Biochimica et Biophysica Acta 1788 (2009) 790–796

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Review

Cell–cell junction formation: The role of Rap1 and Rap1 guanine nucleotide exchange factors

Willem-Jan Pannekoek, Matthijs R.H. Kooistra, Fried J.T. Zwartkruis, Johannes L. Bos *

Department of Physiological Chemistry, Centre for Biomedical Genetics and Cancer Genomics Centre, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands

ARTICLE INFO

Article history:
Received 18 July 2008
Received in revised form 15 December 2008
Accepted 16 December 2008
Available online 29 December 2008

Keywords:
Rap1
Junction
Cell–cell contact
Barrier function
E-cadherin
Actin
C3G
PDZ-GEF
Epac1

ABSTRACT

Rap proteins are Ras-like small GTP-binding proteins that amongst others are involved in the control of cell–cell and cell–matrix adhesion. Several Rap guanine nucleotide exchange factors (RapGEFs) function to activate Rap. These multi-domain proteins, which include C3G, Epacs, PDZ-GEFs, RapGRPs and DOCK4, are regulated by various different stimuli and may function at different levels in junction formation. Downstream of Rap, a number of effector proteins have been implicated in junctional control, most notably the adaptor proteins AF6 and KRIT/CCM1. In this review, we will highlight the latest findings on the Rap signaling network in the control of epithelial and endothelial cell–cell junctions.

Contents

1. Introduction

Rap proteins, which include Rap1 (A and B) and Rap2 (A, B and C), are members of the Ras-like small G-proteins. These monomeric proteins cycle between a GTP-bound (active) conformation and a GDP-bound (inactive) one, thereby allowing signaling pathways to be quickly switched on or off. GTP-binding is facilitated by guanine nucleotide exchange factors (GEFs) that release GDP and allow the more abundant GTP to bind. Inactivation is catalyzed by GTPase activating proteins (GAPs) that enhance the hydrolysis of GTP [1]. The prototypic Ras family members (H-, K- and N-Ras) function in signaling cascades that impinge on proliferation, differentiation and apoptosis. As such, these proteins have great oncogenic potential and are indeed found mutated in 15% of all human cancers [2]. In a screen for suppressors of this transforming activity, Noda and colleagues identified Rap1A as a protein with Ras reverting potential [3]. Rap1A carries an effector domain that is very homologous to that of Ras, suggesting that Rap1A might directly inhibit Ras signaling by competition for Ras effectors. Nowadays, it is commonly accepted that Rap1A as well as the very homologous Rap1B signals largely independently of Ras. Rap1-induced inhibition of transformation by Ras is due to its central role in a signaling network that controls cell polarity [4] and strengthening of cell attachment to both extracellular matrix and neighbouring cells [5]. We have recently reviewed the central role of Rap1 in cell–cell adhesion [6], but much progress has

* Corresponding author.
E-mail address: j.l.bos@umcutrecht.nl (J.L. Bos).

0005-2736/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2008.12.010

been made over the past period. Here we will highlight recent findings with a focus on the role of the various RapGEFs in junction formation.

Epithelia function as physical barriers that regulate transport of substances from and towards underlying tissues. As such, it is essential that the epithelial layer forms an intact sheet, the individual cells of which are tightly attached to one another. Two main cell–cell anchorage systems function to maintain epithelial barrier; the apical tight junction (TJ) and the closely associated adherens junction (AJ) [7]. TJs link neighbouring cells closely together, thereby creating a physical barrier for ions and solutes. Also, TJs limit diffusion of membrane proteins, thereby contributing to apical–basolateral polarity [8]. AJs increase monolayer rigidity by linking the actin networks of neighbouring cells. The central core of AJs is the homophilic interaction of cadherin proteins. Cadherins are single-pass transmembrane glycoproteins of which the extracellular domains interact with opposing cadherins in a calcium-dependent manner. E-cadherin constitutes the AJs of epithelial cells, whereas endothelium expresses the closely related VE-cadherin. Intracellularly, (V)E-cadherin interacts with catenin family members. p120-catenin binds to the juxtamembrane region of (V)E-cadherin to stabilize its plasma membrane localization, whereas α- and β-catenin serve a dynamic role in linking (V)E-cadherin to the actin cytoskeleton [9].

A landmark paper by Knox and Brown for the first time linked Rap1 to AJ formation. In *Drosophila melanogaster*, *rap1* localizes to the AJ. *Rap1* mutant cell clones have aberrant shapes and intermingle with surrounding wild-type cells, indicating a defect in mutual cell–cell adhesion, whereas adhesion to wild-type cells occurs properly. Apical–basolateral polarity is maintained and the formation of septate junctions, which are considered to be analogous to TJs, seems unaffected. However, the junctional proteins DE-cadherin, α-catenin and β-catenin lose their even, circumferential distribution and localize in clusters at one side of the cell [10]. In MDCK cells, dominant negative Rap1 prevents cells from attaching to Fc-E-cadherin. Conversely, disruption of cell–cell contacts by scatter factor (HGF) can be completely inhibited by activation of endogenous Rap1 [11]. Forcing Rap1 into its inactive conformation by ectopic expression of RapGAP does not disrupt mature cell–cell contacts, but dramatically affects junction reformation in a calcium-switch model [12]. Similarly, cells depleted of Rap1A by siRNA only show defective junction formation after replating [13]. Together, these data clearly establish Rap1 as a key regulator of adherens junctions and suggest it functions in the formation process instead of junction maintenance.

2. RapGEFs in junction formation

The notion that Rap1 is involved in the establishment of cell–cell contacts implies that Rap1 is being activated before or during contact formation. Using a FRET-based Rap1 activation construct, activation of Rap1 was found at newly formed cell–cell contacts [14]. Others have used a calcium switch model, in which chelation of extracellular calcium is used to break up the calcium-dependent transinteraction of E-cadherin proteins. Following add-back of calcium, AJs reform synchronously, allowing for biochemical assays. Indeed, Rap1 is activated during early junction reformation and this activation decreases when junctions are restored [15]. These data are in line with the observations that Rap1 functions in junction formation. Intriguingly, activation of Rap1 has also been reported upon disruption of adherens junctions [16]. It is well possible that disruption of AJs serves as a trigger to activate Rap1, thereby inducing junction reformation to maintain epithelial integrity. A first clue to understand this pattern of Rap1 activation may come from the characterization of the Rap1-specific GEFs. To date, six major classes of highly conserved GEFs are discriminated (Fig. 1): C3G (RapGEF1), PDZ-GEFs (RapGEF2 and -6), Epac proteins (RapGEF3, -4 and -5), members of the RasGRP family (RasGRP2 and 3), Phospholipase C epsilon and the atypical RapGEF DOCK4. Four of these GEF families have been implicated in junction regulation.

C3G was the first RapGEF identified with a characteristic catalytic region consisting of a CDC25 homology domain and a Ras exchange motif (REM). Furthermore, it contains proline-rich sequences which bind to the SH3 domain of the adaptor proteins Crk and Grb2. C3G directly interacts with E-cadherin during the initial steps of junction formation [12]. This binding is mutually exclusive with binding of β-catenin to E-cadherin, suggesting that C3G does not bind E-cadherin in mature AJs. Importantly, inhibition of C3G inhibits translocation of E-cadherin to the junctions, indicating a key role of this GEF in the recruitment of E-cadherin [12]. C3G localizes to endosomes [17] and has been proposed to activate Rap1 on intracellular membrane compartments upon internalization of the EGF receptor [18]. Similarly, Rap1 activation can be triggered by E-cadherin internalization [16],

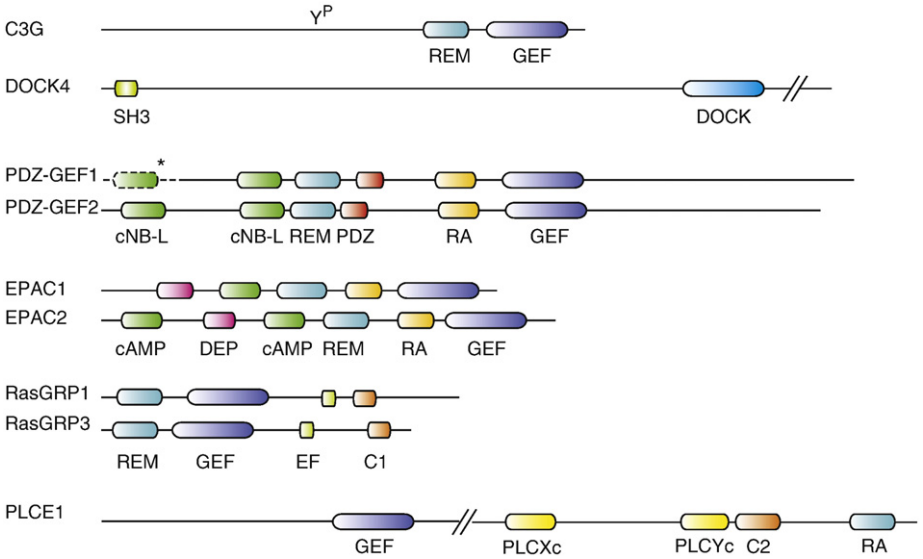

Fig. 1. Schematic representation of the domain structure of RapGEFs. cAMP: cyclic AMP-binding domain; cNB-L: cyclic nucleotide binding domain like; RA: Ras-association domain; REM: domain with unknown function present in most GEFs for Ras-like proteins; DOCK: domain present in proteins with putative GEF function; EF: calcium binding domain; C1: DAG-binding domain; PLCXc: phospholipase C catalytic domain, domain X; PLCYc: phospholipase C catalytic domain, domain Y; C2; Ca^{2+} binding motif. * putative extra nucleotide binding domain.

suggesting C3G binds intracellular E-cadherin to activate Rap1 and induce E-cadherin translocation. C3G was also found to mediate Rap1 activation after engagement of nectins [19]. Nectins are immunoglobulin-like transmembrane proteins that form homophilic and heterophilic dimers via their extra-cellular domains, which have been proposed to induce the formation of AJs [20]. Nectins bind to c-Src at cell–cell contact sites, causing Src activation and subsequent recruitment of C3G [19]. C3G-induced Rap1 activation might also be the switch that stabilizes junctions upon mechanical stress. Indeed, stretching of cells activates Rap1 [21] in a C3G-dependent manner [22]. Interestingly, p130Cas was identified as the first stretch-sensing protein [23] and is well known to form a complex with C3G [24], suggesting p130Cas serves as a sensor that activates Rap1 upon stretching of cells. Whether this mechanism functions in stabilization of cell–cell contacts remains to be determined.

Also the PDZ-GEFs localize to cell junctions and are thus good candidates for Rap1 regulation at these sites. These multidomain proteins have a REM-CDC25 homology domain as catalytic region, a PDZ domain and regions homologous to cyclic nucleotide binding domains. PDZ-GEF1 associates with β-catenin both directly and via the scaffold proteins MAGI-1 and MAGI-2 [14,25,26]. MAGI-1 is localized at cell–cell junctions and is required for contact-induced activation of Rap1, presumably by PDZ-GEF1, although this has yet to be proven [14]. Rap1 activation upon loss of AJs does depend on PDZ-GEF1. The authors propose that PDZ-GEF/Rap1 mediate the repair of AJ [27]. *D. melanogaster* contains a single PDZ-GEF protein and flies that carry mutant dPDZ-GEF show strong dorsal closure defects. However, the AJ components Arm (β-catenin) and DE-cadherin were evenly distributed around the circumference of dPDZ-GEF mutant cells, indicating that AJ integrity is not perturbed by the loss of dPDZ-GEF function. Interestingly, extensions at the lateral cell periphery do point towards a defect in contractility, suggesting that dPDZ-GEF could enhance cell–cell contact tightening by acting on the actomyosin machinery [28].

Recently, PDZ-GEF2 was identified as an essential activator of Rap1 during junction formation both in epithelial and endothelial cells. In contrast to cells inhibited for C3G, cells depleted of PDZ-GEF2 have only slightly decreased E-cadherin levels at the cell surface. However, they do not form mature junctions upon replating, which can be rescued by active Rap1A [13]. This indicates that PDZ-GEF2 functions at a different level in junction formation as C3G (Fig. 2).

A main question remains how PDZ-GEFs are activated. PDZ-GEF1 contains one cyclic nucleotide-binding domain and PDZ-GEF2 even two. However, no effect was observed of cyclic nucleotides on Rap1 activation by PDZ-GEF [29,30]. In neurons, neurotrophins activate PDZ-GEF1 during neurite outgrowth [31] and in splenocytes TNFα-induced M-Ras activates PDZ-GEF2 [32]. Similarly, PDZ-GEF1 is activated and translocated by GTP-bound Rap1 [33]. Therefore, PDZ-GEFs might well function in reiterative GTPase activation and/or positive feedback loops.

DOCK4 is a member of the atypical RhoGEFs, which is found mutated in a subset of human tumors [34]. DOCK4 has GEF activity for both Rap and Rac. Osteosarcoma cells lacking DOCK4 do not form intercellular junctions, but this can be rescued by expression of an active Rap1 mutant, indicating a role for DOCK4 as a RapGEF in junction formation [34]. Recently, it was shown that DOCK4 is activated by RhoG and regulates cell migration via the activation of Rac [35]. Whether RhoG also activates DOCK4 in junction formation is

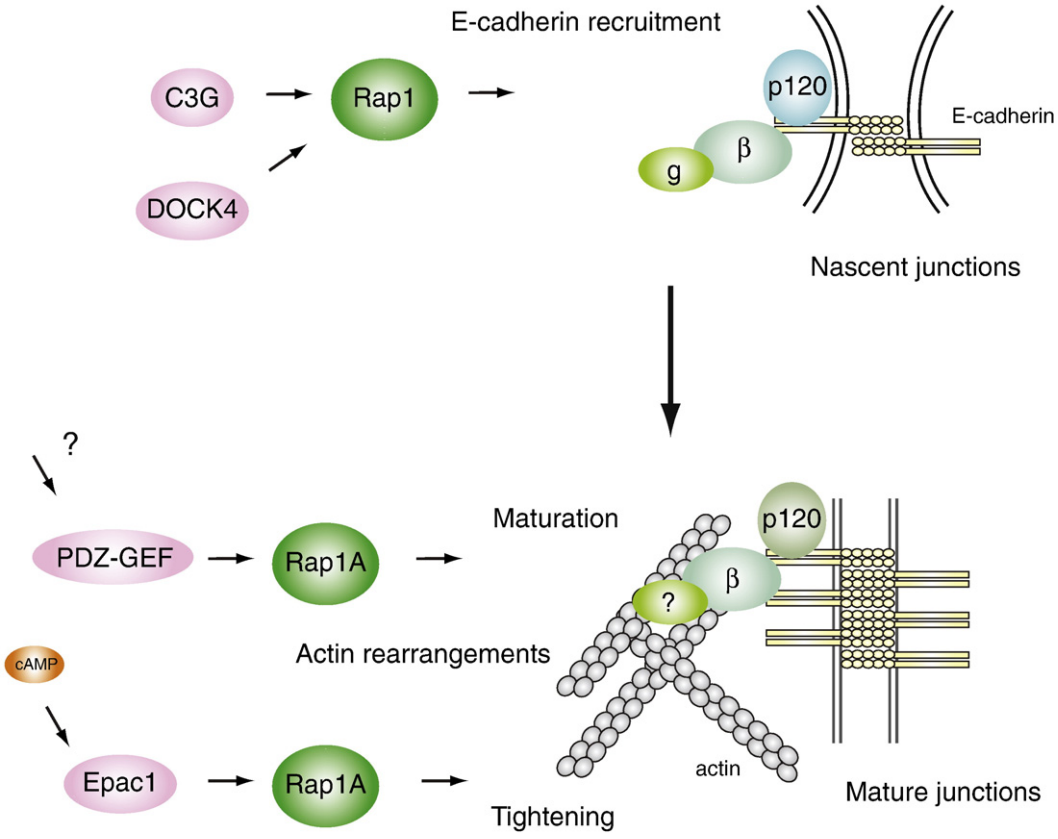

Fig. 2. Model of the differential roles for RapGEFs in junction formation. Activation of Rap1 through C3G and/or DOCK4 is involved in the recruitment of E-cadherin to the plasma membrane and subsequent formation of the initial cell–cell junctions. Secondly, activation of PDZ-GEF is required for the maturation of junctions, presumably through regulation of the actin cytoskeleton. Finally, Epac1 induces tightening of endothelial junctions upon cAMP stimulation in an actin-dependent manner. β: β-catenin; p120: p120-catenin; ?: unknown protein that links AJ and actin cytoskeleton.

currently unclear. Interestingly, RhoG is activated in endothelial cells upon trans-endothelial-migration of leukocytes, a process which requires reorganization of endothelial cell–cell junctions [36].

Lastly, junctional Rap1 can be activated by Epac1. The Epac proteins contain, in addition to the REM-CDC25 homology domain, one or two cAMP binding domains, a DEP (Dishevelled, EGL-10, Pleckstrin) domain and a Ras association (RA) domain. Regulation of junction integrity in endothelial cells by cAMP/ Epac1 is discussed below.

Thus, various RapGEFs are involved in the regulation of cell–cell junctions to activate Rap proteins. These GEFs sense different changes in cell surrounding, like cell–cell contact formation, mechanical stress and leukocyte transmigration.

3. Rap1 in junction formation

Regulation of adherens junctions mainly occurs at two levels: first, tight control exists on the vesicular sorting of E-cadherin, determining its cell surface levels. Second, signaling cascades control the cytoskeletal linkage to E-cadherin, thereby stabilizing the contact [37,38]. Rap1A and Rap1B seem to impinge on both of them in a way that may well be isoform specific [13].

An interesting link between Rap1 and E-cadherin levels was recently found in the nematode *C. elegans*. Homozygous RAP-1 mutant worms show delayed, but normal development [39]. In a genome-wide synthetic lethal screen, *ral-1, sec-5* and *exoc-8* were found to be essential for viability of RAP-1 mutant worms [40]. The mammalian orthologues of these proteins, Ral, Sec5 and Exo84 are known to function in a single signaling cascade that regulates plasma membrane delivery of E-cadherin [41,42]. When fed with RNAi targeting exocyst complex members, RAP-1 mutant embryos show an arrest in hypodermal cell migration, similar to mutants defective in the cadherin–catenin complex [40,43]. The synthetic lethality of Rap1 with the exocyst members suggests that Rap1 functions to stabilize E-cadherin at the cell membrane, the key regulator of which is p120-catenin. Binding of p120-catenin to the juxtamembrane region of E-cadherin prevents E-cadherin internalization [44]. Interestingly, the Rap1 effector AF6 associates with p120-catenin and this binding increases the interaction of p120-catenin with E-cadherin. AF6 prevents non-trans-interacting E-cadherin from being removed from junctions in an *in vitro* assay only when bound to Rap1 or upon deletion of the Ras binding domain from AF6 [45], but dynamics of the AF6/p120-catenin link and its regulation by Rap1 have not been further characterized.

Once E-cadherin homophilically interacts with E-cadherin on neighbouring cells, these proteins become trapped and start signaling to increase cell–cell contact area. These signaling cascades result in dynamic regulation of the cytoskeleton to strengthen the junction (reviewed in [37]). In brief, PI-3-kinase and p120-catenin dependent activation of Rac drives actin dynamics that result in contact extension. Next, the contact is strengthened by direct linkage of the cadherin complex to the actin cytoskeleton. This requires a transition from branched actin to actin cables, brought about by inhibition of Arp2/3 and recruitment of Ena/VASP proteins. This switch is thought to be regulated by dimers of α-catenin. Lastly, trans-interacting E-cadherin induces recruitment and microtubule-dependent phosphorylation of Myosin II, which mediates actomyosin contraction, ultimately resulting in the formation of the typical zonula adherens of polarized epithelial cells [37]. Intermediate stages of junction formation have been visualized in primary keratinocytes, showing E-cadherin-rich filopodial extensions that protrude towards neighbouring cells to form 'adhesion zippers' [46,47]. Interestingly, junction formation of A549 lung carcinoma cells depleted of Rap1A halts at a stage with very similar adhesion zippers. Ultra-structural analysis of the junctions in Rap1A-depleted cells indicates the presence of filopodial extensions that have failed to zipper up. Importantly, surface E-cadherin level was unaltered, suggesting a defect in the cytoskeleton-mediated strengthening of the junctions [13]. Adhesion zippers have also been observed upon inhibition of Arp2/3-mediated actin assembly [48] and in cells depleted of EPLIN, a protein that links the cadherin–catenin complex to F-actin [49]. Together, these reports clearly point to a role of Rap1 in regulating cytoskeletal dynamics to induce extension of nascent contacts. This is in line with observations in mice selectively expressing a dominant-negative Rap1 in differentiating spermatids. These spermatids have normal surface expression of VE-cadherin, but linkage of VE-cadherin to the cytoskeleton is impaired [50].

Thus, Rap1 is clearly important in cell–cell contacts. Interestingly, expressing RapGAP or siRap1A does not perturb a confluent monolayer [12,13]. This suggests that the prime function of Rap1 is in junction formation rather than junction maintenance. However, *rap1* mutant cells in a *Drosophila* wing have perturbed AJs [10] and *C. elegans* RAP-1 seems to stabilize E-cadherin at the cell surface [40], both pointing at a role in maintenance. Possibly, Rap1 serves a subtle role in AJ maintenance that only becomes apparent during certain stages of development and/or conditions that require a quick re-establishment of cell–cell contacts.

Several actin modulating proteins have been shown to be directly regulated by Rap1. First of all, Rap1 can signal to GTPases of the Rho family that are well known for their effects on the actin cytoskeleton and thereby affect cell–cell contacts as well [51]. Rap1 has been shown to induce Rac activation [52] and to mediate relocalization of the RacGEFs Vav2 and Tiam1 during cell spreading [53]. Within the context of junction formation it has been found that a fast-cycling mutant of Cdc42 rescues the effects of RapGAP [12]. Furthermore, TIAM is essential for cell–cell contact maturation [54,55] and nectin engagement activates both Rac and Cdc42 via their respective GEFs Vav2 and FRG in a C3G/Rap1 dependent manner [19,56]. However, how Rap controls junctions through Rho GTPases is currently elusive.

RIAM binds to activated Rap1 and is essential for Rap1 to induce adhesion of T-cells to extracellular matrix [57]. RIAM contains several proline-rich stretches that bind VASP proteins [58] and the actin elongation factor Profilin. Indeed, RIAM induces actin polymerization [57], but its role in junction maturation has not been investigated. In a recent proteomics paper, double-tag purification of Rap1A identified Ena and VASP as specific binding partners [59]. Ena/VASP proteins are essential for several modes of actin modulation at cell–cell contact sites [60], but a functional connectivity with Rap1 has yet to be established.

Another connection of Rap1 to the actin cytoskeleton may be through AF6. This Rap effector binds to a number of junctional proteins, including nectin, ZO-1 and p120. Indeed, AF6 functions to stabilize E-cadherin at the membrane (see above). Furthermore, AF6 directly interacts with actin to mediate E-cadherin dependent cell–cell adhesion. However, the latter functions occurs independently of Rap1 binding [61].

4. Rap1 signalling in endothelial junctions

The endothelium is a highly specialized cell layer that surrounds the entire circulatory system. It controls capillary permeability and diapedesis of leukocytes. As such, the endothelium functions as an important regulator of body homeostasis [62]. The regulation of vascular permeability requires very strict control of junctional integrity. Hence, both assembly and disassembly of endothelial cell–cell contacts are under tight hormonal control [63]. Interestingly, barrier stabilizing agents generally increase intracellular levels of the second messenger cAMP. Dynamic control of endothelial cell–cell junctions by cAMP is mediated by both PKA and Epac1 via independent pathways [64].

Epac proteins are cAMP-responsive GEFs for the Rap family of GTPases. They have been implicated in several physiological processes, such as inflammatory response, secretion, Ca^{2+} signaling, apoptosis,

gene expression and cell adhesion [65,66]. Epac research has been greatly facilitated by the characterization of an Epac-specific cAMP analogue, 8-pCPT-2′-O-Me-cAMP (007) [67]. Treatment of an endothelial monolayer with 007 decreases both basal and thrombin-induced permeability [68–70] and increases trans-endothelial electrical resistance (TER) [15]. Physiological induction of cAMP in pulmonary endothelial cells by prostaglandins or atrial natriuretic peptide (ANP) similarly increases TER in a manner depending on both PKA and Epac1 [71,72].

The importance of Epac1/Rap1 in cell–cell contact tightening has been confirmed *in vivo*. Intradermal injection of 007 prevents both basal and VEGF-induced leakage of Evans Blue from blood vessels [69]. In perfusion experiments with rat venular microvessels, 007 treatment attenuated PAF (platelet activating factor) induced increase in permeability [73], suggesting that cAMP/Epac1/Rap1 blocks increase in vascular permeability via regulation of the AJ *in vivo*.

Several Rap1 effector proteins might mediate the effect on barrier function. As described above, Rac signaling is likely to be downstream of Rap1 and this holds for endothelial cells as well. Similar to Epac1, the RacGEFs Vav2 and Tiam1 are required for ANP and PGE2 induced TER increase [71,72]. Rac1 is activated and translocates to cell–cell contacts when monolayers are stimulated with 007 [74], indicating that Rap1 functions to modulate Rac activity. Recently, the Rap1 binding protein KRIT1 (K-Rev1 Interaction Trapped gene 1, also known as CCM1) was shown to be involved in Epac1/Rap1-induced permeability of endothelial cell–cell junctions [75]. Together with CCM2/MGC4607 and CCM3/PDCD10, KRIT1 constitutes a set of proteins, mutations of which are found in cerebral cavernous malformations. These neurological disorders are characterized by cerebral hemorrhages and vascular malformations in the central nervous system [76]. Mice lacking KRIT1 die because of vascular defects [77] and loss of KRIT1 or CCM2 in zebrafish embryos results in severe dilation of the major vessels [78]. Originally, KRIT1 was identified as an interactor of Rap1 in yeast-two-hybrid assays [79]. In confluent endothelial monolayers, KRIT1 localizes to cell–cell contacts, where it is found in a complex containing the AJ proteins VE-cadherin, β-catenin, α-catenin, p120-catenin, AF6, but not the tight junction marker ZO-1. Depletion of KRIT1 by siRNA disables Epac1/Rap1 to rescue thrombin-induced permeability, establishing KRIT1 as an effector of Rap1 in the tightening of cell-cell contacts. It remains to be elucidated how KRIT1 relays the Rap1 signal towards cell–cell contacts. KRIT1 associates with β-catenin and AF6 in a Rap1-dependent way and siRNA targeting KRIT1 disrupts junctional staining of β-catenin [75], suggesting KRIT1 might stabilize β-catenin at the AJ. Alternatively, KRIT1 regulates endothelial cell shape by microtubule (MT) targeting [80]. Indeed, KRIT1 binds to MTs, which is inhibited by active Rap1. Furthermore, Rap1 activation is associated with increased membrane binding of KRIT1, suggesting that KRIT1 is targeted to the membrane by microtubules where it is captured by Rap1 [81].

Recently, it was shown that Epac1-induced increase in TER of HUVEC monolayers is sensitive to the microtubule disrupting agent nocodazole. Ectopic YFP-Epac1 localizes to microtubules, independent of its activation status. Treatment with 007 induced elongation of microtubules towards the cell periphery. This microtubule growth was also observed in single cells or after overexpression of Rap1GAP, indicating that Epac1 exerts its effect on MTs independent of junctions and Rap1 [82]. Intriguingly, Epac1 binding to MTs seems to suppress activation of Rap1 [83], and disruption of MTs stabilizes cell–cell contacts by increasing the junctional pool of β-catenin [84]. Indeed, β-catenin can be removed from the cell periphery by its association with the MT motor protein dynein [85]. Other junctional proteins can be regulated by MTs as well; p120-catenin binds MTs [86] and focal accumulation of E-cadherin was perturbed by nocodazole [87]. Taken together, these results suggest Epac1 might function as a switch in junction dynamics via its direct effects on MTs and Rap1-mediated effects on actin.

5. Concluding remarks

It is clear that the Rap signaling network plays a critical role in both *de novo* formation and dynamic regulation of cell–cell junctions. Several RapGEFs and Rap1 effector proteins have been proposed to mediate these processes (Fig. 3). A picture emerges in which a certain GEF is preferentially utilized during formation or during remodeling of junctions in a cell type specific manner. Indeed, each of these GEFs is equipped with unique protein domains that are tailored to regulate or localize a GEF under specific conditions. For example, the dynamic nature of adherens junctions in endothelial cells is under the control of the second messenger cAMP and the cAMP-regulated GEF Epac1. PDZ-GEFs may be more important for remodeling junctions during e.g. migration of sheets of cells or junction maturation since it interacts directly with proteins present in junctions like MAGI. While Epac and PDZ-GEF may impinge on a similar process – the stabilization of junctions – C3G and perhaps DOCK4 function in the control of E-cadherin recruitment.

Clearly many questions are still unanswered. For instance, how are the various RapGEFs regulated in time and space to control a dynamic process as junction formation and maintenance? What is the interconnectivity between these GEFs? For instance, PDZ-GEF1 is a GEF for Rap1 but also a Rap1 effector. Is this a direct feedback loop or is this a connection with other GEFs? What is the role of the various Rap1GAPs? Are they general negative regulators or are they also under tight control? Also, what are the critical downstream targets of Rap1 and what is the molecular mechanism by which they control junction formation? It is likely that multiple effectors mediate the Rap effects. Some effectors may regulate vesicular transport, some cytoskeletal dynamics and others may be involved in the connection between the cytoskeleton and junctional proteins. If so, what is the interconnectivity between these different effectors? Finally, most studies point to a major role of Rap1 in junction formation, but since most of the

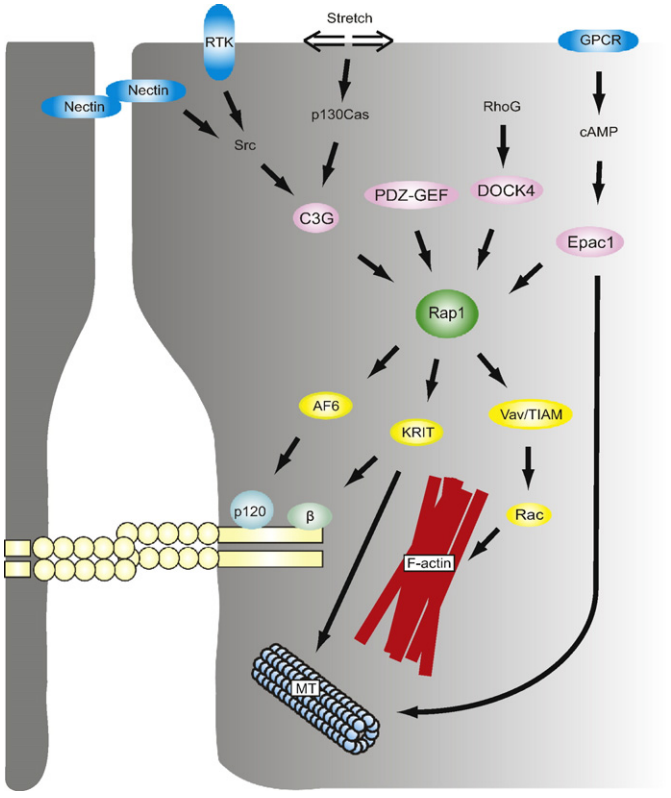

Fig. 3. Overview scheme of Rap1 signalling in cell–cell contacts. See text for details. RTK: Receptor Tyrosine Kinase; GPCR: G-protein coupled receptor; p120: p120-catenin; β: β-catenin; MT: microtubules.

RapGEFs also activate Rap2, it is important to include this family of proteins in the analyses as well.

Acknowledgements

We thank our colleagues in the lab for continuing discussions and support. This work was supported by the Dutch Cancer Society (W.J. P.), the Netherlands Proteomics Centre (M.R.H.K.), the Centre for Biomedical Genetics (F.J.T.Z.) and Netherlands Genomics Initiative/ Netherlands Organisation for Scientific Research (J.L.B).

References

[1] J.L. Bos, H. Rehmann, A. Wittinghofer, GEFs and GAPs: critical elements in the control of small G proteins, Cell 129 (2007) 865–877.
[2] J.L. Bos, ras oncogenes in human cancer: a review, Cancer Res. 49 (1989) 4682–4689.
[3] H. Kitayama, Y. Sugimoto, T. Matsuzaki, Y. Ikawa, M. Noda, A ras-related gene with transformation suppressor activity, Cell 56 (1989) 77–84.
[4] J.C. Schwamborn, A.W. Puschel, The sequential activity of the GTPases Rap1B and Cdc42 determines neuronal polarity, Nat. Neurosci. 7 (2004) 923–929.
[5] J.L. Bos, Linking Rap to cell adhesion, Curr. Opin. Cell Biol. 17 (2005) 123–128.
[6] M.R. Kooistra, N. Dube, J.L. Bos, Rap1: a key regulator in cell–cell junction formation, J. Cell Sci. 120 (2007) 17–22.
[7] C.M. Niessen, Tight junctions/adherens junctions: basic structure and function, J. Invest. Dermatol. 127 (2007) 2525–2532.
[8] S. Tsukita, M. Furuse, M. Itoh, Multifunctional strands in tight junctions, Nat. Rev. Mol. Cell Biol. 2 (2001) 285–293.
[9] A. Hartsock, W.J. Nelson, Adherens and tight junctions: structure, function and connections to the actin cytoskeleton, Biochim. Biophys. Acta 1778 (2008) 660–669.
[10] A.L. Knox, N.H. Brown, Rap1 GTPase regulation of adherens junction positioning and cell adhesion, Science 295 (2002) 1285–1288.
[11] L.S. Price, A. Hajdo-Milasinovic, J. Zhao, F.J. Zwartkruis, J.G. Collard, J.L. Bos, Rap1 regulates E-cadherin-mediated cell–cell adhesion, J. Biol. Chem. 279 (2004) 35127–35132.
[12] C. Hogan, N. Serpente, P. Cogram, C.R. Hosking, C.U. Bialucha, S.M. Feller, V.M. Braga, W. Birchmeier, Y. Fujita, Rap1 regulates the formation of E-cadherin-based cell–cell contacts, Mol. Cell Biol. 24 (2004) 6690–6700.
[13] N. Dube, M.R. Kooistra, W.J. Pannekoek, M.J. Vliem, V. Oorschot, J. Klumperman, H. Rehmann, J.L. Bos, The RapGEF PDZ-GEF2 is required for maturation of cell–cell junctions, Cellular Signalling (2008) In press.
[14] A. Sakurai, S. Fukuhara, A. Yamagishi, K. Sako, Y. Kamioka, M. Masuda, Y. Nakaoka, N. Mochizuki, MAGI-1 is required for Rap1 activation upon cell–cell contact and for enhancement of vascular endothelial cadherin-mediated cell adhesion, Mol. Biol. Cell 17 (2006) 966–976.
[15] E.S. Wittchen, R.A. Worthylake, P. Kelly, P.J. Casey, L.A. Quilliam, K. Burridge, Rap1 GTPase inhibits leukocyte transmigration by promoting endothelial barrier function, J. Biol. Chem. 280 (2005) 11675–11682.
[16] F. Balzac, M. Avolio, S. Degani, I. Kaverina, M. Torti, L. Silengo, J.V. Small, S.F. Retta, E-cadherin endocytosis regulates the activity of Rap1: a traffic light GTPase at the crossroads between cadherin and integrin function, J. Cell Sci. 118 (2005) 4765–4783.
[17] C. Wu, C.F. Lai, W.C. Mobley, Nerve growth factor activates persistent Rap1 signaling in endosomes, J. Neurosci. 21 (2001) 5406–5416.
[18] N. Mochizuki, S. Yamashita, K. Kurokawa, Y. Ohba, T. Nagai, A. Miyawaki, M. Matsuda, Spatio-temporal images of growth-factor-induced activation of Ras and Rap1, Nature 411 (2001) 1065–1068.
[19] T. Fukuyama, H. Ogita, T. Kawakatsu, T. Fukuhara, T. Yamada, T. Sato, K. Shimizu, T. Nakamura, M. Matsuda, Y. Takai, Involvement of the c-Src-Crk-C3G-Rap1 signaling in the nectin-induced activation of Cdc42 and formation of adherens junctions, J. Biol. Chem. 280 (2005) 815–825.
[20] K. Irie, K. Shimizu, T. Sakisaka, W. Ikeda, Y. Takai, Roles and modes of action of nectins in cell–cell adhesion, Semin. Cell Dev. Biol. 15 (2004) 643–656.
[21] Y. Sawada, K. Nakamura, K. Doi, K. Takeda, K. Tobiume, M. Saitoh, K. Morita, I. Komuro, K. De Vos, M. Sheetz, H. Ichijo, Rap1 is involved in cell stretching modulation of p38 but not ERK or JNK MAP kinase, J. Cell Sci. 114 (2001) 1221–1227.
[22] M. Tamada, M.P. Sheetz, Y. Sawada, Activation of a signaling cascade by cytoskeleton stretch, Dev. Cell 7 (2004) 709–718.
[23] Y. Sawada, M. Tamada, B.J. Dubin-Thaler, O. Cherniavskaya, R. Sakai, S. Tanaka, M.P. Sheetz, Force sensing by mechanical extension of the Src family kinase substrate p130Cas, Cell 127 (2006) 1015–1026.
[24] K. Vuori, H. Hirai, S. Aizawa, E. Ruoslahti, Introduction of p130cas signaling complex formation upon integrin-mediated cell adhesion: a role for Src family kinases, Mol. Cell Biol. 16 (1996) 2606–2613.
[25] I.Y. Dobrosotskaya, G.L. James, MAGI-1 interacts with beta-catenin and is associated with cell–cell adhesion structures, Biochem. Biophys. Res. Commun. 270 (2000) 903–909.
[26] A. Mino, T. Ohtsuka, E. Inoue, Y. Takai, Membrane-associated guanylate kinase with inverted orientation (MAGI)-1/brain angiogenesis inhibitor 1-associated protein (BAP1) as a scaffolding molecule for Rap small G protein GDP/GTP exchange protein at tight junctions, Genes Cells 5 (2000) 1009–1016.
[27] S. Asuri, J. Yan, N.C. Paranavitana, L.A. Quilliam, E-cadherin dis-engagement activates the Rap1 GTPase, J. Cell Biochem. 105 (2008) 1027–1037.
[28] B. Boettner, L. Van Aelst, The Rap GTPase activator Drosophila PDZ-GEF regulates cell shape in epithelial migration and morphogenesis, Mol. Cell Biol. 27 (2007) 7966–7980.
[29] H.B. Kuiperij, J. de Rooij, H. Rehmann, M. van Triest, A. Wittinghofer, J.L. Bos, F.J. Zwartkruis, Characterisation of PDZ-GEFs, a family of guanine nucleotide exchange factors specific for Rap1 and Rap2, Biochim. Biophys. Acta 1593 (2003) 141–149.
[30] N. Pham, I. Cheglakov, C.A. Koch, C.L. de Hoog, M.F. Moran, D. Rotin, The guanine nucleotide exchange factor CNrasGEF activates ras in response to cAMP and cGMP, Curr. Biol. 10 (2000) 555–558.
[31] S. Hisata, T. Sakisaka, T. Baba, T. Yamada, K. Aoki, M. Matsuda, Y. Takai, Rap1-PDZ-GEF1 interacts with a neurotrophin receptor at late endosomes, leading to sustained activation of Rap1 and ERK and neurite outgrowth, J. Cell Biol. 178 (2007) 843–860.
[32] Y. Yoshikawa, T. Satoh, T. Tamura, P. Wei, S.E. Bilasy, H. Edamatsu, A. Aiba, K. Katagiri, T. Kinashi, K. Nakao, T. Kataoka, The M-Ras-RA-GEF-2-Rap1 pathway mediates tumor necrosis factor-alpha dependent regulation of integrin activation in splenocytes, Mol. Biol. Cell 18 (2007) 2949–2959.
[33] Y. Liao, T. Satoh, X. Gao, T.G. Jin, C.D. Hu, T. Kataoka, RA-GEF-1, a guanine nucleotide exchange factor for Rap1, is activated by translocation induced by association with Rap1*GTP and enhances Rap1-dependent B-Raf activation, J. Biol. Chem. 276 (2001) 28478–28483.
[34] V. Yajnik, C. Paulding, R. Sordella, A.I. McClatchey, M. Saito, D.C. Wahrer, P. Reynolds, D.W. Bell, R. Lake, S. van den Heuvel, J. Settleman, D.A. Haber, DOCK4, a GTPase activator, is disrupted during tumorigenesis, Cell 112 (2003) 673–684.
[35] K. Hiramoto, M. Negishi, H. Katoh, Dock4 is regulated by RhoG and promotes Rac-dependent cell migration, Exp. Cell Res. 312 (2006) 4205–4216.
[36] J.D. van Buul, M.J. Allingham, T. Samson, J. Meller, E. Boulter, R. Garcia-Mata, K. Burridge, RhoG regulates endothelial apical cup assembly downstream from ICAM1 engagement and is involved in leukocyte trans-endothelial migration, J. Cell Biol. 178 (2007) 1279–1293.
[37] R.M. Mege, J. Gavard, M. Lambert, Regulation of cell–cell junctions by the cytoskeleton, Curr. Opin. Cell Biol. 18 (2006) 541–548.
[38] A.S. Yap, M.S. Crampton, J. Hardin, Making and breaking contacts: the cellular biology of cadherin regulation, Curr. Opin. Cell Biol. 19 (2007) 508–514.
[39] W. Pellis-van Berkel, M.H. Verheijen, E. Cuppen, M. Asahina, J. de Rooij, G. Jansen, R.H. Plasterk, J.L. Bos, F.J. Zwartkruis, Requirement of the *Caenorhabditis elegans* RapGEF pxf-1 and rap-1 for epithelial integrity, Mol. Biol. Cell 16 (2005) 106–116.
[40] E.W. Frische, W. Pellis-van Berkel, G. van Haaften, E. Cuppen, R.H. Plasterk, M. Tijsterman, J.L. Bos, F.J. Zwartkruis, RAP-1 and the RAL-1/exocyst pathway coordinate hypodermal cell organization in *Caenorhabditis elegans*, Embo. J. 26 (2007) 5083–5092.
[41] S. Moskalenko, D.O. Henry, C. Rosse, G. Mirey, J.H. Camonis, M.A. White, The exocyst is a Ral effector complex, Nat. Cell Biol. 4 (2002) 66–72.
[42] M. Shipitsin, L.A. Feig, RalA but not RalB enhances polarized delivery of membrane proteins to the basolateral surface of epithelial cells, Mol. Cell Biol. 24 (2004) 5746–5756.
[43] M. Costa, W. Raich, C. Agbunag, B. Leung, J. Hardin, J.R. Priess, A putative catenin–cadherin system mediates morphogenesis of the *Caenorhabditis elegans* embryo, J. Cell Biol. 141 (1998) 297–308.
[44] K. Xiao, R.G. Oas, C.M. Chiasson, A.P. Kowalczyk, Role of p120-catenin in cadherin trafficking, Biochim. Biophys. Acta 1773 (2007) 8–16.
[45] T. Hoshino, T. Sakisaka, T. Baba, T. Yamada, T. Kimura, Y. Takai, Regulation of E-cadherin endocytosis by nectin through afadin, Rap1, and p120ctn, J. Biol. Chem. 280 (2005) 24095–24103.
[46] A. Vaezi, C. Bauer, V. Vasioukhin, E. Fuchs, Actin cable dynamics and Rho/Rock orchestrate a polarized cytoskeletal architecture in the early steps of assembling a stratified epithelium, Dev. Cell 3 (2002) 367–381.
[47] V. Vasioukhin, C. Bauer, M. Yin, E. Fuchs, Directed actin polymerization is the driving force for epithelial cell–cell adhesion, Cell 100 (2000) 209–219.
[48] S. Verma, A.M. Shewan, J.A. Scott, F.M. Helwani, N.R. den Elzen, H. Miki, T. Takenawa, A.S. Yap, Arp2/3 activity is necessary for efficient formation of E-cadherin adhesive contacts, J. Biol. Chem. 279 (2004) 34062–34070.
[49] K. Abe, M. Takeichi, EPLIN mediates linkage of the cadherin catenin complex to F-actin and stabilizes the circumferential actin belt, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 13–19.
[50] E. Aivatiadou, E. Mattei, M. Ceriani, L. Tilia, G. Berruti, Impaired fertility and spermiogenetic disorders with loss of cell adhesion in male mice expressing an interfering Rap1 mutant, Mol. Biol. Cell 18 (2007) 1530–1542.
[51] K. Takaishi, T. Sasaki, H. Kotani, H. Nishioka, Y. Takai, Regulation of cell–cell adhesion by rac and rho small G proteins in MDCK cells, J. Cell Biol. 139 (1997) 1047–1059.
[52] M. Maillet, S.J. Robert, M. Cacquevel, M. Gastineau, D. Vivien, J. Bertoglio, J.L. Zugaza, R. Fischmeister, F. Lezoualc'h, Crosstalk between Rap1 and Rac regulates secretion of sAPPalpha, Nat. Cell Biol. 5 (2003) 633–639.
[53] W.T. Arthur, L.A. Quilliam, J.A. Cooper, Rap1 promotes cell spreading by localizing Rac guanine nucleotide exchange factors, J. Cell Biol. 167 (2004) 111–122.
[54] A. Malliri, S. van Es, S. Huveneers, J.G. Collard, The Rac exchange factor Tiam1 is required for the establishment and maintenance of cadherin-based adhesions, J. Biol. Chem. 279 (2004) 30092–30098.
[55] A.E. Mertens, T.P. Rygiel, C. Olivo, R. van der Kammen, J.G. Collard, The Rac activator Tiam1 controls tight junction biogenesis in keratinocytes through binding to and activation of the Par polarity complex, J. Cell Biol. 170 (2005) 1029–1037.
[56] T. Fukuyama, H. Ogita, T. Kawakatsu, M. Inagaki, Y. Takai, Activation of Rac by cadherin through the c-Src-Rap1-phosphatidylinositol 3-kinase-Vav2 pathway, Oncogene 25 (2006) 8–19.
[57] E.M. Lafuente, A.A. van Puijenbroek, M. Krause, C.V. Carman, G.J. Freeman, A. Berezovskaya, E. Constantine, T.A. Springer, F.B. Gertler, V.A. Boussiotis, RIAM, an

Ena/VASP and Profilin ligand, interacts with Rap1-GTP and mediates Rap1-induced adhesion, Dev. Cell 7 (2004) 585–595.
[58] A. Jenzora, B. Behrendt, J.V. Small, J. Wehland, T.E. Stradal, PREL1 provides a link from Ras signalling to the actin cytoskeleton via Ena/VASP proteins, FEBS Lett. 579 (2005) 455–463.
[59] L.E. Goldfinger, C. Ptak, E.D. Jeffery, J. Shabanowitz, J. Han, J.R. Haling, N.E. Sherman, J.W. Fox, D.F. Hunt, M.H. Ginsberg, An experimentally derived database of candidate Ras-interacting proteins, J. Proteome. Res. 6 (2007) 1806–1811.
[60] J.A. Scott, A.M. Shewan, N.R. den Elzen, J.J. Loureiro, F.B. Gertler, A.S. Yap, Ena/VASP proteins can regulate distinct modes of actin organization at cadherin-adhesive contacts, Mol. Biol. Cell 17 (2006) 1085–1095.
[61] M. Lorger, K. Moelling, Regulation of epithelial wound closure and intercellular adhesion by interaction of AF6 with actin cytoskeleton, J. Cell Sci. 119 (2006) 3385–3398.
[62] E. Dejana, Endothelial cell–cell junctions: happy together, Nat. Rev. Mol. Cell Biol. 5 (2004) 261–270.
[63] E. Vandenbroucke, D. Mehta, R. Minshall, A.B. Malik, Regulation of endothelial junctional permeability, Ann. N. Y. Acad Sci. 1123 (2008) 134–145.
[64] M.J. Lorenowicz, M. Fernandez-Borja, M.R. Kooistra, J.L. Bos, P.L. Hordijk, PKA and Epac1 regulate endothelial integrity and migration through parallel and independent pathways, Eur J Cell Biol (2008) In press.
[65] J.L. Bos, Epac proteins: multi-purpose cAMP targets, Trends Biochem. Sci. 31 (2006) 680–686.
[66] W.A. Sands, H.D. Woolson, G.R. Milne, C. Rutherford, T.M. Palmer, Exchange protein activated by cyclic AMP (Epac)-mediated induction of suppressor of cytokine signaling 3 (SOCS-3) in vascular endothelial cells, Mol. Cell Biol. 26 (2006) 6333–6346.
[67] J.M. Enserink, A.E. Christensen, J. de Rooij, M. van Triest, F. Schwede, H.G. Genieser, S.O. Doskeland, J.L. Blank, J.L. Bos, A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK, Nat. Cell Biol. 4 (2002) 901–906.
[68] X. Cullere, S.K. Shaw, L. Andersson, J. Hirahashi, F.W. Luscinskas, T.N. Mayadas, Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase, Blood 105 (2005) 1950–1955.
[69] S. Fukuhara, A. Sakurai, H. Sano, A. Yamagishi, S. Somekawa, N. Takakura, Y. Saito, K. Kangawa, N. Mochizuki, Cyclic AMP potentiates vascular endothelial cadherin-mediated cell–cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling pathway, Mol. Cell Biol. 25 (2005) 136–146.
[70] M.R. Kooistra, M. Corada, E. Dejana, J.L. Bos, Epac1 regulates integrity of endothelial cell junctions through VE-cadherin, FEBS Lett 579 (2005) 4966–4972.
[71] A.A. Birukova, T. Zagranichnaya, E. Alekseeva, G.M. Bokoch, K.G. Birukov, Epac/Rap and PKA are novel mechanisms of ANP-induced Rac-mediated pulmonary endothelial barrier protection, J. Cell Physiol. 215 (2008) 715–724.
[72] A.A. Birukova, T. Zagranichnaya, P. Fu, E. Alekseeva, W. Chen, J.R. Jacobson, K.G. Birukov, Prostaglandins PGE(2) and PGI(2) promote endothelial barrier enhancement via PKA- and Epac1/Rap1-dependent Rac activation, Exp. Cell Res. 313 (2007) 2504–2520.
[73] R.H. Adamson, J.C. Ly, R.K. Sarai, J.F. Lenz, A. Altangerel, D. Drenckhahn, F.E. Curry, Epac/Rap1 pathway regulates microvascular hyperpermeability induced by PAF in rat mesentery, Am. J. Physiol. Heart Circ. Physiol. 294 (2008) H1188–1196.
[74] Y. Baumer, D. Drenckhahn, J. Waschke, cAMP induced Rac 1-mediated cytoskeletal reorganization in microvascular endothelium, Histochem. Cell Biol. 129 (2008) 765–778.
[75] A. Glading, J. Han, R.A. Stockton, M.H. Ginsberg, KRIT-1/CCM1 is a Rap1 effector that regulates endothelial cell–cell junctions, J. Cell Biol. 179 (2007) 247–254.
[76] P. Labauge, C. Denier, F. Bergametti, E. Tournier-Lasserve, Genetics of cavernous angiomas, Lancet Neurol. 6 (2007) 237–244.
[77] K.J. Whitehead, N.W. Plummer, J.A. Adams, D.A. Marchuk, D.Y. Li, Ccm1 is required for arterial morphogenesis: implications for the etiology of human cavernous malformations, Development 131 (2004) 1437–1448.
[78] B.M. Hogan, J. Bussmann, H. Wolburg, S. Schulte-Merker, ccm1 cell autonomously regulates endothelial cellular morphogenesis and vascular tubulogenesis in zebrafish, Hum. Mol. Genet (2008).
[79] I. Serebriiskii, J. Estojak, G. Sonoda, J.R. Testa, E.A. Golemis, Association of Krev-1/rap1a with Krit1, a novel ankyrin repeat-containing protein encoded by a gene mapping to 7q21-22, Oncogene 15 (1997) 1043–1049.
[80] M. Gunel, M.S. Laurans, D. Shin, M.L. DiLuna, J. Voorhees, K. Choate, C. Nelson-Williams, R.P. Lifton, KRIT1, a gene mutated in cerebral cavernous malformation, encodes a microtubule-associated protein, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 10677–10682.
[81] S. Beraud-Dufour, R. Gautier, C. Albiges-Rizo, P. Chardin, E. Faurobert, Krit 1 interactions with microtubules and membranes are regulated by Rap1 and integrin cytoplasmic domain associated protein-1, Febs. J. 274 (2007) 5518–5532.
[82] S. Sehrawat, X. Cullere, S. Patel, J. Italiano Jr., T.N. Mayadas, Role of epac1, an exchange factor for rap GTPases, in endothelial microtubule dynamics and barrier function, Mol. Biol. Cell 19 (2008) 1261–1270.
[83] F.C. Mei, X. Cheng, Interplay between exchange protein directly activated by cAMP (Epac) and microtubule cytoskeleton, Mol. Biosyst. 1 (2005) 325–331.
[84] M.J. Lorenowicz, M. Fernandez-Borja, A.M. van Stalborch, M.A. van Sterkenburg, P. S. Hiemstra, P.L. Hordijk, Microtubule dynamics and Rac-1 signaling independently regulate barrier function in lung epithelial cells, Am. J. Physiol. Lung Cell Mol. Physiol. 293 (2007) L1321–1331.
[85] L.A. Ligon, S. Karki, M. Tokito, E.L. Holzbaur, Dynein binds to beta-catenin and may tether microtubules at adherens junctions, Nat. Cell Biol. 3 (2001) 913–917.
[86] M. Yanagisawa, I.N. Kaverina, A. Wang, Y. Fujita, A.B. Reynolds, P.Z. Anastasiadis, A novel interaction between kinesin and p120 modulates p120 localization and function, J. Biol. Chem. 279 (2004) 9512–9521.
[87] S.J. Stehbens, A.D. Paterson, M.S. Crampton, A.M. Shewan, C. Ferguson, A. Akhmanova, R.G. Parton, A.S. Yap, Dynamic microtubules regulate the local concentration of E-cadherin at cell–cell contacts, J. Cell Sci. 119 (2006) 1801–1811.