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Review

Rap1, a mercenary among the Ras-like GTPases

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ABSTRACT

The small Ras-like GTPase Rap1 is an evolutionary conserved protein that originally gained interest because of its capacity to revert the morphological phenotype of Ras-transformed fibroblasts. Rap1 is regulated by a large number of stimuli that include growth factors and cytokines, but also physical force and osmotic stress. Downstream of Rap1, a plethora of effector molecules has been proposed on the basis of biochemical studies. Here, we present an overview of genetic studies on Rap1 in various model organisms and relate the observed phenotypes to *in vitro* studies. The picture that emerges is one in which Rap1 is a versatile regulator of morphogenesis, by regulating diverse processes that include establishment of cellular polarity, cell–matrix interactions and cell–cell adhesion. Surprisingly, genetic experiments indicate that in the various model organisms, Rap1 uses distinct effector molecules that impinge upon the actin cytoskeleton and adhesion molecules.

Introduction

The small Ras-like GTPases form a protein family whose members function as molecular switches regulating many signaling events that control processes like proliferation, differentiation, morphogenesis and apoptosis. In addition to the Ras proteins (H-, K-and N-Ras), this family comprises Rap1, Rap2, R-Ras, TC21/R-Ras2, MRas/R-Ras3, DexRas1/RasD1, RalA/B, Rheb, Rit, and the Rin proteins. The major mode of regulation is via guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). Together, they control GTPase cycling between the active GTP-bound state and the inactive GDP-bound state. GAPs enhance the intrinsic GTPase activity, resulting in hydrolysis of GTP. Inducing the active conformation of GTPases by GEFs results from enhanced release of bound GDP, allowing association with GTP, which is more abundant in the cell (Bos et al., 2007). The various GEFs most likely allow them to actively localize GTP-loading to particular locations in the cell, which thereby result in efficient effector coupling. In addition, the sub-cellular localization and phosphorylation status of a GTPase are factors that may determine how they couple to downstream molecules.

H-, K-and N-Ras proteins are the best-studied members of this family, in part because of their prominent role as oncogenes. Ras proteins are found to be mutated in ~15% of all human tumors (Bos, 1989). In addition, Ras also emerged in various genetic screens in model organisms, where signaling cascades have been delineated on the basis of similar phenotypic traits. Perhaps the two most notable examples are ommatidia development in the *Drosophila* eye and *C. elegans* vulval development (e.g. (Nagaraj and Banerjee, 2004; Wang and Sternberg, 2001)).

The subfamily of Rap proteins was cloned on the basis of homology to Ras (Pizon et al., 1988) and gained interest on the basis of its capacity to reverse the morphological transformation of fibroblasts by Ras (Kitayama et al., 1989). Although this effect of Rap1 over-expression is still not fully understood, a great deal has been learned from cell biological and biochemical experiments (for a recent review, see Raaijmakers and Bos, 2008). In part, such experiments were designed on the basis of genetic studies, especially in *Drosophila*. Interestingly, the function of Rap1 has now been investigated by genetic studies in at least some detail in virtually all model organisms. Indeed, Rap1 has been highly conserved during evolution (Fig. 1). Here, we present an overview of these genetic studies, where Rap1 or its regulators have been disrupted and discuss how the results obtained by genetic approaches relate to *in vitro* studies. We will also describe genetic evidence that links candidate effectors to Rap1.

The small Ras-like GTPase Rap1

Nowadays, the mammalian Rap proteins Rap1A and Rap1B are generally regarded to act independently of Ras. Functions attributed to Rap1 comprise the control of establishment of cell polarity (Schwamborn and Puschel, 2004; Shimonaka et al., 2003), activation of integrin-mediated cell adhesion (Caron et al., 2000; Katagiri et al., 2000; Reedquist et al., 2000; Tsukamoto et al., 1999) and the

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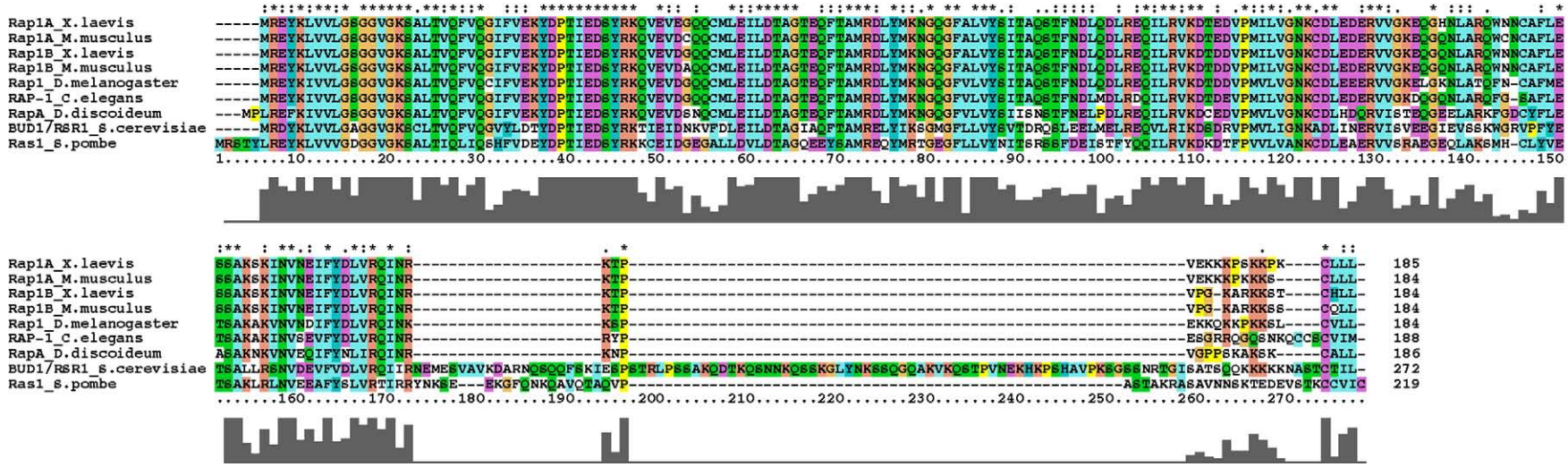

Fig. 1. ClustalX alignment of protein sequences from those species discussed in this review that are most highly related to human Rap1A.

regulation of cell–cell contacts (Hogan et al., 2004; Price et al., 2004). Additionally, Rap1 has been implicated in cell proliferation (Altschuler and Ribeiro-Neto, 1998) and secretion (Crittenden et al., 2004; D'Silva et al., 1998). Biochemical approaches to measure GTP-loading of Rap1 by GEFs have provided clear evidence for the existence of at least five distinct classes of GEFs: C3G (RapGEF1), PDZ-GEF1 and-2 (RapGEf2 and 6), Epac1,-2 and Repac (RapGEF3, 4 and 5), CalDAG-GEF1 (RasGRP2) and Dock4 (reviewed in Pannekoek et al., 2009; see van Dam et al., 2009, for evolutionary aspects). The activity of many of these GEFs has been demonstrated in overexpression studies and/or more directly by *in vitro* GEF assays using purified proteins. It should be kept in mind though, that many of these GEFs are not entirely Rap1-specific, but can activate other GTPases like Rap2 isoforms as well. The same holds true for the negative regulators of Rap1 like Rap1GAPI and-II, Spa1 and SIPA1L1/E6TP1. Almost all candidate effector molecules described for Rap1 to date have initially been isolated on the basis of their binding to GTP-bound Rap1. Again, these effectors may also interact with other Ras-like GTPases, which complicates our understanding of the specificity of GTPases signaling. Genetic studies in model organisms can help to resolve questions about the *in vivo* specificity of GEFs, GAPs and effectors. More importantly, they can disclose physiological processes in which the Rap1 signaling pathway operates.

Saccharomyces cerevisiae

In the budding yeast *S. cerevisiae* establishment of the bud-site, the site where the daughter cell will grow and separate, reveals the polarized organization of the mother cell. The position of the new bud-site is located either at the same side as the previous bud-site or on the opposite side of the cell. After bud-site selection, proteins are recruited for the formation of the bud and the actin cytoskeleton is organized in the direction of the new bud to allow growth. Loss of the single homologue of Rap1 in *S. cerevisiae*, Bud1p/Rsr1p (59% identity, 79% similarity with Rap1A), leads to randomized localization of the bud-site (Cabib et al., 1998; Chant and Herskowitz, 1991). When compared to Rap1, Bud1p has a long C-terminal insertion. This fragment, however, seems not essential for bud site selection (Michelitch and Chant, 1996). Strikingly, also loss of proteins with Bud1p/Rsr1p GEF and GAP activity, Bud5 and Bud2 respectively, leads to random bud-site formation (Bender and Pringle, 1989; Park et al., 1993). This strongly suggests that cycling is important for Bud1 function. Bud-site assembly requires the recruitment of Cdc24p, an exchange factor for the GTPase Cdc42p that binds to GTP-bound Bud1p/Rsr1p. In addition, the scaffold protein Bem1p binds to Cdc24p and to Bud1p/Rsr1p in a GDP-dependent manner and thereby presumably regulates the formation of a complex consisting of Bud1p/Rsr1p and Cdc24p to regulate Cdc42p activity (Park et al., 1997). Thus, in this unicellular organism, activation of Bud1p/Rsr1p is required for positioning the bud site by cell-internal polarity cues by regulating the actin cytoskeleton via a single effector, Cdc24p. There are indications that this function of Bud1/Rsr1p has been conserved during evolution. First, in tissue culture experiments Rap1 has been found to be involved in the establishment of polarity. For instance, in lymphocytes Rap1 is pivotal for chemokine-induced cell polarization characterized by formation of a leading edge and uropod, which both contain a unique set of marker proteins (Shimonaka et al., 2003). Secondly, in hippocampal neurons, Rap1 functions in axon-fate determination (Schwamborn and Puschel, 2004). In both cases, Rap1 seems to function upstream of Cdc42 and to localize and activate the polarity-establishing Par-complex (Gerard et al., 2007; Schwamborn and Puschel, 2004). How Rap1 induces GTP-loading of CDC42 in mammalian cells has not been elucidated, but this may be via direct binding of a CDC42 GEF. Binding of GTP-bound Rap1 to at least two distinct Rho-family GEFs has been reported. In the case of T-cells, Rap1 has been shown to directly bind to the Rac-GEF Tiam1, which also binds to Par3. However, this interaction was suggested to lead to Rac1 activation downstream of CDC42 activity (Gerard et al., 2007). In a separate study, Rap1 has been shown to bind to Vav2 (Arthur et al., 2004), the closest mammalian homologue of the budding yeast protein Cdc24p. This interaction leads to Rac-dependent cell spreading rather than establishment of cellular polarity. It is striking that the binding site of Rap1 on these exchange factors has been reported to reside in the catalytic domain, whereas the binding site for Bud1 has been suggested to be either at the C- (Park et al., 1997) or at the N-terminus (Gulli and Peter, 2001) of CDC24. Taken together, Bud1/CDC24/CDC42 cannot simply be regarded as an evolutionary conserved signaling module. However, it may be derived from an ancestral cascade that linked Ras-like GTPases to Rho-like GTPases and which in vertebrates may have evolved into pathways like Rap1/Vav2/Rac and Rap1/RhoGEF(?)/CDC42.

Dictyostelium discoideum

In the unicellular amoeba *D. discoideum*, Rap1 is presumably an essential gene since no knock-out cells have been obtained (Kortholt et al., 2006). Possibly, such mutants are inviable due to the fact that morphogenetic events in *D. discoideum* are more dynamic as compared to yeast. For example, *D. discoideum* can rapidly respond to chemical signals from its environment by chemotaxis and is able to form multi-cellular aggregates. Functions that have been attributed to Rap1 include the control of cell polarity, cell adhesion, endocytosis, phagocytosis and osmotic stress resistance (Chubb and Insall, 2001; Kortholt and van Haastert, 2008; Rebstein et al., 1997; Seastone et al., 1999). Thus far, three regulators of Rap1 have been characterized, the GEF GbpD (Kortholt et al., 2006) and the GAPs RAPGAP1 (Jeon et al., 2007a) and RapGAPB (Parkinson et al., 2009). Overexpression of active Rap1 induces a more spread and flat cellular phenotype with more peripheral F-actin. These cells adhere more strongly to the extra-cellular matrix and display a substantially decreased chemotaxis (Jeon et al., 2007b; Kortholt et al., 2006; Rebstein et al., 1993). This phenotype is mimicked in cells overexpressing GbpD (Kortholt et al., 2006). In contrast, GbpD-deficient cells are hyperpolar, extend fewer lateral pseudopods but show normal remodeling of the actin cytoskeleton at the leading edge. In one study, increased adhesion seen upon GpbD over-expression was found to depend on the presence of Phg2. Phg2 is a serine/threonine kinase that specifically interacts with GTP-bound Rap1 via its Ras association (RA) domain and loss of Phg2 resulted in diminished cell adhesion (Kortholt et al., 2006). Formation of lateral pseudopods and decreased polarity, however, were found to be Phg2 independent, suggesting the existence of an additional Rap1 effector. In a more recent study, Phg2 was shown to colocalize with active Rap1 at the leading edge of migrating *D. discoideum* cells. Although Phg2 localization was independent of the RA domain and thus of Rap1 binding, the RA domain was required for Phg2 activity. Phg2 was found to induce MyoII phosphorylation, leading edge MyoII disassembly, decreased cortical tension and enhanced protrusive activity at the leading edge. Remarkably, however, in this latter study it was found that Phg2 negative cells have increased cell attachment, which may be due to different assay conditions or stem from the use of a distinct parental wild-type strain (Kortholt et al., 2006) (Jeon et al., 2007a). Loss of RapGAPB leads to enhanced Rap-GTP levels and a concomitant increase in cell–matrix adhesion. While chemotaxis toward cAMP is not affected, RapGAPB negative cells are severely impaired in formation of multi-cellular aggregates, most likely as a consequence of altered cell–cell adhesion. In summary, studies in *D. discoideum* highlight a prominent role for Rap1 in processes like cell adhesion, polarity and migration. Rap1 mediates its effects at least in part via activation of the Phg2 kinase, for which in multi-cellular organisms unfortunately no clear

homologue can be found. Whether in higher eukaryotes Rap1 plays a role in phosphorylation of MyoII has not well been investigated yet.

Drosophila melanogaster

Studies on the function of Rap1 in *Drosophila* have been crucial for elucidation of its role in cell migration and adherens junction formation. Rap1 is an essential gene in *Drosophila*: homozygous Rap1 null mutants die during larval stages while depletion of maternal input causes embryonic lethality. These embryos show various morphogenetic and migration defects described below (Asha et al., 1999). Importantly, none of the observed phenotypes appears to be caused by defects in cell proliferation or specification. Adult flies do not depend on Rap1 for viability although mutant female flies stop producing progeny due to degeneration of the egg chambers. Three morphogenetic events involving cell sheets are affected in embryos devoid of Rap1. These are ventral furrow closure, dorsal closure and head involution. In addition, migration of individual mesodermal and pole cells is defective.

The defects in ventral furrow closure in Rap1-deficient embryos most likely result from the inability of ventral cells to reorganize their actin cytoskeleton in a coordinated fashion. Ventral furrow formation is initiated by apical constriction of the actin-myosin network in mesectodermal cells (Martin et al., 2009). This process starts normally in mutant embryos, but cells fail to come together at the midline, especially in anterior regions. In cross sections, cells appear abnormally shaped and their nuclei, which move basally in wild-type animals, are found at different positions along the apico-basal axis. The Rap1 effector involved in this process is unknown.

Dorsal closure depends on the dorsalward migration of lateral epithelial cells, which elongate along the dorsal-ventral axis, over a structure that is named amnioserosa. The lateral epithelia meet at the dorsal midline to entirely cover the embryo (reviewed in Jacinto et al., 2002). Dorsal closure requires activation of the JNK-pathway that leads to expression of the TGF-β-like DPP protein in leading edge cells. Simultaneously, actin-based filopodial and lamellar protrusions are formed on the dorsal side of these cells. Defective dorsal closure, as seen in Rap1-negative embryos, can be mimicked by expression of dominant negative $Rap1^{N17}$ in lateral ectoderm and resembles the phenotype of Canoe (Cno) mutant embryos (Boettner et al., 2003). Cno is an adaptor protein that can interact with GTP-bound Rap1 via its N-terminal RA-domains. Both proteins co-localize with DE-cadherin in adherens junctions but the localization of Cno does not depend on the interaction with Rap1. Intriguingly, overexpression of wild-type Cno suppresses dorsal closure defects induced by $Rap1^{N17}$, but a Cno isoform that lacks the RA-domains does not. This latter isoform has retained significant activity since it can suppress a Cno loss of function phenotype via activation of the JNK-pathway. Together, these studies indicate that Cno fulfills a Rap1-dependent and a separate, JNK-dependent function in dorsal closure. Boettner and colleagues also demonstrated that the PDZ-GEF homologue dPDZ-GEF (also known as Dizzy or Gef26) functions upstream of Rap1 in this process: dPDZ-GEF loss-of-function mutants have dorsal closure defects and hypomorphic alleles show profound genetic interactions with Rap1 and Cno. How do mutations of genes, encoding proteins that localize to the lateral membrane, interfere with dorsal closure? The failure of epithelial cells to stretch in a dorso-ventral direction as seen in all three mutants, hints at a diminished contractility of the actin cytoskeleton. Indeed, the non-muscle myosin MyoII (encoded by *zipper*) fails to assemble into a continuous cable at the leading edge of the lateral epithelium in these mutants. Possibly, this actin network is connected to adherens junctions where dPDZ-GEF, Rap1 and Cno reside. Furthermore, dPDZ-GEF mutant cells generated during wing development have an increased circumferential size and a less compact MyoII network, suggesting that dPDZGEF may indeed affect MyoII function (Boettner and Van Aelst, 2007).

It should be noted that in all situations described above, adherens junctions have an intact appearance. This is in contrast with the situation in clones of Rap1 mutant cells in the wing. Here, adherens junctions do not encircle epithelial cells, but instead are condensed to one side. The composition of these aberrant adherens junctions appears normal as shown by the presence of DE-cadherin, α-catenin, β-catenin and Cno. In addition, their location along the apicobasal axis is unchanged. An interesting model was proposed where Rap1 is required to reseal the adherens junctions after cell division and does so via counteracting tension-generating components from the unclosed adherens junction (Knox and Brown, 2002). In contrast to wild-type mitotic lineages that usually remain in coherent group, Rap1 deficient wing cells have an aberrant cell shape and disperse into the surrounding wild-type tissue. Since the adherens junction phenotype is observed ahead of dispersion of mutant cells into wild-type clones, this sorting phenotype appears to be the consequence rather than the cause of abnormal adherens junctions. Loss of adherens junctions from a particular side of a cell has also been seen in the testis of dPDZ-GEF mutant flies (Wang et al., 2006). Here, continued proliferation of germ stem cells (GCS) critically depends on their attachment to a group of somatic cells, called the hub. In dPDZ-GEF mutant males, DE-cadherin is strongly diminished at the GCS/hub interface but appears normal between hub cells. As a consequence, GCS loose contact with hub cells and cannot maintain their stem cell identity (Wang et al., 2006).

In vertebrate tissue culture cells, a function for Rap1 in cadherin-mediated cell–cell adhesion has been demonstrated, but an uneven distribution of adherens junctions around the circumference of cells has not been mimicked. Blocking Rap1 in MDCK cells interferes in homotypic E-cadherin interactions (Price et al., 2004) and interferes in the recruitment of E-cadherin to nascent junctions in MCF7 cells (Hogan et al., 2004). In this latter study, CDC42 was postulated as a downstream effector of Rap1, in part based on the observation that an active isoform of CDC42 could overcome the inhibition of Rap1 in junction formation. It remains to be established how direct this effect is. In another study, the homologue of Cno, Afadin (also known as Af6), was suggested to be a critical Rap1 effector in cadherin regulation. Using a cell free assay, Hoshino et al. (2005) found that Afadin inhibits endocytosis of E-cadherin molecules that are not ligated to E-cadherin on neighboring cells. Interestingly, knock down of PDZ-GEF2 in A549 lung carcinoma cells did not drastically diminish E-cadherin levels at the cell surface, but increased the fraction of immature junctions (Dube et al., 2008). Immature junctions can be recognized by their zipper-like appearance upon staining for junctional proteins. Maturation into linear cell–cell junctions requires remodeling of the actin cytoskeleton (Mege et al., 2006), possibly via Myosin VI (Maddugoda et al., 2007). Thus, as seen for integrins (described below), Rap1 may play a role in the connection between cell surface adhesion molecules and the cytoskeleton, which are involved in complex interdependent interactions.

Loss of dPDZ-GEF also affects the shape and migration of macrophages in *Drosophila*. This effect results from the inability of Rap1 to regulate integrins (Huelsmann et al., 2006), but the nature of the downstream Rap1 effector here is elusive. Whether diminished integrin function also underlies the above-mentioned abnormal migration of mesectodermal and pole cells in Rap1 mutant embryos is currently unknown. Effects of Rap1 on integrins had already been demonstrated in various tissue culture cell lines (Caron et al., 2000; Katagiri et al., 2000; Reedquist et al., 2000) and will be discussed in more detail in the section on the function of Rap1 in mice.

Apart from Cno, two other effectors, namely D-raf (Lee et al., 2002; Mishra et al., 2005) and the RalGEF Rgl1 (Mirey et al., 2003) have been postulated to function downstream of Rap1 in *Drosophila.* Direct binding to GTP-bound Rap1 has been demonstrated both for D-raf and Rgl1. D-raf as well as dMEK and dMAPK were found to suppress a rough eye-phenotype induced by overexpression of dPDZ-GEF (Lee et

al., 2002). Surprisingly, elevated levels of Ras that are expected to enhance signaling via the above-mentioned elements of the MAPK pathway had a similar effect. Rap1 and D-raf have also been implicated in differentiation of terminal structures, downstream of the torso tyrosine receptor (Mishra et al., 2005). The idea that Rgl1 functions downstream of Rap1 is based on suppression of larval and eye-specific phenotypes induced by activated Rap1, that is seen upon lowering Rgl1 activity or expression of dominant negative Ral. The simplest explanation is that Rgl1 functions as a direct Rap1 effector. However, the authors keep the option open that Ral operates in a parallel pathway, required for effective Rap1 signaling (Mirey et al., 2003). This would be more similar to the situation in vertebrates. Here, Rap1 can bind with high affinity to various RalGEFs but no direct evidence has been presented that this leads to activation of the latter. For example, stimuli like cAMP analogues that increase the fraction of GTP bound Rap1 leave Ral-GTP levels unaffected (Zwartkruis et al., 1998).

Loss of RapGAP1, the closest homologue of human Rap1GAP does not result in any obvious phenotype, suggesting that it functions in a redundant fashion with another Rap1GAP encoded in the *Drosophila* genome (Chen et al., 1997).

Caenorhabditis elegans

In contrast to *Drosophila*, *rap-1* deficient worms are viable and fertile. During larval stages, the related RAP-2 (a clear homologue of mammalian Rap2) may compensate for the loss of RAP-1. A large fraction of *rap-1;rap-2* homozygous animals die as scrawny animals, unable to complete the molting processes that take place at the transition of the four larval stages (Frische et al., 2007). Their phenotype is very similar to that of null mutants of *pxf-1*, which encodes the *C. elegans* homologue of PDZGEF and activator of Rap1 and Rap2 (Pellis-van Berkel et al., 2005). Electron microscopy of *pxf-1* mutants reveals that their cuticles are abnormal. This is indicative for compromised secretion at the apical side of the outer epithelium of the worm, which is referred to as hypodermis. It is presently unclear if this results from hypodermal polarity defects or an impairment of the secretion process.

Although RAP-1 deficient embryos develop normally, they are highly sensitive to a decreased activity of RAL-1. RNAi for the RAL-1 effectors EXOC-8 and SEC-5 also results in embryonic lethality, while this does not affect wild-type embryos (Frische et al., 2007). In all cases, the stereotypical rows of hypodermal cells, which arise at the dorsal side of the embryo and move toward the ventral side to envelope the embryo in wild-type animals, are completely disorganized. Furthermore, the hypodermal cells in such embryos are hallmarked by the absence of the α-catenin homologue HMP-1 from adherens junctions. In contrast, the localization of the slightly more basally located DLG-1 protein is unaffected. A role for the Ral/exocyst pathway in targeting E-cadherin to the lateral membrane had already been proposed on the basis of studies in *Drosophila* (Classen et al., 2005; Langevin et al., 2005) and vertebrate (Shipitsin and Feig, 2004) cells. Most likely, in the absence of RAP-1, a proper balance between membrane targeting of the cadherin HMR-1 and its internalization cannot be maintained in the developing embryonic hypodermis when RAL-1 signaling is diminished. However, the molecular mechanisms by which RAP-1 exerts its function remain to be identified.

Lower vertebrates: Xenopus laevis and Danio rerio

Although the morphogenetic processes, in which Rap1 functions in flies and worms are well studied, the exact signaling pathways that regulate Rap1 are poorly defined. Interestingly, in *X. laevis* Rap1 was identified as a component of the non-canonical Wnt pathway (Tsai et al., 2007). This pathway is required for convergence extension (CE) movements during gastrulation. CE is a process that depends on localized cell traction, shape changes and migration and results in the transformation of a broad plate of dorsal mesoderm and neurectodermal cells into a more elongated structure (for a review, see Solnica-Krezel, 2005). Wnt8 signaling leads to activation of Casein Kinase I ε (CKIε), a protein that can bind to and phosphorylate the RapGAP SIPA1L1/E6TP1. As a consequence, SIPA1L1/E6TP1 is destabilized and an increase in GTP-bound Rap1 levels is seen. Interfering with Rap1 signaling in *Xenopus* embryos by injection of a CKIε-resistant isoform of SIPA1L1/E6TP1, dominant negative Rap1 or antisense Rap1A/B morpholino oligonucleotides leads to defects in CE. The resulting embryos show defects in the closure of the blastopore and have a short and bended body axis. No defects were observed in β-catenin-induced axis formation. A similar role for Rap1 was described in zebrafish (Tsai et al., 2007).

Rap1 has also been invoked in hingepoint formation during neural tube closure in *X. laevis* (Haigo et al., 2003). Hingepoints are formed by only a few cells of the neural plate that are marked by expression of the actin-binding protein Shroom. In these neural cells, Shroom protein is required for apical constriction, a process most likely mediated by the actin network. Shroom expression has a very similar effect in polarized cultured MDCK cells, but appears inactive in non-polarized cells of the *Xenopus* embryo. Dominant negative $Rap1^{N17}$ and Rap1GAP expression block apical constriction and result in defective neural closure. The exact role of Rap1 in this process or its downstream effector has not been identified.

Rap1 has not yet been extensively studied in zebrafish, but two proteins have been proposed to act as Rap1 effectors here. First, a Cno/Af6-related gene product named Radil was shown to be required for migration of neural crest cells (Smolen et al., 2007). Evidence that this protein functions as a Rap1 effector is mostly based on the specific interaction with GTP-bound Rap1. It will therefore be interesting to see if also genetic data can be obtained that sustain a role for Rap1 itself in neural crest cell migration. Secondly, the homologue of KRIT1 (Krev interactor 1)/CCM1 (cerebral cavernous malformation 1) may function downstream of Rap1 in zebrafish in endothelial junction formation (Gore et al., 2008). Although this study nicely shows synthetic RNAi effects for Rap1 and KRIT1/CCM, they do not form a solid base for epistasis analysis and this putative effector will be dealt with in the section on mouse.

Mus musculus

Rap1 and integrins

The mouse genome encodes two Rap1 genes, Rap1A and Rap1B, which differ by only nine amino acids and are both highly conserved in mammals. Two independent $Rap1A^{-/-}$ mouse strains have been generated and these appear viable and fertile (Duchniewicz et al., 2006; Li et al., 2007). Despite the fact that the immune system is functional and no major abnormalities in the various hematopoietic compartments were detected, isolated Rap1A-deficient cells display a number of anomalities. For example, spleen cells adhere less efficiently to fibronectin or ICAM. The differential adhesion was not due to a difference in the level of cell surface expression of the corresponding receptors (VLA-4 and LFA-1, respectively), suggesting that the process of integrin activation is affected. Furthermore, polarization of LFA-1 in T-cells following CD3 treatment was mildly affected (Duchniewicz et al., 2006). Adhesion of macrophages was also impaired, while random movement of these cells (haptotaxis) was enhanced, possibly as a consequence of less restrained detachment from the extra-cellular matrix. In contrast, directed cell migration of e.g. splenic B and T cells toward chemokines like CCL21 was reduced. Macrophages ingested IgG-coated red blood cells more efficiently, even though they possessed similar numbers of Fc-gamma receptors as their wild-type counterparts (Li et al., 2007). The relatively mild phenotype of Rap1A knock-out mice may result from

functional compensation by the related Rap1B protein, whose presence has been demonstrated in a number of Rap1A-negative cell types described above. Indeed, B-cells from Rap1B null mutant animals also show adhesion defects to ICAM-1, showing that both Rap1 isoforms can perform similar functions (Chu et al., 2008). Taken together, studies with knock-out mice confirm a role for Rap1 in activation of integrins as had previously been described in tissue culture cells (Caron et al., 2000; Katagiri et al., 2000; Reedquist et al., 2000; Tsukamoto et al., 1999) and in transgenic mice expressing a constitutively active Rap1A-V12 in T-cells. In this latter model, T-cells bind more strongly to fibronectin and ICAM-1 via avidity modulation of β_1 and β_2 integrins (Sebzda et al., 2002). Loss of Rap1B also interferes in $\alpha_{IIb}\beta_3$ integrin activation in platelets, where Rap1B is the predominantly expressed isoform. Platelets from Rap1B-deficient mice aggregate less well under the influence of various agonists and show diminished binding of soluble fibronectin to $\alpha_{IIb}\beta_3$ integrin, a direct measure for its activation state. In addition, a delay in the characteristic shape change of platelets was observed for all tested agonists and cell spreading on fibronectin was compromised (Chrzanowska-Wodnicka et al., 2005). An integrin activation defect is also seen in platelets of mice mutant for the calcium and diacylglycerol responsive Rap-1 GEF CalDAG-GEF1 (RasGRP2) (Crittenden et al., 2004). This is in line with the finding that Ca^+ and diacylglycerol lead to GTP-loading of Rap1 in human platelets (Franke et al., 1997). In other cell types or under the influence of other stimuli, Rap1-GEFs distinct from CalDAG-GEF1 appear crucial for integrin activation. PDZ-GEF2 is involved in TNFα-induced integrin activation in splenocytes (Yoshikawa et al., 2007), while C3G has a stimulatory effect on cell adhesion on collagen and fibronectin in mouse embryonic fibroblasts (Ohba et al., 2001).

How do Rap1 proteins mediate inside-out activation of integrins? Clearly, the integrin binding protein talin is essential for activation of integrins and appears to act together with kindlins (Larjava et al., 2008). Interestingly, Rap1 appears to be capable of activating a variety of integrins in many cell types with the notable exception of $\alpha_6\beta_4$ integrin, that is not coupled to the actin cytoskeleton (Enserink et al., 2004). In T-cells and dendritic cells, the Rap1 effector RapL and its splice isoform Nore1 (Rassf5) appear to play a crucial role (Katagiri et al., 2003). GTP-bound Rap1 induces complex formation of RapL with LFA-1 ($\alpha_L\beta_2$), thereby changing its affinity and spatial distribution. RapL is also present in platelets, where it may play an analogous role (Crittenden et al., 2004). The mechanism appears to involve activation of the RapL-bound kinase MST1 (Katagiri et al., 2006). However, RapL is absent from many other cell types that form integrin-mediated contacts with the ECM. In these cells, Rap1 may mediate its action via another effector, named RIAM (Lafuente et al., 2004). This adaptor protein is located at the leading edge of lamellipodia, where it interacts with actin-reorganizing proteins like profiling and Ena/VASP. Overexpression of RIAM induces cell spreading in HEK293 and Jurkat T-cells, whereas reduction of RIAM expression decreases cellular F-actin in Jurkat T-cells. Furthermore, RIAM overexpression was also found to enhance β_1 and β_2 integrin-mediated adhesion to fibronectin, although the molecular mechanism has not been resolved (Lafuente et al., 2004). Using a CHO model system, Han et al. (2006) demonstrated that Rap1-induced complex formation of RIAM and talin unmasks an integrin binding site in talin for the cytoplasmic tail of $\alpha_{IIb}\beta_3$ integrins. It will be interesting to see if genetic disruption of RIAM causes hemostasis defects, similar to that of Rap1B$^{-/-}$ mice or perhaps an even stronger phenotype due to general malfunctioning of integrins.

Rap1 and the vasculature

While the hemostasis phenotype described above for Rap1B mutant animals is not lethal, a bleeding phenotype results in 85% embryonic and perinatal lethality in this strain (Chrzanowska-Wodnicka et al., 2005). Since platelets are not required for embryonic hemostasis, the bleeding phenotype most likely results from a defective vasculature. Indeed, postnatal neovascularization of the retina is delayed in Rap1B mutant animals and microvessel sprouting from aortic rings *in vitro* under the influence of VEGF is impaired (Chrzanowska-Wodnicka et al., 2008). This may be caused by a combination of the observed decreased proliferation and migration of endothelial cells, but a role for smooth muscle cells cannot be excluded. Staining of adherens junctions for the major cadherin isoform in endothelial cells, VE-cadherin, did not reveal any obvious abnormalities. At the molecular level, activation of p38 MAPK by bFGF and VEGF was attenuated in Rap1B mutant endothelial cells (Chrzanowska-Wodnicka et al., 2008). Very similar effects, including impaired FGF2 induced angiogenesis and endothelial cell migration have been found in Rap1A mutant mice (Yan et al., 2008). Also ischemia-induced neovascularization was reduced in Rap1A mutant mice as demonstrated in an neovascularization assay (Carmona et al., 2009).

Mouse embryos homozygous mutant for PDZ-GEF1 provide additional evidence for a vascular function of Rap1. The primary defect in these animals is formation of the vasculature of the yolk sac: blood islands form normally, but fail to fuse and generate a plexus of vascular channels. Also intra-embryonic vasculogenesis is affected, but this might be a secondary defect resulting from the inability of the yolk sac to supply the embryo with nutrients. As a consequence, PDZ-GEF1$^{-/-}$embryos die around embryonic day 10 (E10) (Wei et al., 2007). Hypomorphic C3G mutant embryos die slightly later *in utero* with pronounced hemorrhage in neural tissues (Voss et al., 2003). Remarkably, in these embryos defective recruitment and/or adhesion of smooth muscle cells that support endothelial cells, was observed. Although the authors suggest that abnormal smooth muscle cells are causative for the phenotype, the primary defect may reside in endothelial cells. For example, in case of cerebral cavernous malformation (CCM) hemorrhages in neural tissues are also associated with endothelial cells lacking smooth muscle support and a mouse model system for this disease has established that an endothelial cell-autonomous effect underlies the lack of supporting cells (Whitehead et al., 2009). Another hallmark of CCM (not reported in C3G or Rap1 mutants) are thin-walled endothelial cells that encompass enlarged vascular structures. CCM results from autosomal dominant mutations in one of three genes, named CCM1/KRIT1, CCM2/OSM and CCM3/PCDC10. The proteins encoded by these genes can form a complex and CCM1 has been found to interact with GTP-bound Rap1 (Serebriiskii et al., 1999). Using *in vitro* cultured endothelial cells, Rap1 was found to increase the localization of CCM1/KRIT1 to adherens junctions and stimulate its interaction with junctional proteins like β-catenin and Af-6 (Glading et al., 2007). Conversely, knock down of CCM1/KRIT1 resulted in a disruption of β-catenin localization to adherens junctions and an increase in the permeability of the endothelial cell layer for horse radish peroxidase. Although this appears to be in line with a paucity of endothelial junctions in CCM patients as revealed by EM studies (Clatterbuck et al., 2001), it should be kept in mind that that study revealed many other ultra-structural changes, like the above mentioned lack of supporting cells, a thick collageneous matrix without intervening neural tissues and hemosiderin deposits. In addition, such an analysis in CCM1-and CCM2-deficient zebrafish showed that the thin-walled endothelial cells surrounding dilated vessels contained normal adherens junctions (Hogan et al., 2008). KRIT1 RNAi also drastically changes the cortical actin cytoskeleton into one with prominent stress fibers. A very similar phenotype is seen upon CCM2 knock down, which has been linked to activation of Rho (Whitehead et al., 2009). A reverse, albeit less dramatic effect is seen when Rap1 is activated in human umbilical vein endothelial cells using the Epac1-specific compound 8-CPT-2′O-Me-cAMP (Kooistra et al., 2005). Since this was also seen in sparse cell cultures, it is unlikely to result from KRIT1-mediated junctional strengthening.

In summary, disruption of Rap1A, Rap1B or Rap1-GEFs shows that Rap1 performs various functions in the vasculature of vertebrates. They may include regulation of cell–cell contacts and modulation of the cytoskeleton via CCM proteins. However, additional studies to more precisely define the vascular phenotypes are required to clarify how they relate to those observed in the absence of CCM-proteins, fibronectin or VE-Cadherin (George et al., 1997; Gory-Faure et al., 1999).

Rap1 in tumorigenesis

The first indication that Rap1 may be involved in tumorigenesis, independently of Ras, came from studies where Rap1B was expressed in Swiss 3T3 cells and injection of these cells in nude mice resulted in the formation of solid tumors, without any signs of metastasis (Altschuler and Ribeiro-Neto, 1998). Additional evidence for a role of Rap1 in tumorigenesis came from studies on Rap-GEFs and Rap-GAPs. In BHX-2 acute myeloid leukemic cells, the exchange factor CalDAG-GEF1 is activated leading to enhanced proliferation and morphological transformation (Dupuy et al., 2001). Deletion of SIPA1/SPA1 leads to expansion of bone marrow-derived pluripotential hematopoietic progenitors and chronic myelogenous leukemia in mice (Ishida et al., 2003). In line with this, overexpression of Rap1GAP has been reported to reduce squamous cell carcinoma growth (Zhang et al., 2006). SIPA1L1/E6TP1, a GAP for Rap1 has been identified as a target of the HPV E6 oncoprotein that mediates ubiquitin-dependent degradation leading to enhanced Rap1 activation and cellular transformation (Gao et al., 1999; Singh et al., 2003). Together these studies indicate that overactive Rap1 can enhance the proliferative capacity of certain cell types. However, it is important to note that Rap1 may also prevent tumorigenesis by counteracting metastatic processes. In prostate and ovarian cancer, mutations in an atypical Rap-GEF named Dock4, result in compromised Rap1-activation. Osteosarcoma cells, lacking functional Dock4, fail to form adherens junctions and show enhanced tumor invasion. Expression of Dock4 or $Rap1^{E63}$ in these cells reverses the phenotype (Yajnik et al., 2003). Similarly, SIPA1/SPA1 was identified as modifier of metastasis efficiency: a mutant allele enhances the invasive potential of breast cancer cells to the lung, whereas overexpression of wild-type SIPA1/SPA1 limits metastasis (Park et al., 2005). Similarly, overexpression of Rap1GAP increases invasion by regulating the metallo proteases 2 and 9 in squamous cell carcioma (Mitra et al., 2008). However, loss of Rap1GAP expression has also been linked to an increased ability of prostate cells, showing that cell type-specific contexts are important (Bailey et al., 2009). This may not come as a surprise since multiple pathways affect the cytoskeleton and integrin function in positive and negative fashions and a fine balance is most likely required for metastasis. Finally, Rap1 may contribute to tumorigenesis by enhancement of inflammation. This has been demonstrated in a chemically induced skin-tumor model, where Rap1 acts via PLCε, a Rap1 and Ras binding protein (Ikuta et al., 2008). Altogether, these studies indicate that Rap1 affects proliferative and metastatic properties of cancer cells. In addition, they support the notion that research on the role of Rap1 in processes like migration and cell–cell adhesion may contribute to a better understanding of tumorigenesis.

Concluding remarks and future prospects

Rap1 has been intensively studied during the last decade and biochemical approaches have identified the major regulatory proteins of Rap1. Genetic studies in model organisms have revealed a role for Rap1 and its regulators in various morphogenetic processes via establishment of polarity, regulation of adhesion/migration or cell–cell contact formation. A major challenge is now to understand what the molecular mechanisms are, that allow Rap1 to perform its diverse functions. Remarkably, immediate downstream effectors of Rap1 that have emerged so far from genetic studies differ for the various model organisms (Fig. 2). In yeast a single effector, the CDC42-GEF CDC24, is required for bud-site selection. In *Dictyostelium*, the non-conserved kinase Phg2 affects cell adhesion and cell shape by acting on the cytoskeleton. Cno functions downstream of Rap1 in various morphogenetic processes in *Drosophila*, but additional effectors must exist that are required for e.g. ventral invagination. Rgl1 and D-raf have also been put forward as Rap1 effectors in *Drosophila*. In mice, genetic evidence supports a role for RapL downstream of Rap1 in integrin activation in lymphocytes. Given that RapL expression is mostly confined to lymphocytes, further Rap1 effectors must play a similar role in other cell types. Intriguingly, although many of these effectors appear to act on the actin cytoskeleton, they do so in distinct manners. For example, CDC24 activates CDC42, Phg-2 phosphorylates MyoII, Cno may bind directly to actin and RapL associates with integrins and may indirectly affect the cytoskeleton.

The myriad of proposed Rap1 effectors suggests an uncommon versatile usage of this GTPase. It raises an immediate question about the enormous flexibility required during evolution to generate and adapt to novel effectors. Or should we be more critical when evaluating the presented data that have mostly been obtained in reverse genetic studies and sometimes involve pleiotropic phenotypes? We already indicated discrepancies in experiments with *Dictyostelium* Phg-2 mutants. Complementary biochemical studies are essential to provide solid evidence for an effector role of any given protein. In this respect, more detailed information about the interaction of Bud1 and CDC24 would be welcome. An equally important question is how GTP-bound Rap1 changes the activity of effectors like Phg2 and Cno, whose location is not affected by Rap1. Ultimately, demonstrating a direct change in conformation, post-translational modification or protein complex formation for candidate effectors upon stimulation of Rap1 is needed. This requires sophisticated techniques, like novel mass spectrometry approaches and real time imaging, to study *in vivo* changes of Rap1-associated proteins. Compounds that act rapidly and specifically on a single GEF like 8-

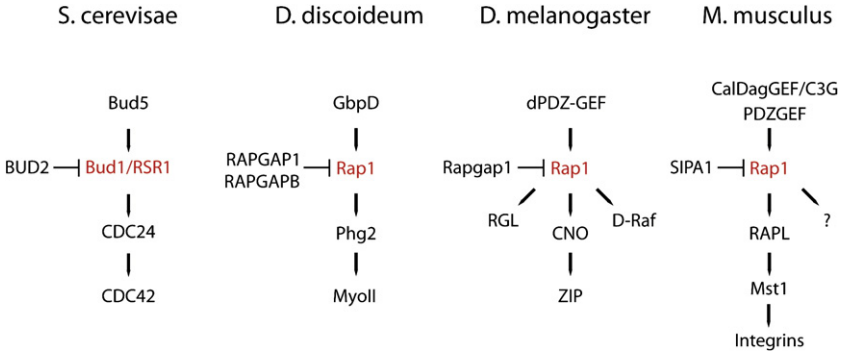

Fig. 2. Overview of the Rap1 orthologs and their upstream and downstream acting proteins as supported by genetic evidence. Proteins, whose function in Rap1 signaling networks is solely based on biochemical evidence, have not been included here.

CPT-2′O-Me-cAMP may turn out to be essential tools here. A second point that should be kept in mind is that genetic studies largely depend on similarities of phenotypes. This is relatively straightforward where proteins function to simply activate a downstream signaling element as in the case of Ras and Raf. However, for proteins like Rap1 whose function may be to localize rather than to drive a biochemical process, the situation is more difficult. In such cases, a protein acts in parallel with other proteins whose diminished function may or may not result in an identical phenotype. Indeed, the phenotype of Bud1 mutants in yeast differs from that of most CDC24 mutant. The studies on Cno in *Drosophila* where mutant proteins are tested in a null background are a good example of how genetics can contribute to a better understanding of the Rap1 signaling network. A similar detailed analysis of the Cno ortholog Af6 in mice may reveal whether this protein functions as a Rap1 effector. Also a comparison of Af6 null mutant mice that die as embryos with ultra-structurally abnormal cell–cell contacts and polarization defects (Zhadanov et al., 1999) with early embryonic lethal Rap1A/Rap1b double mutants (Chrzanowska-Wodnicka et al., 2008) would be of interest.

In conclusion, the enigma of Rap1 and its many putative effectors cannot be solved by genetics only. Genetics will however remain crucial for validating outcomes from biochemical studies and evaluating the suitability of Rap1 and its regulators as drug targets in disease processes like thrombus formation and metastasis.

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