be expected to lead to vascular dysfunction.

In cardiomyocytes, Epac regulates the activity of various cellular compartments and influences calcium handling and excitation-contraction coupling [81]. In cultured rat neonatal cardiomyocytes, PKA and Epac/Rap1 cooperate in cAMP-induced gap junction formation, with PKA signaling promoting gating and Epac/Rap1 increasing recruitment of connexin43 at cell–cell contacts and promoting maturation of AJs [82] (Figure 2). Epac is activated by β-adrenergic receptor (β-AR), a G-protein-coupled receptor that enhances cardiomyocyte contractility in response to acute stress and leads to cardiac hypertrophy and ventricular dysfunction in chronic stress. Following acute β-AR stimulation, Epac-mediated and Rap1-dependent activation of PLC epsilon is critical for Ca^{2+} -induced Ca^{2+} release (CICR) [83]. Epac expression correlates with pathological cardiac hypertrophy [84] and silencing Epac expression blocks the hypertrophic response [85]; however, Epac targets in this case are other small GTPases Rap2B, Rac1 and H-Ras but not Rap1 [86]. Therefore, Rap1 mediates a subset of Epac functions in the heart: regulation of cell-cell junctions and contractility, but there is no evidence for Rap1's direct involvement in promoting hypertrophy.

Rap1 also regulates smooth muscle contractility, albeit by a different mechanism. At a given intracellular concentration of Ca^{2+}, contractility of smooth muscle and fibroblasts is regulated by myosin regulatory light chain (RLC_{20}) phosphorylation, which promotes actomyosin contractility. Agonist-induced activation of small GTPase Rho and its effector Rho kinase (ROCK) leads to increased inhibitory phosphorylation of myosin phosphatase (MLCP) increasing RLC_{20} phosphorylation ("Ca^{2+}-sensitization") [87-89]. In contrast, elevation of intracellular second messenger cAMP or cGMP reduces Ca^{2+}-sensitivity and induces SM relaxation ("Ca^{2+} -desensitization") [90, 91]. We demonstrated that Epac-Rap1 signaling axis contributes to Ca -desensitization of SM and fibroblast contractility [92]. We found that Epac-specific analog reduces agonist-induced contractility, RLC_{20}, and myosin light chain phosphatase phosphorylation in isolated vessels independently of PKA. Further, agonist-induced elevation of cAMP led to activation of Rap1, which was independent of PKA, but inhibited by silencing Epac. Lastly, RhoA activity was reduced by activating Epac

in WT but not Rap1B null fibroblasts, consistent with Epac signaling through Rap1B inhibition of RhoA [92] (Figure 3). These findings demonstrated a novel cAMP-mediated signaling mechanism in which Epac-activated Rap1 leads to desensitization of SM contractility by inhibiting activation of Rho. Additional in vivo studies are needed to address the relevance of this mechanism in regulation of vascular tone.

Genetic models have revealed important roles of Rap1 signaling in promoting angiogenesis and maintenance of vascular stability. Studies in cells have provided some understanding of the underlying mechanisms, in particular, with new insights into regulation of angiogenic responses, where Rap1 acts upstream from VEGFR2; and into the role of Rap1-KRIT1 interactions in meditating endothelial barrier. However, these studies do not fully explain vascular defects observed in the in vivo models of Rap1 deficiency or Rap1's role in the pathophysiology of CCM. Further, emerging studies in cardiac and smooth muscle cells demonstrate that Rap1 is an important regulator of several basic functions, including contractility. The role of Rap1 in smooth muscle cells in regulation of vasculogenesis and vascular tone needs to be addressed in vivo. Additional models that allow temporal and tissue-specific modulation of Rap1 signaling, in particular in pericytes, should help address these outstanding questions.

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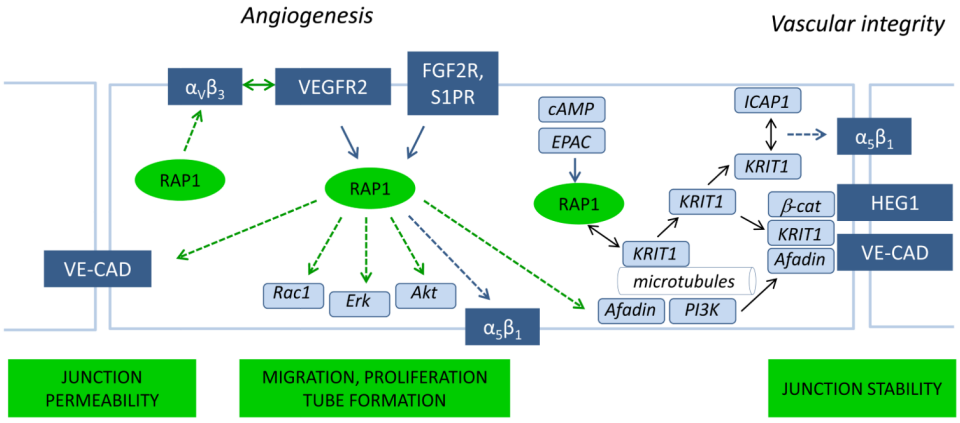

Figure 1.

In endothelium, Rap1 regulates angiogenesis and vascular integrity. Rap1 promotes angiogenic responses to VEGF by promoting VEGF Receptor 2 (VEGFR2) activation, in part via integrin $\alpha_v\beta_3$. Rap1 also acts downstream from VEGF, FGF2 and S1P receptors in signaling pathways leading to EC migration, proliferation, tube formation, and regulation of vascular permeability. Activated by cAMP-dependent GEF, Epac, Rap1 promotes junctional stability by translocating KRIT1, a molecule implicated in cavernous cerebral malformations, from the microtubules to the plasma membrane and cell-cell junctions, where KRIT1 binds Heart-of-Glass (HEG1) receptor and other junctional proteins, including b-catenin (β-cat) and a Rap1GEF, Afadin, promoting junctional stability. KRIT1 at the plasma membrane may also interact with ICAP-1, promoting integrin $\alpha_5\beta_1$ activation. VE-CAD: VE-cadherin.

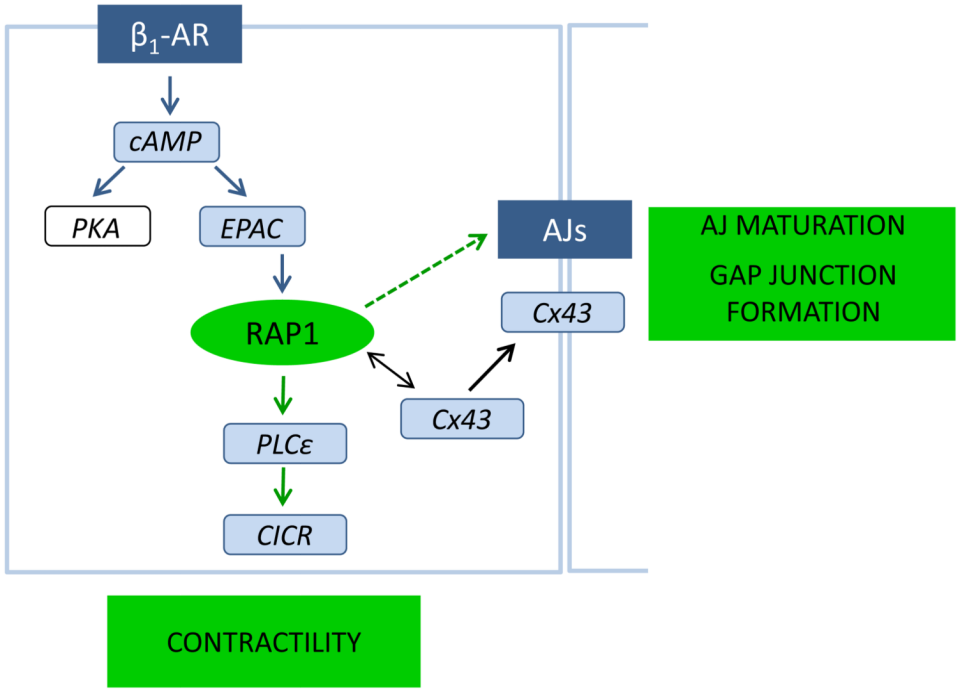

Figure 2.

In cardiomyocytes, Epac-Rap1 axis transmits signals from β_1-adrenergic receptor (β_1-AR) promoting adherens junction (AJs) maturation and gap junction formation by inducing recruitment of connexin 43 (Cx43). Acute stimulation of β_1-AR leads to activation of PLCs, critical for Ca^{2+} -induced Ca release (CICR) and cardiomyocyte contractility, in a Rap1-dependent manner.

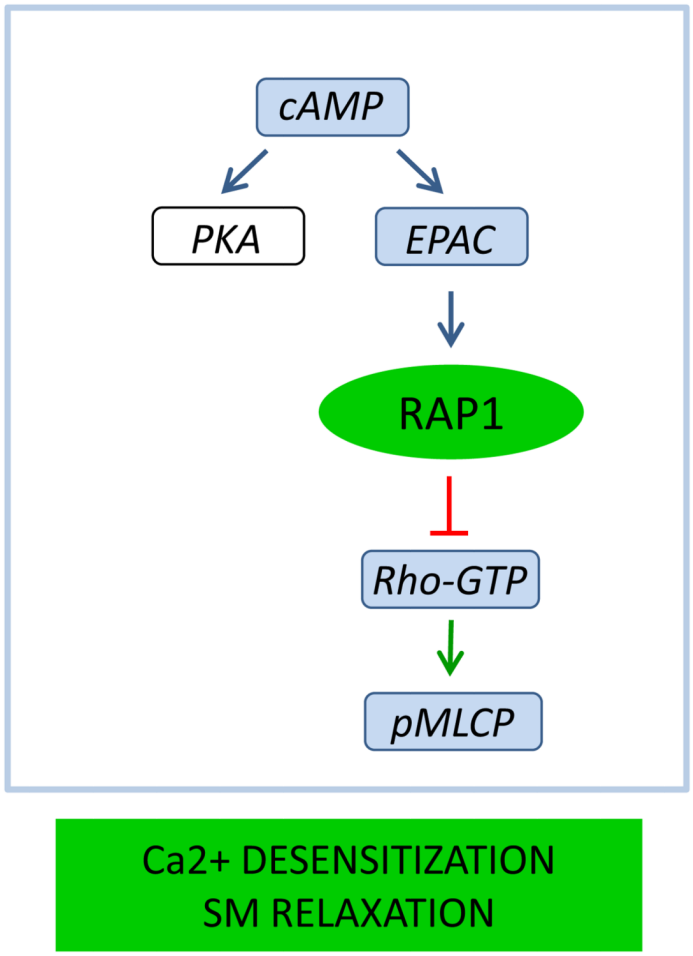

Figure 3.
Rap1 promotes smooth muscle relaxation by mediating Ca^{2+} -desensitization of contraction. Following elevation of cAMP, activated Rap1 inhibits activation of RhoA and downstream signaling leading to myosin light chain phosphatase (pMLCP), resulting in decreased phosphorylation of myosin regulatory light chains (RLC_{20}) and decreased contractility.

Table

Phenotypes of total and tissue-specific murine knockouts of Rap1 isoforms, Rap1-GEFs and Rap1 effector proteins in the cardiovascular system.

Mouse genotype	Mouse phenotype
Rap1b$^{-/-}$	Partial embryonic lethality with bleeding and edema after E12.5 [3]; impaired: angiogenesis [7, 26], platelet function [3], B-cell development and homing to lymph nodes [30, 31] in surviving adults; otherwise normal life span.
Rap1a$^{-/-}$;(on C57Bl6 background)	Partial embryonic lethality with bleeding and edema [4], defect in FGF-mediated angiogenesis [6], normal T-cell and B-cell development but altered myeloid function [4].
Rap1a$^{-/-}$ (on mixed genetic background)	Normal life span, impaired T-cell and B-cell adhesion [5].
Rap1a$^{-/-}$*Rap1b*$^{-/-}$;	Embryonic lethality due to major malformation between E8.5-E10.5 (*M.C.-W. unpublished data*)
Tie2-Cre$^{+/0}$*;Rap1b*$^{f/f}$	Impaired angiogenesis, otherwise normal lifespan [7].
Tie2-Cre$^{+/0}$*; Rap1a*$^{f/+}$*Rap1b*$^{f/f}$	Partial embryonic lethality with bleeding after E12.5; defect in angiogenesis [7].
Tie2-Cre$^{+/0}$*; Rap1a*$^{f/f}$*Rap1b*$^{f/f}$	Embryonic lethality due to hemorrhage between E10.5-E13.5 (*M.C.-W. and Kevin Whitehead, unpublished data*).
PDZ-GEF-1$^{-/-}$	Embryonic lethality after E8.5-E9.5, likely due to failure in yolk sac primary vascular plexus formation [16]
C3G$^{-/-}$	Embryonic lethality before E7.5; defective adhesion and spreading of embryonic fibroblasts [23].
C3G$^{gt/gt}$	Embryonic lethality around E11.5 due to hemorrhage and vascular integrity defects; defective pericyte development [24].
Afadin$^{-/-}$	Embryonic lethality after E9.5 due to developmental defects and loss of structures derived from ectoderm and mesoderm [93].
Tie2-Cre$^{+/0}$*; Afadin*$^{f/f}$	Impaired postnatal angiogenesis in response to VEGF and S-1-P; reduced postnatal viability suggestive of other vascular defects [34]
Ccm1$^{tm1Dmar/tm1Dmar}$(*Ccm1*$^{-/-}$)	Embryonic lethality at mid-gestation; dilatation of brain vessels and vascular defects in branchial arches [48]
Ccm1$^{tm1Dmar/+}$(*Ccm1*$^{+/-}$)	Normal cardiovascular phenotype [48].
Ccm1$^{tm1Dmar/+}$(*Ccm1*$^{+/-}$)*;Trp53*$^{-/-}$	Vascular lesions in the brains similar to CCM lesions [45].
Ccm2$^{-/-}$	Embryonic lethality prior to E10.5, cardiovascular defects [49, 94].
Tie2-Cre$^{+/0}$*; Ccm2*$^{f/f}$	Defective vascular lumen formation and arteriogenesis; pericardial edema and arrested heart development [49, 74, 94, 95].
Nestin-Cre$^{+/0}$*; Ccm2*$^{f/f}$	Normal cerebrovascular phenotype [95].
MX1-Cre$^{+/0}$*; Ccm2*$^{f/f}$	Brain hemorrhages in 7-8 month old adults, following induction of Cre expression at 6-8 weeks of age [49].
Cdh5(*PAC*)*-CreERT2; Ccm2*$^{f/Del}$*; Rosa26-St -Stop*^{f}*-LacZ*	Vascular lesions mimicking human CCM lesions, following induction of Cre expression at P1 [96].