Rap1 and Ral signaling networks in *C. elegans* and mammals

Ester Willemijn Frische

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Rap1 en Ral signaleringsnetwerken in *C. elegans* en zoogdieren

(met een samenvatting in het Nederlands)

Proefschrift

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Promotor: Prof. dr. J.L. Bos

Co-promotor: Dr. ir. G.J.T. Zwartkruis

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"I´ve reason to believe that what I find
is gonna change the face of human kind
and all these years before well I was blind
that´s my conclusion
cause I´m the ..."

dEUS, the Architect

voor mijn moeder en haar moeder

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Introduction

Rap1 and Ral: lessons learned from model organisms.

Abstract

The small GTPases Rap1 and Ral are close homologs of Ras that both regulate important signaling networks. A number of the ideas about the function of these proteins have originated from phenotypes seen upon interfering in their expression in model organisms. Biochemical experiments have frequently confirmed these ideas and at least partially revealed the underlying molecular mechanisms. For Rap1 these functions include regulation of cell polarity, cell adhesion and cell-cell contact formation. Ral is, among others, activated by Ras and forms one of the pathways involved in Ras-mediated tumorigenesis. Ral regulates basolateral protein transport, endocytosis and filopodia formation, but also transcription. Here we will discuss the role of Rap1 and Ral in different model systems and relate this to available biochemical data.

Introduction

The small Ras-like GTPases form a protein family whose members function as molecular switches regulating many signaling processes like proliferation, differentiation, morphogenesis and apoptosis. In addition to the Ras proteins (H-, K-, N-Ras), this family comprises R-Ras, Rheb, Rit, Rin, TC21, DexRas1 and the Rap and Ral proteins. The amino acid residues involved in binding of GTP and effectors are conserved among the members of this protein family. These proteins are regulated via several mechanisms among which are subcellular localization and phosphorylation. However, in general, activation is regulated via the cycling between the active GTP-bound state and the inactive GDP-bound state and this involves guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). The latter enhance the intrinsic GTPase activity, resulting in hydrolysis of GTP. Activation of GTPases involves the activity of GEFs that induce the release of bound GDP, allowing the rebinding of the more abundant GTP (Bos et al., 2007). The Ras proteins are the best-studied of this family since Ras proteins are found to be mutated in ~15% of all human tumors (Bos, 1989). Recently, more interest in other family members has lead to a better understanding of the Rap and Ral proteins. Here we will give an overview on the knowledge of Rap1 and Ral provided by genetic experiments and make a comparison with the available biochemical data of these proteins.

The small Ras-like GTPase Rap1

Rap1 was found in a revertant screen for Ras-induced cell transformation suggesting that Rap1 antagonizes Ras activity (Kitayama et al., 1989). Nowadays, the mammalian Rap proteins Rap1 (A and B) and Rap2 (A, B and C) are

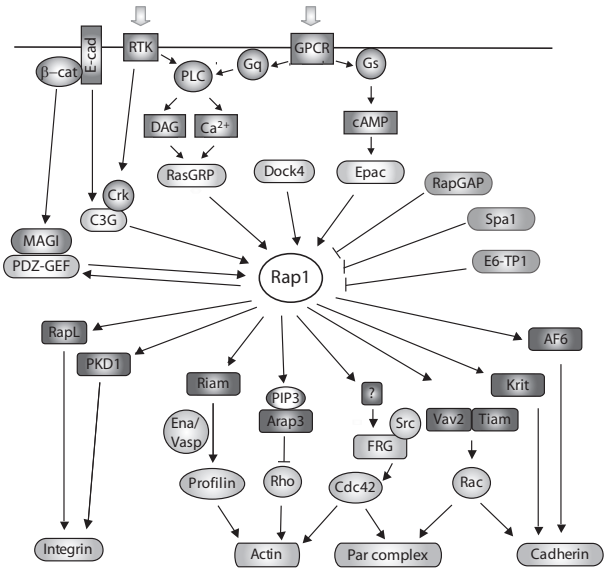

Figure 1: **Overview of the Rap1 signaling network (color image, p124).** Adapted from (Kooistra et al., 2007). DAG, Diacylglycerol; GPCR, G-protein coupled receptor; PIP3, Phosphoinositol-tri-phosphate; PLC, phospholipase C; RTK, Receptor tyrosine kinase.

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) and the 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), secretion (D'Silva et al., 1998) and osmotic stress (Kang et al., 2002). Thus far, many upstream regulators and downstream effectors have been identified in the Rap1 signaling network and multiple proteins are implicated in Rap-1 regulated processes (Figure 1). For instance, Rap1 is involved in the regulation of adherens junctions and multiple exchange factors, C3G, PDZ-GEF1/2, Epac1 and Dock4 have been implicated in this process. AF-6 and KRIT-1 are Rap1 effector proteins in junction formation. Regulation of integrin-mediated adhesion involves the RapGEFs, Epac, PDZ-GEF and CalDAG- GEF1 and the effector proteins RIAM and RAPL, although the latter act via different mechanisms (Han et al., 2006; Kinashi and Katagiri, 2005). Several reviews give an overview on the role of Rap1 in integrin-mediated adhesion (Bos, 2005; Kinashi and Katagiri, 2005) and in adherens junctions formation (Kooistra et al., 2007; Pannekoek et al., in press).

Saccharomyces cerevisiae

The budding yeast *Saccharomyces cerevisiae* requires polarization to establish the bud-site, the site where the daughter cell will grow and separate. 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 homolog of Rap-proteins in *Saccharomyces cerevisiae*, Bud1p/Rsr1p, leads to randomized localization of the bud-site (Cabib et al., 1998; Chant and Herskowitz, 1991). Indeed, also loss of proteins with GEF and GAP activity for Bud1p/Rsr1p, Bud5 and Bud2, respectively, leads to random bud-site formation (Bender and Pringle, 1989; Park et al., 1993). Bud-site assembly requires the recruitment of Cdc24p, an exchange factor for the GTPase Cdc42p, by GTP-bound Bud1p/Rsr1p. In addition, the scaffold protein Bem1p binds to Cdc24p and 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 the establishment of polarity by regulating the actin cytoskeleton via a single effector, Cdc24p. The role of Bud1p/Rsr1p in the establishment of polarity is reminiscent of the observation *in vitro* in mammalian cells where Rap1 has been found to be involved in the establishment of polarity. For instance, in lymphocytes Rap1 is pivotal for chemokine-induced cell polarization (Shimonaka et al., 2003) and in hippocampal neurons for axon-fate determination (Schwamborn and Puschel, 2004). Rap1 activates Cdc42 and thereby localizes and activates the Par-complex (Gerard et al., 2007; Schwamborn and Puschel, 2004). Moreover, Rap1 recruits Vav2 (Arthur et al., 2004), the closest mammalian homolog of Cdc24p, and Tiam1 (Arthur et al., 2004; Gerard et al., 2007), two exchange factors for the regulator of actin dynamics Rac1.

Dictyostelium discoideum

The unicellular amoeba *Dictyostelium discoideum* responds to its environment by adhesion and formation of multi-cellular aggregates. Rap1 controls cell polarity, cell adhesion, endocytosis, phagocytosis and osmotic stress resistance in the amoeba *Dictyostelium discoideum* (Chubb and Insall, 2001; Kortholt and van Haastert, 2008; Rebstein et al., 1997; Seastone et al., 1999). In this organism, Rap1 is presumably an essential gene since no knock-out cells have been obtained (Kortholt et al., 2006). Thus far, two regulators have been identified, the GEF GbpD and GAP RAPGAPA. Overexpression of Rap1 leads to more spread and flat cells that have more peripheral F-actin. Indeed, these cells adhere more strongly to the extracellular matrix (Kortholt et al., 2006; Rebstein et

al., 1993). GbpD-mediated activation of Rap is required for regulation of both cell polarity and cell adhesion. The latter requires the downstream effector Phg2, a serine/threonine kinase whereas the effector for cell polarity establishment is not identified yet (Kortholt et al., 2006) (Figure 2). So in *Dictyostelium,* Rap1 controls two distinct functions, cell polarity and adhesion, via different effectors.

Caenorhabditis elegans

In contrast to Rap1 deficient *Dictyostelium*, *Drosophila* (discussed below) and mice (discussed below), *rap-1* deficient worms are viable and fertile. The presence of two additional Rap-family members, RAP-2 and RAP- 3 may compensate for the loss of RAP-1. The function of RAP-3 has not been analyzed in detail, but might be distinct from the other Rap proteins, given its atypical CAAX-box, consisting of five amino acids and unusual effector domain (Riedl, 2002). A large fraction of the progeny of *rap-1;rap- 2* null animals usually die during larval development and escapers show molting defects and appear scrawny. Null mutants for *pxf-1*, a GEF for RAP-1 and RAP- 2, have a similar phenotype. The molting defects observed in *rap-1;rap-2* mutants are indicative of compromised secretion of cuticle components, suggesting that RAP-1 is required for secretion. Strikingly, embryogenesis is largely normal in *rap-1* and *rap-1;rap-2* null mutants and no indications are seen for integrin- or cadherin-mediated adhesion defects (Pellis-van Berkel et al., 2005). However, *rap-1* null animals arrest during embryonic development upon RAL-1 depletion by siRNA treatment, whereas *rap- 2* null mutants appear normal (Frische et al., 2007). The epidermal cell organization is disturbed in embryos of *rap-1* null animals subjected to *ral-1(RNAi)* and the cadherin-catenin complex is lost from the epidermal cell-cell contacts. Altogether, this shows that RAP-1 and RAP-2 do not simply act redundantly and that RAP-1, although not essential, is involved in regulation of cell adhesion and cell polarity.

Drosophila melanogaster

Studies on the function of Rap1 in *Dro sophila* have been crucial for elucidation of its role in migration and adherens junction formation in addition to the previously in *Saccharomyces,* identified role in the establishment of polarity. Rap1 is an essential gene in *Drosophila* and whereas homozygous Rap1 null mutants die at larval stages, depletion of maternal input is embryonic lethal. These embryos show different morphogenetic and migration defects of which closure of the ventral furrow is the most frequent (Asha et al., 1999; Hariharan et al., 1991). Interestingly, all observed phenotypes appear to be

caused by defects in migration and not by defects in cell specification: nuclear migration to establish cellular organization of cells in the ventral furrow but also migration of the pole-cells and internalized mesodermal precursor cells was disturbed. In post-mitotic cells, Rap1 is not required for viability. Indeed, adult flies do not depend on Rap1 for survival, however mutant female flies stop producing progeny due to degeneration of the egg chambers (Asha et al., 1999). Thus, in *Drosophila,* Rap1 is required for embryogenesis by regulating migration, a process that requires the control of actin cytoskeleton dynamics, cell adhesion and cell polarity. Furthermore, Rap1 has been found to function downstream of the exchange factor PDZ-GEF/Dizzy/gef26 (Huelsmann et al., 2006) in integrin-de pendent cell adhesion, similar to the role of Rap1 in mammalian cells (Caron et al., 2000; Katagiri et al., 2000; Reedquist et al., 2000). Lastly, during dorsal enclosure, a process that depends on migration and elongation of ectodermal cells, PDZ-GEF, Rap1 and Canoe/AF6 are required (Boettner et al., 2003; Boettner and Van Aelst, 2007).

Rap1 also localizes to (Boettner et al., 2003) and regulates adherens junctions in the fly (Knox and Brown, 2002; Wang et al., 2006). Rap1-deficient wing cells have an aberrant cell shape and disperse into the surrounding wild-type tissue, whereas lineage-related cells usually stay in a coherent group. The adherens junctions of Rap1 mutant cells appear normal when forming contacts with wild-type neighboring cells, but the adherens junctions are condensed to one side of the cell when contacting mutant cells. The molecular composition of these adherens junctions does not seem to be affected; *Drosophila* E-cadherin, β-catenin, α-catenin and AF6 are normally present. Since the adherens junction phenotype is observed ahead of dispersion of mutants cells into the wild-type clones, the migration phenotype appears to be the consequence rather than the cause of this phenotype (Knox and Brown, 2002). Altogether, Rap1 functions in cell polarity, cell adhesion and adherens junctions in *Drosophila,* which is in agreement with data obtained in experiments in mammalian cells.

Xenopus laevis

Interfering in Rap1 signaling in *Xenopus* embryos, by injection of antisense Ra p1A/B morpholino oligonucleotides, leads to defects in gastrulation, a process requiring extensive and organized cell movements (for a review(Solnica-Krezel, 2005)). These embryos show defects in the closure of the blastopore and have a short and bended AP-axis. Activation of Rap1 occurs, at least partially, via degradation of the Rap1 specific GAP (SIPA1L1/E6TP1) by Wnt-8 in a casein kinase Iε (CKIε)-dependent manner (Tsai

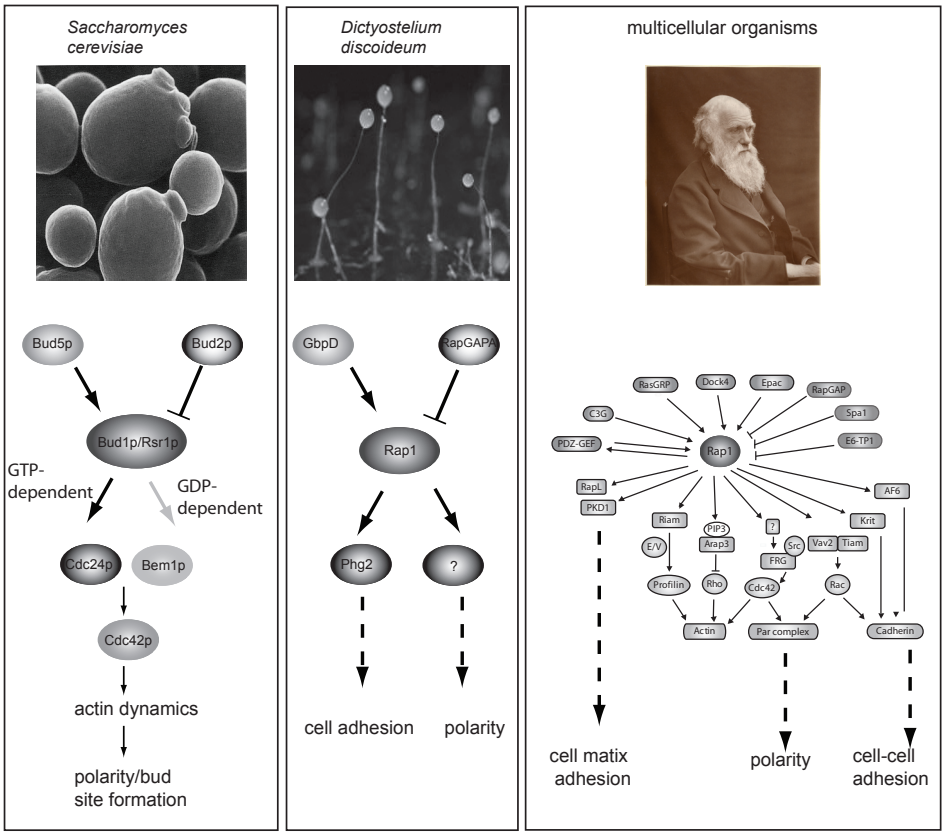

Figure 2: **Overview of the increasing complexity of the Rap1 signaling network from yeast to multicellular organisms (color image p125).**
In yeast, Rap1 regulates cell polarity via a single pathway (picture courtesy of Dr. Alan Wheals, University of Bath). In *Dictyostelium discoideum*, Rap1 controls cell polarity and adhesion via two pathways (picture courtesy of Dr. Kei Inouye, Kyoto University). The Rap1 signaling network regulates several pathways in multicellular organisms (see also figure 1) (Image of Charles Darwin is reproduced with permission from John van Wyhe ed., The Complete Work of Charles Darwin Online (http://darwin-online.org.uk/)).

et al., 2007). Thus, depletion of Rap1 during embryogenesis results in defective cell migration.

Danio rerio

Like in *Xenopus,* Rap1 is implicated in the regulation of gastrulation downstream of the Wnt8-CKIε pathway, independently of β-catenin functioning in zebrafish (Tsai et al., 2007). Knock-down of Rap1B leads to AP-axis elongation defects which are slightly enhanced upon simultaneous depletion of Rap1A, a phenotype suggestive of a role for Rap1 in regulating cell migration during development. Other evidence for a role of Rap1 in migration and cell adhesion is provided by RADIL (AF-6 like) knock-down studies. In zebrafish, RADIL is identified as a Rap1-effector protein and interfering in its

function leads to defects in neural crest cell migration (Smolen et al., 2007).

Mus musculus

Two independent Rap1A$^{-/-}$ mouse strains have been generated and these appear normally viable and fertile (Duchniewicz et al., 2006; Li et al., 2007). Adhesion and migration of leucocytes derived from Ra p1A$^{-/-}$ mice, is affected. In these cells, integrin expression levels at the cell surface are normal, however localization is affected, indicating a cell polarity defect. Strikingly, the immune function of Ra p1A$^{-/-}$ mice is not affected. In contrast to the relatively mild phenotype observed in Rap1A$^{-/-}$ mice, deletion of Rap1B leads to 85% embryonic or perinatal lethality due to a bleeding phenotype (Chrzanowska-Wodnicka et al., 2005). Rap1B regulates neovascularization and microvessel sprouting in angiogenesis (Chrzanowska-Wodnicka et al., 2008). Interestingly, the surviving Rap1B$^{-/-}$ mice are viable with a normal lifespan, although offspring of these mice is smaller compared to wild-type mice. However, these mice show defects that indicate impaired integrin-mediated adhesion. For instance, development of B-cells and T-cell-dependent humoral immunity appears impaired in Rap1B$^{-/-}$ mice, although the function of Rap1B in these cells is not resolved (Chu et al., 2008). In addition, platelet aggregation appeared reduced in a tail bleeding experiment, presumably due to compromised integrin-mediated adhesion. It should be noted that these defects can not explain the bleeding phenotype of the majority of the Rap1B$^{-/-}$ mice, since platelets are not required during embryogenesis (Chrzanowska-Wodnicka et al., 2005). In addition, Rap1A or Rap1B signaling is required for survival since no viable Rap1A$^{-/-}$ and Rap1B$^{-/-}$ progeny was obtained from Rap1A and Rap1B knockout animals (Li et al., 2007). Unfortunately, additional data on these mice are lacking.

Thus, both Rap1A and Rap1B mouse models show defects in integrin-mediated adhesion. This is in line with a mouse model expressing Rap1V12 in T-cells, whose integrin-mediated adhesion was enhanced (Sebzda et al., 2002). Deletion of the Rap-effector protein RAPL leads to a more severe phenotype, but also showed impairment of integrin-mediated adhesion leading to defects in trafficking and homing of lymphocytes (Katagiri et al., 2004). In mouse models of other components of the Rap1 signaling network, similar defects have been described: PDZ-GEF2 deficient mice show impaired integrin-mediated adhesion of splenocytes (Yoshikawa et al., 2007) and also the CalDAG-GEF1 knock-out mice have impaired integrin-mediated adhesion (Crittenden et al., 2004). In this study, integrin-dependent aggregation of platelets was reduced,

a phenotype similar to that of Rap1B$^{-/-}$ surviving mice. These mice reveal an important role for Rap1 signaling in regulation of integrin-mediated adhesion of leucocytes and platelets, whereas integrin expression appeared normal.

Several studies place Rap1 signaling also in vascular development. PDZ-GEF1 knock-out mice die at E9.5 showing defects in the major blood vessels (Wei et al., 2007). The defects in vessel formation might be due to failure of integrin-mediated adhesion and cadherin-mediated cell-cell contact formation (George et al., 1997; Gory-Faure et al., 1999). In line with this, blood vessels and aortic rings derived from Rap1A$^{-/-}$ mice failed to grow or produce new blood vessels, respectively, in response to FGF2 (Yan et al., 2008).

Hypomorphic C3G mice die of heamorrhages and vascular defects at E11.5, but unlike the PDZ-GEF1 mice, the endothelial cells develop normally. In these mice, the vascular supporting cells lack paxillin and β1-integrin-mediated adhesion leading to defective supporting tissue (Voss et al., 2003). The knock-out mouse for the Rap-GAP, SIPA1/SPA1, has impaired hematopoietic stem cell development leading to the expansion of progenitor cells in the bone marrow (Ishida et al., 2003).

Although many studies in tissue culture cells have shown a role for Rap1A and Rap1B in cell-cell contact formation, this was not observed in the above described mouse models. Only mice with disrupted Canoe/AF6, a Rap1 effector protein in *Drosophila*, have defective cell-cell contact formation. These mice die at E10 with reduced cellular polarization and impaired cell-cell junctions (Zhadanov et al., 1999).

Rap1 in tumorigenesis

The first notice that Rap1 may be involved in tumorigenesis, independently of Ras, came form studies where Rap1B was expressed in Swiss 3T3 cells and injection of these cells in nude mice resulted in the formation of tumors (Altschuler and Ribeiro-Neto, 1998). Other evidence for a role for 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 to morphological transformation (Dupuy et al., 2001). Regulation of the activity of Rap by GAPs is also implicated in tumorigenesis: E6-TP1, a GAP for Rap1, is identified as a target of the HPV E6 oncoprotein leading to ubiquitin-mediated degradation and enhanced Rap1 activation and cellular transformation (Gao et al., 1999; Singh et al., 2003). Moreover, deletion of SIPA1/SPA1 leads to expansion of the marrow pluripotential hematopoietic progenitors and chronic myelogenous leukemia (Ishida et al., 2003). Overexpression of Rap1GAP regulates processes in

squamous cell carcinoma with opposite tumorigenic effects: it reduces tumor growth (Zhang et al., 2006b), whereas it increases invasion by regulating the metallo proteases 2 and 9 (Mitra et al., 2008). Additional evidence that Rap1 may also have a tumor suppressive effect comes from studies on the invasive potential of breast cancer cells to the lung. Here, enhanced activity of SIPA1/SPA1 induces the invasive potential (Park et al., 2005). Furthermore, in prostate and ovarian cancer, mutations in Dock4, an atypical Rap GEF, result in compromised Rap1-activation. Osteosarcoma cells, lacking functional Dock4, fail to form adherens junctions and show enhanced tumor invasion. Expression of Dock4 or Rap1-63E in these cells reverses this phenotype (Yajnik et al., 2003). Altogether, these studies indicate that Rap1 may have different effects on the invasive and metastatic capabilities of cancer cells and research on the role of Rap1 in processes like migration and cell-cell adhesion may contribute to a better understanding of Rap1 in tumorigenesis.

Concluding remarks Rap

The studies in model organisms have identified a role for Rap1 in the establishment of polarity, regulation of migration and in cell-cell contact formation. Studies in cell culture have addressed questions on the molecular mechanisms involved in these processes and for example identified integrins as targets for Rap1 signaling. When comparing Rap1 signaling in the unicellular organisms *Saccharomyces cerevisiae* and *Dictyostelium discoideum* and multicellular organisms, a progressive complexity is seen (Figure 2). Yeast requires polarization for bud-site selection, but other Rap-requiring processes are not present. In the more complex organism *Dictyostelium,* regulation of polarization and adhesion is controlled via Rap1. In addition to cell polarity and cell adhesion, in multi-cellular organisms cell-cell contact formation is also regulated by Rap1. Indeed, depletion of Rap1 in *Drosophila* or AF6 in mice results in defects in cell-cell contacts. Importantly, the role of Rap1 in junction formation was only revealed in *Drosophila* upon clonal deletion of Rap1 from wing epithelial cells. Deletion of Rap1 at earlier stages leads to defects in morphogenesis of epithelia and in migration of mesodermal cells, preventing the formation of a viable embryo.

A role for Rap1 in regulation of integrins, as originally found in tissue culture cells, has now been extended to mice, where deficiencies in Rap1 lead to diminished integrin activation in haematopoietic cells and platelets. In *Drosophila*, the Rap1GEF PDZ-GEF/Dizzy/GEF26 regulates integrin function in macrophages and cadherin function in germ stem cells.

Apparently, these adhesion molecules are not in all cells equally important for morphogenesis. Thus, the data obtained with forward genetics show that Rap1 indeed plays a role in cell polarity, cell adhesion and cell/cell contacts, however the molecular mechanisms identified in biochemical experiments can be less prominent *in vivo*.

The small GTPase Ral

The Ral branch, RalA and RalB, of the Ras-like GTPases was identified in simian immortalized B-lymphocytes based on their homology with the Ras proteins (Chardin and Tavitian, 1986). Like other members of the Ras-like GTPase family, Ral proteins are regulated by GEFs (Figure 3). To date, two families of exchange factors for Ral have been identified; one family containing an RBD (Ras-binding domain) (RalGDS, Rgl and Rgl2/Rlf) and a family without RBD domain (RalGPS1 and RalGPS2) (reviewed in (Feig, 2003; Quilliam et al., 2002)). The RBD-containing GEFs can be recruited by Ras to the plasma membrane, thereby bringing Ral and RalGDS to the same cellular compartment (Matsubara et al.,1999). Thus far, no GTPase activating protein for Ral proteins has been identified, although in brain, testis (Emkey et al., 1991) and platelets (Bhullar and Seneviratne, 1996) Ral-GAP activity is measured. Interestingly, other mechanisms of regulation of Ral proteins have been identified. The serine/threonine kinase AuroraA phosphorylates RalA on serine194 and thereby enhances RalA activity (Wu et al., 2005) and dephosphorylation occurs via the phosphatase PP2A at serine183 and 194 (Sablina et al., 2007).

Several Ral effector proteins have been identified and these can be divided in two groups (figure 3), the ones that signal through transcription regulation and those that act directly on vesicle transport and actin dynamics: The first identified Ral-effector was RalBP (Ral binding protein/RLIP76), which is implicated in receptor-mediated endocytosis. RalBP contains a GAP domain that can negatively regulate Cdc42 and Rac *in vitro*, although *in vivo* evidence is limited (Feig, 2003; Park and Weinberg, 1995). Ral regulates secretion of vesicles via the exocyst complex. Ral binds directly to Sec5 and Exoc8, two members of the exocyst complex, and is implicated in basolateral protein transport, endocytosis and filopodia formation (Jin et al., 2005; Moskalenko et al., 2002; Moskalenko et al., 2003; Sommer et al., 2005; Sugihara et al., 2002). Ral may also regulate filopodia formation via another pathway by binding to filamin, an actin filament crosslinker (Ohta et al., 1999).

Transcription regulation via Ral is mediated via several transcription factors, like c-Jun and FOXO (de Ruiter et al., 2000; Essers et al., 2004). However, the direct effector molecule in these

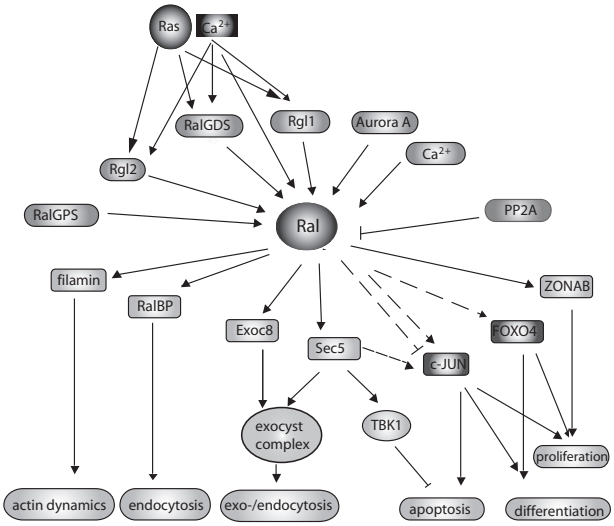

Figure 3: **Overview of the Ral signaling network (color image p124).** RalA effector proteins that bind directly to Ral are depicted in yellow whereas the ones that do not bind directly are depicted in orange. Note that there is no GAP-protein identified, but PP2A may inactivate Ral via dephosphorylation (Sablina et al., 2007).

processes is not identified yet. ZONAB is unique in that it is a transcription factor directly activated via Ral. ZONAB is a Y-box transcription factor and localizes to the nucleus in low-density cells, but upon formation of cell-cell contacts, ZONAB is recruited to the tight junction protein ZO-1 (Frankel et al., 2005). Thus, Ral regulates several signaling pathways and here we will summarize the evidence gained in model systems.

Saccharomyces cerevisiae and Dictyostelium discoideum

Saccharomyces cerevisiae and *Dictyostelium discoideum* do not have a gene encoding for a Ral protein. Phylogenetic analysis revealed that a *ral* gene is present in *Zygomycota,* which are primitive fungi, but not in the *Diamycetes* (containing filamentous fungi, yeasts and mushrooms). The separation of the *Zygomycota* and *Diamycetes* occurred after the separation from the animals and therefore *Diamycetes,* including *Saccharomyces cerevisiae* have lost the *ral* gene during evolution. *Dictyostelium discoideum* does not contain a *ral*-gene indicating that this gene developed after the separation of *Dictyostelium discoideum* from the Animal and Fungi branch (personal communication T.J.P. van Dam).

Caenorhabditis elegans

In *C.* elegans, a single *ral* gene is present encoding the RAL-1 protein. Worms carrying the *ral-1(tm2760)* null allele die during late larval stages (chapter 4) and depletion of RAL-1 by injection of dsRNA leads to embryonic lethality (Sonnichsen et al., 2005). Interestingly, depletion of the single identified Ral-GEF, RGL-1/Ral GDS did not affect viability, suggesting that exchange activity is not essential for Ral signaling. As described above (Rap1

in *C. elegans*), RAL-1 appears to have a role in epithelial cell organization and localization of cadherin and catenin at the cell surface. SEC-5-deficient worms arrest during the L4-stage, whereas hypomorphic *sec-5* alleles develop into adults, but produce only little progeny (Frische et al., 2007). Interestingly, these hypomorphic *sec-5* worms, arrest at the L3-stage in a *rap-1* null mutant background, indicating that the RAL-1/exocyst complex and RAP-1 pathway also genetically interact at later stages of development.

Xenopus laevis

Two Ral-proteins, RalA and RalB, are identified in *Xenopus*. During gastrulation RalB functions downstream of Ras-Ral GDS and expression of dominant negative Ral (S28N) mutants or membrane targeted RalBP leads to failure in blastopore closure (Lebreton et al., 2004; Lebreton et al., 2003). Possibly, blastopore closure defects result from cytoskeletal abnormalities caused by the fact that Ral is no longer able to inactivate Cdc42 via RalBP (Boissel et al., 2007).

Drosophila melanogaster

Only a single *ral* gene is encoded on the X-chromosome of the *Drosophila* genome and Ral is essential during embryogenesis. Strains carrying a hypomorphic *ral* allele are viable but show defects in the development of sensory organs (Balakireva et al., 2006). Interfering in Ral signaling by expression of RalG20V or RalS25N mutants leads to developmental defects, like failure of dorsal closure and disorganized hairs and sensory bristles on the cuticle (Sawamoto et al., 1999a). Additionally, RalG23V expression in the developing eye leads to disruption of the ommatidial organization and to cell shape changes, whereas expression of wild-type Ral had no effect. In these eyes, the actin cytoskeleton was disrupted indicating a role for Ral in regulation of the actin cytoskeleton (Sawamoto et al., 1999b). Although the Ral23V mutants are constitutively GTP-bound, they have been described as interfering mutants and therefore these mutants should be used with care (Feig, 2003). Use of a hypomorphic *ral* allele reveals that defective Ral signaling leads to enhanced apoptosis. Analysis of the bristles, a mechanosensory organ, shows that reduced Ral signaling induced apoptosis of one of the cells in the bristle. Interestingly, Ral-mediated reduction of apoptosis appears to be dependent on the exocyst complex and to link the exocyst complex to Ral-mediated signal transduction via the MAPKKK, MAP4K4 (Balakireva et al., 2006).

Mus musculus

The mouse genome encodes two Ral-proteins and so far, no Ral knock-out mouse model has been described, but

a transgenic mouse expressing RalA28N in the adult central nervous system has been made. In these mice, a block in the formation of the readily releasable pool of synaptic vesicles was observed, confirming *in vivo* that Ral has a role in vesicle formation (Polzin et al., 2002). RalGDS knock-out mice appear normal, suggesting redundant functioning of other Ral-exchange factors (Gonzalez-Garcia et al., 2005). Interestingly, in line with the observation in *Drosophila,* apoptosis in these mice is increased and as a consequence, RalGDS knock-out mice are protected from Ras-induced skin tumor formation (Gonzalez-Garcia et al., 2005). Thus far, no mouse models are present for Ral-effectors, however, mice lacking Sec8, a member of the exocyst complex, are embryonic lethal and die of gastrulation defects (Friedrich et al., 1997). The development of the AP-axis is delayed but appears normal, however developmental defects in the paraxial mesoderm lead to developmental arrest. In addition, the hemoglobin deficit *hbd* mice carry an in-frame deletion of exon 8 of the *Sec15l1* and these mice show defects in exocytosis of the transferrin receptor (Garrick and Garrick, 2007; Lim et al., 2005; Zhang et al., 2006a). It seems likely that due to the in-frame deletion, these are hypomorphic mice or that the *Sec15l2* is partially redundant.

Ral in tumorigenesis

Activation of both RalA and RalB is implicated in tumorigenesis via different mechanisms, which to a large extend requires Ras-mediated activation of Ral via the RBD-containing GEFs (for a review (Bodemann and White, 2008)). RalA is required for anchorage-independent growth of tumor cells, whereas RalB is required for survival of tumor cells. Although several effector proteins have been identified, the involved downstream pathways in tumorigenesis remain to be elucidated. The importance of Ral signaling has been shown in different tumor models where depletion of Ral inhibited tumor formation. For instance, subcutaneous injection of pancreatic cancer cells in nude mice induces tumor formation, but this is reduced upon RalA knock-down (Lim et al., 2006). Likewise, RalA depletion reduces bone metastasis in a prostate cancer model by reducing growth, although it is not clear whether this is due to reduced proliferation or induced apoptosis (Yin et al., 2007). A RalGDS mouse model provides a system to study Ras-induced skin carcinogenesis. In contrast with data previously obtained in tissue culture cells, Ral appears to induce tumorigenesis by inhibiting apoptosis instead of by increasing proliferation (Gonzalez-Garcia et al., 2005).

Concluding remarks Ral

Although the studies on Ral in model systems are limited, they provide important data on the function of Ral signaling. During embryogenesis, Ral seems mostly to affect the actin cytoskeleton, while during later stages, effects on apoptosis become more prominent. Based on biochemical studies, the effect on the actin cytoskeleton might require signaling through RalBP and/or the exocyst complex. However, Ral has also been implicated in adherens junction formation in *C. elegans* (Frische et al., 2007) and the exocyst complex in E-cadherin localization in *Drosophila* (Langevin et al., 2005) and this may be another way by which Ral is involved in embryogenesis. Secondly, Ral signaling is important during tumorigenesis downstream of activated Ras, although the involved downstream pathways are unknown. Recently, an interesting new link has been identified between Ral signaling and tumorigenesis. RalB was shown to activate TBK, an atypical IκB kinase family member, via the exocyst complex member Sec5, thereby reducing apoptosis (Chien et al., 2006). Altogether, it needs further investigation to get a better understanding how the Ral signaling network is activated and regulates processes required in development and tumorigenesis.

Scope of this thesis

The proteins of the Rap1 and Ral signaling networks do not show overlap (Figure 1 and 3) (Bos, 2005; Feig, 2003), however some of the cellular processes controlled by Rap and Ral are similar, like regulation of the actin cytoskeleton, secretion and adherens junction formation. At the start of this research, studies in yeast and *Drosophila* revealed a role for Rap1 in the establishment of polarity, adherens junction formation and in cell migration. In tissue culture cells, Rap1 was identified as a regulator of integrin-mediated adhesion. In the Ral signaling network, Ral GEFs were identified as targets of on cogenic Ras, thereby linking Ral to tumorigenesis. RalBP, filamin, Sec5 and Exoc8 were identified as direct Ral-effector proteins and additionally Ral was implicated in transcription-mediated regulation of processes involving apoptosis, proliferation and differentiation via several transcription factors like c-JUN and FOXO4. In this study, we present a genome-wide synthetic lethal screen and genetic interaction studies in *C. elegans* in order to obtain more insight in the RAP-1 signaling network. In addition, we investigated whether some of these observations could be extended to human tissue culture cells.

In the genome-wide synthetic lethal screen we used a *rap-1* null *C. elegans* mutant and this allowed us to identify

genes functioning in parallel to *rap-1*. Out of these emerged *ral-1* and exocyst complex members and we investigated the synergy between Rap1 and Ral in *C. elegans* (Chapter 2) and in human tissue culture cells (Chapter 3). Furthermore, we show in Chapter 3 that E-cadherin localization is dependent on the RalA/exocyst complex axis in A549 cells. A phenotypic analysis of other genes identified in the synthetic lethal screen in *C. elegans* is presented in Chapter 4. Finally, we investigated the possibility that MIG-10 is a direct effector protein of RAP-1 (Chapter 5).

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2

RAP-1 and the RAL-1/exocyst pathway coordinate hypodermal cell organization in *C. elegans*.

Adapted from:
Ester W. Frische, Wendy Pellis-van Berkel, Gijs van Haaften, Edwin Cuppen, Ronald H.A. Plasterk, Marcel Tijsterman, Johannes L. Bos and Fried J.T. Zwartkruis. EMBO Journal (2007) 26, 5083-5092

Abstract

The small Ras-like GTPase Rap1 has been identified as a regulator of integrin activation and cadherin-mediated cell-cell contacts. Surprisingly, null mutants of RAP-1 in *C. elegans* are viable and fertile. In a synthetic lethal RNAi screen with *C. elegans rap-1* mutants, the Ras-like GTPase *ral-1* emerged as one of seven genes specifically required for viability. Depletion of *exoc-8* and *sec-5*, encoding two putative RAL-1 effectors and members of the exocyst complex, also caused lethality of *rap-1* mutants, but did not affect wild-type worms. The RAP-1 and the RAL-1/exocyst pathway appear to coordinate hypodermal cell movement and elongation during embryonic development. They mediate their effect in part through targeting the α-catenin homolog HMP-1 to the lateral membrane. Genetic interactions show that the RAP-1 and RAL-1/exocyst pathway also act in parallel during larval stages. Together these data provide *in vivo* evidence for the exocyst complex as a downstream RAL-1 effector in cell migration.

Introduction

Cell migration is an important aspect of various biological processes like development and immune responses. Several members of the Ras-like family of GTPases are involved in the control of cell migration. For example, Rap1 affects migration by enhancing integrin-mediated cell-matrix attachment in many different cell types *in vitro* (Caron et al., 2000; Katagiri et al., 2000). In addition, Rap1 may inhibit migration of cells by stimulating the formation of cell-cell contacts via E-cadherin in adherens junctions (Hogan et al., 2004; Price et al., 2004). Also VE-cadherin, present in endothelial cells, is under the control of Rap1 (Fukuhara et al., 2006). The current view is that Rap1 acts locally via different effectors to mediate its effect on integrins and cadherins. For example, the Rap1-binding proteins RIAM, RAPL, TIAM and VAV have all been invoked as Rap1 effectors functioning in enhancement of cell-matrix contacts, whereas AF-6 and Cdc42 are prime candidates for regulators of cadherin in adherens junctions (for a review, see (Bos, 2005; Kooistra et al., 2007)). However, Rap1 can induce cell polarity in suspension cells (Shimonaka et al., 2003) and this polarizing activity of Rap1 may also contribute to the effects seen on integrins and cadherins. A second Ras-like GTPase involved in cell migration is Ral (Rosse et al., 2006; Suzuki et al., 2000). In its active, GTP-bound form, Ral binds to various effectors that play specific roles in migration. For example, Ral can induce filopodia formation by interacting with the actin-bundling protein filamin (Ohta et al., 1999). Filopodia are often found at the leading edge of migrating cells and outgrowing neurites. Furthermore, active Ral can bind to Sec5 (Brymora et al., 2001) and Exoc8 (Moskalenko et al., 2003;

Zhang et al., 2005), two proteins present in the exocyst complex that mediates targeting of E-cadherin to the basolateral plasma membrane. Binding of Ral to Sec5 and Exoc8, which are most likely present in distinct subcomplexes of the exocyst complex, is mutually exclusive. Thus, Ral may enhance the formation of a functional exocyst complex at a specific subcellular site by bringing together different subcomplexes of the exocyst, containing either Sec5 or Exoc8 (Jin et al., 2005). The exocyst complex was originally discovered in yeast as a protein complex required for polarized secretion at the tip of newly formed buds (TerBush et al., 1996). In vertebrate cells, the exocyst complex mediates polarized delivery of membranes from the Golgi apparatus and recycling endosomes to the basolateral membrane, but not to the apical membrane (Lipschutz and Mostov, 2002; Prigent et al., 2003; Yeaman et al., 2004). Among proteins targeted to the basolateral membranes by the exocyst complex are the LDL receptor and E- cadherin (Grindstaff et al., 1998; Langevin et al., 2005; Shipitsin and Feig, 2004). Targeting of E-cadherin to the basolateral membrane is of special importance in epithelial cells that are about to establish cell-cell contacts with other epithelial cells. Once E-cadherin is stabilized at the membrane by homotypic interactions with E-cadherin on neighboring cells, it may help in targeting more exocyst complex to the lateral membrane, thereby enhancing the formation of cell-cell contacts and elaborating cell polarity.

In vivo, the requirement for Ras-like GTPases in cell migration is clearly illustrated by the mutant phenotype of Rap1 mutant flies. Embryos, devoid of Rap1 protein, display defects in ventral furrow closure, head involution and migration of mesodermal and presumptive primordial germ cells (Asha et al., 1999). Thus, both morphogenetic events of complete cell layers as well as individual cell migration are disturbed. Also the migration of a group of specialized follicle cells, called border cells, is under the control of Ras-like GTPases in *Drosophila*. Migration of border cells represents a cadherin-dependent mode of cell movement and requires signaling via Ras and via Ral (Lee et al., 1996). It is presently unclear which Ral effector is required in this process.

A major migration event in *C. elegans* is that of hypodermal cells during embryonic development. Hypodermal cells are the epidermal cells in *C. elegans.* They arise as a series of six rows at the dorsal side of the embryo. The two dorsal-most arrays of cells undergo a process called dorsal intercalation, which organizes them into a single dorsal row. After this process has begun, the ventral-most cells on each side start to migrate ventrally in order to envelope the embryo. In this process of ventral enclosure, the two most anteriorly located cells, named leading cells, are crucial and the first to establish cell-cell contacts with their contra-lateral neighbors ((Williams-Masson et al., 1998); for a review (Simske

and Hardin, 2001)). In between the dorsal and ventral cells a row of lateral epidermal cells, called seam cells, is present. After ventral enclosure is completed, the ovoid embryo elongates about fourfold. This process relies on circumferentially oriented actin bundles that are linked at the apical site of adherens junctions in hypodermal cells. In embryos mutant for the α-catenin homolog HMP- 1 or the β-catenin homolog HMP-2, the circumferential actin bundles in dorsal hypodermal cells detach from adherens junctions, which probably explains the elongation phenotype seen in these animals (Costa et al., 1998).

So far, relatively few gene products have been identified, which play a crucial role in ventral migration of hypodermal cells. Among them are HMP-1, HMP-2 and the cadherin HMR-1, which are physically interacting in the cadherin-catenin complex (CCC). In HMR-1 homozygous embryos, or embryos devoid of maternal HMP-1 or -2 protein, the leading cells do not migrate completely to the ventral side (Costa et al., 1998). Other proteins involved are the APC-related protein APR- 1 that may also be present in the CCC, the inositol 1,4,5-triphosphate receptor ITR-1 and ephrin receptors and ligands (George et al., 1998; Hoier et al., 2000; Thomas-Virnig et al., 2004). These latter proteins are required in the neuroblast cells, over which the hypodermal cells migrate. Also the cytoskeletal regulatory WAVE/ WASP proteins are required for normal hypodermal cell migration (Withee et al., 2004). The interconnectivity of these different genes has not been established. Remarkably, no role for any of the *C. elegans* integrins has been established in hypodermal cell migration (Cox and Hardin, 2004).

In the present study, we have performed a synthetic lethal RNAi screen with a *C. elegans rap-1* mutant, which revealed that *rap-1* mutants are highly sensitive to diminished signaling via the RAL- 1/ exocyst complex pathway. During embryogenesis, the Ras-like GTPases RAP- 1 and RAL-1 act in concert to orchestrate hypodermal cell migration and sorting. Interfering in the Ral-1/ exocyst complex pathway in a *rap-1* mutant background leads to loss of the CCC at adherens junctions. Interestingly, the observed phenotype is more severe than that of CCC mutants alone, indicating additional roles of the Ral-1/ exocyst complex pathway. Furthermore, our screen has identified various other genes that may be involved in the RAL- 1/exocyst complex pathway or otherwise are required for viability of *rap-1* mutants.

Material and Methods

Worms.

General methods for culturing and manipulating worms used were as described (Lewis and Fleming, 1995). Worms were cultured on NGM plates at 20°C. Bristol N2, *dpy-20*(*e1362*), *rap-2*(*vc14*), JR667 [*unc-119*(*e2498*::Tc1) III; *wIs51*], NL4256 *rrf-3(pk1426),* JJ1079 [hmr-1(zu389)/lin-11(n566) unc-75(e950) I], PE97 *hmp-1(fe4),* FZ115 *epac-1(pk1313)*,

FZ181 *rap-1*(*pk2082*), FZ222 *rap-1*(*tm861*), FZ224 [bjIs64[pML902(*dlg-1::GFP*) pRF4], FZ271 *rap-1*(*tm861*); bjIs64[pML902(*dlg-1::GFP*) pRF4], FZ247 *rap-1*(*tm861*); *unc-119*(*e2498::Tc1*)wIs51[*SCM::GFP*; *unc-119*(+)], FZ281 *sec-5*(*pk2358*)/+;+/*dpy-10*(*e128*), FZ282 *sec-5*(*pk2357*)/+;+/*dpy-10*(*e128*). SP636 unc-4(e120) mnDf67/mnC1 dpy-10(e128) unc-52(e444)II, FZ60 bjIs26[hsp-HA-ral-1(G26V); dpy-20; gpa-15::GFP], FZ176 *rap-1*(pk2082); bjIs26[hsp-HA-ral-1(G26V); dpy-20; gpa-15::GFP], FZ245 *rap-1*(tm861); bjIs26[hsp-HA-ral-1(G26V); dpy-20; gpa-15::GFP], FZ208 bjIs61[hsp-HA-ral-1(S31N); dpy-20; gpa-15::GFP], FZ234 *rap-1*(pk2082); bjIs61[hsp-HA-ral-1(S31N); dpy-20; gpa-15::GFP], FZ279 *rap-1(tm861);rap-2(gk11),* FZ233 *rap-1(tm861);dpy-20(e1362),* FZ 311 *rap-1(tm861);dpy-20(e1362) Ex78[let858::rap-1 dpy-20 gpa-15::GFP],* FZ 312 *rap-1(tm861);dpy-20(e1362) Ex78[let858::rap-1 dpy-20 gpa-15::GFP]* .

Generation and detection of mutants

Transgenic animals were obtained by injection of plasmid DNA into the gonads of *dpy-20*(*e1362*) animals, or *rap-1(tm861);dpy-20(e1362)* (Mello *et al., 1991*). FZ311 and FZ312 were made with pPD103.5 containing untagged cDNA sequence of *rap*-1 (C27B7.8). Transgenic arrays were integrated by irradiating animals with 40 Gy of gamma radiation from a 137 Cs source (Way et al., 1991). Target selected mutagenesis for obtaining *sec-5* or *ral-1* mutants was done as described (Cuppen et al., 2007). Mutant animals were out-crossed at least four times to N2 animals before phenotypic analysis. Detection of mutant *rap- 1*(*tm861*) allele was done by nested PCR using the primers 1 GAGAGCCTTCTTCGTTCTGC and 4 GAGTTTCAAAAAACTGTGTG, followed by 2 TCACGGGTCGTCGATTGCAG and 3 GTTAGCCTCCTTTTCATTGAG. The absence of a wild-type allele was confirmed by replacing primers 1 and 2 from the above reaction by two primers complementary to the deleted area from *tm861*: 5 ACGAGAGTTTTTAGTTACAG and 7 AAATGTATCATATCGAGACCG respectively. *sec-5* animals were genotyped with a nested PCR using the primers TCGGAGTCATTGTCAGATTC and GAAGAGCTTTGGGAACCAG, followed by CAGACACAGATGTTCCGTTG and ATGATGTTGGTCAGCATTTG. Digestion with *Acc*I, results for the mutant allele in the disappearance of a 570 bp band.

Determination of viability of N2 and *rap-1(tm861);rap-2(gk11)* was performed by cutting gravid adults in water and collecting the embryos with a drawn-out pipette. Embryos were counted and non-hatched embryos were scored after 24 h.

Constructs.

A full-length cDNA for *ral-1* was obtained by PCR using the primers ral-forward (CTCGAGCATGGCATCGAAAAAAGCAAGC) and ral-reverse (AAAGAATTGTGCAATGCTTC) and cloned into pGEM-T (Promega). From here it was cloned into the pPD49.78 vector which carries the *hsp* 16-2, which had been modified to encode an HA-tag, followed by *Sal*I and *Not*I sites

(pPD49.78HA). The mutations G26V and S31N were introduced by Quick Site Mutagenesis, according to the manufacturer's protocol (Stratagene). The *ral-1(RNAi)-1, rgl-1(RNAi), rap-1(RNAi), rap-2(RNAi), rlbp-1(RNAi)* plasmids contained 500 bp cDNA fragments in L4440. *sec- 5(RNAi)-1* contains basepairs 1816-2655 and *sec-5(RNAi)-2* contains 741-1330 of *sec*-5 cDNA. Other constructs were obtained from the Ahringer RNAi library. *exoc-8(RNAi)-1* corresponds to location 25G8 and *exoc-8(RNAi)-2* corresponds to location 25G9 of the Ahringer library.

Screen.

The synthetic lethal screen was performed essentially as described (van Haaften et al., 2004). However, we used 20-25 L1 larvae per well in 100 µl of $M9^+$ buffer, to which 50 µl of induced bacterial suspension was added. Scoring was done after 7 days either by eye for genome scale screen or by counting the total amount of progeny per well and comparing it to the amount of progeny on control RNAi (L4440) for the small scale screen.

Antibody staining and time lapse recording.

Antibody staining of embryos was performed as described previously (Bossinger et al., 2001). Antibodies used are: α-HMP-1 (clone P1E11, Chemicon), α-AJM-1 (provided by O. Bossinger), mabMH27, mouse, hybridoma supernatant), EEA-1 (provided by B. Grant), RAB-11 (provided by A. Spang) and the Alexa anti-mouse 568 secondary antibody (Jackson ImmRes. Lab). Embryos were visualized under a Zeiss Axioskop 2. For time lapse recordings, L4 animals were put on a RNAi feeding plate on day 1. On day 3, adults were moved to a fresh plate and embryos were collected after a two hour egg lay. Embryos were mounted on a 3% agarose pad in $M9^+$ buffer, covered with a coverslip and sealed with Vaseline.

Heat shock protocol and Western blotting.

For heat shock experiments,

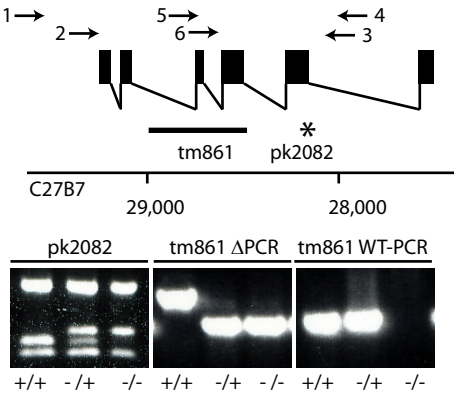

Figure 1: **Schematic representation of the genomic organization of the *rap-1* locus and detection of mutant alleles.**
Filled boxes indicate exons and the line underneath the graphic representation indicates the position of the deletion in *rap-1(tm861)* animals and the star indicates the amber codon (amino acid 130) in the *pk2082* allele. Numbers indicate the position of the *rap-1* locus on the C27B7 cosmid. Below are PCR reactions shown, done on single worm lysates that demonstrate the presence of the wild-type allele in +/+ and +/- worms and the absence of the wild-type allele in -/- worms. An arrowhead indicates a 170 bp Sau3A product that is characteristic for the *pk2082* allele. Arrows indicate the position of the PCR primers used for detection of the deletion and wild-type alleles.

TABLE I **Overview of genes found in the synthetic lethal screen with *rap-1(pk2082).***

Localization	Gene	Mammalian homolog	N2	*rap-1 (pk2082)*	*rap-2 (gk11)*	*epac-1 (pk1313)*
90G7	*ral-1* *Y53G8AR.3*	RalA, RalB	21(549) 45(1068)	0(0) 0(0)	36(702) 23(408)	19(344) 36(640)
25G8	*exoc-8* *Y105E8B.2*	EXOC8	38(984) 55(1314)	2(26) 14(189)	55(1087) 69(1204)	21(377) 97(1721)
2C2	*phi-24* *F23C8.6*	CHMP1B	146(2198) 88(1038) 26(826)	22(433) 30(256) 9(158)	93(2300) 83(1141) 68(1200)	139(2699) 50(813) -
16E8	*sur-6* *F26E4.1*	PR55/B	59(892) 47(550) 47(1533)	4(75) 0(0) 1(13)	44(1101) 51(696) 55(973)	47(912) 53(614) 46(740)
44D3	*vhp-1* *F08B1.1*	MKP7	91(2165) 166(3150)	0(0) 8(72)	38(935) 24(327)	163(2350) 95(2282)
101G7	*him-3* *ZK381.1*	HORMAD1	54(819) 108(1259) 107(3479)	14(276) 35(296) 7(121)	58(1424) 68(1203) -	51(984) 126(1472) 55(884)
104B8	*C49H3.8*	ARP10	137(2068) 74(2393) -	27(529) 80(681) 35(583)	79(1934) 100(1378) 71(1257)	109(2129) 123(1437) 111(1785)
146B2	*C01B7.1*	Zn-finger protein	117(1760) 102(1208) 43 (1373)	9(168) 7(64) -	62(1545) 96(1319) 17(299)	99(1928) 90(1054) 20(330)

Numbers represent the percentage of progeny relative to those found on control RNAi for each strain as determined in rescreening experiments. Numbers in brackets are total numbers of worms per well at day 7. Successive lines represent independent experiments.

hermaphrodites were allowed to lay eggs for two hours (day 0). One hour after removal of the adults, the plates were incubated at 33°C for 30 minutes. On day 1, 2 and 3 a 2-hour heat shock was given. Detection of HA-tagged RAL-1 in lysates of heat-shocked animals was done by immunoblotting using the 12CA5 mAb against HA (Pellis-van Berkel et al., 2005).

Results

RAP-1 and RAP-2 are not required during C. elegans embryonic development.

In addition to our previously described *rap-1(pk2082)* allele, a second *rap-1* allele (*tm861*) has been isolated, in which a 549 bp deletion removes exon 3 and part of exon 4, leaving only the first 42 amino acids of RAP-1 intact (Figure 1). Both homozygous mutant strains are viable and fertile, although progression through larval stages is delayed ((Pellis-van Berkel et al., 2005) and data not shown). Like most *rap-1*(*pk2082*);*rap-2(gk11)* animals, the majority of *rap-1*(*tm861*);*rap-2(gk11)* animals die during late larval stages. The small fraction of double homozygous adults, derived from either *rap-1*(*pk2082*/+);*rap-2(gk11)* or *rap-1*(*tm861*/+);*rap-2(gk11)* animals, is egg laying defective. Surprisingly, inside such animals viable offspring is present. We therefore isolated embryos from N2 or *rap-1(tm861);rap- 2(gk11)* animals and determined the percentage of non-hatched embryos. Although

the percentage of non-hatched *rap- 1(tm861);rap-2(gk11)* embryos was higher (19%, n=340) as compared to wild-type embryos (3%, n=365), the majority of double mutants hatched normally. Of the embryos that did not hatch, the majority (73%) had elongated more than twofold. This showed that whereas RAP- 1 or RAP-2 is required for normal viability in late larval stages there is no strict requirement for RAP-1 and RAP-2 during embryogenesis.

Synthetic lethal screen with rap-1 mutants. The normal development of *rap-1* mutants in *C. elegans* contrasts the situation in *Drosophila* and this suggests that other signaling pathways functionally compensate for the *rap- 1* pathway. To investigate this option, a genome-wide synthetic lethal RNAi screen was performed using the Ahringer library (Kamath et al., 2003; van Haaften et al., 2004). Apart from wild-type and *rap-1*(*pk2082*) mutants, *rap-2*(*gk11*) and *epac-1*(*pk1313*) mutants were included in this screen. *epac-1*(*pk1313*) mutants carry a deletion in the homolog of the cAMP-dependent Rap specific guanine nucleotide exchange factor (GEF) Epac (T20G5.5; unpublished results) (de Rooij et al., 1998). Foods that caused specific lethality with one of the mutant strains, were re-screened using the same mutants in the case of *rap-2* and *epac-1* or with both *rap-1*(*pk2082*) and *rap-1*(*tm861*) (all data in supplementary data set). In this rescreen no genes emerged that caused clear and reproducible synthetic lethality with *rap-2* or *epac-1*. In contrast, seven genes caused specific synthetic lethality of *rap-1* mutants (Table I). These include the MKP7 homolog *vhp-1* (F08B1.1) (Mizuno et al., 2004) and a gene with unknown function C01B7.1. Interestingly, *ral-1* and *exoc*-8 were found to encode for homologs of vertebrate exocyst complex members. Finally, the genes encoding the phosphatase *sur-6* (Sieburth et al., 1999), the vesicle sorting protein *phi-24* (Howard et al., 2001) and *him-3,* known to be involved in meiosis, gave synthetic lethality, but with variable results for *rap-1(tm861)*. To exclude the possibility that an additional mutation present in both *rap-1* strains was responsible for the observed synthetic lethality, two independent *rap-1(tm861)* strains, with *rap-1* under control of the general *let-858* promoter, were generated. Although the level of RAP-1 was too low for detection on blot, we observed a partial rescue of

TABLE II ***rap-1(tm861); dpy-20* animals carrying transgenic *rap-1* (rescue 1 and 2) are less sensitive to *exoc-8(RNAi)* than *rap-1(tm861); dpy-20* animals.**

Strain	control RNAi	*exoc-8 (RNAi)-1*	Control RNAi (%)
wild-type	62	70	113
rap-1(tm861); dpy-20	57	10	17
rescue 1	61	36	59
rescue 2	65	86	132

Numbers in the first and second column are absolute numbers of viable progeny after a 16 hour egglay. Numbers are mean values of the progeny 15 (rescue strain 1 and 2) or 10 (wild-type and *rap-1(tm861); dpy-20* hermaphrodites. The third column shows the percentage of the amount of progeny on *exoc-8(RNAi)* in comparison with animals subjected to control RNAi.

the phenotype when these worms were subjected to *exoc-8(RNAi)* food as L1-stage animals (Table II).

We focused on RAL-1 and the exocyst complex member Exoc-8 since their vertebrate homologs RalA and Exoc8 have previously been reported to directly interact: in its active GTP-bound form, RalA binds Exoc8. Therefore, we retested the effects of RNAi of other exocyst complex members on the viability of both *rap-1* mutants (Supp. Table I). No or only very limited effects were scored

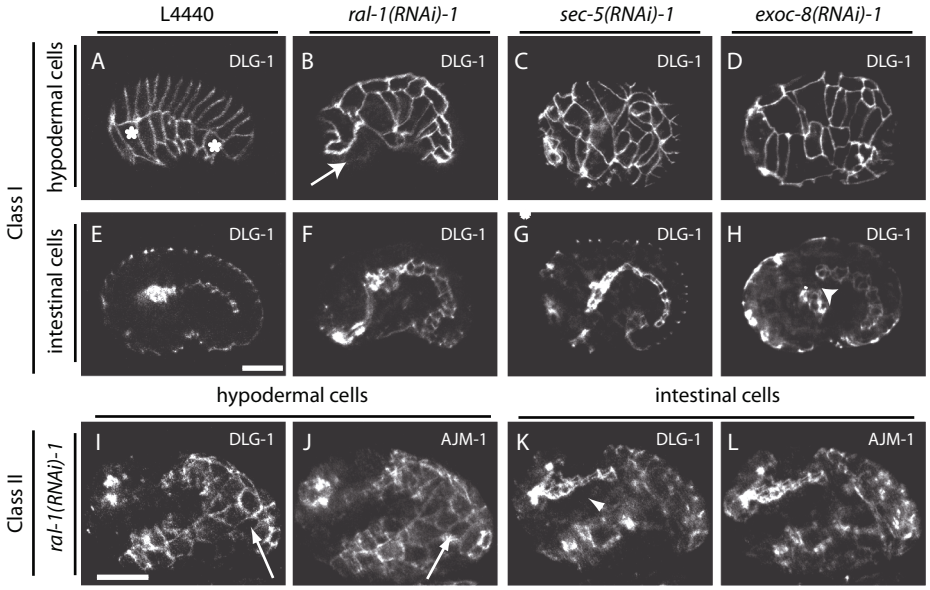

Figure 2. **Phenotype of *rap-1* mutant embryos carrying the *dlg-1*::GFP gene subjected to control, *ral-1*, *sec-5* or *exoc-8(RNAi)*.**

Phenotypes of *rap-1* mutant embryos carrying the *dlg-1*::GFP marker (FZ271) that were derived from animals subjected to RNAi for *ral-1*, *sec-5* or *exoc-8*. DLG-1 indicates *dlg-1*::GFP expression, AJM-1 indicates immunofluorescence staining using the MH27 antibody. Genes used for RNAi are indicated above each panel. L4440 is the empty vector control RNAi. A-H show class I embryos, I-L class II embryos. A-D and I-J show focal planes to visualize hypodermal cells, E-H and K-L are focal planes at the level of the gut. The most anterior and posterior visible seam cells are marked with *. In all cases AJM-staining was performed (only shown for class II, subjected to *ral-1 (RNAi)*) which demonstrated clear colocalization with DLG-1::GFP).

Class I embryos are characterized by halted migration of hypodermal cells as examplified by a *ral-1(RNAi)* embryo (arrow in B) or misalignment of hypodermal cells as shown here for a *sec-5* and *exoc-8(RNAi)* embryo (C+D). Halted migration can be discerned from early stages of migration by comparison of the developmental stage of the gut. Note that during normal hypodermal cell migration (A+E) the gut has not yet extended along the entire length of the embryos, in contrast to the gut of the *ral-1(RNAi)* embryo, which has established clear adherens junctions and shows a more mature pharynx (B+F). In some cases a gap between intestinal and pharyngeal cells is observed (arrowhead H). In class II embryos hypodermal cells do not envelope the entire embryo (arrow I,J) and a clearly recognizable gut structure is found on the outside of the embryo (arrowhead K). All embryos are oriented such that their pharynx is on the left side. Scale bar is 10 µm.

for *sec-6*, *sec-8*, *sec-15, sec-3* and *exoc-7*. Although not found to be synthetic lethal in the screen, quantification showed less progeny on *sec-10(RNAi)*. Finally, also *sec-5(RNAi)* resulted in specific synthetic lethality with both *rap-1* alleles.

In vertebrates, Sec5 is identified as a member of the exocyst complex and also as a direct downstream effector of Ral. Other Ral effectors include RalBP, involved in endo- and exocytosis, and the actin-bundling protein filamin. For each of these effectors a single homolog was found in the *C. elegans* genome (*rlbp-1* (T23G11.5), *flna-1* (C23F12.1)). In addition, a single homolog for the family of RALGEFs is found, RGL-1 (F28B4.2). We tested the effect of RNAi feeding of L1 larvae on plate for each of these genes. *ral-1* and *sec-5(RNAi)* prevented *rap-1*(*pk2082*) from producing viable offspring, while *rlbp-1(RNAi)* had no effect. *flna-1(RNAi)* resulted in a high number of sick animals, but this was also seen with wild-type animals (Supp Figure 1). Together, these results suggest that full functionality of the *ral-1/sec-5/exoc-8* pathway is required in *rap-1* mutants, but not in wild-type worms. Further evidence comes from the observation that also *rgl-1(RNAi)* reduces the number of *rap- 1* offspring, while no effect was seen on wild-type worms (Supp. Figure 1A and data not shown). A drawback of RNAi studies is that the efficacy of RNAi can be variable. However, when the same set of RNAi constructs was tested in a more RNAi sensitive background using the *rrf-* 3 strain, similar results were observed (Supp. Figure 1B+C). Strikingly, a further reduction was seen in the number of *rap- 1;rrf-3* mutants on *rgl-1(RNAi)*.

Comparative analysis of ral-1/ sec-5/ exoc-8(RNAi) in rap-1 mutants.

To obtain more definitive proof that *ral- 1*, *sec-5* and *exoc-8* operate in the same pathway in *C. elegans,* we compared the phenotypes of *rap-1* mutants after RNAi feeding on plate. When *rap-1* L1 larvae are subjected to RNAi for any of these genes, most animals make it to adulthood but are sluggish and largely sterile. In contrast, when L4 larvae were used at the start of RNAi, the resulting adults appeared healthy. Initially, they produce viable offspring but then start to shed many embryos that do not hatch. Inspection of these embryos showed a phenotypic series that can be roughly classified into two types (Figure 2). In class I embryos, the hypodermis still covers the entire embryo, but little morphogenesis is seen following the comma stage. Class II embryos appeared more disorganized and hypodermal cells are clustered and do not envelope the embryo. Hypodermal cell junctions have been characterized in much detail and consist of a single electron dense junction, in which an apical and basal domain can be discerned by immunofluorescence. The apical domain of the junction harbors the CCC, whereas the basal domain contains the membrane-associated protein DLG-1 and its binding partner AJM-1. We used DLG-1 and AJM-1 localization to visualize adherens junctions in the

hypodermis and developing gut, which allows the simultaneous evaluation of the development of both tissues relative to each other (Segbert et al., 2004). The pattern and organization of hypodermal cells in *rap-1* mutant worms on control RNAi is identical to that of wild-type worms and the dorsal, seam and ventral cells form regular arrays of cells (Figure 2A, reviewed in (Simske and Hardin, 2001)). In contrast, many of the class I embryos obtained with *ral-1*, *sec-5* or *exoc-8(RNAi)* are characterized by a failure of cells to align in the stereotyped pattern of dorsal, seam and ventral cells (Figure2B-D and Supp. Figure 2). 3D-animation indicates that this results from defects in both dorsal intercalation and ventral migration (data not shown). In other class I embryos, migration appears to have stopped before reaching the ventral midline. Analysis of pharyngeal and intestinal cells of these embryos showed that these tissues are not affected (Figure 2F-G), although in rare cases abnormalities were seen (Figure 2H). Overall, the phenotypes of *ral-1*, *sec-5* or *exoc-8(RNAi)* are very similar. In class II embryos, DLG-1 and AJM-1 staining showed the presence of disorganized patches of hypodermal cells at various sites, but both proteins still colocalized (Figure 2I-L, *sec-5* and *exoc-8(RNAi)* data not shown). The intestine of these embryos was frequently recognizable on the exterior, indicating that despite the severe morphogenetic defect, cell specification of this tissue had occurred normally (arrowhead Figure 2K). To study cell specification of hypodermal cells in class II embryos we used the seam cell marker SCM::GFP, which is specifically expressed in 10 hypodermal seam cells on each lateral side from the 1.5-fold stage onwards (Koh and Rothman, 2001). GFP-positive cells were detected in the aberrantly localized hypodermal cell clusters following *ral-1*, *sec-5* or *exoc-8(RNAi)* feeding of *rap-1*(*tm861*) animals, showing that specification of at least a subset of hypodermal cell fates had occurred in these embryos (Figure 3, showing a single focal plane). However, the number of SCM::GFP positive cells was reduced in some cases. Whether this is the consequence of altered cell division, defects in specification or loss to cell death is presently not clear. In conclusion,

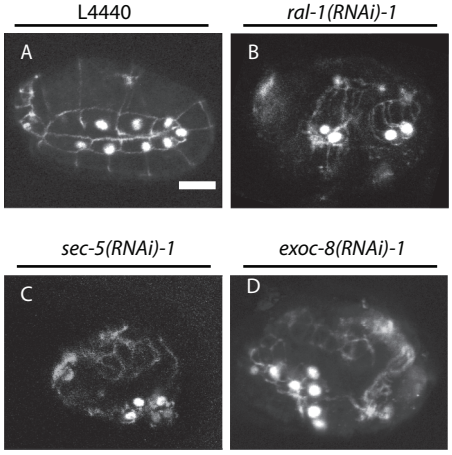

Figure 3: **Phenotype of *rap-1* mutant embryos carrying the *SCM::GFP* gene subjected to control, *ral-1, sec-5* or *exoc-8(RNAi)* (color image p124).** Expression of the seam cell marker SCM::GFP in nuclei of the disorganized hypodermis of class II embryos. Hypodermal cell borders are visualized using the MH27 antibody. Only one focal plane is shown per condition, seam nuclei are also present in other slices (data not shown). Scale bar is 10 µm.

the phenotype of *rap-1* mutants on *ral-1*, *sec-5* and *exoc-8(RNAi)* is consistent with the fact that these latter three genes operate in a single pathway involved in hypodermal cell organization.

Localization of HMP-1.

Certain aspects of the RNAi phenotypes like the arrested migration of hypodermal cells seemed consistent with a defect in CCC function. We therefore investigated if HMP-1 was normally located at adherens junctions. HMP-1 colocalized with DLG-1 in wild-type on control, *ral-1, sec-5* or *exoc-8 (RNAi)* and *rap-1* mutants on control RNAi. In contrast, in almost all *rap-1* mutant embryos on *ral-1, sec-5* or *exoc-8(RNAi),* a diffuse distribution of HMP-1 was observed (Figure 4). So these gene products are required for targeting HMP-1 to adherens junctions or for stabilizing it there. This diffuse staining was clearly distinct from that of EEA-1 (early endosome) and RAB-11 (recycling endosome) staining (Andrews and Ahringer, 2007; Poteryaev et al., 2007) (Supp. figure 3). Genetic disruption of CCC members has been shown to result in elongation defects. We therefore performed a limited time lapse analysis of *rap-1* mutants on *ral-1(RNAi)*. Elongation in such embryos is almost completely blocked, while the development of the intestine and pharynx is not disturbed (Figure 5). Loss of HMR-1 or maternal and embryonic HMP-1 or -2 causes migration defects of hypodermal cells, but has not been reported to cause dorsal intercalation defects or to result in an abnormal alignment of dorsal, lateral or ventral cells (Costa et al., 1998). In this respect, the defects seen in *rap-1* mutants after *ral-1*, *sec-5* or *exoc-8(RNAi)* appear more severe. Possibly, deletion of *rap-1* enhances the phenotype of *hmr-1* mutants. Alternatively, the RAL- 1/ exocyst complex pathway targets more proteins than those of the CCC to the membrane, which are also involved in cell migration. To investigate this, we constructed *hmr-1;rap-1* double mutants. Double homozygous embryos, derived from *rap-1(tm861);hmr-1(zu389/+)* hermaphrodites did not differ from those derived from *hmr-1(zu389/+)* hermaphrodites (Supp. Figure 4). Due to the fragility of *hmr-1* embryos, we were not able to stain sufficient numbers of embryos for adherens junction markers. Since loss of maternal HMP-1 protein from *hmp-1* homozygous embryos strongly enhances their phenotype to resemble that of *hmr-1* embryos, we also investigated the effect of loss of RAP-1 in *hmp-1(fe4)* embryos. Again, double homozygous embryos could not be discerned from *hmp-1* mutants (Supp. Figure 4). Together, these results indicate that the aberrant organization of hypodermal cells in *rap-1* embryos seen after *ral-1*, *exoc-8* or *sec-5(RNAi)* is not a simple compound phenotype of *rap-1* and members of the CCC.

Genetic interactions between the RAP-1 and RAL-1/exocyst complex pathway.

To obtain additional proof for genetic interactions between the RAP-1 and

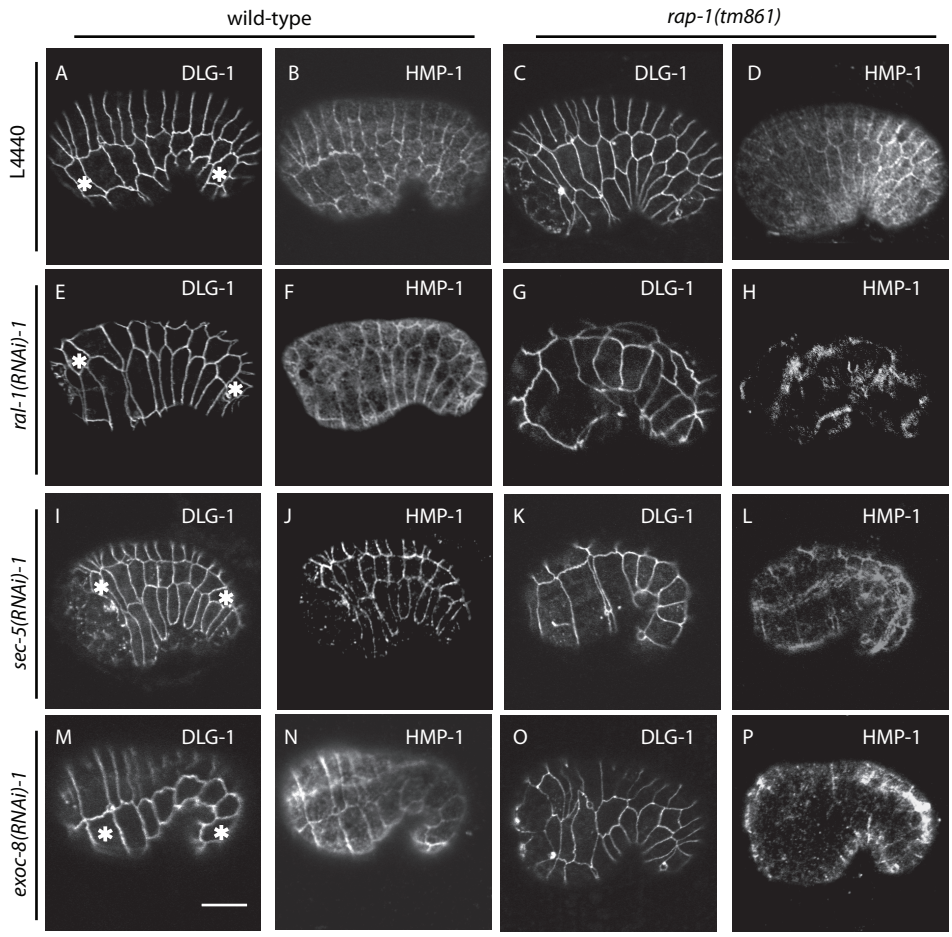

Figure 4: **Localization of HMP-1 in wild-type or *rap-1* mutant embryos subjected to control, *ral-1*, *sec-5* or *exoc-8(RNAi)*.**
dlg-1::GFP and HMP-1 staining in a lateral view of wild-type carrying *dlg-1*::GFP (FZ224) (A-B, E-F, I-J, M-N) and *rap-1* mutant embryos (FZ271) (C-D, G-H, K-L, O-P), derived from animals subjected to either control L4440 (A-D), *ral-1(RNAi)* (E-H), *sec-5(RNAi)* (I-L) and *exoc-8(RNAi)* (M-P). Pictures show hypodermal cells in which DLG-1 indicates *dlg-1*::GFP expression and HMP-1 indicates immunofluorescence staining using the P1E11 antibody. The most anterior and posterior visible seam cells are marked with *. Scale bar is 10 µm.

RAL- 1 pathways in *C. elegans*, we studied three independent mutants. *sec-5*(*pk2357*) and *sec-5*(*pk2358*) carry the same amber mutation at amino acid position 389 and were obtained with target selected mutagenesis (Cuppen et al., 2007) (Figure 6). Animals, homozygous for *sec-5*(*pk2357*) or *sec-5*(*pk2358*) become adult but produce only few embryos that elongate normally but never progress to the L2 stage. These *sec-5* mutations are hypomorphic based on the fact that when placed over a deficiency (*mnDf67*) most adults do not produce elongated embryos. The *sec-5*(*tm1443*) allele is most likely a null since it has a 385 bp deletion

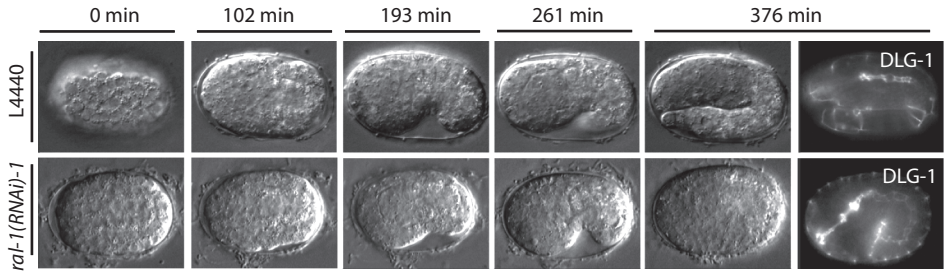

Figure 5: **Time lapse analysis of *rap-1* mutant embryos subjected to control and *ral-1(RNAi)*.**
Time lapse recordings of embryos derived from *dlg-1*::GFP;*rap-1(tm861)* mutant (FZ271) embryos on control L4440 (upper panels) or *ral-1(RNAi)* (lower panels). Last timepoint also shows DLG-1::GFP expression in the gut of the same embryo.

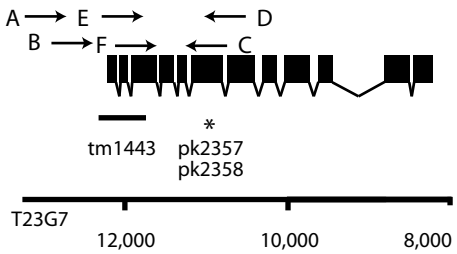

Figure 6. **Schematic representation of the genomic organization of the *sec-5* locus.**
Filled boxes indicate exons and the line underneath the graphic representation indicates the position of the deletion in *sec-5(tm1443*) animals and the star indicates the stop codon (amino acid 389) in the *pk2357* and *pk2358* allele. Numbers indicate the position of the *rap-1* locus on the T23G7 cosmid. Arrows indicate the position of the PCR primers used for detection of the deletion and wild-type alleles.

removing the first two exons and part of the third (Figure 6). Indeed, *sec-5(tm1443)* mutants have a more severe phenotype: homozygotes never produce progeny and die as late L4-stage larvae.

When introduced into a *rap-1*(*tm861*) mutant background, the phenotype of all *sec-5* alleles was clearly enhanced, showing RAP-1 and SEC-5 act in different pathways. *sec-5(pk2357);rap-1(tm861)* double homozygotes arrested as late L3 or early L4 larvae (n=23, confirmed by PCR). Importantly, *sec-5(tm1443);rap-1(tm861)* die at the L2 (25%) or L3 (72%) stage. This phenotype is less severe than that of *rap-1(tm861)* animals on *sec-5(RNAi)*. This is most likely due to maternal input of SEC-5 since *sec- 5(tm1443)* homozygous embryos shed by heterozygous animals on *sec-5(RNAi)* die as embryo (data not shown).

For *ral-1* we did not identify mutants with a stop codon. As an alternative, we generated transgenic animals, in which the dominant negative RAL- 1(S31N) or the constitutively GTP-bound RAL- 1(G26V) are under the control of a heat-shock promoter. Despite being constitutively GTP-bound, mutations corresponding to RAL-1(G26V) (like Ral G23V in mammals), have been described as interfering mutants (Feig, 2003; Moskalenko et al., 2002). Expression of either mutant protein (Figure7A) did not severely affect these transgenic animals, although it took slightly longer to reach adulthood as compared to heat shocked, wild-type worms. In contrast, expression of RAL-1 mutant proteins in

a *rap-1* mutant background induced a clear larval arrest, which was strongest for RAL- 1(G26V) (Figure 7B, C). Notably, whereas expression of HA-RAL-1(G26V) only delays development in wild-type worms, *rap-1* worms expressing the same protein never reach adulthood. The effects of expression of Ral-1(G26V) in a *rap-2*(*gk11*) background were not significantly different from those observed in wild-type worms, showing the specificity of the effect of RAL-1(G26V) on *rap-1* animals (data not shown). Together these data show that the RAP-1 and RAL- 1/exocyst complex pathway also interact during other stages of the life cycle.

Discussion

Synthetic lethal screens are an efficient approach towards identification of signaling pathways that function in a redundant fashion to mediate critical functions during development or any other stage of an organism's life cycle (Tong et al., 2001; van Haaften et al., 2004; Withee et al., 2004). Here we have used an RNAi feeding-based method to screen for signaling routes that function in conjunction with the RAP-1 pathway in *C. elegans.* Seven genes were identified, whose full function is required for two distinct *rap-1* mutants to produce viable offspring. These genes are synthetic lethal with two independent *rap-1* mutations, strongly suggesting that

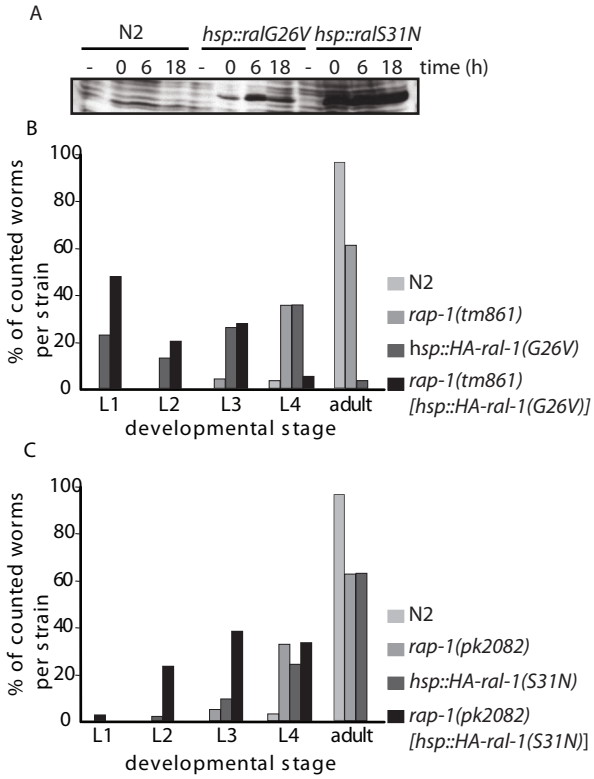

Figure 7. **Effect of heat-shock driven expression of RAL-1 proteins in wild-type and *rap-1* mutant backgrounds**.
(**A)** Western blot of total lysates from L1 larvae. Larvae were lysed without heat shock (-) or at 0h (0), 6 h (6) or 18 h (18) after a 2h heat shock. Probing was done with the 12CA5 antibody, which recognizes the HA-tag at the N-terminus of over-expressed proteins. (**B+C)** Embryos subjected to heat shock were allowed to develop 5 days before determination of developmental stage. Percentage of animals at the different developmental stages of the total number of hatched animals is shown for overexpression RAL-1(G26V) and RAL-1(S31N) in panel B and C respectively.

mutations in *rap-1* rather than a closely linked mutation is responsible for the observed effect. Indeed, we obtained a partial rescue of the phenotype on *exoc-8(RNAi)* with lines expressing *rap-1* from a heterologous promoter. Synthetic lethality on *ral-1(RNAi)* that is generally more robust, was not rescued, which we contributed to the low levels of RAP-1 expression from this transgene (data not shown). High levels of RAP-1 expression appear not to be tolerated in *C. elegans* as is also seen for Rap1 in tissue culture cells (data not shown).

Not all genes may function in the same pathway (see below), but based on literature, it seems likely that *ral-1, sec- 5* and *exoc-8* do so: in vertebrate cells, active Ral directly binds to the exocyst complex members Sec5 or Exoc8 (Brymora et al., 2001; Moskalenko et al., 2003), which results in targeting of proteins to the basolateral membrane (Moskalenko et al., 2002; Shipitsin and Feig, 2004). The fact that Ral binds to Sec5 or Exoc8 in a mutually exclusive manner may hint to two distinct pathways. In addition, Ral collaborates with Sec5 in the formation of filopodia (Sugihara et al., 2002). Analysis of embryos derived from *rap-1* mutant worms, grown on *ral-1*, *sec-5* or *exoc-8(RNAi)*, showed that they displayed virtually identical phenotypes. This strongly suggests that also in *C. elegans* these genes function together in hypodermal cell migration.

Rap1 has been claimed to activate the Ral pathway in *Drosophila* by direct interaction with the Ral GEF RGL (Mirey et al., 2003). Also *C. elegans* RAP-1 and RGL-1 interact in a yeast two-hybrid assay (J. Riedl and F.Z., unpublished results). Although our studies did not exclude the option that RAP-1 contributes to activation of RAL-1 in *C. elegans*, our current hypothesis is that RAP-1 has a distinct function in parallel to that of RAL-1. This is based on the fact that RAP- 1 null mutants have only a very mild phenotype under normal conditions, indicating that RAP-1 is not a major activator of the RAL-1/exocyst complex pathway. Furthermore, interfering at the level of RAL-1 by expression of mutant RAL-1 proteins has a strong effect in *rap- 1* mutants but not in wild-type animals. It is possible that RAP-1 converges with the RAL-1 pathway at the level of the exocyst complex or below. Since no interactions of RAP-1 with the exocyst complex have been documented we favour the latter option. In agreement with this, we find that RAP-1 clearly enhances the phenotype of *sec-5* mutations. .

The defects observed in *rap-1* embryos following *ral-1*, *sec-5* or *exoc-8(RNAi)* are most prominent in hypodermal cell migration during ventral enclosure. It may be that in the most severely affected class II embryos also gastrulation movements are abnormal, but this has not been investigated in any detail. Various causes may underlie the improper alignment and defective migration of hypodermal cells. For example, hypodermal cells need to be specified correctly in order to accommodate to their correct position. Abnormal cell

specification, leading to defects in dorsal intercalation, ventral enclosure and elongation, has been proposed to underlie the phenotype of *apr-1* mutants (Hoier et al., 2000). However, cell specification is unlikely to cause the migration defects found in *ral-1(RNAi)*, *rap-1* embryos. First, even in class II embryos, expression of a seam cell marker could be detected, which demonstrates that at least some specification within the hypodermis has occurred. Secondly, we did not observe any abnormal patterning of hypodermal cells before the onset of migration, in contrast to what was reported for *apr-1* mutants (data not shown). Finally, the Ral- 1/exocyst complex pathway was found to regulate migration in tissue culture cells, which is unlikely to depend on cell fate specification (Rosse et al., 2006).

A more likely explanation for the observed phenotype is the inability of hypodermal cells to change their adhesive properties that can support the extensive reorganization of cell-cell contacts required for dorsal intercalation and ventral migration. Cell-cell adhesion is of fundamental importance in cell sorting, migration and cell shape changes. This is exemplified in *Drosophila*, where loss of DE-cadherin in border cells blocks migration and in follicle cells has dramatic effects on cell sorting (Lee et al., 1996; Niewiadomska et al., 1999; Pacquelet and Rorth, 2005). Even loss of DE-cadherin from part of the cell circumference, as seen in clones of Rap1 mutant cells following cell division in the wing, changes their sorting behavior (Knox and Brown, 2002). Indeed, our analysis of proteins present in adherens junctions revealed the absence of HMP- 1 and thus most likely the complete CCC (Costa et al., 1998). This indicates that cell adhesive properties have changed. Loss of HMP-1 from adherens junctions upon interfering in the RAL-1/exocyst complex route is in line with recent studies in vertebrate tissue culture cells and *Drosophila* (Classen et al., 2005; Langevin et al., 2005; Shipitsin and Feig, 2004). It should be noted however, that in *C. elegans* loss of HMP-1 from adherens junctions upon *ral-1(RNAi)* was only seen in *rap-1* worms and not in wild-type worms. Thus, upon *ral-1* depletion, RAP-1 is crucial for targeting the CCC to the lateral membrane or stabilizing it. In mammalian cells, Rap1 has been shown to promote E-cadherin interaction at newly forming adherens junctions in tissue culture cells, while it seems not required for the maintenance of mature junctions (Hogan et al., 2004; Price et al., 2004). Possibly, Rap1 acts by inhibiting endocytosis of E-cadherin proteins, which are not yet ligated to E-cadherin on other cells. In order to do so, Rap1 is suggested to interact via the actin binding protein AF-6 with p120 catenin, an established stabilizer of adherens junctions (Hoshino et al., 2005). Although it is attractive to explain the lack of HMP-1 in adherens junctions in terms of the combined effect of diminished targeting via the RAL-1/exocyst complex and enhanced internalization, due to lack of RAP-1, the

situation may be more complex. It should be kept in mind that during reorganization of cell-cell contacts, cadherin is recycled via endosomes (Bryant and Stow, 2004). Indeed, disturbing the function of these endosomes in the *Drosophila* wing during the period that cells organize themselves into hexagonal arrays, results in a loss of DE-cadherin at selective cell contact sites (Classen et al., 2005). Consequently, loss of HMP-1 may not result from effects of RAP-1 on endocytosis, but on sorting events in recycling endosomes or exit from such endosomes. The situation is further complicated by the observation that the exocyst complex member Sec5 functions not only in exocytosis, but also in endocytosis (Sommer et al., 2005; Sonnichsen et al., 2005). Indeed, it is currently unclear if and how the exocyst complex interacts with the recycling endosomes. In this respect, *phi-24* may be an interesting hit from our screen since this protein is homologous to the endosomal protein CHMP1 (Howard et al., 2001) which is involved in vesicular sorting.

In contrast to HMP-1, DLG-1 and AJM-1 localized normally to adherens junctions and this is consistent with the finding that the localization of these latter proteins occurs independently of the CCC (Costa et al., 1998; Koppen et al., 2001). In addition, it shows that the RAL-1 and RAP-1 pathways affect only the transport of a subclass of proteins present at the basolateral membrane. Differential effects of inhibition of the exocyst complex on transport to the basolateral membrane have previously been shown in *Drosophila*, where DE-cadherin transport is blocked but delivery of the septate junction protein Coracle is normal (Langevin et al., 2005). Importantly, class I embryos differ from *hmr-1* embryos in that the shape and alignment of hypodermal cells is clearly abnormal. Therefore, the synthetic lethal phenotype observed cannot be simply attributed to loss of the CCC at the adherens junction. Double mutant analysis demonstrates that the phenotype of *hmr-1* or *hmp-1* embryos does not become more severe if *rap-1* is simultaneously absent. Consequently, the RAL-1/exocyst complex pathway is not only required for targeting the CCC but has additional functions as well.

It will be interesting to learn if and how the other genes uncovered in the synthetic lethal screen, fit in the RAL-1/exocyst complex pathway. At least one gene, *vhp-1,* has been indirectly linked to Ral signaling. VHP-1 is a phosphatase for the stress-induced kinases KGB-1 and PMK-1, which are homologous to Jnk and p38 MAPK, respectively (Mizuno et al., 2004). Previously, Ral has been found to activate the Jnk-pathway in tissue culture cells (de Ruiter et al., 2000) while in *Drosophila* it was found to act as a negative regulator of Jnk (Sawamoto et al., 1999). Therefore, *vhp-1* may act together with RAL-1 in other processes, possibly with different RAL-1 effectors.

In conclusion, a synthetic lethal screen has been used as a starting point to learn more about genes, that function in a redundant fashion with RAP-1. Our

data demonstrate a functional overlap for the Ras-like GTPases RAP-1 and RAL-1 during various phases of the life cycle. Using RNAi to time the interference in the RAL- 1/exocyst complex pathway, we identify both GTPases as novel elements in *C. elegans* hypodermal cell migration.

Acknowledgements

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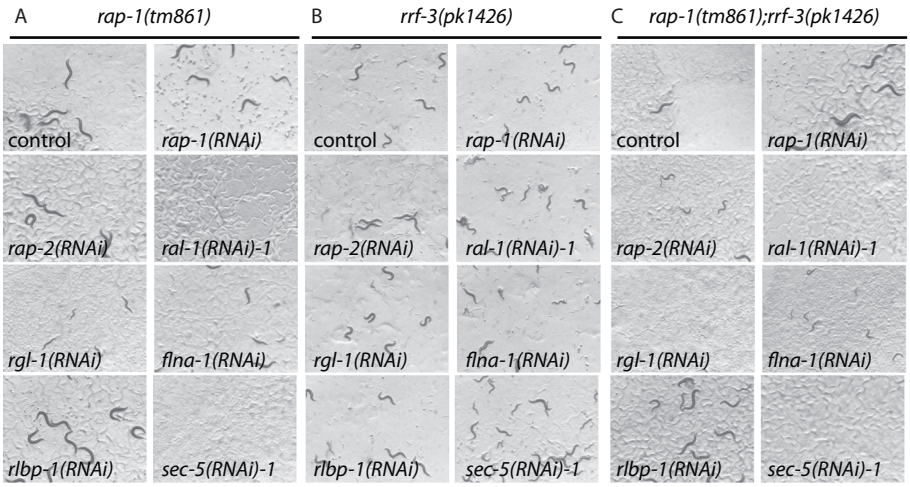
Supplementary data

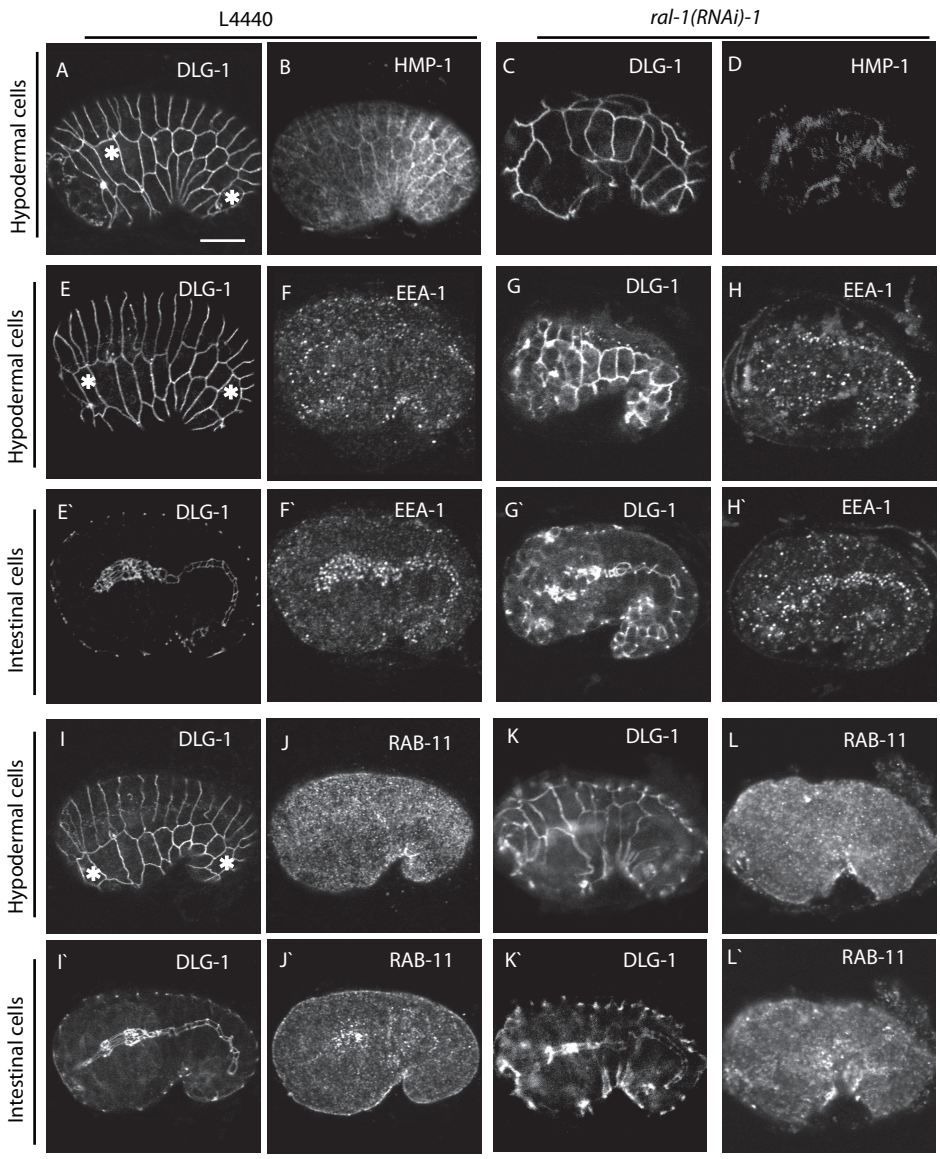


Supplementary Figure 1**: RNAi efficiency in wild-type and *rrf-3* background worms.**
rap-1, rrf-3 or *rap-1;rrf-3* strains were grown on *E. coli* HT115 cells induced to express RNAi constructs against the indicated RNAs. Pictures show a typical area of a plate.

SUPPLEMENTARY TABLE I **Quantification of the amount of progeny upon RNAi for exocyst complex members.**

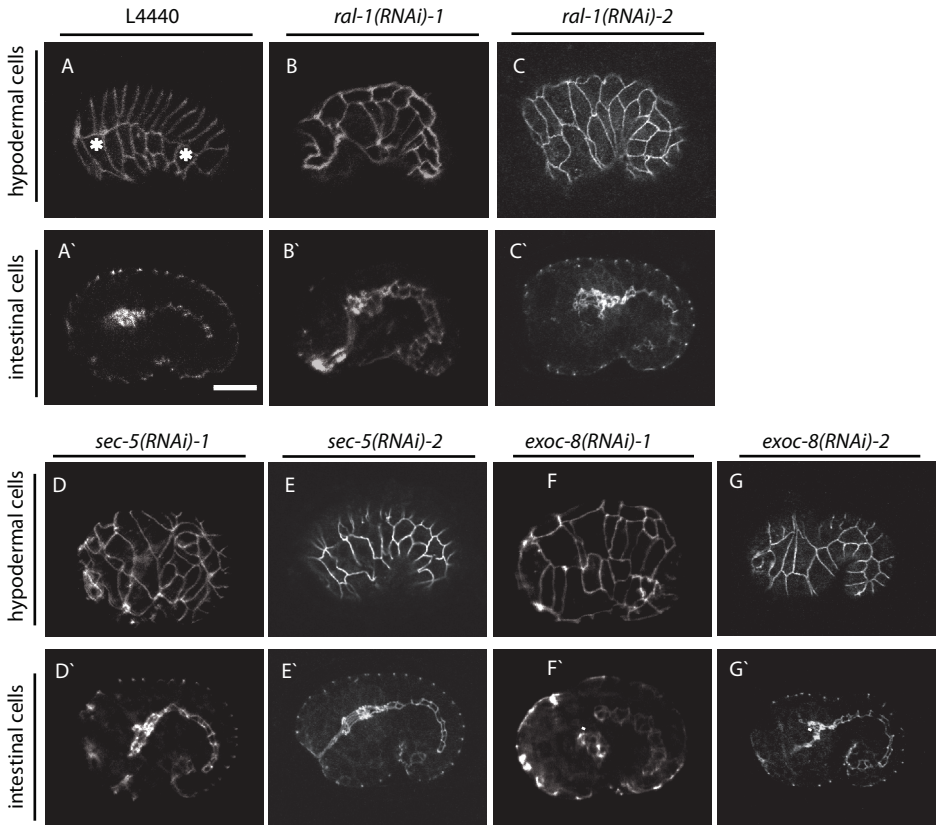
Gene	Mammalian homologue	N2	*rap-1 (pk2082)*	*rap-1 (tm861)*	*rap-2 (gk11)*	*epac-1 (pk1313)*
sec-5 T23G7.4	Sec5	73(1872) 82(1955)	0(6) 3(48)	12(214) 8(110)	66(1295) 21(362)	46(848) 76(1336)
exoc-8 Y105E8B.2	Exoc8	38(984) 55(1314)	2(26) 14(189)	58(995) 47(677)	55(1087) 69(1204)	21(377) 97(1721)
exoc-7 C43E11.8	Exoc7	108(2840) 79(2057)	40(537) 45(632)	73(1250) 102(1457)	61(1204) -	101(1850) -
sec-15 C28G1.3	Sec15	103(2458) 72(1357)	37(619) 81(741)	83(1572) 103(1277)	96(2337) 198(2663)	112(2687) 96(1378)
sec-3 F52E4.7	Sec3	55(1295) 121(2304)	31(522) 46(417)	57(1073) 91(1125)	55(1334) 77(1035)	124(2966) 110(1588)
sec-10 C33H5.9	Sec10	66(1565) 105(2001)	6(104) 31(285)	48(901) 21(255)	48(1177) 173(2336)	62(1483) 122(1760)
sec-8 Y106G6H.7	Sec8	96(2502) 118(3091)	26(345) -	79(1325) 60(858)	107(2112) -	79(1445) 120(2120)

Numbers represent the percentage of progeny relative to those found on control RNAi for each strain as determined in rescreening experiments. Numbers in brackets are total numbers of worms per well at day 7. Successive lines represent independent experiments.

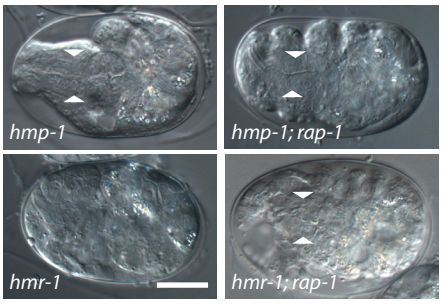


Supplementary Figure 3: **Localization of HMP-1, EEA-1 and RAB-11 in *rap-1* mutant embryos subjected to control or *ral-1(RNAi)*.**

dlg-1::GFP and HMP-1, EEA-1 or RAB-11 staining in a lateral view of *rap-1* mutant embryos carrying *dlg-1*::*GFP* (FZ271), derived from animals subjected to either control L4440 (A-B, E-F, I-J) or *ral-1(RNAi)* (C-D, G-H, K-L). Panel A-D is copied from Figure 4 for comparison of HMP-1 localization. Pictures show hypodermal cells or intestinal cells (marked with `) in which DLG-1 indicates *dlg-1*::GFP expression. The most anterior and posterior visible seam cells are marked with *. Scale bar is 10 µm.



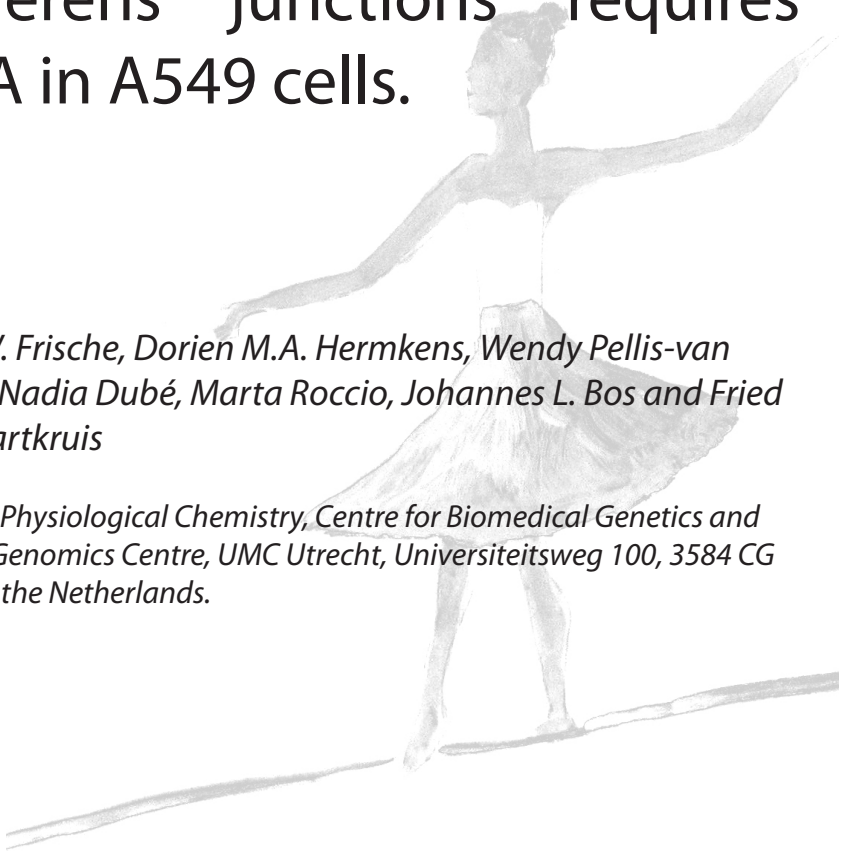
Supplementary Figure 2. **Phenotype of *rap-1* mutant embryos carrying the *dlg-1*::GFP gene subjected to control, *ral-1, sec-5* or *exoc-8(RNAi).***
Phenotypes of *rap-1* mutant embryos carrying the *dlg-1*::GFP marker (FZ271) that were derived from animals subjected to RNAi for *ral-1*, *sec-5* or *exoc-8*. DLG-1 indicates *dlg-1*::GFP expression. Genes used for RNAi are indicated above each panel. A-C, D-E and F-G show focal planes to visualize hypodermal cells, A`-C`, D`-E` and F`-G` are focal planes at the level of the gut. The most anterior and posterior visible seam cells are marked with *. In all cases AJM-staining was performed (data not shown) which demonstrated clear colocalization with DLG-1::GFP. Scale bar is 10 µm.



Supplementary Figure 4**: *hmp-1(fe4)* and *hmr-1(zu389)* embryos in a wild-type or *rap-1*(*tm861*) background.**
Phenotype of *hmp-1(fe4)* and *hmr-1(zu389)* embryos in a wild-type or *rap-1* background. Arrow heads depict the position of the pharynx. Scale bar represents 10 µm.

3

E-cadherin localization at the adherens junctions requires RalA in A549 cells.



Ester W. Frische, Dorien M.A. Hermkens, Wendy Pellis-van Berkel, Nadia Dubé, Marta Roccio, Johannes L. Bos and Fried J.T. Zwartkruis

Dept. of Physiological Chemistry, Centre for Biomedical Genetics and Cancer Genomics Centre, UMC Utrecht, Universiteitsweg 100, 3584 CG Utrecht, the Netherlands.

Abstract

In *C. elegans*, RAP-1 and the RAL-1/exocyst complex are required during embryogenesis for the localization of the cadherin-catenin complex to cell-cell contacts. Here, we investigated the role of Rap1 and RalA in E-cadherin localization in the human cell lines A549, DLD1 and MCF7 that are derived from different tumors. It was shown previously that depletion of Rap1A in A549 cells resulted in immature adherens junctions, but only had a limited effect on the presence of E- cadherin in adherens junctions. Here we show that in these cells, RNAi-mediated knock-down of RalA strongly reduced the amount of E-cadherin in the adherens junctions. Activation of Rap1 did not restore E-cadherin localization, suggesting that Rap1 and RalA operate in different pathways. In contrast, in DLD1 and MCF7 cells, depletion of RalA and/or RalA and Rap1 together did not affect the levels of E-cadherin or adherens junction morphology. Importantly, knock-down of the exocyst complex members, Sec8 and Exoc8 as well as the upstream RalA-activator RalGDS, also strongly reduced E-cadherin levels in A549 cells. Interestingly, the loss of E-cadherin from the cell-cell contacts did not disrupt the adherens junctions due to the presence of N-cadherin.

Introduction

The core proteins of the adherens junctions (AJ) are the classical cadherins (hereafter referred to as cadherins), which are interconnecting the cells by forming Ca^{2+}-dependent homophilic interactions. Localization and stabilization of cadherins is tightly regulated via exocytosis and endocytosis. Work in model organisms revealed a role for Rap1 in the regulation of AJs. In *Drosophila*, loss of Rap1 in the epithelium of the wing disrupts the hexagonal cellular packaging and AJs are not located round the circumference, but rather cluster at one side of the cell. Cell-cell contacts between wild-type and mutant cells form normally, indicating that Rap1 is involved in localization of E- cadherin to the cell surface where it can be stabilized via homophilic interactions (Knox and Brown, 2002). Recently, we have shown that in *C. elegans rap-1* null mutants are fully viable and fertile, but depend on signaling via RAL-1 and the exocyst complex for survival. Depletion of RAL-1 or members of the exocyst complex in these *rap-1* null mutants causes defects in hypodermal cell migration and sorting, leading to embryonic lethality. In these animals, the localization of HMP-1/α-catenin is disturbed leading to disruption of the cadherin-catenin complex (Costa et al., 1998) suggesting a role for RAP-1, RAL-1 and the exocyst complex in localization of these molecules to the lateral membrane (Frische et al., 2007). As well, Rap1 is involved in cell-cell contact regulation

in mammalian cells (for a review see (Kooistra et al., 2007)). Rap1 is required for initial cell-cell contact formation (Hogan et al., 2004) and is involved in regulation of E-cadherin endocytosis (Hoshino et al., 2005). Moreover, Rap1 exchange factors affect AJ dynamics; C3G binds directly to E-cadherin and this is most prominent during initial AJ formation (Hogan et al., 2004), whereas PDZ-GEF2 is required for AJ maturation (Dube et al., 2008). Moreover, expression of the atypical RapGEF, DOCK4, induces AJ formation in an osteosarcoma cell line (Yajnik et al., 2003). In endothelial cells, activation of Epac1 increases junction maturation and thereby decreases endothelial permeability.

The small GTPase Ral also affects E- cadherin localization; expression of an active mutant, RalA72L, increases the rate of transport of newly synthesized E- cadherin in MDCK cells to the cell surface (Shipitsin and Feig, 2004). In E- cadherin trafficking, the exocyst complex is likely a candidate to act downstream of Ral based on studies in *C. elegans* (Frische et al., 2007), exocytosis of Weibel-Palade bodies (Rondaij et al., 2008) and on insulin secretion in pancreatic β-cells (Lopez et al., 2008; Tsuboi et al., 2005). It also transports the glucose transporter GLUT4 to the cell surface (Chen et al., 2007). The exocyst complex consists of eight proteins; Sec3, Sec5, Sec6, Sec8, Sec10, Exo70 and Exoc8 and was originally identified in yeast where the complex is involved in docking of vesicles to the plasma membrane (TerBush et al., 1996). In higher eukaryotes, the exocyst complex is involved in vesicle delivery from the trans Golgi network (TGN) (Yeaman et al., 2001) and recycling endosomes (RE) (Langevin et al., 2005; Prigent et al., 2003) to specific sites at the basolateral membrane (Grindstaff et al., 1998; Vega and Hsu, 2001). RalA interacts directly with two members of the exocyst complex, Sec5 and Exoc8, and thereby affects protein transport. In *Drosophila*, recycling of E-cadherin is dependent on a functional exocyst complex (Langevin et al., 2005). Also in mammalian cells, the exocyst complex is involved in E- cadherin localization (Yeaman et al., 2004). Interfering in exocyst complex functioning results in an accumulation of the cargo proteins in intracellular vesicles (Grindstaff et al., 1998; Prigent et al., 2003; Vega and Hsu, 2001; Yeaman et al., 2001). However, little is known about the molecular mechanism of exocyst complex functioning in other model systems and about the upstream signals activating Ral and the exocyst complex.

Here, we have investigated in human cell lines whether, like in *C. elegans*, Rap and Ral act synergistically on the regulation of E-cadherin at the cell surface. We therefore investigated the effect of depletion of Rap1A, Rap1B and

RalA in A549, DLD1 cells. Whereas neither depletion of RalA nor depletion of Rap1 and RalA affected E-cadherin levels in DLD1 cells, knock-down of RalA in A549 cells was already sufficient to decrease the total amount of E- cadherin and the E-cadherin at cell-cell contacts. In line with previous studies, knock-down of members of the exocyst complex resulted in a phenotype that resembled RalA-depleted cells. Moreover, our data also suggest that the Ral-exchange factor RalGDS is involved in E-cadherin localization. Strikingly, loss of E-cadherin did not disrupt the AJs due to the presence of other cadherins indicating that the RalA/exocyst complex regulates the localization of only a subset of cadherins.

Material and Methods

Cell lines and culture.

A549 and DLD1 cells were maintained in RPMI media (BioWhittaker) and MCF7 in DMEM media (BioWhittaker) and all three supplemented with 10% FBS (Cambrex), 1.2 mM L-Glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin (BioWhittaker). A549 cells expressing Epac1 or canine GFP-E-cadherin were created by infecting A549 cells with ecotrophic virus containing Epac1 or GFP-E-cadherin respectively. The Epac1 or E-cadherin gene was linked via an IRES sequence to a zeocin resistance gene. Forty-eight hours after infection, cells were placed under selection with zeocin (0.2 mg/ml) to select for Epac1 or E-cadherin expressing cells. Monoclonal A549-Epac1 cell lines (B14 cell-line) expressing moderate levels of Epac1 and A549 cells expressing relatively high amount of GFP (C4 cell-line) were single-cell sorted from these polyclonal cell lines by FACS.

Antibodies.

Antibodies used are anti-E-Cadherin (HECD-1), N-cadherin and anti-PAN-cadherin (AbCam); anti-Tubulin and anti-Vimentin (Oncogen); anti-Rap1 and anti-RalGDS (Santa Cruz); anti-RalA, anti-Sec8, β-catenin and p120-catenin (BD Biosciences); anti-GAPDH (Chemicon); anti-HA (12CA5); Alexa Fluor 488 anti-human CD324 (anti-E-cadherin; clone 67A4) (BioLegend); Alexa Fluor 488 Mouse IgG1, κ isotype control (clone MOPC-21) (BioLegend); anti-mouse and anti-rabbit Alexa 488 and 568 (Molecular Probes).

siRNA transfections.

siRNA duplexes were obtained from Dharmacon and single siRNA transfections were performed with Oligofectamin (Invitrogen) according to manufacturers recommendation. Cells were incubated in minimal medium, 16 hours after transfection medium was replaced with normal medium without penicillin and streptomycin. Cells were

grown to confluency and replated on glass coverslips and for preparation of cell lysate and, if applicable, for FACS samples. Rap1A/B and RalA knock-down experiments in DLD1 cells were performed using Amaxa (Lonza) using buffer L. $2x10^6$ cells were transfected using 50nM siRNA duplexes against Rap1A and Rap1B and 30 nM siRNA duplexes RalA. 48 hours after transfection cells were replated on collagen coated (10 µg/ml, BD Biosciences) glass coverslips and grown to confluency. Different oligo's were tested for efficient RalA knock-down: RalA #1 GACAGGUUUCUGUAGAAGA; RalA#3C AGAGCUGAGCAGUGGAAUG and On-target plus SMARTpool RalA (L-009235). Oligo's, SMARTpool and combination of oligo's were tested. All induced RalA specific knock-down using RalA and RalB specific antibodies, but a combination of oligo #1 and #2 was the most efficient and used in experiments described here (not shown). Other On-target plus SMARTpools: cdh-1(L-003877); Sec-8(L-017357); Exoc-8(L-018001); RalGDS(L-005193); Rgl1(L-008387); Rgl2(L-009321); Rap1B(L-010364); Rap1A(L-00362).

Quantifications.

Western blot quantifications are determined using ImageJ. Band intensities are determined and numbers represent the ratio compared to the Tubulin signal.

Immunofluorescence.

Cells were fixed in 4% formaldehyde, permeabilized in 0.1% Triton X-100 and blocked with 2% BSA in PBS. Coverslips were incubated with the indicated primary antibodies (3 hours), followed by incubation with Alexa antibodies (1 hour) and mounted on glass slides. Images were acquired on a Zeiss Axioskop 2.

E-cadherin cell surface levels.

siRNA transfected A549 cells were replated and collected 24 h later in cold PBS containing 2% FBS on ice. Cell suspensions were incubated with Alexa Fluor 488 antihuman CD324 (anti-E- cadherin; clone 67A4) or Alexa Fluor 488 Mouse IgG1, κ isotype control (clone MOPC-21). Data acquisition was done on a FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software, and analysis was performed using WinMDI 2.9 software.

Results

RalA but not Rap1A/B, is required for normal levels of E-cadherin in A549 cells.

In *C. elegans,* knock-down of *ral-1* in *rap-1* null mutants leads to loss of the cadherin-catenin complex from the AJ. Here, we investigated whether Rap1 and Ral also act synergistically on E- cadherin localization in the human lung carcinoma (A549), colon carcinoma (DLD1) and breast cancer (MCF7) cell lines. Staining for E-cadherin revealed that DLD1 and MCF7

cells showed regular junctional staining, whereas the staining in A549 was much more irregular. RNAi mediated knock-down of RalA in A549 cells resulted in an almost completely abolished E- cadherin localization at AJs comparable to knock-down of E-cadherin by siRNA. In contrast, we did not observe an effect on junctional staining of E- cadherin in DLD1 or MCF7 cells (Figure 1A). In the A549 cells, E-cadherin did not appear in an intracellular pool and we therefore determined the amount of E- cadherin by Western blotting. Depletion of RalA reduced the total amount of E-cadherin whereas these levels were hardly affected in DLD1 and MCF7 cells (Figure 1B). Apparently, knock-down of RalA in A549, but not in DLD1 and MCF7 cells, is sufficient to reduce E-cadherin localization from the AJs.

We next investigated whether for DLD1 cells additional depletion of Rap1 reduces the localization of E- cadherin at the AJs. However, treatment of DLD1 cells with Rap1A/B and RalA siRNA hardly affected E-cadherin localization at the junctions (Figure 2A). Although the knock-down

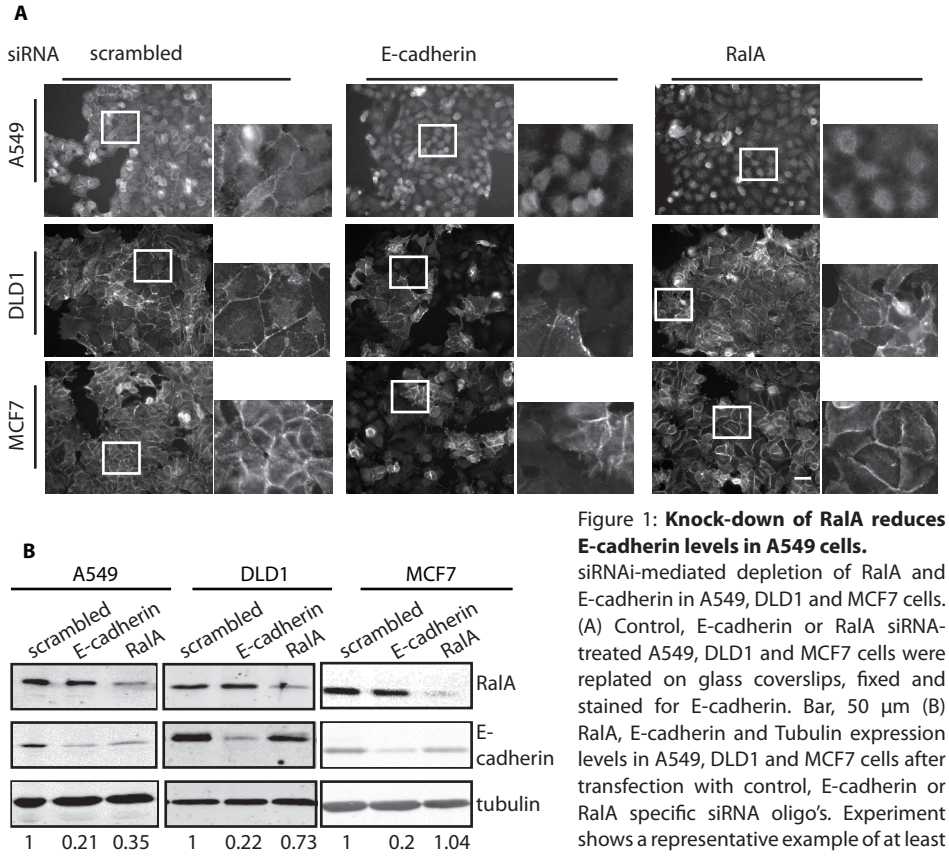


Figure 1: **Knock-down of RalA reduces E-cadherin levels in A549 cells.** siRNAi-mediated depletion of RalA and E-cadherin in A549, DLD1 and MCF7 cells. (A) Control, E-cadherin or RalA siRNA-treated A549, DLD1 and MCF7 cells were replated on glass coverslips, fixed and stained for E-cadherin. Bar, 50 µm (B) RalA, E-cadherin and Tubulin expression levels in A549, DLD1 and MCF7 cells after transfection with control, E-cadherin or RalA specific siRNA oligo's. Experiment shows a representative example of at least n=4. Quantifications represent E-cadherin levels relative to control treated cells.

of Rap1A/B and RalA was not complete, these data suggest that Rap1A/B and RalA were not essential for E-cadherin localization in DLD1 cells. Unfortunately, we have not been able to obtain simultaneous knock-down of Rap1A/B and RalA in MCF7 cells.

To investigate whether activation of Rap1 could rescue the effect of RalA knock-down in A549 cells, we made use of A549 cells stably expressing the Rap1 exchange factor, Epac1. In these cells, Rap1 can be activated with the cAMP analog, 8-pCPT-2'O-Me-cAMP (007). Previously we have shown that stimulation of control treated cells (scrambled siRNA) by 007 results in maturation of the AJs (Dube et al., 2008). However, activation of Rap1 by 007 either simultaneously with the RalA siRNA treatment or before replating the cells on coverslips, did not restore E-cadherin levels as determined by Western blot and immunofluorescence (Figure 2B and data not shown). Thus, in DLD1 cells, Rap1A/B do not synergize with RalA to control the localization of E-cadherin localization at the AJs, whereas in A549 cells, RalA is essential for E-cadherin localization, a phenotype that cannot be rescued by activation of Rap1A/B. From this, we conclude that Rap1 and RalA do not synergize to control the localization of E- cadherin in these cells.

RalA is required for normal levels of E- cadherin at the basolateral membrane.

We next investigated whether the absence of E-cadherin at the cell-cell contacts in RalA- depleted A549 cells may result from the inability of cells to form AJs. Therefore, we stained cells for other junctional markers like β-catenin (Figure 3A). β-catenin was normally localized, showing that the AJs were not disrupted. This result also suggested that other cadherins were present in A549 cells, as could be shown by PAN-cadherin staining (Figure 3A). Indeed, total N-cadherin levels were not affected upon RalA depletion (Figure 3B). Knock-down of E- cadherin increased the signal with the PAN-cadherin antibody (Figure 3A, blot right panel) indicating that the expression or the stability of other cadherins may be increased upon reduced expression of E- cadherin.

Since the knock-down of RalA did not lead to a clear appearance of an intracellular pool of E-cadherin, it remained possible that E-cadherin is diffusely localized at the membrane and is, therefore, difficult to detect by immunofluorescence. The cell surface localized E-cadherin can be measured by flow cytometry using Alexa Fluor 488 labeled anti-E-cadherin antibodies on living cells (Dube et al., 2008). We observed that knocking-down RalA in a monolayer reduces the amount of cell surface localized E- cadherin compared to control cells (70% ±9

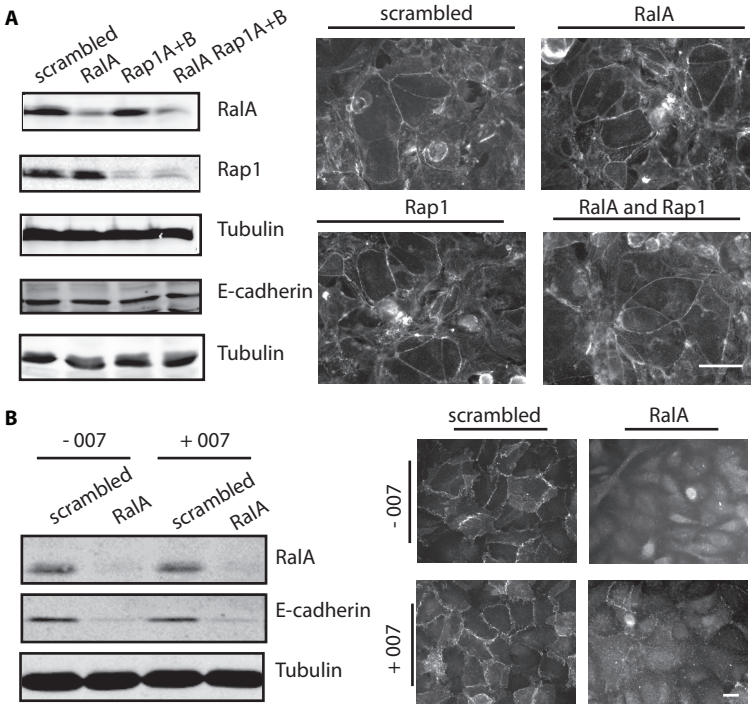
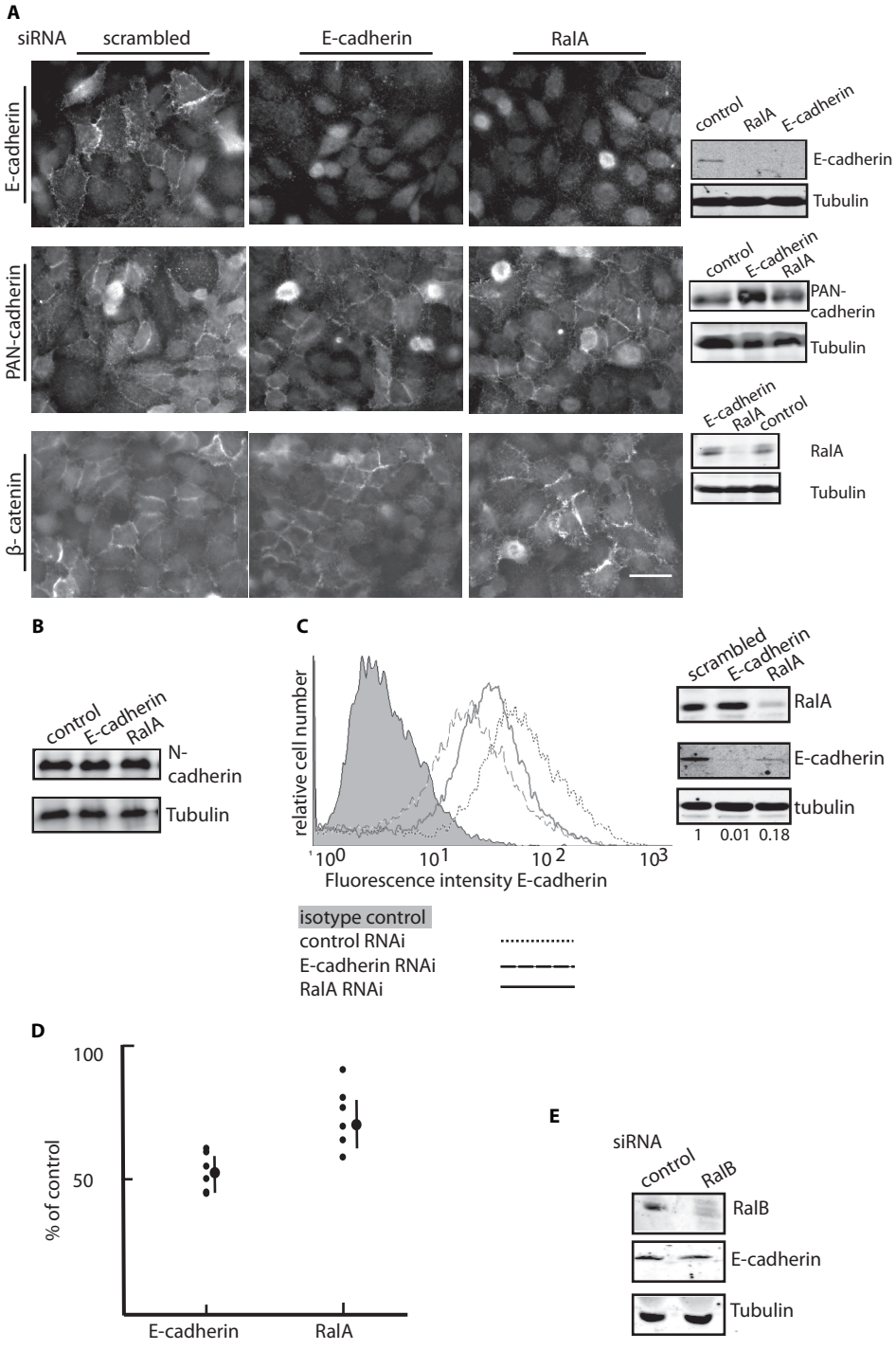


Figure 2: **Rap1 and RalA do not act synergistically in A549, DLD1 and MCF7 cells**
(A) RalA, Rap1 and E-cadherin expression levels in DLD1 cells. Left panel: Cells are transfected with control, E-cadherin, RalA, Rap1A and Rap1B (Rap1A/B) or RalA together with Rap1A/B specific siRNA oligo's. Tubulin levels were used as a loading control. Right panel: E-cadherin immunostaining of DLD1 cells treated with siRNA as indicated. (B) A549-Epac cells were treated with and without 8-pCPT-2'O-Me-cAMP (007) simultaneously with the siRNA treatment. Left panel, expression levels of RalA and E-cadherin in cells treated with control or RalA siRNA oligo's in the presence or absence of 007. Tubulin expression is used as a loading control. Right panel, E-cadherin immunostaining of A549-Epac cells, control or 007 treated. Bar, 50 µm.

Figure 3: **Knock-down of RalA reduces the membrane-bound fraction and total amount of E-cadherin in A549 cells (color image (C) p124).**
A549 cells are treated with control, E-cadherin and RalA siRNA oligo's and E-cadherin localization is determined. (A) Cells were grown in a monolayer on glass coverslips and stained for E-cadherin, PAN-cadherin and β-catenin. Bar, 50 µm. Blots show the expression levels of cadherins, E-cadherin and RalA. In this experiment, knock-down of RalA appears to result in complete E-cadherin knock-down in contrast to other experiments (Figure 1B), this may be due to high background staining in this blot. (B) Expression levels of N-cadherin and Tubulin in A549 cells treated with control, E-cadherin or RalA oligo's. (C) E-cadherin localization at the cell surface of siRNA treated cells growing in a monolayer. E-cadherin was stained with Alexa Fluor 488 anti-human CD324 antibody and analyzed by flow cytometry. Alexa fluor 488 Mouse IgG1, κ isotype control antibody demonstrates negligible background binding (filled peak). The FACS profile shows a representative example and the inset shows expression levels of RalA, E-cadherin and tubulin after transfection with siRNA oligo's in this experiment. (D) The geometric mean fluorescence of E-cadherin and RalA siRNA treated cells is presented as a percentage of remaining E-cadherin of their respective control siRNA-treated samples (scrambled). The graph shows the results of six independent experiments (•), the mean (●) 53±6 and 70 ±9 (mean ±STD) for E-cadherin and RalA respectively, bars represent standard deviation (STD). (E) Expression levels of RalB, E-cadherin and Tubulin in A549 cells treated with control or RalB oligo's.


A
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remaining E-cadherin) (Figure 3C-D). Importantly, the amount of cell surface located E-cadherin in RalA depleted cells was higher than in E-cadherin knock-down cells (53% ±6 compared to 70% ±9 remaining E-cadherin respectively in E-cadherin and RalA knock-down cells) (Figure 3C-D). Knock-down of RalB, which is highly similar to RalA (85% identical) did not affect E-cadherin levels (Figure 3E). These data show that E-cadherin localizaton at the cell surface , but not of all cadherins, is largely dependent on RalA in A549 cells.

The exocyst complex regulates E-cadherin localization.

Previously, we have shown that RAL-1 and the exocyst complex are involved in localization of the cadherin-catenin complex at the cell-cell contacts in *C. elegans* (Frische et al., 2007). Here we investigated whether the exocyst complex is also involved in regulating E- cadherin localization by knocking-down members of the exocyst complex, Sec8 and Exoc8 using siRNA. At least for Sec8, we could show efficient depletion by siRNA treatment in A549 cells (Figure 4B). Interfering with the function of the exocyst complex reduced the total amount of E-cadherin as determined by Western blotting (Figure 4B) and E- cadherin was not detected at the AJs by immunofluorescence (Figure 4D). In addition, the amount of E-cadherin at the cell surface was reduced compared to control cells as examined by FACS (Figure 4A and C) (56% ± 23 and 61% ±15 remaining E-cadherin for Sec8 and Exoc8, respectively). In contrast, knock-down of RalBP, another effector protein of RalA, does not affect the total or cell surface levels of E-cadherin (data not shown), indicating that RalA acts via the exocyst complex on E-cadherin localization. Depletion of Sec8 more strongly reduced the E-cadherin levels than Exoc8, which might reflect different knock-down efficiencies or functions in the exocyst complex. Altogether, these data show that depletion of components of the exocyst complex leads to a similar phenotype as depletion of RalA and, therefore, suggest that RalA and the exocyst complex act together in localizing E-cadherin at cell-cell contacts in A549 cells.

The guanine nucleotide exchange factor RalGDS regulates E-cadherin localization.

Next, we investigated whether any of the RBD-containing Ral GEFs, RalGDS, Rgl or Rgl2/Rlf, were involved in the regulation of E-cadherin localization. Similar to the previous experiments, we treated A549 cells with siRNA against RalGDS, Rgl or Rgl2/Rlf and determined the total and cell surface amount of E-cadherin and analyzed the presence of E-cadherin in cell-cell contacts by immuno-fluorescence. Depletion of RalGDS and

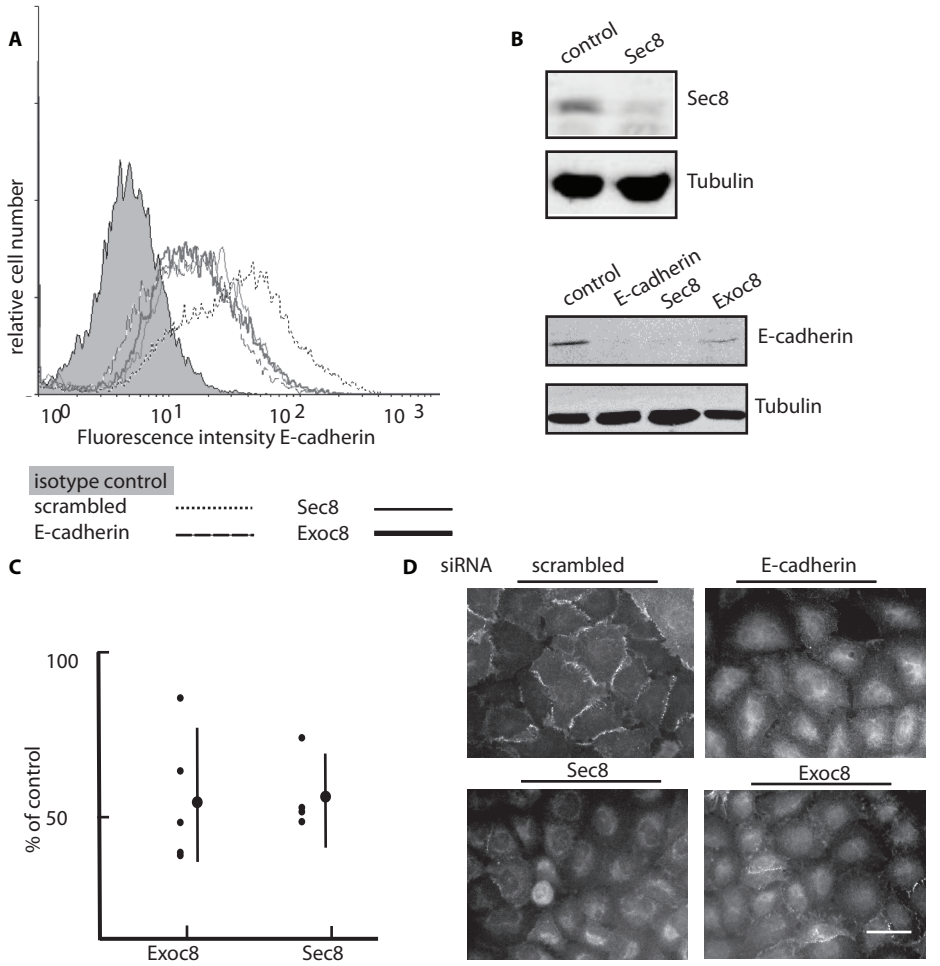

Figure 4: **Knock-down of Sec8 or Exoc8 reduces the total and membrane-bound fraction of E- cadherin (color image (A) p124).**

A549 cells were treated with control, E-cadherin, RalA, Sec8 or Exoc8 oligo's and E-cadherin localization was determined. (A) Cells were grown in a monolayer and the amount of cell surface localized E-cadherin was determined by flow cytometry after staining with Alexa Fluor 488 anti-human CD324 antibody. Alexa fluor 488 Mouse IgG1, κ isotype control antibody demonstrates negligible background binding (filled peak). A representative result is shown. (B) Expression levels of Sec8 and E-cadherin after indicated siRNA treatment. (C) The geometric mean fluorescence of Exoc8 and Sec8 siRNA treated cells as determined in FACS experiments is presented as a percentage of remaining E-cadherin of their respective control-treated samples. The graph shows the results of at least four independent experiments (•), the mean (●) 61±15 and 56 ±23 (mean ±STD) for Exoc8 and Sec8 respectively, bars represent the standard deviation. (D) Cells were grown on glass coverslips and stained for E-cadherin and phalloidin. Bar, 50 µm.

RalA resulted in a similar phenotype: the total amount of E-cadherin was reduced as determined by Western blot. In addition, examination of the amount of E-cadherin at the cell surface by FACS (62% ±7 remaining E- cadherin) and

immunofluorescence showed reduced levels in RalGDS-depleted cells to a similar extent as observed in RalA-depleted cells (Figure 5A-C and 3C). Due to a lack of appropriate antibodies to detect Rgl1 and Rgl2/Rlf we have not been able to determine the efficiency of knock-down. However, if the knock-down was efficient, these data indicate that Rgl1 and Rgl2/Rlf are not essential for E-cadherin localization. Together these results show that RalGDS is required for normal E- cadherin localization and suggest that RalGDS activates RalA in order to regulate E- cadherin localization.

A549 cells express epithelial and mesenchymal marker proteins.

The differential effect of RalA RNAi on E- cadherin levels in A549, DLD1 and MCF7 cells prompted us to investigate if we could detect clear differences in E- cadherin or associated proteins between these cell lines. As expected, on the basis of immunofluorescence data (Figure 1A), A549 cells expressed considerably less E-cadherin than MCF7 and DLD1 cells. As assesed by Western blotting, the PAN-cadherin antibody showed that the major cadherin in MCF7 and DLD1 cells migrated faster than the cadherin most prominent in A549 cells (Figure 6A). Moreover, the size of the cadherin expressed in DLD1 and MCF7 cells corresponded with the protein detected by the E-cadherin specific antibody and the one expressed in A549 cells with the protein detected by the N-cadherin antibody (Figure 6A). Using a P-cadherin specific antibody we failed to detect proteins, suggesting that P- cadherin is not expressed in these cell lines (data not shown). In addition, expression of β-catenin was lower in A549 cells compared to MCF7 and DLD1 cells. p120-catenin binds to the juxtamembrane region of cadherin and plays an important role in stabilizing E-cadherin at the membrane (Yap et al., 1998). The major isoform of p120-catenin expressed in A549 cells migrated slower than those detected in DLD1 and MCF7 cells (Figure 6A). Previously, the longer p120 isoform 1 has been found to be mostly expressed in mesenchymal cells whereas the shorter isoform number 3 is more abundant in epithelial cells (Keirsebilck et al., 1998; Mo and Reynolds, 1996). In line with this, we found that vimentin, a mesenchymal marker, is clearly expressed in A549 cells (Figure 6A). To investigate if the different E-cadherin levels might underlie the sensitivity of A549 cells for depletion of RalA, we made use of A549 expressing exogenous E- cadherin. Strikingly, the knock-down of RalA had no effect on the localization of E-cadherin in these cells (Figure 6B). This may be due to the exogenous expression of E-cadherin or this may indicate that the sensitivity for RalA-depletion may be caused by the low

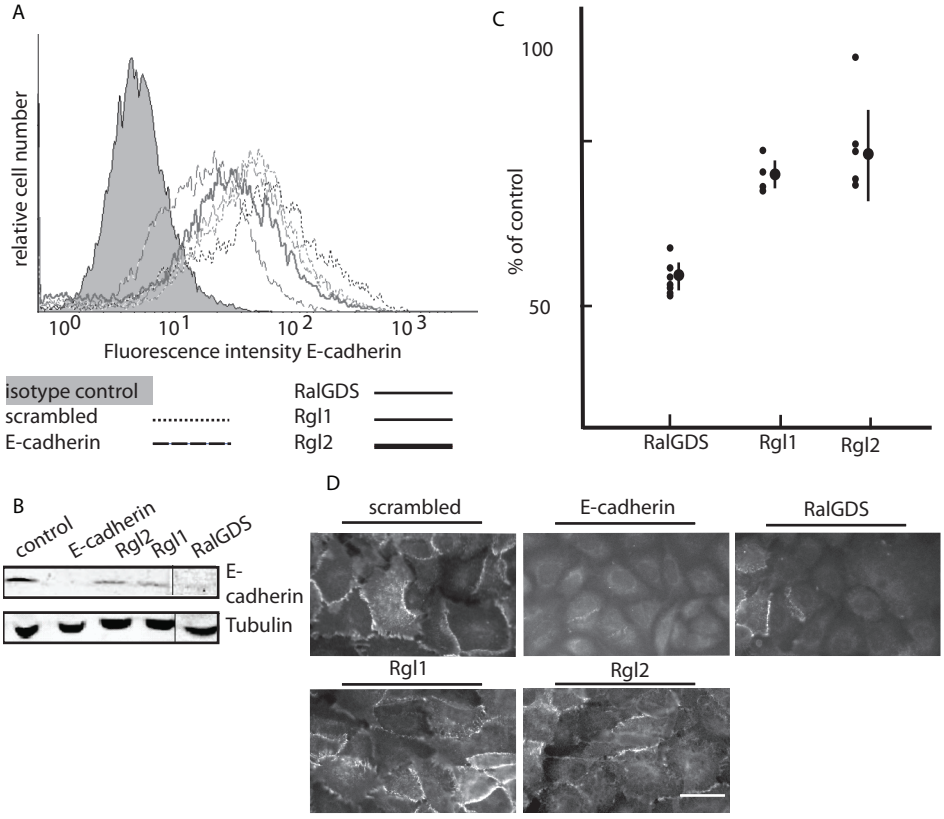

Figure 5: **Knockdown of the RalGEFs suggests RalGDS activates RalA leading to normal E-cadherin localization (color image (A) p124).**
A549 cells were treated with control, E-cadherin, RalA siRNA, RalGDS and Rgl1 oligo's and E-cadherin localization was determined. (A) Cells were grown in a monolayer and the amount of cell surface localized E-cadherin was determined by flow cytometry after staining with Alexa Fluor 488 anti-human CD324 antibody. Alexa fluor 488 Mouse IgG1, κ isotype control antibody demonstrates negligible background binding (filled peak). A representative example is shown. (B) Western blot shows the amount of E-cadherin after siRNA-treatment as indicated. Data originate from a single blot but lanes are removed between the lanes showing the results of Rgl1 and RalGDS siRNA treated cells. (C) Representation of the geometric mean fluorescence of RalGDS, Rgl1 and Rgl2 siRNA treated cells as determined in at least four FACS experiments. Numbers represent the percentage of remaining E-cadherin of their respective control-treated samples, in single experiments (•) and the mean (●) where bars represent the standard deviation. Remaining fluorescence is for RalGDS 62±7, Rgl1 and 86 ±7 and in Rgl2-treated cells 93±21 (mean ±STD). (D) Cells were grown on glass coverslips and stained for E-cadherin. Bar, 50 μm.

expression level of E- cadherin in normal A549 cells.

Discussion

In this study, we analyzed the role of RalA in targeting E-cadherin to AJs in three distinct tumor cell lines, namely A549, MCF7 and DLD1. In MCF7 or DLD1 cells, RNAi-mediated knock-down of RalA had no effect on E-cadherin levels at the AJs whereas in A549 cells, RalA is required for correct localization of E-cadherin at

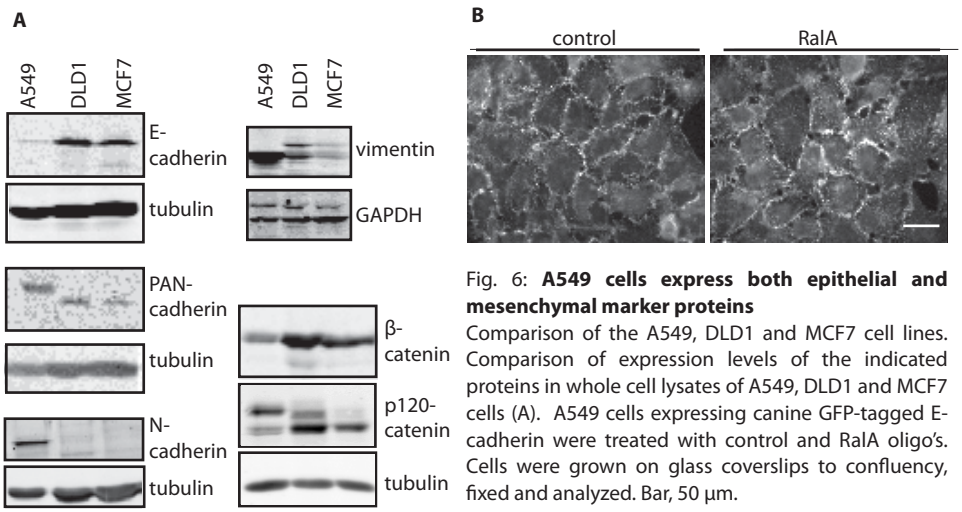

Fig. 6: **A549 cells express both epithelial and mesenchymal marker proteins**
Comparison of the A549, DLD1 and MCF7 cell lines. Comparison of expression levels of the indicated proteins in whole cell lysates of A549, DLD1 and MCF7 cells (A). A549 cells expressing canine GFP-tagged E-cadherin were treated with control and RalA oligo's. Cells were grown on glass coverslips to confluency, fixed and analyzed. Bar, 50 µm.

AJs. Also, knock-down of two members of the exocyst complex, Sec8 and Exoc8, abolishes E-cadherin accumulation at AJs, suggesting that the exocyst complex functions downstream of RalA in A549 cells in this process. Finally, knock-down of the Ral exchange factor RalGDS results in a similar phenotype.

At present, it is unclear why MCF7 and DLD1 cells are less dependent on RalA activity than A549 cells for E-cadherin localization. The morphology of the AJs of MCF7 and DLD1 cells as well as the presence of molecular markers (Figure 6), indicate that they have more typical epithelial features than A549 cells. It is possible that we observe a cell-type specific effect or that the level of E- cadherin is a determining factor, since ectopically expressed dog GFP-tagged E-cadherin is normally localized at AJs in RalA-depleted A549 cells. However, we cannot exclude that this GFP-tagged E-cadherin induces expression of other proteins that stabilize E-cadherin at AJs as it has been described before (Seidel et al., 2004). Alternatively, the GFP-tag could interfere in normal E-cadherin regulation.

In *C. elegans*, knock-down of RAL-1 in animals deficient for RAP-1 prevents localization of HMP-1/α-catenin and thereby disrupts HMR-1/cadherin localization (Frische et al., 2007). Based on these results, we concluded that in *C. elegans* RAP-1 functions in parallel to that of RAL-1. Also in A549 cells, Rap1 and RalA appear to act in different pathways since we were unable to rescue depletion of RalA in A549 cells by selective activation of Rap1 via Epac1. RalA has been shown to affect the rate of transport of newly synthesized E- cadherin to AJs (Shipitsin and Feig, 2004) and may be important in the recycling of E-cadherin via the exocyst

complex (Langevin et al., 2005). Rap1, on the other hand, has been postulated to inhibit E-cadherin internalization via AF6 and p120 catenin (Hoshino et al., 2005). We have shown previously that knock-down of Rap1B only had a mild effect on the level of E- cadherin in AJs in A549 cells, while Rap1A was required for maturation of AJs (Dube et al., 2008). However, it should be kept in mind that over-expression of Rap1GAP in Ovcar cells (Price et al., 2004) and MCF7 cells (Hogan et al., 2004) results in more severe effects on E-cadherin localization in AJs, possibly indicating a role for Rap2 proteins. Interestingly, the phenotype of RalA knock-down was clearly different from the previously described effect of depletion of Rap1A (Dube et al., 2008). Staining of the AJs with e.g. a β-catenin antibody did not show the presence of immature junctions. The previously described knock-down of Rap1B mildly reduced the amount of E- cadherin (Dube et al., 2008), however we did not observe additional E-cadherin reduction upon simultaneous Rap1B and RalA knockdown in comparison to RalA-depleted cells. It will be interesting to compare the phenotype that we observed with RalA and Rap1B siRNA treated cells and to investigate whether depletion of Rap1B only affects E- cadherin or also N-cadherin levels in A549 cells.

The minimal effect of depletion of Rap1A/B and RalA in DLD1 cells in comparison with the phenotype observed in *C .elegans* could be due to incomplete knock-down, to the presence of other pathways in DLD1 cells that contribute to E-cadherin localization or to differences in the model systems used. In *C. elegans*, the process of epidermal sheet formation and dorsal enclosure is very dynamic and presumably requires tight regulation of HMR-1/E-cadherin localization which is likely to be less important in tissue culture cells.

It is not clear whether the reduction of the total amount of E-cadherin in RalA-depleted cells is due to a failure in newly synthesized E-cadherin transport or to aberrant sorting during recycling of E- cadherin (for a review see (Bryant and Stow, 2004)). Although the quantitative data from the FACS and Western blotting may not be directly comparable, they suggest that in RalA-depleted cells the amount of cell surface localized E- cadherin was less reduced compared to the total amount of E-cadherin. This difference might be explained by aberrant membrane targeting. For instance, if E- cadherin molecules are targeted to the apical membrane and cannot make homophilic interactions, it may result in increased endocytosis and to enhance E- cadherin degradation. This might result in a more prominent reduction of the total amount of E- cadherin than cell surface localized

E- cadherin.

It is interesting to note that RalA RNAi selectively affects E-cadherin, but not other cadherins in A549 cells. Thus far, relatively little is known about the differential regulation of the different types of cadherins. Formation of N- cadherin containing cell-cell junctions appears not to be sensitive to Rap1GAP over-expression and, in addition, N- cadherin does not interact with the Rap1 GEF C3G (Hogan et al., 2004). Finally, the E3-ligase, Hakai, that targets E-cadherin for degradation, is not acting on N-cadherin and so will require other degradation signals (Fujita et al., 2002). If any of these differences explains the different effect on cadherin localization upon RalA-depletion, remains to be investigated. However, differential trafficking of cadherins might have a function in cadherin switching, a process where cells change their cadherin expression profile during development. This is an important mechanism during morphogenesis to allow the separation of different cell types from one another. In addition, cell type switching is a phenomenon more often observed in cancer cell lines (reviewed in (Gumbiner, 2005; Hazan et al., 2000; Wheelock et al., 2008). It will be interesting to determine if and how cadherins are differentially regulated and if these profiles may provide a basis for treatment of tumors.

Acknowledgements

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Screening for new players in RAP-1 and RAL-1-mediated pathways.

Ester W. Frische[1], Wendy Pellis-van Berkel[1], Edwin Cuppen[2], Margarita Camacho[1], Johannes L. Bos[1] and Fried J.T. Zwartkruis[1].

[1]Dept. of Physiological Chemistry, Centre for Biomedical Genetics and Cancer Genomics Centre, UMC Utrecht, Universiteitsweg 100, 3584 CG Utrecht, the Netherlands; [2]Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.

Abstract

In a genome-wide RNAi screen with a *C. elegans* strain mutant for *rap-1*, we previously identified seven genes that caused a synthetic lethal phenotype. Out of these, *ral-1* and *exoc-8* act together with *rap-1* in positioning and migration of hypodermal cells during development. Interfering in the RAP-1 and RAL-1-mediated pathways lead to loss of the adherens junctions in the hypodermal cells. Here we present our preliminary studies, indicating that some of the remaining synthetic lethal genes do not act in the RAL-1/EXOC-8/SEC-5 pathway. A surprising outcome of these experiments is that RAP-1 is required for the viability of animals in which the activity of the JNK/p38 MAPK pathway is aberrant. Furthermore, we investigated the option to set up a reverse synthetic lethal screen to identify novel elements of the RAP-1 pathway.

Introduction

Synthetic lethality is the phenomenon in which the simultaneous disruption of two genes is lethal whereas the disruption of either one is not. Synthetic lethality indicates that two genes function redundantly, based on sequence homology or in parallel pathways to mediate a single biological process. Synthetic lethal screens have been a powerful tool to identify new genetic interactions. The technique was originally developed in yeast where crossing a mutant cell with a library consisting of several other mutants allowed the identification of essential genes in a specific mutant background (reviewed in (Ooi et al., 2006)). Ultimately, a genetic interaction map may be build based on systematic genome-wide analysis of these synthetic lethal profiles. The development of genome-wide RNAi libraries in *C. elegans* (Fraser et al., 2000; Kamath et al., 2003) made it feasible to perform similar screens *in vivo* in a multicellular organsism. We performed such a screen using null mutants of the Rap1 signaling network (Frische et al., 2007). Rap1 is a very close homolog of Ras and was found in a revertant screen for Ras-induced cell transformation in tissue culture cells suggesting that Rap1 antagonizes Ras activity (Bourne et al., 1990; Kitayama et al., 1989). Since then, several studies have shown that Rap1 functions independently of Ras in processes like integrin-mediated adhesion, cell-cell contact formation, neuronal polarity and outgrowth and vesicle trafficking (for reviews (Bos et al., 2001; Kooistra et al., 2007; Menasche et al., 2007; Pannekoek et al., in press). The big challenge today is to understand how the signaling networks around Rap1 are organized to mediate such complex processes. We identified seven

genes that were synthetic lethal with two independently derived *rap-1* null alleles in *C. elegans,* but not with mutants of the related *rap-2* gene or *epac-1* that encodes for a cAMP-regulated exchange factor for RAP-1 and RAP-2. Previously, we have demonstrated that the RAL-1/exocyst complex pathway acts in parallel to RAP-1 in hypodermal cell organization (Frische et al., 2007). In this screen, three other genes were identified, *vhp-1, phi-24* and *sur-6* that we choose to investigate further.

SUR-6 is a regulatory subunit of the PP2A holoenzyme, a member of ubiquitously expressed serine-threonine phosphatases that are involved in many different signaling processes (reviewed in (Janssens and Goris, 2001). In *C. elegans*, this protein positively regulates Ras-mediated signaling and acts downstream of LET-60/Ras and upstream of LIN-45/Raf, but the mechanism remains unclear (Kao et al., 2004; Sieburth et al., 1999). In human cells, PP2A Aβ negatively regulates RalA by dephosphorylation of Ser183 and Ser194, but the biological function remains to be determined (Sablina et al., 2007). *vhp-1* encodes for the dual specificity phosphatase VHP-1 that acts on threonine and tyrosine residues. *In vivo*, VHP-1 dephosphorylates a specific set of MAP kinases; the JNK-like KGB-1 and the p38-like PMK-1 (Mizuno et al., 2004). Indeed, the closest human homolog of VHP-1, MKP-7, is also a specific regulator of JNK and p38-like MAP kinases (Tanoue et al., 2001). In *C. elegans*, both the KGB-1 and PMK-1 pathways are involved in stress-induced signaling. The NSY-1/SEK-1/PMK-1 signaling module is required for pathogen resistance whereas survival in the presence of heavy metals is dependent on the MLK-1/MEK-1/KGB-1 pathway (Kim et al., 2002; Mizuno et al., 2004). In both these pathways, VHP-1 functions as a negative regulator (Kim et al., 2004; Mizuno et al., 2004). In addition, these pathways are connected via MEK-1 that activates PMK-1 in addition to KGB-1 (Figure 4A). Furthermore, KGB-1 phosphorylates VHP-1, but so far no *in vivo* functions for this phosphorylation have been described (Mizuno et al., 2004). Finally, PHI-24 is homologous to human CHMP1, a protein involved in endosomal recycling via multivesicular bodies.

Here, we show that the phenotype of SUR-6, VHP-1 and PHI-24 depletion in a *rap-1* null background is not identical to the synthetic lethal phenotype of RAP-1 and RAL-1. We investigated the observed synthetic lethality between RAP-1 and VHP-1 in more detail and RAP-1 did not appear to control the heavy metal stress response. Strikingly, RAP-1 is an essential protein in *mek-1* and *kgb-1* mutant animals indicating a putative role for RAP-1 in regulation of this pathway. Finally, we performed preliminary studies to investigate the options for a reverse synthetic lethal screen to identify new

components of the RAP-1 signaling network.

Material and Methods

Worms.

General methods for culturing and manipulating worms were as described unless otherwise stated (Lewis and Fleming, 1995). Worms were cultured on NGM plates at 20°C. Strains used: Bristol N2, FZ222 *rap-1*(*tm861*), FZ224 [bjIs64[pML902(*dlg-1::GFP*) pRF4], FZ271 *rap-1*(*tm861*); bjIs64[pML902(*dlg-1::GFP*) pRF4], FK171 *mek-1(ks54)*; FZ293 [*mek-1(ks54); rap-1(tm861)/dpy-20(e1362)*]; *kgb-1(ku21);* FZ291 [*kgb-1(km21); rap-1(tm861)/dpy-20(e1362)*]; KU4 *sek-1(km4)*; FZ276 *ral-1(pk2355);* FZ280 *ral-1(pk2356);* FZ289 [*ral-1(pk2355);rap-1(tm861)];* FZ290 [*ral-1(pk2356);rap-1(tm861)]; rgl-1(tm2255); FZ228 [rap-1(tm861);kyIs140[str-2::GFP lin-15]*. *rap-1(tm861)* was detected as previously described (Frische et al., 2007). *ral-1(p2355)* and *ral-1(pk2356)* alleles were used with primers as described in the *mutant isolation* section and digested with *Mfe I* and *Acc I* respectively. Other mutants were detected in a nested PCR by the following primers: *rgl-1(tm2255),* set 1: forward CGACAAATTGTGATGTGCTGG and reverse CCTCGATGAATTGAAGAGCC, set 2: forward CCATGCTCAGCAGCCTTCTCG and reverse CCTGTTCTAGAATCTTTCTCG. *mek-1(ks54),* set 1: forward TCCGCACTCGCCCATCAT and reverse CAATGCCACGATGACTAGGC, set 2: forward CGGCAAACCACTCCTCGACG and reverse TCCACATTTTCCAGCACCGC. *kgb-1(km21),* set 1: forward CAGCACTTTCACTATTCCAACTCG and reverse CCTTGGGAAACGCGAAAGACGGC, set 2: forward AAACCTTAGTTTTCTGAATGCCG and reverse AGCAAAAATGAATCCGCCACTCC.

Analysis embryos and larvae.

L4-stage larvae were transferred to a plate containing bacteria expressing dsRNA and incubated at 15 ˚C (day1). Adults were transferred to a fresh plate for a 2-hour egg-lay and embryos were allowed to develop for 2 hours (day4). Embryos were mounted on a 2% agarose patch and comma stage embryos were analyzed. Larvae were transferred to a 2% agarose patch containing sodium-azide. Animals were analyzed using a Zeiss Axioskop2.

Screen and siRNA.

Screening was essentially performed as described in chapter 2 (Frische et al., 2007)). Adults were bleached to collect eggs and these were allowed to hatch overnight in M9 medium. L1 stage larvae were incubated at 20˚C on OP50 plates before they were transferred as L4-stage larvae to screening plates (day 1). Bacteria were grown overnight in 1 mM IPTG containing NGM and used to

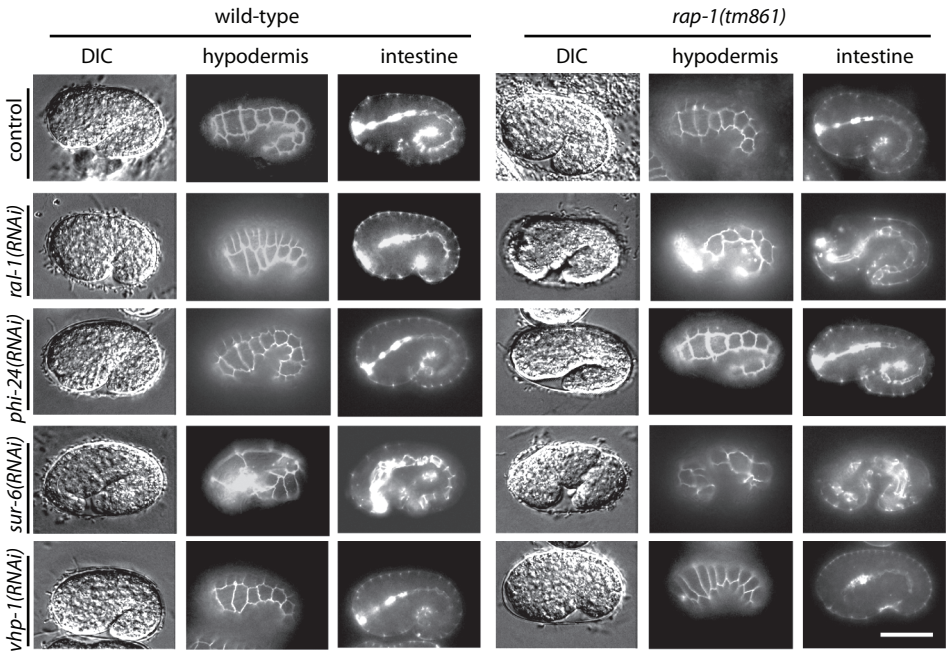

Figure 1: **Phenotype of *rap-1* null mutant embryos carrying the *DLG-1::GFP* gene subjected to *ral-1, phi-24, sur-6* or *vhp-1(RNAi)*.**
L4-stage wild-type or *rap-1* mutant animals expressing DLG-1::GFP were subjected to *ral-1, phi-24, sur-6* or *vhp-1(RNAi)* and embryos of timed egg-lays of these animals were collected for analysis. Panels show DLG-1::GFP expression and the corresponding DIC picture of comma stage embryos.

inoculate screening plates. Incubation on siRNA food was performed at 15 ˚C and adults and progeny were scored at day 4. Plates number 31-52 were used.

Mutant isolation.

Nested PCR primersets for the identification of mutants were designed according to the TILLING protocol (Cuppen et al., 2007). Primers for *ral-1* mutant identification were designed on exon 5, set 1: forward AATTTTAACCCGAATTTTGTG and reverse TCAGCCGAAAACTTATTTCC. Set 2: tgtaaaacgacggccagtCGAAAAGCTTCGATTTTTAGAC and reverse aggaaacagctatgaccatAATTTTTCGCTTTTCAGCTC. Lowercase sequences are invariant sequences required for direct sequencing.

Heavy metal resistance.

L4-stage larvae were transferred to fresh (<1 week old) NGM plates containing no or $CuSO_4$ with 25, 50, 100 or 200 µM final concentration. Adults were transferred to a fresh plate and allowed to lay eggs for 2 hours. Adults were removed, embryos counted and incubated at 20˚C. After 4 days, the amount of adults were scored.

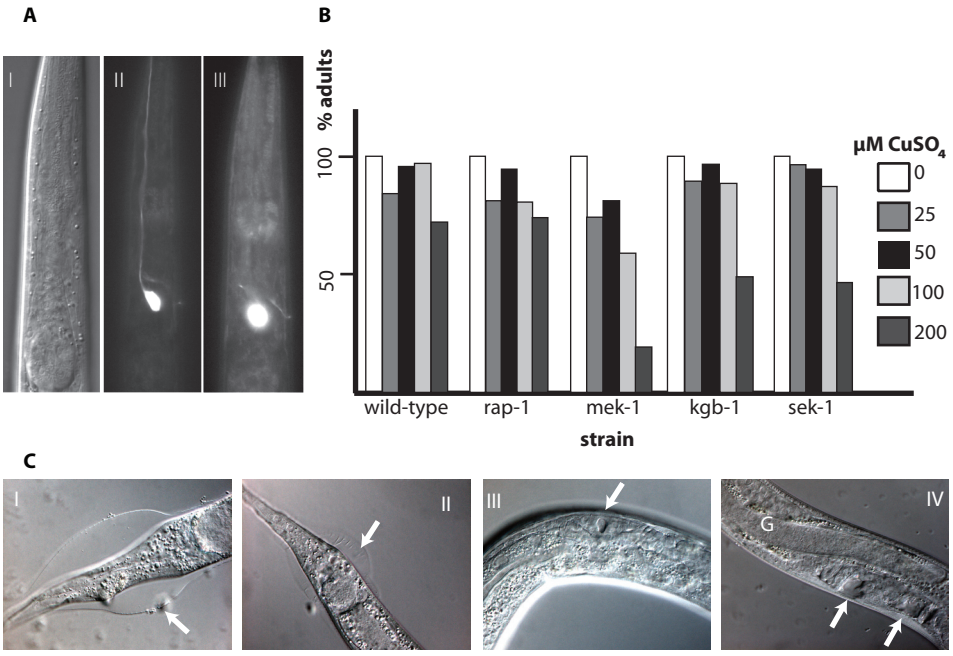

Figure 2**: Phenotype of *rap-1(tm861);mek-1(ks54)* mutants, STR-2 expression in *rap-1(tm861)* animals and heavy metal stress sensitivity (color image (A) p125).**
(A) Expression of the odorant receptor STR-2 using a GFP reporter strain in the olfactory AWC neuron in a *rap-1(tm861)* mutant animal (I). These animals have a wild-type expression pattern; only one neuron expresses STR-2 (II) whereas the other olfactory AWC neuron is GFP-negative (III). (B) Heavy metal stress sensitivity assay. Percentage of animals that reach adulthood in 4 days is normalized to the non-$CuSO_4$ containing condition for each cell line. A representative example is shown (for each strain at least three independent experiments were performed). (C) Analysis of hypodermal defects in L3/L4-stage *rap-1(tm861);mek-1(ks54)* mutants. (CI-II) Molting defects. The arrow points at the old cuticle that is still attached. (CIII-IV) vacuoles are present underneath the cuticle (arrows). In some cases the gut lumen is enlarged (G in the picture).

GTP-loading.

Cos7 cells were transfected with HA-tagged versions of *ral-1*, carrying the mutations indicated. Cells were metabolically labeled with [^{32}P]-orthophosphate and lysed and processed essentially as described in (Wolthuis et al., 1997). Ral-proteins were extracted from the cell lysate by anti-HA immunoprecipitation, nucleotides were eluted and separated by thin layer chromatography. GTP-GDP ratios were determined using a Phosphorimager.

Results and Discussion

The phenotype of phi-24, sur-6 or vhp-1 depletion is not identical to ral-1 knock-down in rap-1 null mutants.

A genome wide RNAi screen using *rap- 1* mutants lead to the identification of seven genes that are essential for survival of these mutants. Two of these genes, *ral-1* and *exoc-8*, function in one complex, which is a strong indication for

the functionality of this screen. Here we analyzed in more detail the phenotype induced by depletion of some other hits of the screen, SUR-6, PHI-24 and VHP-1. To this end, we transferred *rap-1(tm861)* L4-stage larvae to plates containing bacteria expressing dsRNA against these genes and analyzed their progeny. Since these proteins might act together with RAL-1 we first compared the phenotype of comma stage embryos to the previously described phenotype of offspring of *rap*-1 null mutants on *ral-1(RNAi)*. In these latter embryos, hypodermal cell organization is disturbed at the comma stage (Figure 1 and Frische et al., 2007). In contrast, *rap-1* null mutants on *phi-24* or *vhp-1(RNAi)* appeared normal and indistinguishable from wild-type animals at the comma stage with hypodermal cells organized in rows of dorsal, lateral and ventral cells as visualized by DLG-1::GFP (Figure 1). Therefore, the defects causing the synthetic lethality occur later in development. Depletion of SUR-6 led to compromised development both in wild-type and in *rap-1* null animals and most embryos did not become twofold. DLG-1::GFP was localized at cell-cell contacts of hypodermal, intestinal and pharyngeal cells, however the position of these cells inside the embryo was aberrant (Figure 1). Strikingly, *sur-6(RNAi)* on agar-plates did not show the strong difference between wild-type and *rap-1* null animals that was seen in the screen performed in liquid. Whether this is due to the difference of culture conditions (plate versus liquid) or to the timing at which RNAi was started (L4 versus L1) is unclear. Although we could not control for knock-down efficiencies, these results do not support the idea that VHP-1, PHI-24 or SUR-6 act in the RAL-1/exocyst complex pathway but act independently in parallel to RAP-1.

rap-1 null mutants are synthetic lethal with the phosphatase vhp-1 and with the kinases mek-1 and kgb-1.

Given the previously described links between Ral and the JNK pathway (de Ruiter et al., 2000; Sawamoto et al., 1999) we decided to further investigate the sensitivity of *rap-1(tm861)* mutants to *vhp-1(RNAi)*. VHP-1 is a negative regulator of two stress-induced kinases, namely KGB-1 and PMK-1. Worms homozygous for the presumptive null allele *vhp-1(km20)* do not proceed past the L3 stage. Interestingly, *vhp-1(km20)* null mutants are normally viable in a *mek-1(ks54)* or *kgb-1(km21)* null background (Mizuno et al., 2004), indicating the importance of balanced signaling in this pathway. In a wild-type genetic background, *vhp-1(RNAi)* is not lethal but results in animals that are sick, move slowly or have a burst vulva (Kamath et al., 2003; Maeda et al., 2001)(our own data).

The simplest explanation for the selective enhancement of *rap-1(tm861)* animals for

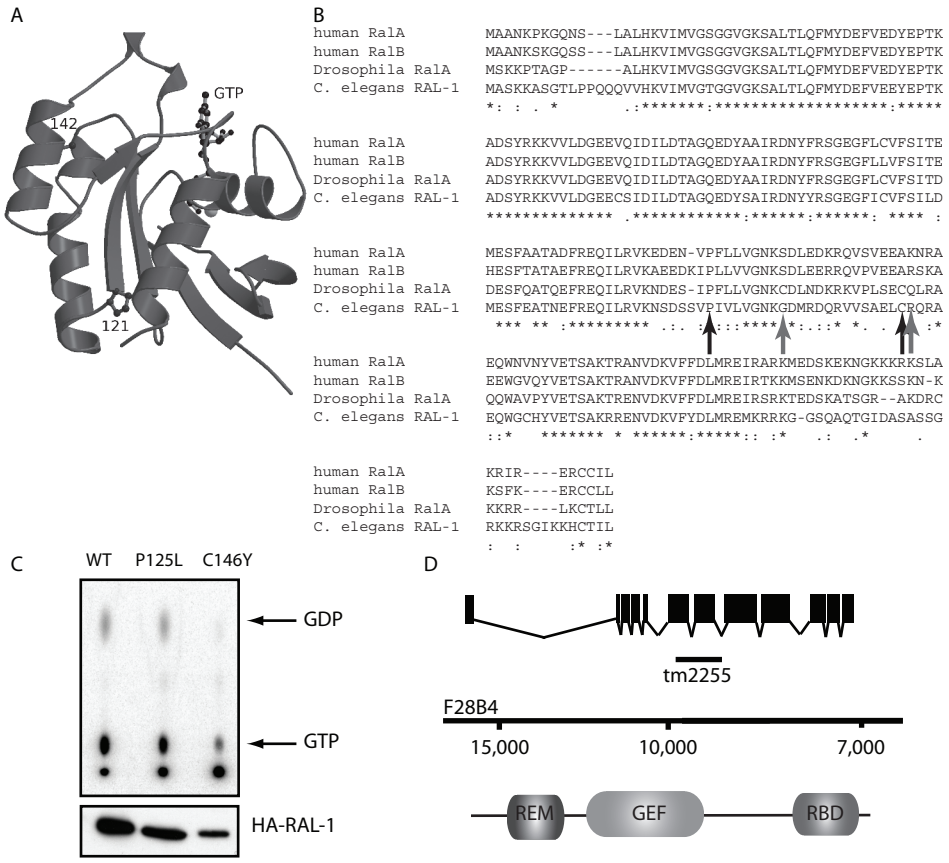

Figure 3: **Characterization of *ral-1(pk2355)* and *ral-1(pk2356)* (color image (A+D) p125).**
(A) Graphic representation of the mutated sites in RAL-1 for which the corresponding *ral-1* alleles were isolated. (B) Alignment of *C. elegans ral-1* with *Drosophila* RalA and human RalA and RalB. Black arrows point at the mutated sites in *ral-1(pk2355)* and *ral-1(pk2356)*, grey arrows point at the mutants that were not isolated (Table I). (C) Upper panel: GDP/GTP loading of RAL-1 mutants. Arrows indicate the position of the nucleotides after separation. Bottom panel: Expression levels of the RAL-1 proteins. (D) Schematic representation of the *rgl-1* locus. Filled boxes represent exons and the bar indicates the area of the out-of-frame deletion and insertion of the *tm2255* allele. Numbers indicate the position on the F28B4 cosmid. Below is a graphic representation of the domain structure of RGL-1.

vhp-1(RNAi) is that RAP-1 like VHP-1 acts as a negative regulator of KGB-1 and/or PMK-1. Modulation of the NSY-1/SEK- 1/ PMK-1 pathway has been reported to alter expression of the odorant receptor *str-2* in olfactory AWC neurons. Normally, *str-2* is expressed in either one of the two AWC neurons, but diminished activity of this stress kinase pathway leads to expression in both neurons, while loss of expression is seen upon over-activation. Introducing a *str-1::GFP* reporter strain in *rap-1(tm861)* mutant animals however revealed a wild-type expression pattern (Figure 2A), indicating that at least in AWC neurons, RAP-1 does not alter PMK- 1

activity.

KGB-1 and PMK-1 are stress kinases that protect animals when growing in the presence of heavy metals. Also, their upstream kinases, SEK-1 and MEK-1 are required for heavy metal resistance. We therefore investigated whether RAP-1 controlled heavy metal stress resistance by comparing the growth of *rap-1(tm861)* mutants to wild-type, *mek-1(ks54)*, *sek-1(km4)* and *kgb-1(km21)* mutants in heavy metal containing growth conditions. We incubated L4-stage larvae on plates containing no or different concentrations of $CuSO_4$ until adulthood. Progeny of a four-hour egg-lay was collected and the percentage of adults was determined after four days. Development was severely disturbed in *mek-1(ks54)*, *sek-1(km4)* and *kgb-1(km21)* animals at 200 µM $CuSO_4$, but wild-type animals and *rap-1(tm861)* mutants were much less affected (Figure 2B). Although these data show that, as expected, loss of RAP-1 does not lead to diminished $CuSO_4$ resistance, a negative influence of RAP-1 on these stress kinases may be more readily detectable in double mutants of e.g. *mek-1(ks54);rap-1(tm861)* as previously shown for *mek-1(ks54);vhp-1(km20)* animals (Mizuno et al., 2004). Strikingly, we were not able to generate *mek-1(ks54);rap-1(tm861)*, *kgb-1(km21);rap-1(tm861)* or *sek-1(km4);rap-1(tm861)* mutants. For example, homozygous *mek-1(ks54)* mutants, derived from *rap-1(tm861)* heterozygous animals, arrested during late larval stages and showed hypodermal abnormalities like molting defects and vacuoles in hypodermal cells (Figure 2C). This sensitivity of *rap-1(tm861)* mutant animals to loss of the phosphatase VHP-1 as well as its opposing kinases MEK-1, SEK-1 and KGB-1 is remarkable and indicates the importance of balanced stress kinase signaling for these animals.

TABLE I **Overview of the mutations causing an amino acid substitution found in a TILLING approach to identify *ral-1* mutants.**

Mutation	Description
R147Q	The side-chain of arginine147 points outside the protein, the mutation to a glutamine most likely will not affect the structure.
P125S	Proline125 makes a specialized turn, this may be disrupted by a mutation to a serine.
P125L	Proline125 makes a specialized turn, this may be disrupted by a mutation to a leucine.
G133S	The turn that glycine133 makes, may be disturbed by a mutation to a serine due to the longer side-chain.
C146Y	The side-chain of cysteine146 points into a hydrophobic pocket, mutation into a tyrosine may affect the conformation of the pocket due to the bigger side-chain.

None of the mutations affects the effector or nucleotide binding interface.

Identification of additional mutants synthetic lethal with rap-1(tm861). An interesting aspect of synthetic screens is their reversibility, i.e. mutants that cause synthetic lethality with *rap-*

1(tm861) maybeusedtoidentifyadditional elements from the RAP-1 pathway. Work described in this and other chapters identified three such genes, namely *unc-34* (chapter 5), *kgb-1* and *mek-1*. We tried to extend this number by investigating genes from the RAL-1/exocyst complex pathway. Mutants carrying the null allele *ral- 1(tm2760)* have a partially deleted exon 3 but these animals are not viable (S. Mitani mutant database and our data, not shown). Therefore, we tried to isolate a hypomorphic *ral-1* mutant by TILLING using an existing EMS library (Cuppen et al., 2007). In this screen, five mutant alleles were identified (Table 1) of which we decided to isolate the alleles with the P125L and C146Y mutation based on the analysis of the RalA crystal structure (Jin et al., 2005). The proline at position 125 makes a turn that most likely will be disturbed by a mutation to a leucine. The side-chain of the cysteine at position 142 points inside a hydrophobic pocket. A mutation into a tyrosine is expected to disrupt the structure since the side-chain is too big for the hydrophobic pocket. However, both mutations most likely do not affect the effector-binding domain (Figure 3A). We isolated and crossed these mutants four times with wild-type animals, to remove any additional mutations. Both mutants appeared fully viable and fertile, but unfortunately did not show any obvious phenotype when introduced in a *rap-1(tm861)* mutant background. This prompted us to check the proteins encoded by these mutants for GTP loading in comparison with wild-type RAL-1 (Figure 3B). As expected from the outcome of the genetic experiments, GTP-loading appeared not to be affected. As an alternative, we obtained a mutant for *rgl-1* (kind gift of Dr. S. Mitani), the gene encoding for the guanine nucleotide exchange factor for RAL-1. The *rgl-1(tm2255)* allele is a putative null allele, due to an out of frame deletion and insertion (Figure 3D) and is viable and fertile. Interestingly, *rgl-1(tm2255);rap-1(tm861)* double mutants are not viable and die as L3 or L4 larvae, indicating that this mutant may be useful for a *rap-1* reverse screen.

Reverse screening for new players in the RAP-1 signaling network.

We started with a reverse screen for RAP- 1 pathway elements using *unc- 34(e566)* based on the observation that *unc-34(e566);rap-1(tm861)* double mutants are not viable (chapter 5). We performed the screen in duplo in an RNAi screening approach according to the screen described previously (chapter 2). Only those RNAi foods that were lethal in both wells were scored as synthetic lethal. However, after screening 22/200 plates of the Ahringer RNAi library, we stopped screening since the amount of hits was too high; 2.8% caused lethality and 7.4% caused a severe reduction of

TABLE II **Overview of genes found in the RNAi screen that caused less progeny (<50%) with strains carrying *mek-1(ks54)* and *kgb-1(km21)* and appeared normal in wild-type and *unc-34(e566)* animals.**

Location	Gene	Description/best human BLASTP hit
Enzyme		
48F8	*T02G5.7*	Acetyl-CoA acetyltransferase, ACAT1
50B1	*B0495.2*	Isoform SV10 of PITSLRE serine/threonine-protein kinase CDC2L1
50E10	*T01H3.3*	Permease of the major facilitator superfamily / Solute carrier family 17
50F5	*ZK669.1/tag-341*	Chimaerin and related Rho GTPase activating protein
50C1	*B0228.5/ trx-1*	Thioredoxin
Receptor		
32D9	*K07E8.5*	Somatostatin receptor typel (mammalian), Fmrf receptor (fly)
46A12	*ZK84.1*	Mucin-1 receptor
Transcription factor (related)		
52D10	*C34C6.8/ ceh-7*	Homeodomain transcription factor / human ventral anterior homeobox 2
Other		
49H9	*R07G3.3/ npp-21*	Nuclear pore complex-associated protein TPR
40E6	*M151.4*	Early endosome antigen I
50H4	*T05H10.5/ ufd-2*	Ubiquitin fusion degradation protein-2
31A9	*C01B12.8*	Forkhead-associated (FHA) phosphopeptide binding domain 1 isoform 8
50B3	*B0495.5*	Highly conserved protein containing a thioredoxin domain / isoform 2 of Spermatogenesis-associated protein 20 precursor
50E3	*T09A5.8/ cex-2*	Heterochromatin-associated protein HP1 and related CHROMO domain proteins / Isoform 1 of M-phase phosphoprotein 8
Unknown function		
31C7	*C50D2.6*	No description, no clear mammalian homolog
40E9	*F19B10.1*	No description, no clear mammalian homolog
40H3	*F12E12.1*	No description, no clear mammalian homolog
50D1	*C06A8.8*	No description, no clear mammalian homolog
50D8	*T09A5.1*	No description, no clear mammalian homolog
51C10	*F35C11.4*	No description / Uncharacterized protein C9orf125
52D5	*C34C6.3*	No description, no clear mammalian homolog

progeny. We concluded that this strain was too sensitive for this screening approach in liquid (data not shown). In addition, we used the *mek-1(ks54)* and *kgb-1(km20)* alleles for a RAP-1 reverse screen. In order to identify genes that act in parallel to the JNK-like pathway, we only scored for foods synthetic lethal with both mutants. First, we used the small-scale RNAi-library (Chapter 5, Table I), but none caused synthetic lethality in both strains (data not shown). Next, we screened 11% of the library, where we also did not identify RNAi foods causing synthetic lethality in both strains. Only one RNAi food caused synthetic lethality in either one of the strains: dsRNA against *suf-1*, a gene encoding for a subunit of the mRNA cleavage and polyadenylation complex, was synthetic lethal with the *kgb- 1(km21)* strain. However, we identified 22 genes that reduced brood-size (<50% reduction) in both the *mek- 1(ks54)* and *kgb-1(km21)* mutants (Table II). The encoded proteins are involved in various processes and it will be interesting to see if and how these proteins act in parallel to RAP-1. Due to time constraints, we were not able to proceed with this screen or to perform a preliminary screen with *rgl-1(tm2255)* mutants.

Concluding remarks

Genes with synthetic lethal interactions may have very different functions that together are required for viability. A careful comparison of the lethal phenotype can provide a first classification of genes into groups that may operate within a single biochemical pathway. Although a phenotypic analysis indicates that SUR-6, PHI-24 and VHP- 1 do not operate in the RAL-1 signaling network, our results are too preliminary to conclude this. Based on literature, the three genes studied here (Figure 1) may all be involved in the Ral signaling network: In tissue culture cells, PP2A is involved in regulating Ral activity by dephosphorylation (Sablina et al., 2007) and SUR-6 may have the same function. PHI-24 is a homolog of CHMP1 and is implicated in the regulation of endocytosis by the formation of multivesicular bodies (Howard et al., 2001). Since the exocyst complex is not only involved in exocytosis but also in endocytosis (Oztan et al., 2007; Sommer et al., 2005), PHI-24 may play a role together with the exocyst complex. Most supportive evidence for a link with the function of Ral exists for VHP-1, the phosphatase for the JNK- and p38-like pathway (Figure 4A). In mammalian cells, Ral-1 has been implicated in activation of c-JUN by Src and JNK (de Ruiter et al., 2000) (Figure 4C). In contrast, in *Drosophila* Ral negatively regulates JNK activation (Balakireva et al., 2006; Sawamoto et al., 1999), whereas it simultaneously activates the p38-pathway, possibly via Sec5 (Figure 4B). Such a complex interaction between

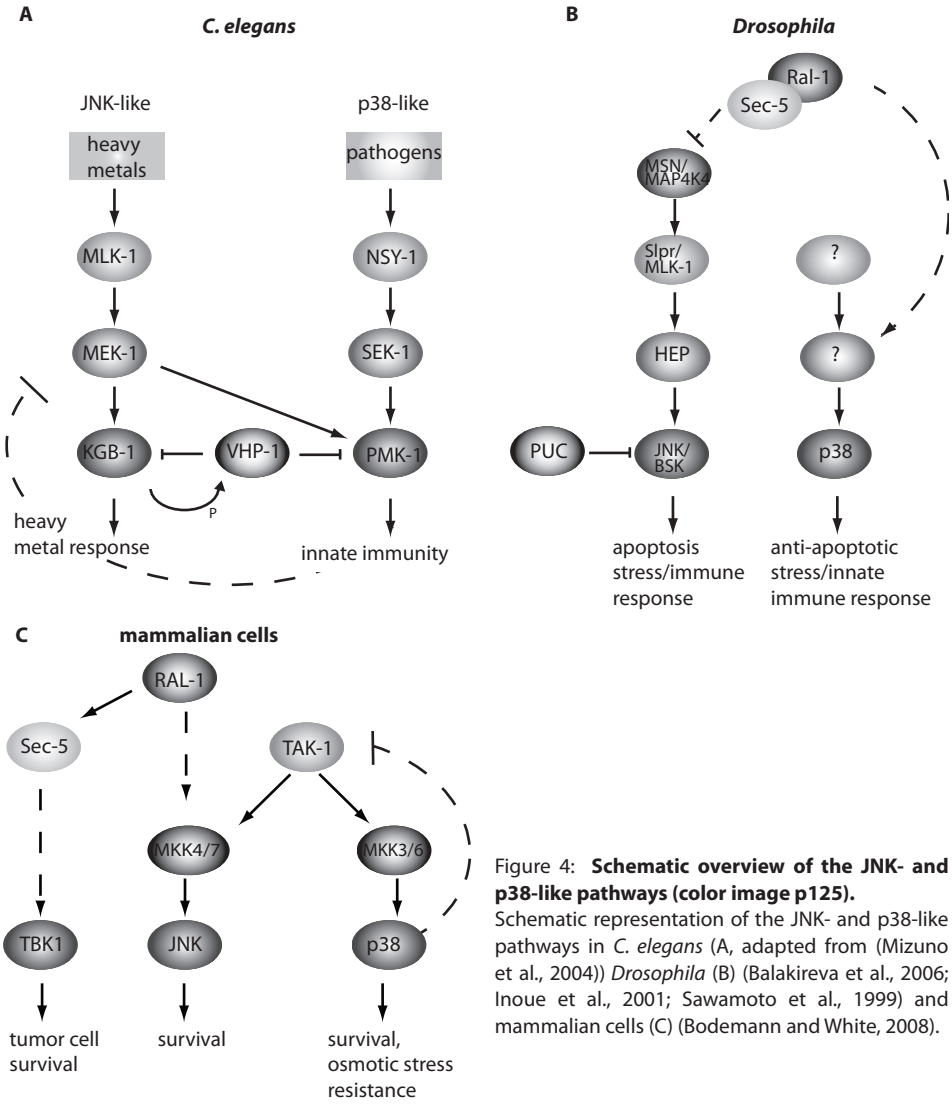

Figure 4: **Schematic overview of the JNK- and p38-like pathways (color image p125).**
Schematic representation of the JNK- and p38-like pathways in *C. elegans* (A, adapted from (Mizuno et al., 2004)) *Drosophila* (B) (Balakireva et al., 2006; Inoue et al., 2001; Sawamoto et al., 1999) and mammalian cells (C) (Bodemann and White, 2008).

the JNK and p38 pathways is also seen in mammalian cells, where p38 negatively feeds back on JNK via their shared upstream activator TAB1/TAK1 (Cheung et al., 2003) (Figure 4C). The peculiar synthetic lethality of *rap-1(tm861)* mutants with the kinases *mek-1(ks54)* and *kgb-1(km21)* on one hand and the phosphatase *vhp-1(km20)* on the other, may reflect feedback interactions in the KGB-1 and PMK-1 pathways and highlights the importance of correct stress kinase activity. In this respect it is interesting to note that in mammalian cells Rap1 has been postulated as an activator of p38 (Chrzanowska-Wodnicka

et al., 2008; Sawada et al., 2001).

The RNAi screen with *rap-1* null mutants has identified new interesting leads for both the RAP-1 and RAL-1 signaling network. A reverse screen to identify new players in the RAP-1 signaling network was not successful when *unc-34(e566)* mutants were used. The limited screen performed with *mek-1(ks54)* and *kgb- 1(km20)* mutants indicates that these would be a better starting point for a reverse screen. Although we did not find genes causing synthetic lethality, we identified 22 RNAi foods that caused a reduced brood-size with both mutants. However, since many signals are integrated on JNK and p38 it seems likely that also genes from other pathways will be identified. In this respect, the *rgl- 1(tm2255)* might be the most promising mutant to screen for proteins in the RAP-1 signaling network.

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5

C. elegans RAP-1 and MIG-10 function in separate pathways.

Ester W. Frische, Wendy Pellis-van Berkel, Maartje A. Gorte, Johannes L. Bos and Fried J.T. Zwartkruis

Dept. of Physiological Chemistry, Centre for Biomedical Genetics and Cancer Genomics Centre, UMC Utrecht, Universiteitsweg 100, 3584 CG Utrecht, the Netherlands.

Abstract

Axon outgrowth requires the actin cytoskeleton to respond to extracellular cues in order to migrate correctly. In *C. elegans*, MIG-10 is together with UNC-34 required for axon outgrowth and viability. The mammalian MIG-10 homologs, RIAM and Lpd, and the UNC-34 homologs Ena, Vasp and Mena have been shown to interact and stimulate membrane protrusions via actin polymerization. Furthermore RIAM, but not Lpd is an effector of the small GTPase Rap1. Here we investigated whether RAP- 1 and MIG-10 act in a single pathway regulating actin dynamics. *rap-1(tm861)* null mutants require, like *mig-10(ct41)* mutants, UNC-34 for survival. Interestingly, MIG-10 and RAP-1 also depend on each other: *mig-10(ct41);rap-1(tm861)* animals are sick and produce little progeny. Moreover, we show that defects in axon outgrowth of the touch neurons in *rap-1(tm861)* and *mig-10(ct41)* null animals are different. Lastly, the synthetic lethal profiles of these mutants are different. Altogether, these data indicate that RAP-1 and MIG-10 function in separate pathways.

Introduction

Regulation of actin dynamics is crucial for several processes like morphogenesis, migration and metastasis and is tightly regulated in time and place. Two closely related proteins, RIAM and Lamellipodin (Lpd) are both involved in regulating actin dynamics. These proteins share the same domain structure consisting of a Ras-binding (RA) domain, a PH-domain and an EVL binding motif. Although only RIAM binds to Rap1 via its RA-domain, both RIAM and Lpd bind Ena/VASP via their EVL binding motifs (Krause et al., 2004; Lafuente et al., 2004). It is suggested that Ena/VASP proteins regulate actin dynamics by blocking capping proteins to bind to the barbed ends of actin filaments and thereby enhance elongation of actin fibers. Indeed, Ena/VASP molecules localize to sites of actin remodeling at the tips of lamellipodia and filopodia (Trichet et al., 2008). Overexpression of RIAM leads to cell spreading and lamellipodia formation, similar to that seen upon Rap1 activation (Lafuente et al., 2004). In addition, RIAM is involved in integrin-mediated adhesion via activation of Talin (Han et al., 2006). Lpd is located at the tips of membrane protrusions and overexpression of Lpd increases the lamellipodial protrusive velocity (Krause et al., 2004). In *C. elegans*, a single RIAM/Lpd homolog is encoded on the genome by *mig-10*. Like RIAM/Lpd, MIG- 10 contains an RA and a PH domain and binds to the Ena/VASP homolog UNC-34. MIG-10 overexpressed in human HEK293 cells induces lamellipodia formation and colocalizes with F-actin (Quinn et al., 2006). *mig-10(ct41)* null mutants are viable and fertile but have

defective migration of neurons and excretory canals (Chang et al., 2006; Quinn et al., 2006). In the absence of the guidance cues UNC- 6/ netrin or SLT- 1/ Slit, overexpression of MIG-10 in the touch neurons leads to multipolar axon outgrowth. This indicates the contribution of additional pathways downstream of UNC-6/netrin or SLT-1/Slit to axon outgrowth (Quinn et al., 2006). UNC-34 is the only member in *C. elegans* of the Ena/VASP protein family and is also involved in regulating actin dynamics. Although brood size is decreased, embryogenesis of *unc-34(gm104)* null mutants appears normal (Forrester and Garriga, 1997; Withee et al., 2004). However, during larval stages, neuronal cell migration and axon outgrowth is aberrant due to decreased filopodia formation (Withee et al., 2004), but lamellipodia formation through UNC-6/netrin signaling was not affected in these mutants (Chang et al., 2006). The observed defects in *unc-34* mutants are enhanced in strains deficient for WSP-1 or WVE-1, both members of the actin regulating WASP protein family and homologous to Wasp and Wave respectively (Withee et al., 2004).

Both UNC-34 and MIG-10 function downstream of UNC-40/DCC, the receptor that senses axon guidance cues. Other proteins that function in the UNC-40/DCC signaling network are the actin binding protein UNC-115/AbLIM and CED-10/ Rac (Chang et al., 2006; Gitai et al., 2003; Quinn et al., 2006). Recently, CED-10/Rac has been shown to be involved in the asymmetric MIG-10 localization, required for growth cone formation (Quinn et al., 2008). Interestingly, double mutants of the predicted null alleles *unc-34(e566)* (Brenner, 1974) and *mig-10(ct41)* (Manser and Wood, 1990) are not viable and arrest early in development. Escapers show severe axon guidance defects, indicating that UNC-34 and MIG-10 act in parallel pathways downstream of UNC-40/DCC (Chang et al., 2006; Manser et al., 1997; Quinn et al., 2006).

Rap1, an interactor of RIAM, also functions in the establishment of polarity and in regulation of axon outgrowth. Cultured rat hippocampal neurons initially have several indistinguishable neurites, but during development, one of them differentiates into an axon. This process of axon determination requires the specific localization of Rap1B at the tip of the neurite that gains axon-fate. The asymmetrically localized Rap1B recruits the Cdc42-Par3-Par6-aPKC complex specifically to this neurite and thereby establishes the polarity required for further axon maturation (Schwamborn and Puschel, 2004). Studies in PC12 cells and rat cortical neurons show that activation of Rap1 leads to increased neurite length and dendritic complexity (Chen et al., 2005; Hisata et al., 2007). Also, the Rap activating proteins Epac1

and -2 and PDZ-GEF1 are involved in axon outgrowth regulation (Hisata et al., 2007; Murray and Shewan, 2008).

Here, we use genetics to investigate the connection between MIG-10 and RAP-1. Like the previously described *unc-34(e566);mig-10(ct41)* animals, we show that *unc-34(e566);rap-1(tm861)* double mutants are not viable. Furthermore, *rap-1(tm861);mig-10(ct41)* animals are also not viable, suggesting that MIG-10 and RAP-1 are likely to function in the same process, but not in the same pathway. In line with this, we observed that the defects of *rap-1(tm861)* null mutants in touch neuron development are different from those in *mig-10* deficient animals. Additionally, we found that the synthetic lethal profiles of *rap-1(tm861)* and *mig-10(ct41)* null mutants do not overlap. Altogether, these data show that MIG-10 and RAP-1, at least partially, act in separate pathways.

Material and Methods

Worms.

General methods for culturing and manipulating worms used were as described (Lewis and Fleming, 1995). Worms were cultured on NGM plates at 20°C. Strains used: Bristol N2, FZ222 *rap-1*(*tm861*), BW315 *mig-10(ct41)*, FZ224 [bjIs64[pML902(*dlg-1::GFP*) pRF4], FZ271 *rap-1*(*tm861*); bjIs64[pML902(*dlg-1::GFP*) pRF4], JR667 [*unc-119*(*e2498*::Tc1) III; *wIs51*], FZ247 *rap-1*(*tm861*); *unc-119*(*e2498::Tc1*)wIs51[*SCM::GFP*; *unc-119*(+)], CF700 muIs32[*mec-7::GFP*], FZ209 *rap-1(tm861)*; muIs32[*mec-7::GFP*].

Detection of mutant *mig-10(ct41)* allele was done by nested PCR using the primers TGTTTGAATTTTCAGAATCCGC and TGTTTCTTCTCACAATCCAACC, followed by AACTCAACTCTAGTAATGGTGG and GCACAAATGTACACAAAGAATCC. PCR products are digested with the Hpy188 endonuclease yielding one 800 basepair mutant or two 400 basepair fragments for mutant or wild-type fragments, respectively. Detection of *rap-1(tm861)* is as described previously (Frische et al., 2007). The presence of the *unc-34(e566)* allele was followed by selecting animals with a movement (unc) phenotype.

Neuronal analysis.

Young adults expressing GFP under the control of the *mec*-7 promoter were analyzed either for their PLMR, ALMR and AVM or for the PLML, ALML and PVM depending on their orientation on a 2% agarose patch with sodiumazide. Analysis was performed using a Zeiss Axioskop 2.

Analysis embryonic development.

L4-stage larvae were transferred to a plate containing bacteria expressing dsRNA and incubated at 15 ˚C (day 1). Adults were transferred to a fresh plate for a 2-hours egg-lay and embryos were allowed to develop for another 2 hours (day 4). Embryos were mounted on 2%

agarose patch and analyzed using a Zeiss Axioskop 2.

Screen and siRNA.

Clones B8, C4, C9, C10, and D3 contain 500 bp cDNA fragments and C11 contains basepairs 1816-2655. H3 and H4 were obtained from the cDNA based Vidal-library, H6-8 were a kind gift of O. Bossinger. The other clones were collected from the Ahringer RNAi library (Kamath et al., 2003). All clones were confirmed by sequencing.

Screening was essentially performed as described previously (chapter 2 (Frische et al., 2007)) Adults were bleached to collect eggs and these were allowed to hatch overnight in M9 medium. L1 stage larvae were incubated at 20˚C on OP50 plates before they were transferred as L4-stage larvae to screening plates (day 1). Bacteria were grown overnight in 1 mM IPTG containing NGM and used to inoculate screening plates. Incubation on siRNAi food was performed at 15 ˚C and adults and progeny were scored at day 4.

Results

rap-1(tm861) null mutants require UNC-34 and MIG-10 for viability.

Double mutants of *mig-10(ct41)* and *unc-34(gm104* and *e566)* arrest during larval stages, while escapers show severe defects in neuronal development (Chang et al., 2006; Quinn et al., 2006) (our data not shown). If RAP-1 and MIG-10 operate in a linear pathway, we predict that also *unc-34(e566);rap-1(tm861)* animals will not be viable and therefore we made *unc-34(e566);rap-1(tm861)* double mutants. Although some of these mutants develop into adults and produce some progeny that die as L1-stage larvae, other animals die at late larval stages. The fact that the phenotype of *unc-34(e566);rap-1(tm861)* is less severe than that of *unc-34(e566);mig-10(ct41)* animals could be explained by different

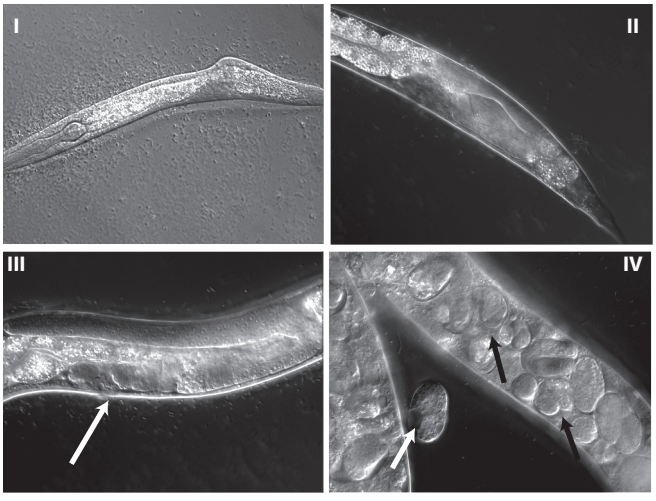

Figure 1: ***rap-1(tm861);mig-10(ct41)* embryos and animals show different defects.**
DIC images of animals derived from *rap-1(tm861)+/-;mig-10(ct41)-/-* hermaphrodites. Some animals have a hump as represented by this L2-stage larva (A), migration of gonads is aberrant (B) or vulva-development is affected (arrow) (III). Embryonic development is also affected; showing vacuoles (white arrow) or defective elongation (black arrow) (IV).

levels of maternal input for MIG-10 and RAP-1 (see also below). To test the relation between RAP-1 and MIG-10 we generated *mig-10(ct41);rap-1(tm861)* animals. Interestingly, the majority of these double homozygotes dies within the first week showing different phenotypes (Figure 1). Animals that become adult have a reduced brood size (14 larvae ±15 STD, n=20), making it practically impossible to maintain a double mutant strain. In agreement with this, *mig-10(ct41)* L1-stage larvae treated with *rap-1(RNAi)* did not make it until adulthood, even though knock-down of RAP-1 is not efficient with this construct (data not shown, M.G.). Taken together, *unc-34(e566), mig-10(ct41)* and *rap-1(tm861)* single mutants appear viable and fertile, but double mutants of these genes are either sick or not viable. These data suggest that MIG-10 does not act as a direct RAP-1 effector and that RAP-1 acts in a parallel pathway to UNC-34 and MIG-10.

rap-1(tm861) and mig-10(ct41) show different defects in touch neuron development.

MIG-10 deficient animals have defects in touch neuron development and we therefore analyzed the touch neuron development in *rap-1(tm861)* animals. To this end, touch neuron axons were visualized using a strain with a GFP-reporter gene under the control of the touch neuron-specific *mec-7* promoter in wild-type and *rap-1(tm861)* mutant animals. Touch neurons in *rap-1(tm861)* mutants showed defects in length and number of their extensions (Figure 2). The orientation of the ALMR, PLMR and AVM (Figure 2A) and the ALML, PLML and PVM (Figure 2B) was examined. The most prominent defect is an additional extension of the ALMR (56% of anterior located defects, Figure 2A III) and the ALML (28% of posterior located defects, Figure 2B III).

Other defects were observed less frequently (Figure 2) such as lenghtened extensions of the PLMR axon in the midbody (9%, Figure 2A II) or defects in outgrowth length of the PLML (19%) (Figure 2B II). Defects observed in *mig-10(ct41)* mutants are failure of the AVM and PVM to extend ventrally but this was not seen in the *rap-1(tm861)* mutants. Therefore, we conclude that RAP-1 is involved in the regulation of the axon length and in the number of extensions of the touch neurons and that *rap-1(tm861)* mutants do not have the same phenotype as *mig-10(ct41)* mutants in neuronal development.

rap-1(tm861) and mig-10(ct41) are synthetic lethal with different genes.

Two genes are synthetic lethal if deletion of either of these genes is not affecting viability whereas deletion of both genes is lethal. If, for a given gene, the set of

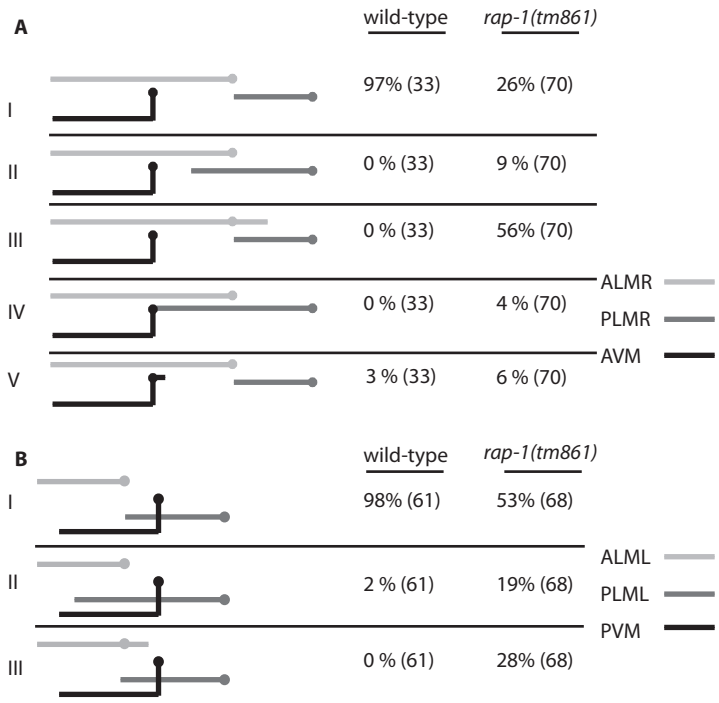

Figure 2**: RAP-1 affects axon guidance of *C. elegans* touch neurons**
Axon guidance defects in *rap-1(tm861)* mutants. Schematic overview of normal and defective axon outgrowth. In numbers is the percentage of defective axon outgrowth of wild-type and *rap-1(tm861)* mutants and in parentheses is the number of animals scored (A-B). II – V of section A and II – III of section B represent the different observed outgrowth defects anterior (A) and posterior of the vulva (B) respectively. The pharynx is orientated at the left of the scheme.

genes causing synthetic lethality (which we will refer to as the synthetic lethal profile), is identical or has significant overlap with that of another gene, these genes are likely to act in a single pathway. Here, we screened for genes that are synthetic lethal with *mig-10(ct41)* and compared them with the synthetic lethal profile of the *rap-1(tm861)* mutant, that had previously been determined (Frische et al., 2007). In order to do so we performed a small scale RNAi-screen with the *mig-10(ct41)* mutant using a library containing proteins with an RA-domain, other small Ras-like GTPases and proteins likely to be involved in Rap1 signaling (Table I). Additionally, the genes that were synthetic lethal with *rap-1(tm861)* mutants were tested. To this end, worms were synchronized and allowed to grow until the L4-stage on normal OP50 bacteria when they were transferred to agar-plates containing RNAi food. Progeny of these worms were scored for viability and number in two independent experiments. Strikingly, none of the genes identified in the screen with *rap-1* appeared synthetic

TABLE II **Overview of genes found in the two independently performed synthetic lethal screen with *mig-10(ct41)* or with *rap-1(tm861)* and *rap-1(pk2082).***

rap-1(tm861) and rap-1(pk2082)			*mig-10(ct41)*		
Gene	Sequence name	Mammalian homolog	Gene	Sequence name	Mammalian homolog
ral-1	*Y53G8AR.3*	RalA, RalB	*cdc-42*	*R07G3.1*	Cdc42
exoc-8	*Y105E8B.2*	EXOC8	*let-60*	*ZK792.6*	K-Ras
phi-24	*F23C8.6*	CHMP1B	-	*R02D3.5*	GGTase I/FTase
sur-6	*F26E4.1*	PR55/B	-	*M57.2*	GGTase II
vhp-1	*F08B1.1*	MKP7			
him-3	*ZK381.1*	HORMAD1			
ztf-12	*C01B7.1*	Zn-finger protein			
-	*C49H3.8*	ARP10			

The screen with the *mig-10(ct41)* mutant was performed with the small scale RNAi-library (Table I), whereas the Ahringer RNAi library was used to screen for synthetic lethals with the *rap-1* mutants (see also Chapter 2).

lethal with *mig-10(ct41)* mutants. Foods that affected *mig-10(ct41)* mutants were *cdc- 42, let-60, R02D3.5* and *M57.2* (Table II). CDC-42 and LET-60 are homologs of two well-studied small GTPases Cdc42 and Ras respectively. The other two genes are involved in prenylation, a type of posttranslational modification common for small GTPase family members. *R02D3.5* encodes for the α-subunit of the geranylgeranyltransferase type I that also functions as a farnesyltransferase. *M57.2* is a homolog of another geranylgeranyltransferase, the α-subunit of the type II transferase. The effect of knockdown for these genes in wild-type and *mig-10(ct41)* null background was further analyzed using two different marker strains. One strain expresses DLG- 1::GFP and allows analysis of hypodermal, intestinal and pharyngeal cells. The other strain carries GFP-positive seam cells. The phenotype of the siRNA treated animals was compared to control-treated embryos. Embryos of *mig-10(ct41)* mutants subjected to *cdc-42, let-60, R02D3.5* or *M57.2(RNAi)* were analyzed and defects were observed in comma and twofold stage embryos, whereas control-treated *mig-10(ct41)* mutants appeared normal. Of *mig-10(ct41)* animals treated with *cdc-42(RNAi),* 68% of the progeny was aberrant whereas only ~10% of the progeny on *let-60, R02D3.5* or *M57.2(RNAi)* appeared abnormal. Next we further analyzed the phenotype caused by

TABLE I **RNAi library**

	1	2	3	4	5	6	7	8	9	10	11	12
A	*L4440*	W03F11.6	*C09E10.2*	*F10E9.6*	K05B2.2	F17H10.3	H09G03.2	T10H10.1	F25H9.5	F56A6.2	T24F1.3	
		afd-1/AF-6	*dgk-1/ DAG-kinase*	*mig-10/ RIAM/Lpd*	*RA domain containing*	*Sorting nexin-17*	*frm-8/ PDZK10*	*Hum-6/ myosin*	*frm-10/krit-1*	*hum-7*	*rapL*	*pos-1*
B	*L4440*	B0334.8	*F39B1.1*	*F31B12.1*	Y73B6A.5	ZK470.5	F49E7.1	F28B4.2	Y71G12B.11a	M01E11.7	C48G7.3	
		age-1/PI3K p110	*PI3K*	*plc-1/ phospholipaseC*	*lin-45/raf-1*	*Nck-1/ Dock*	*rme-/ VPS9*	*rgl-1/ RalGDS*	*talin*	*tag-163/ tensin*	*tag-333/ rin-1*	*pos-1*
C	*L4440*	C51F7.1	-	C25D7.7	F54C8.5	T27F2.2	F53A10.2	T14G10.2	T23G11.5	C27B7.8	T23G7.4	
		frm-7/ FERM-domain	-	*rap-2*	*rheb-1*	*spa-1*	*rapGAP*	*pxf-1/ PDZ-GEF*	*rlbp-1/RalBP*	*rap-1*	*sec-5*	*pos-1*
D	*L4440*	Y105E8B.2	Y53G8AR.3	C43E11.8	C01G8.5	F52A8.6	-	Y106G6H.7	W02B9.1	F09C3.1	-	
		exoc-8	*ral-1*	*exoc-7*	*erm-1/ezrin*	*ras like*	-	*sec-8*	*hmr-1/ cadherin*	*pes-7/ IQ GAP*	-	*pos-1*
E	*L4440*	W03F11.6	F53A10.1	C54A12.4	-	R07G3.1	T22C8.8	-	F23H11.4a	C04D8.1	T20G5.5	
		AF-6	*RapGAP*	*drn-1*	-	*cdc-42*	*vab-9/BCMP1*	-	*ARAP-3*	*RhoGAP*	*Epa-1/ Epac*	*pos-1*
F	*L4440*	R02D3.5	-	M57.2	R05G6.10	C33H5.9	C08F8.7	ZK792.6	K01A6.2	K03D3.10	-	
		farnesyltrans-ferase, α subunit	-	*geranyltransferase, type II, α subunit*	*CalDAG-GEF*	*sec-10*	*rap-3*	*let-60/Ras*	*magi-1*	*rac-2*	-	*pos-1*
G	*L4440*	F26D11.11	F22E12.2	Y32F6B.3	C14A11.7	F52E4.7	C28G1.3	C25A11.4	C35B8.2	C23F12.1	C09F12.1	
		let-413	*Chw-1/Rho like*	*crp-1/ cdc-42 like*	*ras like*	*sec-3*	*sec-15*	*ajm-1/ JAM-1*	*vav-1*	*filamin*	*clc-1*	*pos-1*
H	*L4440*	F08B1.1	Y105C5B.21	F25B3.3	F33A8.3	R13H4.4	K05C4.6	C25F6.2	-	-	-	
		vhp-1/ MKP7	*Jac-1/p120 catenin*	*CalDAG-GEF*	*cey-1*	*hmp-1/ α-catenin*	*hmp-2/ β-catenin*	*dlg-1/ MAGUK*	-	-	-	*pos-1*

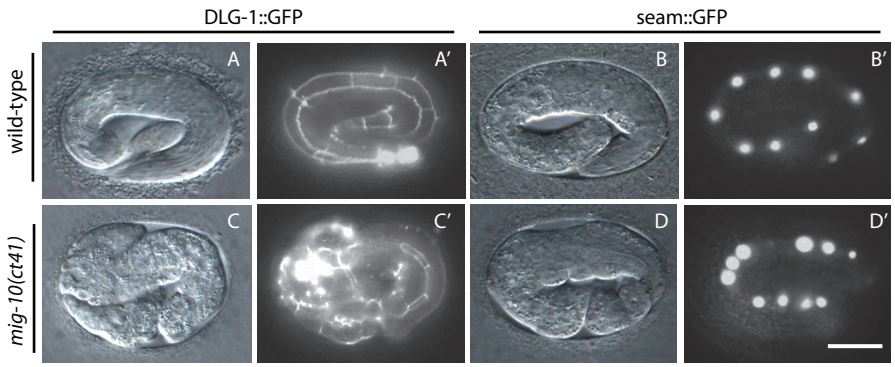

Figure 3: **Localization of DLG-1::GFP or seam cells in *mig-10(ct41)* embryos subjected to *cdc-42(RNAi).*** DIC and epifluorescence images of lateral views of embryos of wild-type (upper row) or *mig-10(ct41)* (bottom row) animals subjected to *cdc-42(RNAi)*. Animals express DLG-1::GFP (left two columns) or the seam cell marker SCM::GFP (right two columns). DIC images show disruption of the embryonic structure of *mig-10(ct41)* embryos (C) whereas wild-type embryos appear normal (A). Intestinal and hypodermal DLG-1 localization in wild-type embryos is normal (A' and data not shown). DLG-1 expression in *mig-10(ct41)* embryos shows a recognizable gut structure but also DLG-1 localization in undefined areas in the embryo suggesting a disrupted hypodermis (C'). Wild-type worms, expressing GFP in the seam cells show the normal seam cell distribution and number of ten seam cells at each lateral side on *cdc-42(RNAi)* food (B'). In the *mig-10(ct41)* background, the number of seam cells appears normal, but the regular distribution is lost (D'). Scale bar is 10 µm.

cdc- 42(RNAi) in *mig-10(ct41)* mutants. In these embryos, development appeared normal until the comma stage. However, at later stages, embryos appeared elongated but with an aberrant shape (Figure 3 A,C). In these embryos, the DLG-1 expression pattern was disturbed suggesting defects during hypodermal cell development or ventral enclosure. Similarly, the position of the seam cells was affected (Figure 3C). Although we did not perform rescue studies, these data suggest that CDC-42 acts in parallel to MIG-10 during embryonic development. In summary, the synthetic lethal profiles of these *mig-10(ct41)* and *rap-1(tm861)* mutants did not indicate that MIG-10 and RAP-1 function in the same pathway.

Discussion

Here, we present three lines of evidence that MIG-10 does not act in a single pathway downstream of RAP- 1 in *C. elegans.* Both RAP-1 and MIG- 10 play a role in axon outgrowth, but *rap- 1(tm861)* and *mig-10(ct41)* null mutants have different phenotypes. Whereas in *mig- 10(ct41)* mutants the touch neurons fail to migrate ventrally, in *rap-1(tm861)* animals the length and number of extensions were affected. Furthermore, whereas single *rap- 1(tm861)* and *mig- 10(ct41)* mutants appear normal, *rap- 1(tm861);mig- 10(ct41)* double mutants are not viable. Finally, the synthetic lethal profile of the *rap- 1(tm861)* and *mig- 10(ct41)* mutants are clearly distinct.

MIG-10 is the single homolog of mammalian RIAM and Lpd and the *mig-10* gene produces two isoforms MIG- 10A and MIG-10B. Both isoforms are expressed in neurons and no isoform specific function has been determined (Manser et al., 1997; Quinn et al., 2006). The mammalian homologs Riam and Lpd are both involved in the formation of membrane protrusions by regulating actin dynamics. RIAM interacts with active Rap1 and both overexpression of RIAM or Rap1V12 induces cell spreading and membrane protrusions (Lafuente et al., 2004). Additionally, knock-down of RIAM interferes in Rap1-mediated integrin activation in Jurkat cells (Lafuente et al., 2004). In *C. elegans,* MIG- 10 is not required for viability as shown by the *mig-10(ct41)* allele that contains an amber mutation disrupting both isoforms. Interestingly, the phenotype of *mig-10(ct41)* null mutants is mild and is different from integrin mutants in *C. elegans* (Cox and Hardin, 2004). In line with our data suggesting that RAP-1 and MIG-10 act in different pathways is a very recent *in vitro* binding study suggesting that MIG-10 and RAP-1 do not interact directly (Quinn et al., 2008). Also, in a yeast-two hybrid setting no interaction was observed (F.Z.).

Here we show for the first time that RAP- 1 plays a role in neuronal development in an intact animal. *rap- 1(tm861)* null mutants show abnormalities in axon outgrowth and to a minor extend in axon pathfinding in the *C. elegans* touch neurons. This is in line with studies in mammalian tissue culture cells where Rap1 is also implicated in neuronal polarity (Schwamborn and Puschel, 2004), length (Anneren et al., 2000; Lu et al., 2000) and in dendritic branching (Chen et al., 2005). Based on the genetic interaction between *rap-1(tm861)* and *unc-34(e566),* it is possible that loss of *unc- 34* enhances the neuronal phenotype of *rap-1*, but this was not analyzed due to time constraints. In the current model of touch neuron axon outgrowth, MIG-10 and UNC-34 act downstream of the UNC-40/DCC receptor and appear to have overlapping but distinct functions. MIG-10 and UNC- 34 directly interact but *mig-10(ct41);unc-34(e566)* are not viable (Chang et al., 2006; Quinn et al., 2006), indicating that MIG-10 and UNC-34 may act in the same pathway but also have a separate function. UNC- 34/Ena induces filopodia formation via regulation of actin dynamics (Krause et al., 2003; Withee et al., 2004; Yu et al., 2002). The site of filopodia formation appears to be determined by CED-10/Rac that binds and thereby localizes MIG-10 asymmetrically (Quinn et al., 2008). In addition, also WVE-1/WAVE, WSP-1/WASP and UNC-115/AbLIM are involved in regulation of actin polymerization during axon outgrowth (Shakir et al., 2008). From this, it becomes clear that protrusive

activity in axon outgrowth is mediated by various pathways and therefore genetic analysis is complicated.

The small-scale RNAi screen with *mig-10(ct41)* revealed that knock-down of CDC-42 in these animals induced embryonic lethality and that DLG-1 localization was disturbed in twofold stage animals. This suggests that MIG-10 might also have a function during morphogenesis. On the other hand, these data, together with the observation in mammalian cells where Cdc42 acts downstream of Rap1B in axon outgrowth (Schwamborn and Puschel, 2004), suggest that Cdc42 might also be involved in axon outgrowth in *C. elegans* touch neuron development.

Here, we propose that RAP-1 and MIG-10 act in distinct pathways based on a comparison of their synthetic lethal profile. Thus, we reason that null mutants of genes acting in the same pathway, will require identical proteins for survival and therefore will have the same synthetic lethal profile. None of the genes found in the screen with *rap-1(tm861)* (chapter 2) were synthetic lethal with *mig-10(ct41)* in this screening setup and vice versa. This makes it unlikely that the different phenotype of *mig-10(ct41);unc-34(e566)* and *rap-1(tm861);unc-34(e566)* double mutants is simply explained by a difference in maternal input of RAP-1 and MIG-10. Furthermore, we propose that also RAP-1 is involved in axon outgrowth regulation, most likely in parallel to the UNC-34 or MIG-10 pathways. Given the fact that RAP-1 and MIG-10 do not appear to interact, we suggest that MIG-10 might be a closer functional homolog of Lpd than of RIAM.

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6

Discussion

The small GTPase Ras has been extensively studied for its role in tumorigenesis, but more recent developments highlight the importance of two other Ras-like family members, Rap1 and Ral. The latter is involved in processes requiring polarized protein transport such as filopodia formation, exocytosis and also in transcription e.g. via c-JUN. Interestingly, Ral is implicated in tumorigenesis and this requires, at least in some situations, Ras-mediated Ral GEF activation (Bodemann and White, 2008). Rap1 was originally identified to counteract Ras activity, however recent evidence shows that Rap1 acts independently of Ras in several processes like cell polarity, adherens junction formation and integrin-mediated adhesion. Several upstream and downstream proteins have been identified in the Rap1 and Ral signaling cascades, but additional, yet to be identified proteins may also play a role.

In this study we used a genetic approach to obtain more insight in Rap1-mediated cellular processes. A genome-wide genetic screen in *C. elegans* revealed that RAP-1 acts in parallel to several pathways among which are the RAL-1/exocyst complex and the JNK-like pathway. We showed in A549 cells that depletion of RalA leads to a loss of E-cadherin from the adherens junctions whereas N-cadherin localization is unaffected. In contrast, in DLD1 cells, E-cadherin localization to the adherens junctions was not dependent on RalA and Rap1. Finally, we provided genetic evidence that MIG-10 and RAP-1 function in separate pathways.

Screening for players in the RAL-1 and RAP- 1 signaling network.

The establishment of genome-wide RNAi libraries in *C. elegans* provides an excellent tool for synthetic lethal studies. When two genes are synthetic lethal this means that they either act redundantly or in parallel biological pathways. We turned to this technique to study Rap signaling using *rap-1, rap-2* and *epac-1* null mutants. Although we did not observe any specific synthetic lethal phenotype with the *rap- 2* and *epa-1* mutants, we identified seven genes essential for survival of the *rap-1* null mutants; *ral-1, exoc-8, phi-24, sur-6, vhp-1, him-3* and *C01B7.1*.

Null mutants for MEK-1/MAPKK, KGB- 1/JNK and UNC-34/ENA are not viable in a *rap-1* null background and may therefore be suitable candidates for a RAP-1 revertant screen (Chapter 4). An initial screen for ~10% of the Ahringer RNAi library revealed that the *unc-34* null mutant was too sensitive for siRNA-mediated knock-down and is therefore not a good candidate for a genome-wide screen. In this screen, we did not observe synthetic lethality with the *mek-1* and *kgb-1* mutants, but these strains may be used for further screening. The null mutant for RGL-1/RalGDS may provide

us with a useful mutant to screen for proteins in the RAP-1 signaling network; *rgl-1(tm2255);rap-1(tm861)* animals arrest during the third larval stage and embryos of *rap-1* null mutants subjected to *rgl-1(RNAi)* or *ral-1(RNAi)* have a similar phenotype. It will be interesting to see whether an RNAi screen with the *rgl-1(tm2255)* mutant identifies other proteins involved in hypodermal cell organization and especially in adherens junction formation.

Synthetic lethality in model systems.

In this study we obtained multiple genes that encoded different types of proteins that were synthetic lethal with RAP- 1. But what does this mean and what is the molecular basis of the observed synthetic lethality? In order to address these questions, we undertook several approaches. First of all, we investigated the synthetic lethal phenotype by making double mutants instead of using RNAi. In addition, we performed a phenotypic analysis of the affected animals to classify the hits in groups of similar effects. For instance, this was the first indication that RAL-1 and the exocyst complex act synergistically with RAP- 1. However, this approach may not be conclusive due to the variation in timing or level of knock-down and this may complicate the phenotypic comparison. Lastly, we turned to mammalian tissue culture cells for two reasons: analysis of the synthetic lethal phenotype may contribute to a better understanding of human cells and tissue culture cells allow for a biochemical characterization of the connectivity of pathways. This approach might be straightforward if a synthetic lethal phenotype is observed, however when this is not the case this might be due to several reasons. First, the deletion of genes in a developing organism might have a different effect than in tissue culture cells. For instance, the dynamics of E-cadherin localization at the cell surface during epidermal sheet formation in a developing organism might be very different from epidermal cells growing in a monolayer in a tissue culture dish. On the other hand, the regulation of cellular processes has become more complex between *C. elegans* and mammals. Therefore, even if the connectivity of two pathways is conserved throughout evolution, the synthetic lethal phenotype may not be observed due to compensation of other pathways in the more complex organism.

RAP-1 and RAL-1 act synergistically in C. elegans.

In Chapter 2, we show that RAP-1 and RAL-1 act synergistically during embryogenesis on hypodermal cell organization and that they are important in locating the HMR-1/E-cadherin and HMP-1-2/ α- / β-catenin complex. Moreover, this

study also revealed a role for RAP-1 in embryogenesis in *C. elegans*. Several studies in mammalian cells have shown the involvement of the Rap1 signaling network in controlling adherens junction dynamics (Pannekoek et al., in press). Thus far, only two proteins, AF6 and KRIT1 have been implicated in the regulation of cell-cell contacts downstream of Rap1. In endothelial cells, KRIT1 is involved in Rap1-dependent tightening of adherens junctions, possibly via binding to β-catenin (Glading et al., 2007). AF6 binds to p120-catenin and may thereby increase the interaction of E-cadherin and p120-catenin. *In vitro* studies indicate that the interaction of Rap1 with AF6 reduces endocytosis of E-cadherin, presumably via the enhanced interaction with p120-catenin (Hoshino et al., 2005). Another interesting observation is the genetic interaction in *Drosophila* between Rap1 and the deubiquitinating enzyme FAM (Li et al., 1997). FAM is implicated in post-Golgi trafficking of E-cadherin and β-catenin (Murray et al., 2004) and may provide another mechanism for Rap1 to regulate adherens junctions.

RalA is required for the localization of E-cadherin to the adherens junctions

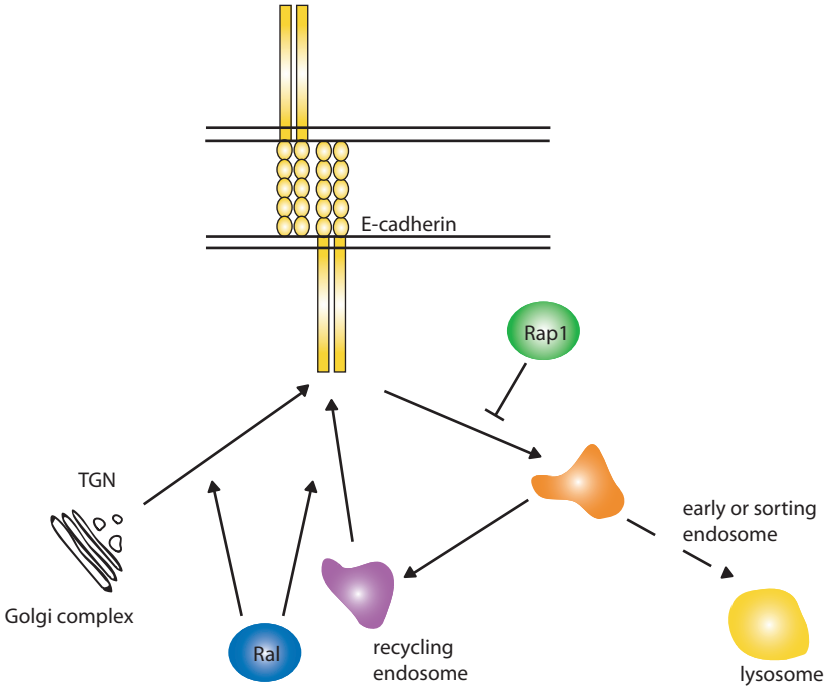

Figure 1: **Model for the role of Rap1 and RalA in E-cadherin localization in *C. elegans*.**
Adapted from (Bryant and Stow, 2004). Ral is involved in transport of E-cadherin to the cell surface from the TGN and/or the recycling endosome whereas Rap1 inhibits endocytosis of E-cadherin. Data from *C. elegans* suggest that RAP-1 and RAL-1 are also involved in the transport of other proteins (Frische et al., 2007).

in A549 cells (Chapter 3). Although others have shown that the expression of activated Ral mutants leads to an increased rate of E-cadherin transport (Shipitsin and Feig, 2004), the complete loss of E-cadherin, as found in A549 cells, is much more dramatic. Additionally, the exocyst complex is involved in exo-and endocytosis and is required for transport of *Drosophila* E-cadherin from the recycling endosome to the cell surface (Langevin et al., 2005) (discussed below). Thus, the embryonic defects we observed in *rap-1* null mutants subjected to either *ral-1, exoc8* or *sec-5(RNAi)* might be due to increased endocytosis of non-trans-interacting HMR-1/E-cadherin due to a lack of RAP-1 and decreased transport of E-cadherin to the cell surface in animals lacking a functional RAL-1/ exocyst complex pathway (Figure 1). Embryos of *rap-1* null animals subjected to *ral-1(RNAi)* arrested earlier than *hmr-1*/E-cadherin or *hmp-1*/α-catenin null embryos indicating an additional role for RAP-1 and RAL-1. We did not observe the synergy between Rap1 and RalA in mammalian cells and this may be due to several reasons. First, multiple Rap and Ral isoforms may act redundantly or secondly, the regulation of E-cadherin localization is more complex involving multiple pathways that act in parallel in order to control adherens junctions. Lastly, the synthetic lethality in *C. elegans* is observed during hypodermal enclosure, a process requiring regulation of migration and cell-cell contacts. It might be that specifically during this developmental stage, signaling via RAP- 1 and RAL-1 is crucial whereas other processes are less dependent on both these pathways. This is illustrated by the function of Rap1 in *Drosophila* where Rap1 is essential in development, but can be omitted for the survival of adults (Asha et al., 1999).

Ral mediates E-cadherin localization via the exocyst complex.

Sec5 and Exoc8 are direct effectors of Ral (Moskalenko et al., 2002; Moskalenko et al., 2003) and bind in a mutually exclusive manner (Jin et al., 2005). Ral might activate the exocyst complex by bringing the Sec5- and Exoc8 containing subcomplexes together. Recent data suggest that Ral is required for localization of the exocyst complex and for targeting of cargo to the exocyst complex in rat prostate tumor cell lines (Spiczka and Yeaman, 2008). The Ral-exocyst complex interaction appears to be important in polarized vesicle transport, for instance in transport of the glucose transporter GLUT4 upon insulin stimulation (Chen et al., 2007). We show in *C. elegans* that depletion of Ral or the exocyst complex results in a similar phenotype and that in at least one mammalian cell line, the Ral/exocyst complex axis is involved in E-cadherin localization (Chapter 2

and 3). Until very recent, the activating protein for the RalA/exocyst complex pathway was not identified but our data indicate that the exchange factor RalGDS acts upstream of RalA in E-cadherin localization, suggesting a RalGDS/RalA/exocyst complex axis. This is further supported by the recently identified role of RalGDS in RalA-exocyst complex-mediated Weibel-Palade body secretion (Rondaij et al., 2008). Activating signals for the Ral/exocyst complex pathway obviously can include Ras but also other signals may be involved. Indeed, studies in insulin-mediated GLUT4 transport have shown that RalA activation is PI-3kinase-dependent (Chen et al., 2007). Furthermore, calmodulin is implicated in secretion of Weibel-Palade bodies and GLUT-4 containing vesicles, via RalGDS (Rondaij et al., 2008) or RalA (Chen et al., 2007) respectively. Finally, the GTPase R- Ras stimulates exocytosis via the Ral GEF Rgl2 and might thereby also regulate the RalA-exocyst complex pathway (Takaya et al., 2007). It will be interesting to investigate what signals are involved in RalGDS-RalA-exocyst complex-mediated E-cadherin localization.

Thus far, multiple levels of regulation of cadherin trafficking and membrane localization have been identified (for a review, see (Bryant and Stow, 2004)). For instance, endocytosis of E-cadherin is induced directly via modification of E- cadherin by signals such as ubiquitination by Hakai (Fujita et al., 2002) or tyrosine phosphorylation by Src (Palacios et al., 2005). Both signals induce endocytosis of E-cadherin and targeting for lysosomal degradation. On the other hand, modification of p120-catenin upon, e.g. EGFR stimulation (Mariner et al., 2004) also affects the binding of p120-catenin to E-cadherin and therefore its stability on the plasma membrane.

The exocyst complex has been implicated in both exocytosis and endocytosis of proteins, but thus far the signals that regulate the exocyst complex in these processes are not well understood. In yeast, the exocyst complex is implicated in docking of vesicles to the cell surface, however it is not clear how the exocyst complex binds to specific sites on the plasma membrane. A recent study in yeast indicates that the interaction of Sec3 with Cdc42 and phosphatidylinositol-4,5-bisphosphate is important for association with the plasma membrane (Zhang et al., 2008). Targeting of E- cadherin via the exocyst complex to the correct membrane site presumably involves sorting signals. For instance, the cytoplasmic tail of E-cadherin contains sorting signals required for lateral membrane targeting, such as a dileucine motif and these signals may vary among the different cadherin-proteins (Miranda et al., 2001). Recently, the scaffold protein IQGAP was found to bind to members of

the exocyst complex and to be essential for exocyst complex-mediated secretion in invadopodia (Sakurai-Yageta et al., 2008). IQGAP is also localized at the adherens junctions (Kuroda et al., 1998) and may be involved in targeting proteins to the correct membrane domain via the exocyst complex. Another protein that might act together with the exocyst complex in the regulation of E-cadherin is the GTPase Arf6. Arf6 is implicated in endocytosis and protein recycling to the membrane (Gillingham and Munro, 2007) and more specifically in endocytosis of E-cadherin (Kon et al., 2008). Previously, it was shown that Arf6 interacts with Sec10 and suggested that Arf6-mediated delivery and insertion of recycling membranes requires the exocyst complex (Prigent et al., 2003). Interestingly, in a yeast-two hybrid setting, Rap1 interacts with Sec15, suggesting that Rap1 might also have a function in regulation of the exocyst complex. Most studies on the trafficking of cadherins have focused on E-cadherin in MDCK cells, but there are indications for differential regulation of cadherins; N-cadherin does not bind to the Rap exchange factor C3G (Hogan et al., 2004) and is not endocytosed via the E3-ligase Hakai (Fujita et al., 2002). Furthermore, our data indicate that whereas E-cadherin localization requires RalA signaling, localization of N-cadherin does not depend on RalA signaling. It will be interesting to see whether differential cadherin regulation involves exo- and/or endocytic pathways and how the RalA/exocyst complex pathway is involved.

The RalGDS family of Ral GEFs forms one of the downstream signaling pathways of activated Ras and indeed in pancreatic tumor tissue or cell lines, RalA-GTP levels are elevated in comparison with normal pancreatic tissue or cell lines (Lim et al., 2005; Lim et al., 2006). The oncogenic capacity of Ras is, at least in part, mediated via RalA, but the involved downstream signaling pathway(s) remain(s) to be uncovered (for a review, see (Bodemann and White, 2008)). On the other hand, the positive contribution of Ral to the establishment of adherens junctions is remarkable, since adherens junctions are generally regarded as anti-invasive (Cavallaro and Christofori, 2004). This discrepancy might be due to the extensive RalA and RalB signaling network: activation of RalA is involved in anchorage-independent growth, whereas RalB acts anti-apoptotically (reviewed in (Chien and White, 2003)). It is suggested that RalA-mediated anchorage-independent growth requires signaling through the transcription factor ZONAB. The RalA-ZONAB interaction retains ZONAB in the cytoplasm and thereby relieves the proliferative restraints that are normally regulated via ZONAB (Frankel et al., 2005). The anti-apoptotic effect of RalB activation might occur

through activation of TBK1, a kinase that is normally involved in host defense gene expression. Constitutive activation of RalB induces TBK1 activity and thereby cell survival (Chien et al., 2006). Interestingly, RalB-mediated TBK1 activation requires Sec5 and is tethered to the exocyst complex, however this pathway may be independent of canonical exocyst complex function. The RalA and RalB-mediated effects on tumorigenesis may however also require other signaling pathways, for example regulation of JNK activity.

C. elegans MIG-10 does not only operate in a RAP-1 regulated pathway.

C. elegans MIG-10 and mammalian RIAM and Lamellipodin are proteins with a similar domain structure and although all three contain an RA domain (Chang et al., 2006), Rap1 only binds to RIAM (Lafuente et al., 2004). MIG-10 and RAP-1 appear to function in separate pathways based on the results we present in Chapter 5, which is in line with the observation that MIG-10 and RAP-1 do not bind in an *in vitro* binding assay (Quinn et al., 2008). Studying the function of these proteins in actin regulation might be very complex as is illustrated by studies on *C. elegans* axon outgrowth. Here, multiple pathways are involved in regulating actin dynamics including WSP-1/WASP, WVE-1/WAVE, UNC-34/Ena, UNC-115/abLIM and MIG- 10. Whereas WSP-1/WASP appears to mediate actin dynamics via MIG-2/RhoG, WVE-1/WAVE and MIG-10 appear to act downstream of CED-10/Rac (Lundquist et al., 1998; Quinn et al., 2008; Shakir et al., 2008). During axon outgrowth, CED- 10 localizes asymmetrically and recruits MIG-10 via a direct interaction leading to actin rearrangements required for membrane protrusion (Quinn et al., 2008). It will be interesting to see whether Rac is involved in localization of Lpd to sites of membrane protrusion in mammalian cells. Previously, Rap1 has been shown to induce cell spreading by localizing the Rac1-exchange factors Vav2 and Tiam to the cell periphery in mammalian cells (Arthur et al., 2004). However this appears not to be the case in *C. elegans* since our data and others (Quinn et al., 2008) show that RAP-1 and CED-10/Rac have distinct functions in axon outgrowth regulation.

Multiple pathways are likely to be involved in the regulation of complex processes like adherens junction formation. The combination of genetic studies and cell biological assays taught us that the RAP-1 and RAL-1-mediated signaling pathways both act on the regulation of adherens junctions. It will be interesting to see how these and other pathways integrate on the regulation of E-cadherin and how E-cadherin localization is spatially and temporally regulated via Rap1, RalA and the exocyst complex.

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Summary

Of the family members of the small Ras-like GTPases, Ras-proteins are the best studied since ~15% of all human tumors contains a mutation in any one of the Ras-proteins. However, more recent focus on two other small Ras-like GTPases, Rap1 and Ral, revealed their important function in regulation of cellular dynamics. Rap1 signaling is involved in cell polarity, integrin-mediated cell adhesion and cell-cell contact formation whereas Ral functions in endocytosis, exocytosis and in filopodia formation. Moreover, activation of Ral is involved in tumorigenesis, presumably via the inhibition of apoptosis and by mediating anchorage-independent growth. Studying model organisms has provided important lessons on the function of the Rap1 and Ral signaling networks. In **Chapter 1,** these have been summarized and related to available biochemical data.

In a genome-wide RNAi screen for synthetic lethality with *rap-1* null *C. elegans* mutants, RAL-1 emerged (**Chapter 2)**. Two effector proteins of RAL-1, SEC-5 and EXOC-8, were also synthetic lethal with the *rap-1* null mutants. SEC-5 and EXOC-8 are both members of the exocyst complex, which is involved in endocytosis and in exocytosis of proteins to specific membrane compartments. Progeny of *rap-1* null mutants subjected to either *ral-1, sec-5* or *exoc-8(RNAi)* arrested during embryonic development with a similar phenotype: hypodermal cell migration and organization was disturbed leading to failure of ventral enclosure. Furthermore, HMP-1/α-catenin was lost from the cell-cell contacts in these hypodermal cells, which resulted in disruption of adherens junctions. Finally, genetic studies with the *rap-1* null animals and *ral-1* and hypomorphic *sec-5* mutants indicated that the RAP-1 and RAL-1/exocyst complex also act in parallel during larval development. Together, these data show that the RAP-1 and RAL-1/exocyst complex pathway act synergistically in *C. elegans* development. In order to investigate whether this synergy was also present in other model systems, we turned to human tissue culture cells, A549, DLD1 and MCF7 cells (**Chapter 3**). E-cadherin levels at the adherens junctions were not much affected in Rap1- and RalA-depleted DLD1 cells, but strikingly E-cadherin levels were largely reduced upon depletion of RalA in A549 cells. Moreover, depletion of the exocyst complex members Sec8 and Exoc8 and of the Ral activator RalGDS led to a similar phenotype. Thus, in A549 cells the RalGDS/RalA/exocyst complex pathway is crucial for localization of E-cadherin to adherens junctions. Interestingly, depletion of RalA did not lead to loss of N-cadherin and due to this differential

effect of RalA on cadherins, adherens junctions were still present.

In the aforementioned synthetic lethal screen with the *rap-1* null mutant, we also identified *phi-24, sur-6, vhp-1, him-3* and *C01B7*. In **chapter 4**, we investigated the phenotype of *rap-1* null mutants subjected to *sur-6, phi-24* or *vhp-1(RNAi)*. Progeny of these animals arrested at different stages than observed for *rap- 1* null animals on *ral-1(RNAi)*. Next, we investigated the synthetic lethality of RAP-1 and VHP-1 in more detail. VHP-1 is a phosphatase for KGB-1 and PMK- 1, which are JNK- and p38-like kinases respectively. The KGB-1 and PMK-1 pathways are involved in stress signaling and interfering in their function reduces heavy metal stress resistance or changes the expression of the odorant receptor STR-2 in the olfactory AWC neurons. However, deletion of RAP-1 neither affected heavy metal stress resistance nor the expression of STR-2. Strikingly, *rap-1* null mutants do not only require the presence of the phosphatase VHP-1 but also of the opposing kinases MEK- 1, SEK- 1 and KGB-1. Finally, we used the *mek-1* and *kgb-1* null mutants in a revertant screen to identify new players in the RAP-1 signaling network. Thus far, we did not obtain synthetic lethal genes with the *mek-1* and *kgb-1* null mutants, but we identified 22 genes with a reduced brood-size in both the *mek- 1* and *kgb-1* null mutants. Additionally, we identified the viable *rgl-1(tm2255)* mutant, encoding a Ral-GEF, as a possible mutant for a reverse screen.

In **chapter 5**, we addressed the question if in *C. elegans,* MIG-10 is an effector protein for RAP-1. The mammalian homologs for MIG-10, Riam and Lamellipodin regulate actin dynamics and Riam has been shown to bind to Rap1. Mutants for *mig- 10* appear viable and fertile but require the presence of UNC-34/Ena. The data presented in chapter 5 show that *rap-1* null mutants also require *unc-34* for survival. Interestingly, also *rap-1;mig-10* double mutants appear sick and produce little offspring, indicating that RAP-1 and MIG-10 do not operate in a single pathway. This is further supported by the little overlap of the synthetic lethal profiles of *rap-1* and *mig-10* null mutants.

In **chapter 6,** the findings described in this thesis and their implications for the understanding of the role of Rap1 and Ral in regulating cellular dynamics are discussed.

Samenvatting (ook voor niet ingewijden)

Achtergrond

Het menselijk lichaam is opgebouwd uit microscopische kleine onderdelen, de cellen. Ons lichaam is opgebouwd uit cellen die vele verschillende functies kunnen hebben en ze bevatten o.a. voedingsstoffen, eiwitten en het erfelijk materiaal (DNA). Een cel zou je kunnen vergelijken met een fabriek, waarvan het bouwplan gevormd wordt door het DNA en eiwitten de machines zijn die de fabriek maken en draaiende houden. In deze vergelijking kunnen genen, dit zijn onderdelen van het DNA, voorgesteld worden als het bouwplan voor deze machines. Een enkel gen is dan het bouwplan voor een enkele machine. Er wordt geschat dat mensen ongeveer 30.000 verschillende genen hebben. Eiwitten zijn essentieel voor het functioneren van de cel en kunnen verschillende functies hebben. Ze zorgen bijvoorbeeld voor de structuur, deling, stofwisseling of voor het contact met de omgeving van een cel. Alle eiwitten samen maken de cel en het bouwplan hiervoor ligt vast in het DNA.

Mutaties in het DNA zijn te vergelijken met fouten in de bouwtekening. Ze kunnen fouten opleveren in de eiwitten en dus grote gevolgen hebben voor het functioneren van de cel. Hoewel de cellen van een individu allemaal hetzelfde erfelijke materiaal bevatten, hebben ze een andere functie bijvoorbeeld in spieren, de lever of de huid. Dit is mogelijk omdat in deze cellen verschillende genen actief zijn waardoor verschillende eiwitten aangemaakt worden. De aanwezigheid van eiwitten moet goed gereguleerd worden om er bijvoorbeeld voor te zorgen dat cellen op het juiste moment gaan delen en zich op de juiste manier gaan specialiseren. Daarnaast is het belangrijk dat deze eiwitten met elkaar kunnen communiceren en signalen aan elkaar kunnen doorgeven (een signaleringsroute). Het vakgebied dat zich hiermee bezig houdt wordt wel moleculaire signaaltransductie genoemd.

Aangezien onderzoek naar de werking van cellen niet in mensen gedaan kan worden, zijn er modelsystemen nodig. Een van de mogelijkheden is het gebruik van menselijke cellen die in een schaaltje in het lab groeien. Een andere mogelijkheid is het gebruik van modelorganismen, aangezien andere organismen ook opgebouwd zijn uit cellen en erfelijk materiaal hebben dat vergelijkbaar is met dat van de mens. Modelorganismen die sterk verwant zijn aan de mens zijn muizen en ratten. Hoewel ze meer van de mens verschillen dan de rat en de muis, worden ook andere organismen zoals de fruitvlieg (*Drosophila melanogaster*), een worm (*Caenorhabditis elegans*) of bakkersgist (*Saccharomyces cerevisiae*) als

model gebruikt voor de werking van het menselijk lichaam.

Het onderzoek dat in dit proefschrift beschreven wordt, gaat over signaaltransductie van 2 eiwitten: Rap1 en Ral. Deze eiwitten behoren tot dezelfde familie van eiwitten en dit betekent dat Rap1 en Ral erg op elkaar lijken qua vorm, maar ook dat ze op dezelfde manier communiceren met andere eiwitten. Beide eiwitten zijn betrokken bij het doorgeven van signalen die uiteindelijk leiden tot veranderingen in de cel. Rap1 is betrokken bij de regulatie van polariteit in cellen (bijvoorbeeld: wat is de voor- en wat is de achterkant van een cel) en het hechten van cellen aan elkaar of aan de omgeving. Ral reguleert andere processen zoals het transport van eiwitten in de cel en de aanmaak van sommige andere eiwitten. Daarnaast is Ral van invloed op het ontstaan van sommige tumoren, hoewel het nog niet duidelijk is hoe Ral dit effect geeft.

Dit proefschrift

Zowel Rap1 als Ral zijn eiwitten die niet alleen in de mens een belangrijke rol spelen, maar ook in andere organismen zoals in de worm en in de fruitvlieg. In **hoofdstuk 1** geef ik een overzicht van de kennis die is verkregen door het bestuderen van Rap1 en Ral in modelorganismen. Hieruit blijkt dat Rap1 en Ral meerdere functies hebben in de cel, maar ook dat deze vergelijkbaar zijn in verschillende organismen. Daarnaast blijkt uit het onderzoek naar tumoren dat de signaleringsnetwerken waarin Rap1 en Ral functioneren, mogelijk betrokken zijn in processen die een rol spelen in kanker.

In dit proefschrift gebruik ik verschillende technieken om onderzoek te doen naar de rol van Rap1 en Ral in het functioneren van cellen. Voor dit onderzoek heb ik zowel geïsoleerde menselijke cellen (**hoofdstuk 3**) gebruikt, maar ook een compleet organisme, namelijk *C. elegans*. Dit modelorganisme is een rondworm van ongeveer 1 mm groot en komt van nature in de grond voor. De eitjes van deze worm kunnen worden bevrucht door zelfbevruchting (de meerderheid van de wormen is hermafrodiet) of door het mannetje (ongeveer 0.05% van de populatie) **(hoofdstuk 2, 4 en 5)**.

Een van de belangrijkste experimenten die in dit poefschrift beschreven staat is een screen die ik heb uitgevoerd om andere eiwitten te vinden die betrokken zijn bij de Rap1 en Ral signaleringsroute. Deze screen is te vergelijken met een zoektocht naar 'iets' op de bouwtekening zoals ik die hierboven heb beschreven. In plaats van een aantal criteria op te stellen, ga je het hele bouwplan af in de hoop te vinden wat je zoekt. Dat is ook wat we gedaan hebben: een worm die geen Rap1 meer heeft is niet ziek, blijkbaar is Rap1 niet nodig in de worm. In deze screen hebben we onderzocht

of in een worm die geen Rap1 heeft het weghalen van een ander, nog onbekend, eiwit wel lethaal is. We hebben bijna alle eiwitten een voor een weggehaald en bekeken wat hiervan het gevolg is. In deze studie hebben we speciaal gezocht naar die eiwitten waarvan het weghalen geen effect heeft in een normale worm, maar wel lethaal is in wormen die geen Rap1 meer hebben. Dit wordt synthetisch (van synthese, samen) lethaal genoemd, dus 2 gebeurtenissen (en niet een van deze gebeurtenissen alleen) samen zorgen ervoor dat de worm niet meer kan overleven. Deze gegevens geven meer inzicht in welke signaleringsroutes samenwerken met die van Rap1. In **hoofdstuk 2** laat ik zien dat wormen die geen Rap eiwit meer hebben afhankelijk zijn van Ral, maar ook dat ik met behulp van deze screen andere eiwitten heb kunnen identificeren die samen met Ral in een signaleringsroute functioneren, deze eiwitten behoren tot een groep van eiwitten, het exocyst complex. De ontwikkeling van nakomelingen van wormen die geen Ral en geen Rap eiwitten meer hebben stopt voortijdig. Een van de defecten die optreedt, is dat het contact tussen de huidcellen van deze embryo's niet goed wordt gevormd waardoor de huid niet goed ontwikkelt. In **hoofdstuk 3** heb ik gebruik gemaakt van menselijke cellen die we in een petrischaal kunnen laten groeien om te onderzoeken of Rap1 en Ral mogelijk op een vergelijkbare manier samenwerken in de mens. Voor dit onderzoek heb ik cellen gebruikt die onderling vergelijkbare contacten maken als de huidcellen van de worm, maar met een verschillende oorsprong: long- (A549) en darmkanker (DLD1) cellen. In mensen is het eiwit E-cadherine, een van de belangrijkste bouwstenen voor het maken van contact tussen twee cellen. Experimenten in darmcellen laten zien dat Rap1 en Ral niet essentieel zijn voor het contact tussen deze cellen. In longcellen was de situatie nog anders: deze cellen waren voornamelijk afhankelijk van Ral en nauwelijks van Rap1 voor het vormen van celcontacten via E-cadherine. Net zoals in de worm werken eiwitten van het exocyst complex samen met Ral in de bouw van het contact tussen cellen. De reden dat we verschillende resultaten hebben verkregen in wormen en menselijke cellen kan verschillende oorzaken hebben: Misschien de meest voor de hand liggende oorzaak is dat Rap1 en Ral anders functioneren in wormen en mensen. Een andere verklaring kan zijn dat de ontwikkeling van een organisme (het wormenembryo in dit geval) hele andere processen nodig heeft dan een groep cellen in een schaaltje.

In **hoofdstuk 4** beschrijf ik het onderzoek naar andere eiwitten die we hebben gevonden in de screen met wormen die het Rap1 eiwit niet meer hebben: PHI-24, SUR-6 en VHP-1. Het effect van

het weghalen van deze eiwitten is niet hetzelfde als het weghalen van Ral. Hoewel dit verschillende oorzaken kan hebben, is het een indicatie dat ze niet in dezelfde signaleringsroute opereren (maar Ral en het exocyst complex dus wel). In dit hoofdstuk besteed ik de meeste aandacht aan de relatie tussen Rap1 en VHP-1. VHP-1 zorgt voor het inactiveren van twee signaleringsroutes in de worm en draagt daarmee bij tot het regelen van de juiste balans in een cel. Verder onderzoek toonde aan dat wormen die geen Rap1 hebben, zowel VHP-1 (de rem) als SEK-1, MEK-1 en KGB- 1 (het gaspedaal) nodig hebben en dit geeft aan dat ook Rap1 betrokken is bij het regelen van de juiste balans.

De humane eiwitten RIAM en Lammelipodin en het *C. elegans* eiwit MIG-10 lijken op elkaar en spelen een rol in de regulatie van het actine cytoskelet. Dit is een netwerk van eiwitketens, dat zorgt voor stabiliteit en mobiliteit van een cel. Aangezien studies in cellen hebben aangetoond dat RIAM gereguleerd wordt door Rap1, heb ik onderzocht of MIG-10 inderdaad door Rap1 wordt gereguleerd in wormen. De resultaten in **hoofdstuk 5** geven aan dat Rap1 en MIG-10 een onafhankelijke functie hebben in de worm. In **hoofdstuk 6** worden de bevindingen en de mogelijke implicaties voor de rol van Rap1 en Ral in cellulaire signaleringsroutes bediscussieerd.

Curriculum vitae

Ester Frische werd geboren op 16 februari 1979 in Purmerend. Na het behalen van het VWO-diploma aan het Jan van Egmond College te Purmerend in 1997, begon zij in hetzelfde jaar met de opleiding Bioprocestechnologie aan Wageningen Universiteit. Gedurende deze opleiding werd onderzoekservaring opgedaan in de groep van Prof. Dr. John van der Oost op de afdeling Microbiologie van Wageningen Universiteit en in de groep van Prof. dr. Titia Sixma onder begeleiding van Dr. ir. Joyce Lebbink op de afdeling Molecular Carcinogenesis van het Nederlands Kanker Instituut. Tot slot werd een stage gedaan in de groep van John Hancock, PhD onder begeleiding van Angus Harding, PhD te Brisbane, Australië aan het Institute for Molecular Bioscience. In maart 2004 werd de studie afgerond (*cum laude*) en in september 2004 werd begonnen als AIO in de groep van Prof. dr. Hans Bos onder begeleiding van Dr. ir. Fried Zwartkruis bij de afdeling Fysiologische Chemie van het Universitair Medisch Centrum te Utrecht. De resultaten van dit onderzoek staan beschreven in dit proefschrift.

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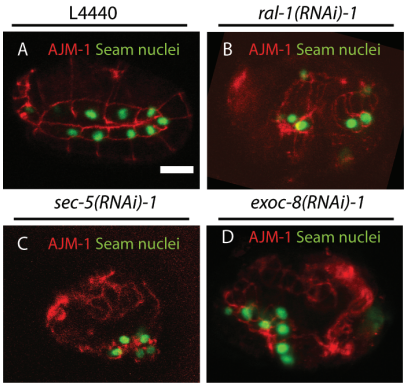
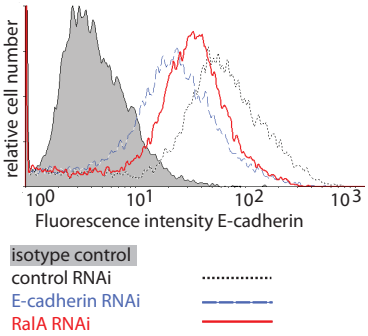
Color Figures

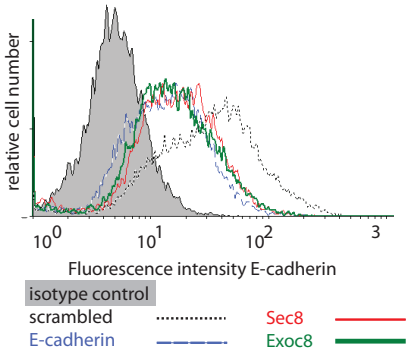
Chapter 2, figure 3



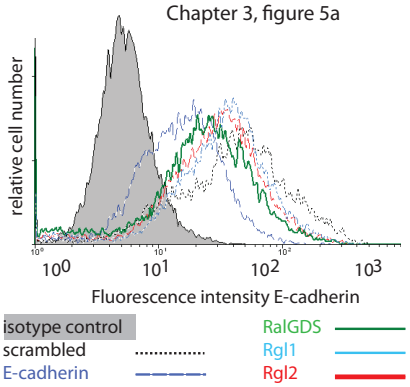
Chapter 3, figure 3c



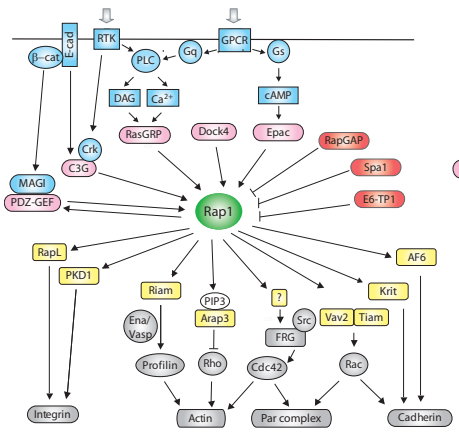
Chapter 3, figure 4a



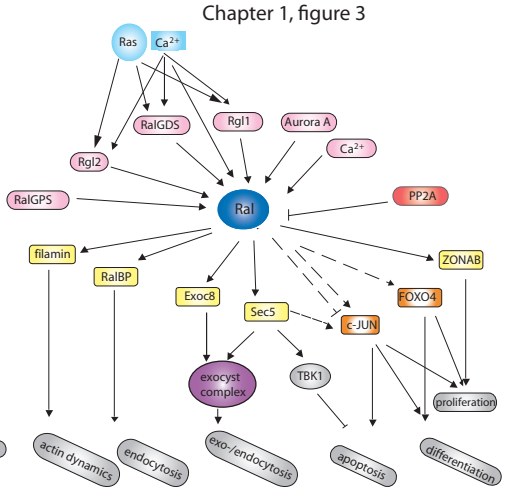
Chapter 3, figure 5a

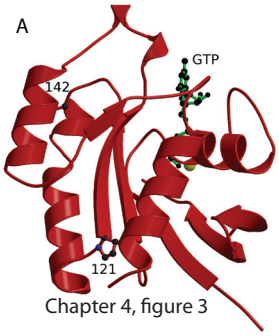


Chapter 1, figure 1

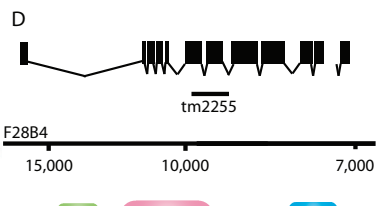


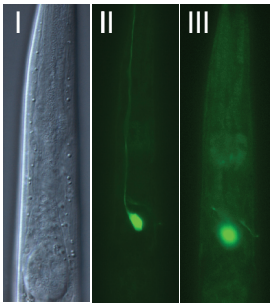
Chapter 1, figure 3





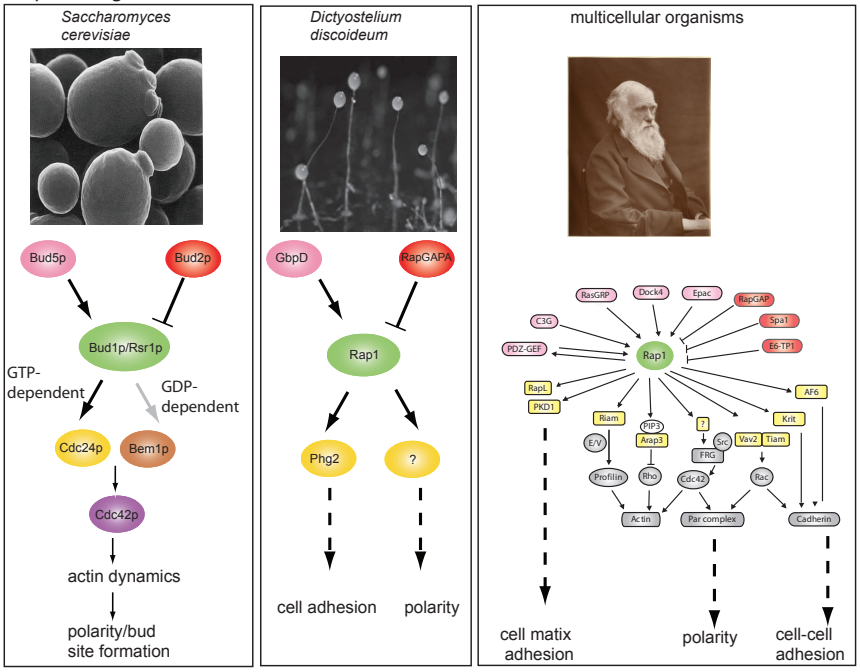
Chapter 4, figure 3





Chapter 4, figure 2

Chapter 1, figure 2



Chapter 4, figure 4