Does Rap1 mix and match?

Anneke Post

Supervisor:
Dr. F.J.T Zwartkruis

Master program Biology of Disease
University Utrecht

18-05-2009

SUMMARY

Rap1 is a monomeric G protein belonging to the Ras superfamily. As other G proteins, Rap1 cycles between a GTP-bound, active state and a GDP-bound, inactive state. Rap1 was first discovered to inhibit the transforming properties of Ras. Later it was found to have several functions. Depending on the subcellular context, Rap1 can either inhibit the Raf/MEK/ERK pathway by binding Raf-1 in an inactive complex, or activate the Raf/MEK/ERK pathway by activating B-Raf. The subcellular pool of Rap1 at the plasma membrane is responsible for its effects on the Raf/MEK/ERK pathway.
Besides the Raf/MEK/ERK pathway, Rap1 has been found to be involved in integrin activation and adherens junction formation. Rap1 can activate integrins by enhancing its affinity and avidity. Furthermore, Rap1 is responsible for the maturation of adherens junctions by inducing cadherin recruitment to newly forming adherens junctions.
Adherent cells depend on the adherence to the extracellular matrix in order to allow proliferation. This is mediated through integrins. On the other hand, adherens junctions inhibit proliferation in order to inhibit unchecked growth. Thus, extracellular growth signals must ne well coordinated with signals from integrins and cadherins in order for proper proliferation to take place. The scope of this thesis is to determine whether Rap1 could interconnect growth signals activating the Raf/MEK/ERK pathway and integrin activation and/or adherens junction formation, allowing coordination of these separate events.

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1. THE RAP1 PROTEIN.

1.1 Introduction.

Rap1, a monomeric G-protein, involved in a diverse array of pathways, was discovered by Noda et al.[1] in 1989 in a search for proteins that could suppress the oncogenic effect of K-Ras. In their search they transfected v-Ki-Ras transformed NIH 3T3 cells with a cDNA library developed from human fibroblasts. Normally, NIH 3T3 fibroblasts carrying the Kirsten murine sarcoma virus, containing the v-Ki-Ras gene, transform into malignant cells. However, they found that a small number of colonies had become resistant to the transforming abilities of the v-Ki-Ras protein after transfection with the cDNA. They first determined that these cell lines had an abundance of a $p21^{ras}$-like protein. Later this protein was characterized as K*rev*-1 (Kirsten-*ras*-revertant-1) and it was found to have high similarity to other *ras* proteins.[2] Since then many groups have reported Rap1 to suppress the actions of Ras by capturing its effector protein, Raf, and forming an inactive complex.[3] Simultaneously, the group of Pizen et al. found two new proteins in their search for Ras homologues. These proteins were characterized as Rap1 and Rap2. Later, the protein characterized as K*rev*-1 was determined to be the same protein as Rap1.[4, 5]

Rap1 is found in two isoforms, Rap1a and Rap1b, which share 95% sequence identity with each other. Furthermore, no apparent differences in cellular function have been found for Rap1a and Rap1b. Rap1 has 50% sequence identity to Ras and belongs to the Ras superfamily of G proteins.[4] As most research has focused on Rap1 this thesis will focus on the effects of Rap1 protein alone without distinguishing between its two isoforms.

Like all G proteins, Rap1 exerts its function by cycling between an inactive, guanine nucleotide diphosphate (GDP) bound state and an active, guanine nucleotide triphosphate (GTP) bound state. Speed and timing of this cycle is regulated by guanine exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs induce the dissociation of bound GDP from Rap1. An excess of GTP within the cell results in the Rap1 protein to bind GTP, resulting in the activation of Rap1. GAPs regulate the deactivation of Rap1 by increasing the hydrolyzing activity of Rap1, which intrinsically is very low.[6]

Rap1 activation occurs in response to stimulation of several membrane receptors such as G-protein coupled receptors, tyrosine receptors, cytokine receptors and cell-adhesion molecules. Stimulation of these receptors results in the activation of Rap1 via second messengers such as cAMP, Ca^{2+} and diacylglycerol (DAG).

1.2 Structural domains.

Rap1 consists of several distinct domains. These domains are involved in the binding of the guanine nucleotide, the hydrolysis of GTP, binding of GAPs, GEFs and effector proteins and localization of Rap1. The main domains involved in nucleotide binding, hydrolysis and GAP, GEF and effector binding are the P-loop with its GXXXXGK/S motif (found in all guanine binding proteins, amino acids 10-17)[7] and the switch I (amino acids 25-40) and switch II (amino acids 57-75) domains.[8, 9] These switch domains are highly mobile regions that change conformation depending on their nucleotide bound state.

1.2.1 GTP hydrolysis.

The duration of GTP binding by Rap1 determines the specificity, timing and intensity of a cellular process. Therefore, not only the binding of GTP itself, but also the regulation of GTP hydrolysis is important in determining the outcome of Rap1 activation. Much of the

information on GTP binding and hydrolysis by Rap1 has come from comparison with studies on Ras. The GTP binding pocket of both Ras and Rap1 consists of the P-loop and the switch I and switch II regions. As does Ras, Rap1 binds guanine nucleotides in complex with a Mg^{2+}-ion. Mg^{2+} increases the affinity of Rap1 for guanine nucleotides by interactions with the γ- and β-phosphates of GTP and GDP, respectively, and by interactions with Rap1.[10]

In order to allow hydrolysis of GTP, a nucleophilic water molecule must enter the GTP-binding pocket and engage an interaction with the γ-phosphate of GTP, thereby inducing the hydrolysis of GTP. During the hydrolysis a negative charge develops over the nucleotide, decreasing the activating energy for the hydrolysis. The GTP-binding pocket contains several hydrogen bond donors and positive charges that stabilize the γ- and β-phosphates of GTP, thereby supporting and stabilizing the transition state of the nucleotide and accelerating hydrolysis. The P-loop amides (residues 13 through 16) and the Mg^{2+}-ion show extensive electrostatic interactions and hydrogen bonding with the non-bridging oxygen atoms of the β-phosphate and with the β-phosphor itself. This can stimulate and stabilize a negative charge shift from the γ-phosphate to the β-phosphate, which is necessary for hydrolysis.[11] Further stabilization of the transition state is achieved by the correct orientation of the water molecule in respect to the γ-phosphate. For Ras this is facilitated by residue Gln61, located within the switch II region. Binding of GTP by Ras results in a conformational change, causing Gln61 to dip in to the GTP-binding pocket. Hydrogen bonds with the nucleophilic water molecule allow it to position the water molecule inline with the γ-phosphate of GTP in order for it to attack the phosphate group.[11]

The intrinsic catalytic rate of GTPases is very low and thus GAPs are needed to enhance the reaction rate and terminate activation. The GAPs for Ras, Rho and Ran contain a positively charged arginine finger, which protrudes into the GTP-binding pockets of these G-proteins. The positive charge of this residue further stabilizes the negative charge on the nucleotide. Binding of a RasGAP to GTP-bound Ras also stabilizes the conformational change of switch II, thereby further positioning Gln61.[11]

Although Ras and Rap1 are highly homologous, there are some differences which have a major effect on the mechanism of GTP hydrolysis. First, in contrast to Ras, Rap1 has a threonine amino acid at position 61 and therefore cannot align the water molecule inline with the γ-phosphate. Furthermore, unlike the GTPases themselves, the GAPs for Rap1 and Ras show little homology. GAPs for Rap1 do not contain an arginine finger. However, RapGAPs do have an asparagine residue at position 290, referred to as the Asn-thumb. This Asn-thumb protrudes into the GTP-binding pocket, much like the arginine finger does, and is believed to mimic the actions of Gln61 in Ras, thus orienting the nucleophilic water molecule inline with the γ-phosphate.[6, 11, 12]

The Rap1 T61Q mutant increases the intrinsic GTPase activity of Rap1 to that of Ras, indicating that the substitution of Gln61 by Thr in Rap1 accounts for the lower intrinsic GTPase activity of Rap1 compared to Ras. Other mutations of Thr61 have little effect on the intrinsic GTPase activity suggesting that the residue plays no part in hydrolysis.[13, 14] The regions of Rap1 interacting with its GAPs have been located to the P-loop, the switch I region and the switch II region, including residue Thr61.[6]

1.2.2 GDP dissociation.

In contrast to RapGAPs, which do not show sequence homology to RasGAPs, RapGEFs are homologous to RasGEFs. Both Rap1 and Ras specific GEFs contain an REM (Ras exchange motif) domain and a catalytic CDC25 homology domain.
In analogy with the Arg-finger and Asn-thumb, the catalytic domain of Rap1GEFs forms a glutamic finger, also referred to as the HI-hairpin. Upon complex formation of Rap1 with its GEF, the glutamic finger is projected into the nucleotide binding region. The insertion of this

hairpin structure causes switch I to be displaced[8] and the side chains of the HI-hairpin consequently block the binding sites of the Mg^{2+}-ion and the α-phosphate of the nucleotide, thereby resulting in the dissociation of the bound nucleotide.[15] The REM domain regulates the catalytic activity through interactions with the hairpin.[16]

Mutational analysis has pointed out three regions within the Rap1 protein to be important for GEF recognition and binding: the switch I region (amino acids 25-40) the switch II region (amino acids 57-75) and the stretch of amino acids 100-110.[8, 9] Certain residues within this region, as well as outside of these regions, account for GEF specificity.

1.2.3 Effector binding.

As mentioned above, binding of GTP by Rap1 induces a conformational change. This change in conformation allows Rap1 to bind to its effector proteins, through which Rap1 exerts its effects.

Effector proteins have been found to have a 10^3 higher affinity for GTP-bound than GDP-bound Rap1. Most research on the binding surface between Rap1 and its effectors has been done on the Rap1-Raf complex. The interaction between the two proteins is mediated through two anti-parallel β-sheets of each protein, forming an apparent continuous β-sheet structure. The region of Rap1 forming the interface encompasses residues 21-41, which overlaps with the switch I.[17, 18] Effector specificity is determined by the residues within the effector binding region, with the most important role for residue Lys31.[18]

Binding of effectors to Rap1 results in the inhibition of the release of the bound GTP. This effect has been determined as the guanine dissociation-inhibitory (GDI) effect. Somewhat surprisingly, the guanine nucleotide binding pocket is not located near the effector binding region. Tyr32, within the switch I region and the effector binding domain, is a very mobile nucleotide, presumably switching in and out of two conformations. Upon effector binding Tyr32 is thought to be stabilized in a conformation in which it interacts with the γ-phosphate of the bound triphosphate. This stable binding of the effector prevents the dissociation of GTP. Upon hydrolysis the disruption of the tight interaction of Tyr32 with the γ-phosphate results in a highly mobile Tyr32 residue again, probably weakening the interactions of Glu31 and Asp33 with residues from the Raf protein. This, together with the disruption of the interactions of residues Thr35 and Gly60 (found within the switch I and switch II regions, respectively) with the γ-phosphate, is presumed to result in a conformational change resulting in the dissociation of the effector protein from Rap1.[17]

1.2.4 Cellular localization.

Another domain within Rap1, which is homologous to Ras, is the CAAX motif. This C-terminal Cys-Ali-Ali-Xaa amino acid sequence (Ali stands for aliphatic residue) is rendered to post-translational modifications, allowing association with membranous components. Rap1 has been shown to be located towards perinuclear membranes, most likely the membranes of the Golgi apparatus, as Rap1 has been costained with Golgi membrane markers[19], as well as to the plasma membrane.[20] Depending on which sub-cellular pool of Rap1 is activated and which effector proteins are bound, distinct actions will occur (see §3.4.1.1)

1.3 Physiological effects of Rap1 signalling.

Rap1 has been reported to play roles in a divergent array of processes (fig.1). Since its discovery as a repressor of K-ras,[2] several research groups have reported the inhibitory effect of Rap1 on Ras signalling.

Rap1 and Ras have been found to have a highly similar effector binding domain, indicating that Ras and Rap1 may have common effector proteins. The inhibitory actions of Rap1 on Ras

signalling could be by trapping the effector proteins of Ras in inactive complexes, or causing their translocation to a region distinct from the Ras pool, resulting in the inability of activation by Ras. Indeed, Rap1 is able to bind Raf-1, a direct effector protein of Ras. Activation of Raf-1 by active Ras induces the activation of the Raf/MEK/ERK pathway. However, in contrast to Ras, Rap1 forms an inactive complex with Raf-1 thereby squelching the effects of Ras.[21] Besides a negative effect of Rap1 on the pathway activated by Ras, Rap1 has also been reported to activate the Raf/MEK/ERK pathways. Whereas binding of Raf-1 by Rap1 seems to leave the proteins in an inactive complex, the binding of Raf-1's homologue, B-Raf, by Rap1 creates an active complex, resulting in the activation of the Raf/MEK/ERK pathway by Rap1.[22]

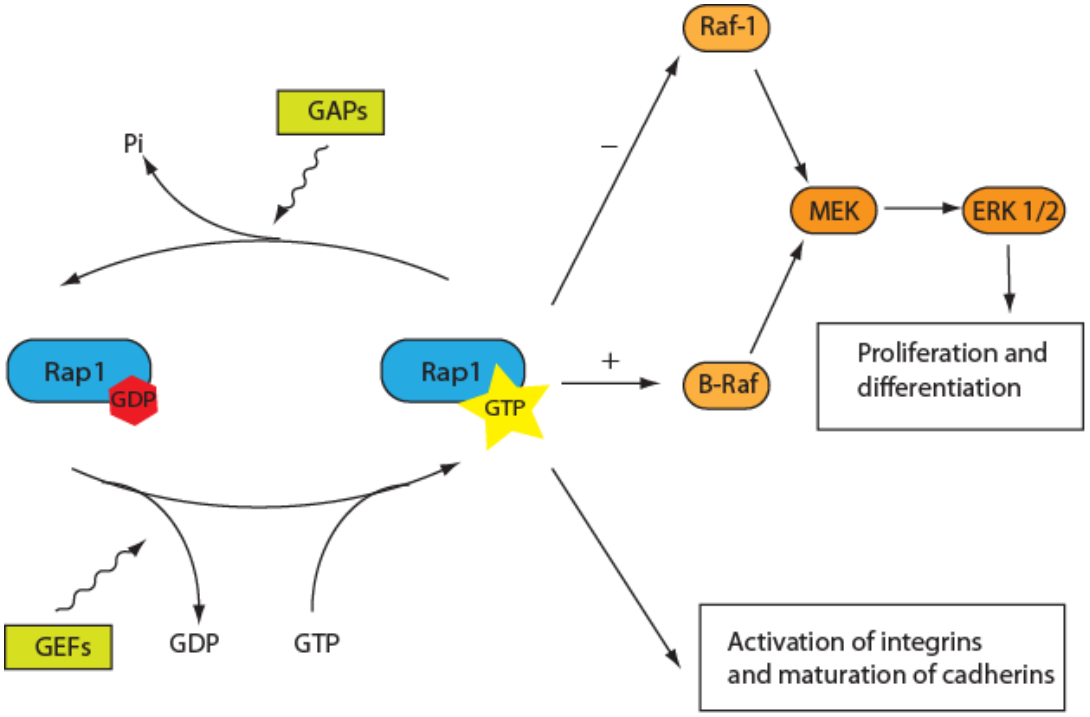

Figure 1. Scheme of the regulation of Rap1 by GAPs and GEFs and the distinct outcomes of the activation of Rap1.

Although Rap1 was first only thought to have inhibitory roles on Ras signalling, the presence of GEFs and GAPs specific for Rap1, and the distinct cellular localizations of Ras and Rap1, suggested that Rap1 might have other cellular functions distinct from Ras signalling. Indeed, research has suggested a role for Rap1 in cell-adhesion via integrins and cadherins.

Rap1 has been found to play an important role in inside-out activation of integrins that associate with the actin cytoskeleton.[23] Integrin activity is regulated by recruitment of integrins to the membrane by means of recycling, by redistribution and clustering of integrins, referred to as avidity, and by induction of conformational changes resulting in the change of its affinity. Rap1 has been implicated in all three of these processes.[24-30]

Besides its role in cell-matrix adhesion, Rap1 has also been implicated in cell-cell adhesion. Formation of immature cell-cell junctions results in the activation of Rap1. The activated Rap1 is required for maturation of these junctions by directing E-cadherin to the junctions and activating and attaching the cadherins to the cytoskeleton.[31]

So far two major roles for Rap1 have been discovered, regulation of the Raf/MEK/ERK pathway and subsequent proliferation and differentiation, and regulation of cell adhesion, both to the extracellular matrix as well as cell-cell adhesion.

It has been well established that benign growth can only take place in the presence of cell adhesion to the extracellular matrix. Integrins, through outside-in signalling, can influence cell-cycle progression through its modulation of several transduction pathways, including the Raf/MEK/ERK pathway. Once adhesion is interrupted cell-cycle progression is inhibited. Cells that do continue to proliferate generally progress into tumor formatting cells. Besides matrix adhesion, signalling from growth factors is also necessary for proliferation. Thus, in the absence of one of these two signal transduction pathways proliferation should not take place. Therefore, there must be some kind of communication between these different pathways.

Although signalling by integrins, allowing proliferation, is mediated through outside-in signalling, integrins must first be activated in order to form adhesion complexes with the extracellular matrix. Rap1 is implicated in both the activation of integrins as well as in the Raf/MEK/ERK pathway. This raises the question: Is their one single signal that, through Rap1, coordinates the activation of integrins together with the activation of the Raf/MEK/ERK pathway to ensure that proliferation takes place? Or are there distinct signals for the activation of integrins through Rap1 and the activation or inhibition of the Raf/MEK/ERK pathway through Rap1?

In contrast, formation of cell-cell adhesions, with the involvement of E-cadherins, induces a signalling cascade that inhibits Raf/MEK/ERK signalling. Again, could one single signal, activating Rap1, result in the maturation of cell-cell adhesion and simultaneously inhibit proliferation through inhibiting activation of the Raf/MEK/ERK pathway? Or are these separated events, regulated by separate signals and separate second messengers?

The presence of multiple Rap1GEFs and Rap1GAPs, the distinctive distribution of these proteins both within the cell and over tissues and the implication of Rap1 in diverse signalling pathways, suggest that the effect of Rap1 depends on the subcellular contexts it finds itself in. However, could the activation of Rap1 in one subcellular pool coordinate two seemingly separate pathways, i.e. the Raf/MEK/ERK pathway and integrin activation or adherens junction maturation? If so, it would be likely that one single signal activating Rap1, simultaneously modulates cell adhesion and proliferation. Furthermore, this would suggest that one single Rap1GEF is responsible for the activation of a Rap1 pool capable of modulating both pathways.

2. REGULATION OF RAP1 ACTIVITY.

The activity of Rap1 is determined by the cyclic state between the GDP-bound and the GTP-bound Rap1. Hormones, cytokines, growth factors and signalling molecules on the one hand activate Rap1 through there actions on guanine nucleotide exchange factors, GEFs, inducing dissociation of GDP in order to allow GTP to bind, and on the other hand inhibit the activity of Rap1 by activating GTPase activating proteins, GAPs, which increase the intrinsic GTPase activity of Rap1 (fig.2).[32]

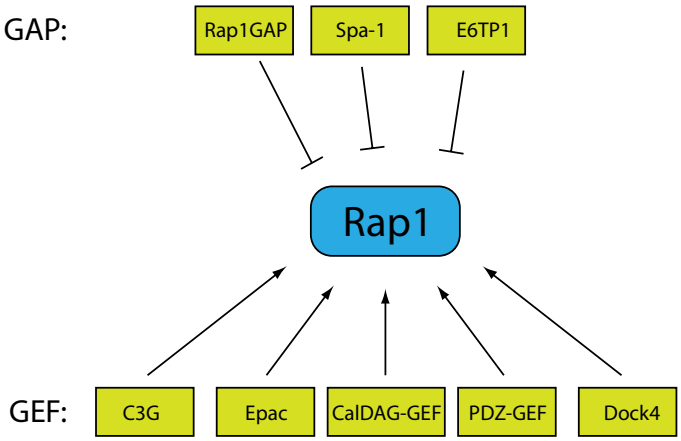

Figure 2. Overview of all GAPs and GEFs for Rap1.

2.1 GEFs

In order for G proteins to be activated, the proteins must replace their bound GDP by GTP. This replacement is catalyzed by a family of proteins called guanine exchange factors (GEFs), which increase the dissociation rate of the nucleotide. Several GEFs have been identified for Rap1, all containing the CDC25-homology domain. The CDC25 homology domain is the catalytic domain of GEF's, catalyzing the release of GDP, thereby allowing the G protein to bind the more abundant GTP.

2.1.1 C3G

C3G (CRK SH3-domain-bindining guanine-nucleotide releasing factor) was the first exchange factor found for Rap1. It mediates the activation of Rap1 through a signalling cascade consisting of tyrosine kinases. C3G contains a catalytic CDC25 homology domain at its C-terminus. The central region contains proline rich regions which have the ability to bind SH3 domains of various proteins.[33] C3G is found in complex with Crk. Upon phosphorylation of receptor tyrosine kinases the Crk-C3G complex is recruited to the membrane and interacts with the phosphorylated tyrosine residues on the receptor via the SH3 domain of Crk. This results in the activation of C3G, allowing it to activate Rap1.[34]

Besides mediating the activation of Rap1 via direct interactions with membrane receptors, C3G can also mediate the activation of Rap1 via cytoplastic adaptor signals. The first adaptor protein in this cascade is the Src protein. Cytoplasmic Src is phosphorylated by PKA. Phosphorylated Src then recruits and phosphorylates another group of cytosolic adaptor proteins, such as Cas, Sin and Cbl, resulting in the recruitment of the Crk-C3G complex and the activation of Rap1. As PKA is activated by cAMP and is the activator of Src, the activity of Rap1 can indirectly be regulated by cAMP.[34]

C3G expression has been detected in many tissues, with high abundance in the adult skeletal muscle and placenta and the fetal brain and heart and with low abundance in the liver.[35] Inactive C3G spreads throughout the cytoplasm with some prominence at the perinuclear regions and the plasma membrane. Activated C3G accumulates mainly at the Golgi apparatus, the subcortical actin cytoskeleton[33] and the plasma membrane.[36]

2.1.2 Epac

Besides the regulation of Rap1 activity by cAMP via PKA, Rap1 activity can also be regulated by cAMP in a PKA-independent manner, by a protein called Epac. This exchange protein directly activated by cAMP (Epac) is a guanine nucleotide exchange factor specific for Rap1 and Rap2. The Epac family consists of three members, Epac1, Epac2 and Repac. Epac1 is widely expressed whereas Epac2 expression is restricted to the brain, pituitary, adrenal gland and liver. Epac1 has four distinct domains, a cAMP binding domain, a DEP domain (Dishevelled, Egal-10 and Pleckstrin), a REM domain (Ras exchange motive) and a CDC25-homology domain.[37, 38] Epac2 differs from Epac1 in having an extra cAMP binding domain at its N-terminus, however the biological importance of this domain is unknown. In the unbound state the cAMP binding domain is an auto-inhibitory domain, inhibiting the catalytic CDC25-homology domain of Epac. This CDC25-homology domain mediates the exchange activity of Epac. The DEP domain is responsible for the membrane localization of Epac. Epac has been found to localize at the plasma membrane, perinuclear membranes and mitochondrial membranes. Epac2 is implicated in cAMP mediated exocytosis whereas Epac1 is implicated in Ca^{2+}-induced Ca^{2+} release and cell adhesion mediated via integrins and cadherins. In contrast to Epac1 and Epac2, Repac lacks a regulatory domain and therefore it may be constitutively active.[38]

Epac1 is ubiquitously expressed with the most abundant expression being in the brain, spinal cord, ovaries, pancreas, spleen, kidney and thyroid, whereas Epac2 expression is limited to the adrenal gland, liver, islets of Langerhans and certain regions of the brain.[38]

2.1.3 CalDAG-GEF

The members of the CalDAG-GEF family contain a N-terminal CDC25 homology domain, centrally contain two tandem repeats of EF-hand Ca^{2+} -binding motifs, and C-terminally contain a DAG (diacylglycerol) binding domain.[39] CalDAG-GEFI is specific for Rap1 and R-ras and is regulated by Ca^{2+}. CalDAG-GEFII is specific for Ras and R-ras and CalDAG-GEFIII is specific for Rap1, Rap2, Ras and R-ras. Both CalDAG-GEFII and CalDAG-GEFIII are translocated to the plasma membrane upon binding of DAG. It therefore seems plausible that CalDAG-GEFI is the guanine nucleotide exchange factor responsible for the activation of Rap1 via the second messenger Ca^{2+}, whereas CalDAG-GEFIII is the guanine nucleotide exchange factor responsible for the activation of Rap1 via the second messenger DAG.[39-42]

CalDAG-GEF proteins are highly expressed in brain and hematopoietic cells.[32, 39, 42] Upon activation the protein translocates towards the plasma membrane.[39, 43]

2.1.4 PDZ-GEF

PDZ-GEF is found in two isoforms in mammals, PDZ-GEF1 and PDZ-GEF2.[44, 45] These proteins contain a PDZ (PSD-95/DlgA/ZO-1) domain, an RA domain, a CDC25-homology domain, a REM domain and a PDZ domain containing a GLGF-binding motif.[44, 45] Furthermore it contains a domain with weak homology to cAMP binding domains. Experiments have indicated, though, that cAMP does not increase the activity of the GEF. This domain does however have an inhibitory effect on the GEF activity.[44] The RA domain of PDZ-GEF1 is responsible for the translocation of the protein to perinuclear domains, including the Golgi apparatus. The RA domain of PDZ-GEF2 on the other hand is responsible for translocation to the plasma membrane.[46] Both PDZ-GEFs are specific for Rap1 and Rap2.[44-46] Interestingly, although it is not exactly clear what induces PDZ-GEF, it has been found that active Rap1 itself can induce PDZ-GEF activity, suggesting a positive feedback loop.[47]

PDZ-GEF1 is ubiquitously expressed throughout the body, whereas PDZ-GEF2 is expressed in the heart, brain, placenta, lung and liver.[44, 46]

2.1.5 Dock4

Dock4 (dedicator of cytokenesis 4) belongs to the CDM gene family encoding small GTPase regulators. Dock4 consists of a N-terminal SH3 region, an extended homology region including the DHR1 and DHR2 domains (Dock homology region) and a C-terminal proline rich region, within which also a consensus sequence can be found for the SH3 domain of Src.

DOCK4 is localized to the plasma membrane and has been shown to be widely expressed. DOCK 4 can interact with the N-terminal SH3 domain of the CrkII adaptor protein. So far, Rap1 has been the only GTPase found to be activated by DOCK4.[48]

2.2 Phosphorylation by kinase A

Phosphorylation of Rap1 by PKA has also been reported. However, whether this phosphorylation results in activation of Rap1 is unclear. In vitro research has indicated that phosphorylation of Rap1 by PKA does not affect the binding affinity of guanine nucleotides by Rap1. Also, no effect has been found on the intrinsic GTPase hydrolysis rate, nor on the Rap1GAP stimulated hydrolysis.[10]

2.3 GAPs

To terminate the activity of Rap1, the bound GTP is hydrolyzed into GDP. Rap1 has a very low intrinsic GTPase activity because it does not contain a glutamine residue at position 61, which is conserved amongst other GTPases. Therefore, Rap1 needs GTPase activating proteins (GAPs) to induce the hydrolysis of GTP.

2.3.1 Rap1GAP

Rap1GAPI was the first GAP identified to be specific for Rap1. Simultaneously with their report on the first, specific Rap1GAP, Rubinfeld et al.[49] reported to have found a second transcript identical to the first one, but containing a duplicated amino acid sequence at the C-terminal tail, later to be distinguished as Rap1GAPII. Rap1GAPII can interact with the G_{α}-subunit of trimeric G proteins through its additional domain. This results in the translocation of Rap1GAPII to the plasma membrane upon activation of the G-protein.[50]
Neither Rap1GAP isoforms has similarities with known GAPs specific for other members of the Ras GTPase superfamily.[49] The Rap1GAPs consist of a N-terminal dimerization region and a C-terminal catalytic region.[12]

Rap1GAPI is expressed in several tissues in the fetus, in the placenta and in the adult brain. Rap1GAPII is mainly expressed in the fetal and adult brain, heart, liver and kidney.[49, 50]

2.3.2 Spa-1

Spa-1 (signal-induced proliferation associated gene-1, also referred to as Sipa-1) contains a catalytic GAP-related domain (GRD), highly homologous to that of Rap1GAP. This domain increases the GTPase activity of the G protein, thereby promoting the hydrolysis of the bound GTP. N-terminally to the GRD Spa-1 has a proline rich region with a possible SH3-binding domain. The C-terminal region contains a leucine zipper-like motif.[51] Spa-1 is a specific GAP for Rap1 and Rap2.

Spa-1 is located to the perinuclear region. Spa-1 is mainly expressed in hematopoietic tissues and it has been found that the expression of Spa-1 and Rap1GAPs segregate amongst

tissues, meaning that where Spa-1 expression is high Rap1GAP expression is low or absent and vice-versa.[51]

2.3.3 E6TP1

E6TP1 (E6-targeted protein 1) was discovered in a search for target proteins of the HPV (human papillomavirus) E6 oncoprotein. E6TP1 exhibits high homology with other Rap1GAPs, the highest homology being with SPA-1. E6TP1 consists of a GAP domain, a leucine zipper region and a C-terminal PDZ domain.[52] E6TP1 has GAP activity specific for Rap1 and Rap2. The PDZ domain of E6TP1 does not play a role in the activity of the GAP. PDZ domains, however, are known protein-protein interaction domains and usually locate proteins to submembranous locations.[53] It is therefore possible that the PDZ domain of E6TP1 regulates the subcellular localization of the GAP, thereby terminating the effect of Rap on a specific pathway.

3. RAP1 AND THE Raf/MEK/ERK PATHWAY.

Rap1 has been implicated to play a role in a diverse array of pathways, including cell proliferation and differentiation. Extracellular stimulatory signals induce proliferation and differentiation by inducing signalling pathways that result in the expression of genes essential for proliferation and differentiation. One major pathway responsible for this is the Raf/MEK/ERK pathway. Many contradictory results have been published about Rap1's role in this pathway. Whereas Rap1 was first discovered as a protein inhibiting the action of Ras in oncogenesis, by inhibiting the Raf/MEK/ERK pathway, later publications have reported Rap1 to have a stimulatory effect on the pathway.

3.1 Raf/MEK/ERK

The MAP kinase cascade is a well conserved signalling cascade, transducing signals from the membrane to the nucleus. The Raf/MEK/ERK pathway has been implicated in a diverse variety of cellular functions, such as proliferation, differentiation, cell-cycle arrest and apoptosis. The distinct outcome of the pathway is thought to depend on the duration and intensity of activation of the pathway, as well as on other pathways that are activated in parallel. Furthermore, the outcome of the pathway can be determined by which isoform of each protein in the pathway is involved.

In vertebrates, for each protein in the pathway, several isoforms exist. ERK and MEK are both found in two isoforms, ERK1 and ERK2, and MEK1 and MEK2. On the other hand, for the Raf proteins three isoforms exist, A-Raf, B-Raf and Raf-1, making the pathway more complex. A-Raf is the poorest activator of MEK and is the least characterized isoform. Raf-1 is ubiquitously expressed throughout the body, whereas B-Raf has a less uniform expression pattern, with the highest expression in neuronal tissue. However, of the three isoforms B-Raf is the most efficient activator of MEK and even in tissues where the expression of B-Raf is low, the protein is still considered to be the main activator of MEK.[54] B-Raf is found in a variety of isoforms resulting form alternative splicing. These isoforms also display a tissue specific expression pattern.[54]

Raf proteins are serine/threonine kinases and are activated by a series of events. First, Raf needs to be bound by an activated member of the Ras superfamily, resulting in the translocation to the plasma membrane. Following this, Raf proteins dimerize forming homomeres or heteromeres with other Raf subtypes. This is then followed by phosphorylation and dephosphorylation of several sites, resulting in an active protein complex.[54]
At the level of activators of the Raf proteins, the pathway is even more complicated. Many GTPases of the Ras super family have the ability to bind and activate Raf proteins. The GTPases may differ in the Raf-subtype specificity, in their efficiency of activating the specific Raf subtype and in their subcellular localization, possibly reflecting the divergent array of outcomes of activation of ERK.[55]

Activated ERK translocates to the nucleus and can directly phosphorylate transcription factors.[54] Depending on the nuclear context, this can have several outcomes. One of the targets of ERK is fos. Fos is a member of the AP-1 transcription factor family, which are known to induce cyclin D1, thereby promoting cell-cycle progression by stimulating G1/S-phase transition.[56]

3.2 Negative regulation of Raf/MEK/ERK by Rap1

As has been mentioned previously, Rap1 was identified in a screen for proteins able to revert a *ras*-induced transformed cell line. When Rap1 was discovered several groups were investigating the mechanisms of malignant cell transformation. For this, the group of Noda et al.[1] transformed NIH 3T3 cells by infecting them with a Kirsten murine sarcoma virus (Ki-MSV). The viral oncogene, v-Ki-*ras*, induces malignant transformation. Following transformation, the cells were transfected with a cDNA library formed from human fibroblasts. Colonies were selected that had lost one or more of the properties associated with v-Ki-*ras* induced transformation. Noda et al. found that these colonies were morphologically different from the parental transformed cells and showed contact-inhibited growth, whereas before the parental transformed cells had lost density-dependent growth inhibition.[1] Since the discovery of Rap1 as an inhibitor of the actions of Ras, many groups have tried to elucidate the mechanisms if this.

Cook et al.[57] demonstrated that the phosphorylated state of ERK1 and ERK2, due to stimulation by LPA or EGF, is mediated through a Ras-dependent pathway in Rat1 fibroblasts. They also showed that the constitutively active Rap1 mutant, RapV12, inhibits the level of ERK 1 and ERK2 phosphorylation, thus indicating that Rap1 counteracts the effects of Ras. The activation of Ras itself was not inhibited by RapV12, indicating that the interference of Rap1 happens down stream from Ras.[57] Lin et al.[58] showed that this interference results in inhibition of proliferation. For this they transfected Heb3B cells with wild-type Rap1. Cells overexpressing Rap1 did not increase their cell number after reaching confluency, although Heb3B/Neo control cells kept on increasing their cell number until they became saturated. When transfected into Balb/c nude mice, Heb3B/Neo cells induced 100% tumorigenesis, whereas the Heb3B/Rap1 transfected cells showed no tumorigenesis at all. This anti-oncogenic effect of Rap1 was contributed to the inhibition of ERK1 and ERK2 phosphorylation, as both insulin and TPA were no longer able to induce the phosphorylation of these proteins in Heb3B/Rap1 cells.[58]

Similarly, stimulation of NIH 3T3 cells with forskolin, a stimulator of adenylyl cyclase, resulted in the activation of Rap1. This stimulation of Rap1 was able to inhibit the mitogenic and proliferative effects induced by EGF stimulation and mediated through the Raf/MEK/ERK pathway. Treatment with H89 and Rap1GAP1 abolished the inhibitory effect of Rap1, indicating that the inhibitory effect of cAMP on proliferation was mediated through PKA and Rap1, respectively.[3]

Vossler et al.[59] showed that the interference of Rap1 in the activation of the Raf/MEK/ERK pathway by Ras was downstream of Ras and upstream of MEK, pointing toward Raf as the effector of Rap1 in this pathway. Activation of Rap1 does not result in a decreased level of active Ras, however, a reduction in Raf phosphorylation and thus activation was seen upon Rap1 activation.

As mentioned before, Rap1 and Ras show a high degree of homology, however, Ras is capable of activating Raf-1, whereas Rap1 cannot. Several reports indicate that the inhibitory effect of Rap1 on the activation of the Raf/MEK/ERK pathway is by sequestering Raf-1 in an inactive complex.[3, 21, 60] Indeed, in Hek293 cells it was confirmed that endogenous Rap1 inhibits the formation of a Ras/Raf-1 complex by binding Raf-1. cAMP can inhibit the recruitment of Raf-1 by Ras in a PKA-dependent manner. It was shown that this was a consequence of the activation of Rap1 by PKA. Although the effect of direct phosphorylation of Rap1 by PKA is unclear, PKA may also induce Rap1 activation by inducing the activation of the Rap1GEF C3G. The constitutively active Rap1 mutant, RapV12, also had an inhibitory effect on Raf-1 and inhibition of Rap1 activity by overexpressing Rap1GAP1 abolished the inhibitory effects of Rap1 on the formation of the Ras-Raf-1 complex.[3]

It was later demonstrated by Carey et al.[21] that the inability of Rap1 to activate Raf-1 depends on which cellular domain Rap1 directs Raf-1 to. It has been indicated that for the activation of Raf-1 the protein needs to be phosphorylated on residue Tyr341 and subsequently on residue Ser338. Phosphorylation of Tyr341 results in the translocation of Raf-1 to raft microdomains where phosphorylation of Ser338 can be induced. Carey and coworkers demonstrated that whereas Ras could support the phosphorylation of Tyr341, triggering the complex to translocate to the raft microdomains, Rap1 was unable to support this phosphorylation step, resulting in the redirection of Raf-1. Due to this redirection, Raf-1 is no longer phosphorylated at Ser338, rendering it inactive.[21]

For the colonic adenocarcinoma cell line HT29 it was found that stimulation of the formation of cAMP by vasoactive intestinal peptide (VIP) resulted in the inhibition of proliferation in the presence of serum.[61] Later reports by the same group demonstrated a stimulatory effect of VIP on proliferation in the absence serum. As will be discussed later, these inhibitory and stimulatory effects are most likely mediated through Rap1 activation. The outcome is probably dependent on the expression level of the 95-kDa B-Raf splice variant in the presence and absence of serum.[62]

In vivo evidence for the inhibitory role of Rap1 on ERK activation and proliferation came from $SPA^{-/-}$ mice. Antigen-stimulation of T cells results in the activation of Rap1 and T cell receptor (TCR) stimulation results in the phosphorylation of ERK. Due to the absence of SPA-1, an increased amount of Rap1-GTP develops in T cells in response to antigen-stimulation. This increase in Rap1-GTP inhibits ERK phosphorylation after subsequent TCR stimulation.[63]

3.3 Positive regulation of Raf/MEK/ERK by Rap1

Although Rap1 has been implicated in the repression of proliferation by its inhibitory role on Ras signalling, in recent years much evidence has been found for a positive role of Rap1 on proliferation. Depending on the cell type, Rap1 has been found to be able to induce the phosphorylation of B-Raf and consequently of MEK and ERK and thereby stimulate proliferation.[22, 59, 62, 64-70] However, the stimulation of the Raf/MEK/ERK pathway by Rap1 seems to be cell type specific. Perhaps not surprisingly, this pattern in cell type dependence seems to correspond with the pattern found for cAMP.

Altschuler et al.[64] have reported to see a decreased doubling time and an increased saturation density on growth curve assays for Swiss 3T3 fibroblasts overexpressing Rap1, pointing toward a proliferative role for Rap1. They showed that the mitotic effect of Rap1 was achieved by promoting the entry into the S phase.[64]

Further evidence for the mitotic effects of Rap1 have come from the oncoproteins BCR/ABL and RET/PTC. These oncoproteins result in hyperproliferation by bypassing the need for stimulatory impulses such as growth factors and bypassing the need for elevation of cAMP levels in certain cell types. The fusion of the BCR and ABL genes, also known as the Philadelphia chromosome, results in the BCR/ABL fusion product which is found in 95% of the patients with chronic myeloid leukemia (CML) and 30-40% of the patients with lymphoblastic leukemia. BCR/ABL protein constitutively activates a variety of intracellular signalling cascades involved in hematopoietic, including the Ras/Raf-1/MEK/ERK pathway and the PI3K/Akt pathway. It has been found that the cytokine receptor for IL-3 and erythropoietin, besides activating Ras, can activate Rap1, Rac and the Rho family GTPases. This is mediated by the adaptor protein CrkL, which is known to be one of the major substrates for BCR/ABL.[67] TonB210 cells express BCR/ABL when treated with DOX. Stimulation of TonB210 cells with IL-3 or transfecting them with BCR/ABL results in proliferation of these cells and reduces apoptosis. Mizuchi et al.[67] showed that upon

stimulation with DOX the activity of Rap1 increases compared to cells treated with IL-3. In CML cells, treatment with an inhibitor of the ABL kinase activity reduces the activity of Rap1.[66] Although Ras has been found to play the major role in the activation of Elk-1 (a downstream effector of ERK) by BCR/ABL, Rap1 has also been found to contribute to this activation. A dominant negative Rap1 mutant, as well as overexpression of Spa-1 are both able to reduce the level of activity of Elk-1 in BCR/ABL expressing cells by reducing Rap1, MEK and ERK activity.[66, 67] Furthermore, expression of Spa-1 reduces proliferation and reduces the viability of these cells, suggesting that the phosphorylation of ERK by active Rap1 results in cell proliferation and an increased viability.[66] Mizuchi et al.[67] have shown that BCR/ABL induces both Raf-1 and B-Raf, confirming that both Ras and Rap1 might play a role in the activation of the Raf/MEK/ERK pathway by BCR/ABL.

A second strong model for the proliferative effects of Rap1 are the human papillary thyroid carcinoma (PTC) cells. PTCs often feature the RET/PTC oncoprotein, which is formed by the fusion of the intracellular kinase domain with the N-terminus of heterologous proteins. This oncoprotein can dimerize, creating an autophosphorylating protein. Autophosphorylation results in the activation of Rap1 in HEK293 cells. Furthermore, CrkII and C3G were found to coprecipitate with RET/PTC and Rap1, suggesting that activation of Rap1 by RET/PTC is mediated through the Rap1GEF C3G. Indeed, a C3G mutant devoid of its GEF ability inhibited the activation of Rap1 by RET/PTC, supporting that the CrkII/C3G complex mediates the actions of RET/PTC on Rap1.[65] The activation of Rap1 by RET/PTC via CrkII/C3G, results in the phosphorylation of B-Raf, MEK and ERK, as a mutant CrkII, C3G or Rap1 can all independently reduce the levels of phosphorylation.[65] Supporting this model, human papillary thyroid carcinoma cell lines, expressing RET/PTC endogenously, show high levels of Rap1 activity. In these cell lines RET/PTC-Gab1-CrkII-C3G protein complexes were found, indicating that this is an *in vivo* protein complex responsible for the activation of Rap1. In these cell lines it was found that inhibiting the activity of Rap1 reduced the level of DNA synthesis, indicating that Rap1 has a positive role in cell growth.[65]

As has been mentioned before, Rap1 can only induce the B-Raf isoforms of the Raf family. B-Raf is found in many splice variants. Fujita et al.[22] have reported that only those cells expressing the 95-kDa B-Raf splice variant showed an increased activity of ERK upon the increase of cAMP concentration, as represented by phosphorylation of ERK. In bone cell-derived cell lines lacking the 95-kDa B-Raf isoforms, Rap1 inhibited ERK activation in a cAMP dependent fashion.[22] This suggests that Rap1 can only activate the 95-kDa isoforms of B-Raf and that only in those cells that express this isoforms of B-Raf, Rap1 activation results in activation of ERK and the stimulation of proliferation. Consequently, this suggests that the anti-proliferative effects of Rap1 will only be seen in those cells lacking the 95-kDa isoform. Indeed, the inhibition of ERK in the 95-kDa B-Raf lacking cells results in the inhibition of proliferation in these cells. These cells do express a 62-kDa B-Raf isoforms.[22] To strengthen the hypothesis that the proliferative effect of Rap1 is dependent on the B-Raf isoform, Fujita et al.[22] have transduced the 95-kDa B-Raf lacking bone cells with 95-kDa B-Raf. Stimulation of these cells by forskolin converts the negative effect of cAMP on proliferation into a positive stimulator.

In normal human keratinocytes (NHK) it has been found that under subconfluent circumstances cAMP can induce ERK phosphorylation and NHK proliferation, whereas under confluent circumstances cAMP inhibits proliferation by inhibiting the Raf/MEK/ERK pathway. Takahashi et al.[71] indicated that subconfluent NHKs express both the 95-kDa as well as the 62-kDa isoforms of B-Raf. Interestingly, they found that once confluent, NHKs no longer express the 95-kDa isoforms of B-Raf and that this coincides with an anti-proliferative effect. This supports the hypothesis that Rap1 is only able to activate the 95-kDa isoforms of

B-Raf. This switch is splice variant expression suggests a possible new model in the regulation of density-dependent inhibition of proliferation. Interestingly, Alleaume et al.[62] found for the colonic adenocarcinoma cell line (HT29) that stimulation with vasoactive intestinal peptide (VIP) in the absence of serum resulted in an increase in ERK phosphorylation and proliferation. Stimulation by VIP results in increased intracellular cAMP levels and in the activation of Rap1, as indicated by its GTP-loaded state. Contradictory, the same group previously reported an inhibitory effect of VIP on proliferation of HT29 cells in the presence of serum.[61] Whether this was due to the inhibition of ERK phosphorylation was not reported. However, these contradictory findings may resemble what was reported for the NHK cells. Perhaps switching from serum-deprivation to serum results in a switch from the 95-kDa B-Raf isoform to the 62-kDa B-Raf isoform, resulting in a switch from stimulation to inhibition of proliferation. It was determined that the HT29 cells in the absence of serum express the 95-kDa B-Raf.[62] It would be of interest to determine whether this is also expressed or not in the presence of serum.

Interestingly, Schmitt et al.[3] showed that for Hek293 cells activation of Rap1 by cAMP does result in the formation of the inactive Rap1/Raf-1 complex. However, Hek293 cells do express B-Raf and therefore it would be expected that the stimulation of Rap1 by cAMP in these cells results in the activation of ERK. Indeed previous results from this group confirms this.[72]

Insight into how Rap1 is able to activate B-Raf came from experiments from Carey et al.[21] in which they tried to elucidate why Ras was able to activate Raf-1, whereas Rap1 was not. As has been described in the previous section, they reported that Rap1 was not able to support the phosphorylation of Tyr341 in Raf-1, inhibiting the translocation to specific membrane locations needed for further activation of Raf-1. Interestingly, B-Raf contains an aspargine in stead of a tyrosine residue, mimicking the phosphorylation of Tyr341 in Raf-1, resulting in a constitutively active B-Raf. For activation of MEK and ERK, B-Raf needed to be targeted to the membrane which was achieved by binding active Rap1 or Ras. [21] Although, several groups have reported to see a stimulatory effect of Rap1 on proliferation, the reported modes of activation of Rap1, resulting in this effect have been contradictory. Some groups have reported a PKA-dependent activation of Rap1 by cAMP,[59, 68], whereas other groups have reported that this activation is PKA-independent.[62] For instance, the stimulation of cAMP by VIP in HT29 cells resulted in the phosphorylation of ERK in a PKA-independent manner.

The activation of ERK1 and ERK2 by cAMP was also confirmed by Vossler and coworkers.[59] However, in contradiction to what was reported by Alleaume and coworkers, Vossler et al. reported that the activation of ERK by cAMP and Rap1 was dependent on PKA. Treatment of the PC12 cells with H89 inhibited the activation of ERK, indicating that the activation was due to cAMP and that this effect was mediated by PKA. They proposed that this was due to the direct phosphorylation of Rap1 by PKA on serine 179 as RapD179, a mutant with its serine residue replaced by an aspartic acid thereby mimicking phosphorylation of Rap1, was able to activate ERK. However, as has been discussed earlier, whether phosphorylation of Rap1 activates the protein is unclear. It is possible that whereas the activation of Rap1 through PKA is dependent on C3G, the phosphorylation may augment the efficiency of Rap1, for instance by strengthening its interactions with its effectors, accounting for its seemingly increased activity. Vossler et al.[59] demonstrated that the activation of Rap1 induced the recruitment and association of B-Raf to the cell membrane, which was dependent on GTP-loading of Rap1.[59] Ribeiro-Neto et al.[68] also reported that phosphorylation of Rap1 by PKA results in proliferation in thyroidal cells. They showed that Rap1 needed to be phosphorylated by PKA as well as have GTP bound to transmit the mitogenic signal.[68] What the exact role of this phosphorylation was in inducing the mitotic signal was not determined.

In a later report this group was able to confirm that cAMP, through Rap1 activation, can result in proliferation. In mice, chronic elevation of thyroidal cAMP resulted in clusters of proliferating cells. This enhanced proliferation is due to the S phase entry by active Rap1. After 1 year of sustained treatment with TSH, which enhances the level of thyroidal cAMP and the activity of Rap1, mice were found to develop multilobular, hyperemic glands showing signs of thyroid follicular cell carcinoma characterized by invasion of the surrounding tissues and vessels.[69]

3.4 Rap1 activation pathway and its outcome.

It may be clear by now that, depending on the cellular context, Rap1 can positively or negatively regulate the activity of ERK, depending on the presence of the 95-kDa isoforms of B-Raf. However, this does not mean that every activation pathway of Rap1 in 95-kDa B-Raf containing cells automatically results in B-Raf activation. In other words, whether activation of Rap1 results in activation of B-Raf and consequently ERK, depends on how Rap1 is activated.

3.4.1 GEFs

3.4.1.1 Epac

It has been debated whether Rap1 could mediate the effects of cAMP on the Raf/MEK/ERK pathway. Although much evidence exists on its role in the activation and inhibition of the Raf/MEK/ERK pathway, there have also been reports indicating that Rap1 has no influence on the pathway at all.[73, 74] cAMP mediates its effect through several effectors, including PKA and Epac. PKA has been reported to have a direct effect on the Raf/MEK/ERK pathway. Contradictory, the activation of Rap1 by cAMP, through Epac, has been shown to have no effect on Raf/MEK/ERK. Stimulation of both NIH 3T3 cells and Chinese hamster ovary (CHO) cells with cAMP inducing agents or cAMP analogues activated both Rap1 and ERK. However, inhibiting PKA with H89 did inhibit ERK activation but did not inhibit Rap1 activation. Furthermore, the Epac specific cAMP analogue (8CTP-2Me-cAMP) did activate Rap1 but had no effect on ERK activation. From this it was concluded that Rap1 had no effect on the Raf/MEK/ERK pathway.[74] However, research done by Wang et al.[36] demonstrated that the effects of Rap1 activation depend on which subcellular pool of Rap1 is activated. They demonstrated that the activation of Epac, which is located perinuclear, results in the activation of a perinuclear pool of Rap1 which does not influence ERK activation. However, when redirecting Epac to the plasma membrane, activation of Epac by 8CTP-2Me-cAMP resulted in the activation of a pool of Rap1 located at the plasma membrane. The activation of this pool of Rap1 did result in the activation of ERK in PC12 cells.[36]

3.4.1.2 C3G

As has been mentioned in chapter 2, it has been shown that PKA can directly phosphorylate Rap1. What the consequences of this phosphorylation are and whether it activates Rap1 is unclear. However, it has been demonstrated that elevation of the cAMP levels results in Rap1 activation and that in some instances this is mediated through PKA.[3] Whether this is due to direct or indirect activation by PKA is not clear. Schmitt et al.[75] have shown that the activation of Rap1 by PKA happens through the RapGEF C3G. Upon stimulation with forskolin cAMP levels rise resulting in the activation of PKA. PKA in turn phosphorylates Src, which phosphorylates Cbl on tyrosine, which is located towards the plasma membrane. Upon tyrosine phosphorylation of Cbl, a Crk/C3G complex is recruited to

the plasma membrane and is activated. The activated C3G is then able to activate Rap1. In NIH 3T3 cells this pathway is responsible for the inhibition of the Raf/MEK/ERK pathway as mutants of any of the proteins in this pathway abolish the inhibitory effect.[3, 75] Interestingly, this research has also indicated that only the membrane fractions of NIH 3T3 cells, treated with forskolin, were able to activate Rap1, suggesting that the Rap1 pool situated at the plasma membrane is the pool responsible for the effects of Rap1 on ERK activation, due to cAMP elevation.[75] Indeed, Wang et al.[36] confirmed that cAMP's effects on ERK activation can be mediated by the activation of the plasma membrane pool of Rap1 and that the activation of this pool of Rap1 happens through C3G.

3.4.1.3 CalDAG-GEF

There are a variety of reports linking an increase in intracellular Ca^{2+} and DAG and an increase in ERK activation.[76, 77] Ca^{2+} and DAG are ligands for CalDAG-GEFI and CalDAG-GEFII. Guo and coworkers were able to demonstrate that in PC12 cells the activation of CalDAG-GEF by Ca^{2+} resulted in the activation of Rap1, which in turn activated B-Raf and consequently MEK and ERK. Stimulation of PC12 cells with carbachol, resulting in increased cytoplasmic Ca^{2+} concentrations allowed them to immunoprecipitate a CalDAG-GEF/Rap1/B-Raf cassette.[76] As mentioned in chapter 2, CalDAG-GEF is located toward the plasma membrane upon activation[39], suggesting that it couples the plasma membrane pool of Rap1 to the Raf/MEK/ERK pathway.

3.4.1.4 PDZ-GEF

In *Drosophila* PDZ-GEF was shown to be involved in the proper development of the eye. Loss-of-function mutations of PDZ-GEF or Rap1 result in similar eye phenotypes, with a reduction in photoreceptor cell number, suggesting that these two proteins are involved in the same pathway resulting in photoreceptor cell proliferation. Furthermore, it was determined that PDZ-GEF is capable of activating the Raf/MEK/MAPK pathway in Drosophila, as the dominant negative mutants D-Raf, dMEK and dMAPK were able to inhibit the activation of MAPK by overexpression of PDZ-GEF.[78] Noteworthy is that the photoreceptor cells of the eye originate from the neural tube. For neuronal cells it is known that they express B-Raf in humans. This suggests the possibility that PDZ-GEF is able to regulate the activity of the Raf/MEK/ERK pathway through Rap1 and B-Raf. Confirming this, Liao et al.[47] reported that activation of PDZ-GEFI resulted in the activation of Rap1 and consequently of B-Raf. Interestingly, they also reported that the activation of PDZ-GEFI resulted in the translocation of the GEF to the perinuclear region where it activated Rap1. However, Wang et al.[36] demonstrated that the perinuclear pool of Rap1 was incapable of modulating the Raf/MEK/ERK pathway. Most likely this difference in results is due to the experimental procedures. Whereas Wang and coworkers investigated the effects of endogenous GEFs and Rap1[36], Liao and coworkers transfected Cos7 cells with constructs containing PDZ-GEFI, Rap1 and B-Raf.[47]

3.4.1.5 Dock4

The role of the Rap1GEF, Dock4, in the Raf/MEK/ERK pathway is currently unknown. Although Dock4 has been reported to contain a loss-of-function mutation or to be downregulated in certain tumours[79, 80], this does not mean that this is due to a decreased inhibition of the Raf/MEK/ERK pathway by Rap1. The progression of tumorigenesis due to the loss of Dock4 function may well be due to the aberrant AJ regulation.

3.4.2 GAP

3.4.2.1 Rap1GAPI

If Rap1 inhibits the activation of the Raf/MEK/ERK pathway, one would expect that inhibition of Rap1 abolishes this effect. NIH 3T3 cells treated with EGF show an increased phosphorylation of ERK. When treated with forskolin, ERK phosphorylation is abolished, which is mediated by the activation of Rap1. Indeed, inhibition of Rap1 activity by overexpression of Rap1GAPI abolishes this inhibitory effect on ERK phosphorylation.[3] In 293T cells activating Rap1GAPII resulted in an increased activation of ERK due to the inhibition of Rap1. Interestingly, in contrast to the previous report it was found that Rap1GAPI had no effect on ERK activity.[50] Melanomas are associated with a decreased expression of Rap1GAP. This decrease in Rap1GAP expression correlates with an increased level of active Rap1, an increased ERK phosphorylation and increased proliferation. As epithelial cells express B-Raf[71], this is most likely due to the activation of the B-Raf/MEK/ERK pathway by Rap1.[81]

3.4.2.2 Spa-1

BCR/ABL expression in TonB210 cells induces the activation of Rap1 which results in the activation of the Raf/MEK/ERK pathway. Overexpression of Spa-1 in these cells abolishes this activation of Raf/MEK/ERK.[82] Furthermore, *in vivo* evidence for the effect of Spa-1 on ERK activation comes from Spa-1-difficient mice. Spa-$1^{-/-}$ mice show a reduced level of ERK phosphorylation in T cells, indicating that the inhibition of Rap1 activity in T cells is necessary for ERK phosphorylation.[63] In contrast these mice show an increase in pluripotent hematopoietic progenitors.[83] This indicates that Spa-1 is capable of inactivating the Rap1 pool involved in modulating the activity of the Raf/MEK/ERK pathway.

3.4.2.3 E6TP1

E6TP1 was discovered as a target for the E6 protein in the human papillomavirus type 16. E6TP1 is targeted for degradation by E6. The reduced levels of E6TP1 result in increased levels of Rap1 and increased proliferation.[53] However, whether this is mediated through activation of Rap1 and the Raf/MEK/ERK pathway is unknown.

4. RAP1 AND CELL ADHESION.

When Rap1 was first discovered it was thought that its main function was to antagonize Ras. However, as several GEFs and GAPs specific for Rap1 were discovered it seemed plausible that Rap1 would have other functions, distinct from antagonizing Ras. Indeed, one of these turned out to be the control of cell adhesion. Rap1 is now known to play a role in both cell-matrix adhesion through integrins and cell-cell adhesion through cadherins (fig. 3).

4.1 Integrin activation.

Integrins interact with the extracellular matrix, intracellular proteins and the cytoskeleton, forming both a structural connection as well as a bidirectional signaling pathway over the membrane at focal adhesions. They are involved in signal transmission over the plasma membrane through inside-out and outside-in signaling, which regulates several cellular processes including proliferation, migration, apoptosis, differentiation and gene expression.[84, 85]

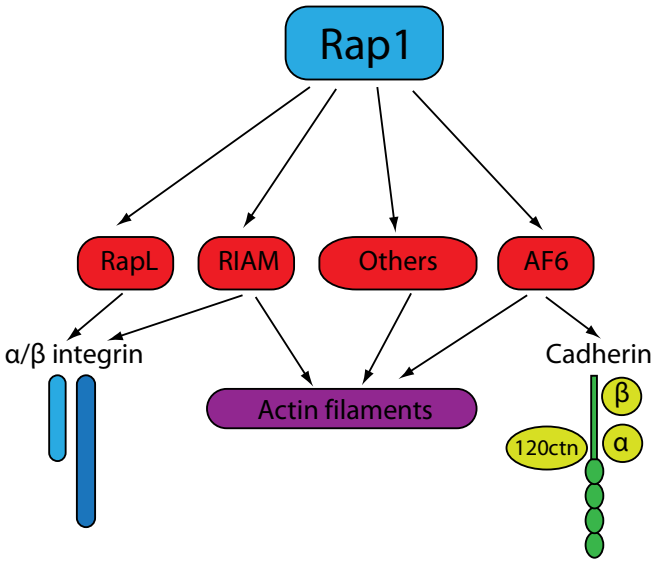

Figure 3. Rap1 is involved in integrin activity and adherens junction formation through cadherin.

Integrins are glycosylated transmembrane heterodimers consisting of an α and a β subunit. 18 α subunits and 8 β subunits exist, which together can form 24 distinct heterodimers. Both the α and the β subunit consist of a large extracellular domain, a single transmembrane α-helix and a short cytoplasmic domain.[86] Non-covalent binding between an α and a β subunit retains them in a low-affinity state. Activation of integrins through inside-out signaling transforms them into a high-affinity state. Disruption of the non-covalent binding between the α and a β subunits is needed for activation.[84]

The cytoplasmic domains of integrins play an essential role in integrin activation. Binding of the cytoplasmic tail of the β subunit by the adaptor protein talin seems to be the final step in integrin activation for several types of integrins. Talin is a cytoskeletal protein, binding both integrins and actin, as well as actin-binding proteins. Talin is thought to induce the activation of integrins by disrupting the interaction between the α and the β tails. This dissociation is thought to be a two-step process in which talin first binds the β tail with high affinity, allowing talin to subsequently bind a membrane proximal site with low affinity, where it disrupts the interaction between the α and the β subunits, resulting in activation. Interestingly, whereas inside-out activation of integrins needs the two subunit to dissociate, outside-in activation does not.[84]

4.2 Rap1 regulates inside-out signaling.

Rap1 has been found to play a major role in the conversion of cellular signals into integrin activation. Rap1 is capable of activating all integrins associated with the actin cytoskeleton, which include integrins containing the β1, β2 and β3 subunits[25, 27-30, 87]. Besides

inducing integrin activity (affinity), Rap1 is also involved in enhancing integrin clustering, known as avidity.[26]

Most research on the role of Rap1 in integrin activation has used leukocytes as a cell model. Normally integrins are kept in an inactive state in leukocytes. Through inside-out signaling integrins can be activated, inducing cell-matrix adhesion. The change from non-activated integrins to activated integrins is easily visible, making leukocytes an adequate model.

Katagiri et al. were able to demonstrate directly that Rap1 is involved in integrin activation. They were able to show that activation of Rap1 resulted in the activation of LFA-1 (integrin $\alpha_L\beta_2$) in leukocytes, in a phosphatidylinositol-3-OH kinase (PI3K) independent manner.[28] Similarly, Reedquist and coworkers demonstrated that Rap1 could mediate the activation of both LFA-1 and VLA-4 (integrin α4β1) by CD31 stimulation. Transfection of Jurkat T cells with the constitutively active Rap1V12, C3G or an inactive Rap1GAP resulted in increased CD31-induced binding to ICAM and VCAM, indicating the activation of both LFA-1 and VLA-4, respectively. Coordinately, transfection with the inactive Rap1N17, a Rap1GAP or the RBD of RalGDS, which binds active Rap1 and prevents it from associating with its effectors, abolishes the activation of integrins. It was shown that the increased adhesion was probably due to an increased integrin affinity, without any effects on integrin expression.[29] As for LFA-1 and VLA-4, Rap1 also increases the affinity of CR3 (αMβ2)[27], VLA-5 (α5β1)[87] and GPIIb-IIIa (αIIbβ3)[25], whereas Sebzda et al. were able to demonstrate that Rap1 also regulated the activity of β1 and β2 integrins by regulating integrin avidity[30]. Interestingly, the regulation of integrin activity by Rap1 could also explain the flat revertant phenotype described by Noda et al.[88] in the identification of Rap1.

Work from Han and coworkers demonstrated that RIAM, an effector of Rap1, is essential for integrin activation by Rap1.[89] RIAM (Rap1-GTP-interacting adaptor protein) was first discovered by Lafuente et al.[90] in a search for Rap1-interacting molecules. Like Rap1, RIAM is widely expressed. Overexpression of RIAM induces cell spreading and integrin activation of the β1 and β2 subtypes. In Jurkat T cells activation of Rap1 induces the activation of integrins and consequently adhesion. Knock-down of RIAM in these cells abolishes the activating effects of Rap1, suggesting that Rap1 induces integrin activation through RIAM. Furthermore, knock-down of RIAM displaces Rap1-GTP from the plasma membrane, indicating that RIAM is necessary for the translocation of Rap1 to the plasma membrane.[90] Interestingly, Rap1 activation induces the translocation of talin from the cytoplasm to the plasma membrane, suggesting a Rap1-RIAM-talin complex. Indeed, activation of Rap1 induces the colocalisation of RIAM and talin at focal adhesions. Interestingly, as focal adhesion sites mature they become devoid of RIAM.[89] As it is known for talin that it is involved in integrin activation by inducing a conformational change resulting in increased binding affinity, it is plausible that RIAM is the effector of Rap1 mediating its effects on integrin affinity.

RapL (regulator of cell adhesion and polarization enriched in lymphocytes) is another effector of Rap1, necessary for LFA-1 activation. RapL is most abundantly expressed in lymphoid tissues and is generated as a splice variant of Rassf5, which is expressed in the brain. Both *in vitro* and *in vivo* RapL is bound by Rap1-GTP. The activation of RapL by active Rap1 induces clustering of LFA-1 (avidity) and increases its binding affinity. Unactivated RapL is found in the cytoplasm and in the perinuclear regions. Activation of RapL results in its translocation toward the plasma membrane. RapL can then recruit LFA-1 to this location resulting in clustering. This is thought to be mediated through the αL integrin subunit.[91] These findings suggest that RapL might be the effector mediating the effects of Rap1 on avidity.

4.3 Rap1 activation pathway for integrins.

The role for Rap1 in integrin activation has been firmly established. However, in order to determine whether Rap1 could form a link between the proliferative state of a cell and its adhesiveness, it is necessary to look at the mode of regulation of Rap1. Which GEFs and which GAPs are involved in the regulation of cell adhesion?

4.3.1 GEFs

4.3.1.2 Epac

cAMP is able to induce cell adhesion. Previously it has been reported that this is a PKA dependent process.[92] However, cAMP is also known to activate Rap1 activity through Epac and active Rap1 is involved in integrin activation, suggesting the possible involvement of Epac-Rap1 pathway in mediating cAMP induced adhesion. Activation of integrins in OvCar3 cells by the cAMP analogue 8-Br-cAMP is not affected by the PKA inhibitor H89, indicating a PKA-independent action. Furthermore, the Epac-specific cAMP analogue 8CTP-2Me-cAMP is capable of activating integrins through activation of endogenous Epac and Rap1.[93] It was later demonstrated that the increased adhesion of OvCar3 cells, mediated by the Epac-Rap1 pathway, was due to the activation of the α3β1 integrins, whereas active Rap1 had no effect on the α6β4 integrin. Interestingly, as for all integrins described so far, α3β1 integrins are coupled to the actin cytoskeleton, whereas α6β4 integrins are coupled to intermediate filaments. This suggests that Rap1 is solely involved in the activation of integrins coupled to the actin cytoskeleton.[23]

4.3.1.2 C3G

The role of the CrkL-C3G complex in integrin activation has already been demonstrated before Rap1 had been implicated in the activation of integrins. Arai et al.[24] have demonstrated that in the hematopoietic 32D cells CrkL could induce the activation of VLA-4 and VLA-5 and enhance adhesion to fibronectin through C3G. For this the GEF activity of C3G was necessary. Furthermore, R-Ras could only partially explain the effect of CrkL and C3G, leaving the possibility for Rap1 to mediate the effects.[24] Later, Nolz and colleagues demonstrated that the activation of β1 integrins upon T cell receptor (TCR) stimulation, through Rap1, was dependent on the activation of CrkL and C3G by the TCR.[94] Similarly, Takahashi et al.[95] demonstrated the activation of β3 integrins, by recruiting them to leading edges upon stimulation of NIH 3T3 cells with platelet derived growth factor (PDGF). They demonstrated that this coincided with the translocation of CrkL-C3G and the Rap1 effector RalGDS to these leading edges. Furthermore, Rap1GAP and knock-down of Rap1 could inhibit this, indicating that the increased avidity was indeed mediated through Rap1 activation.[95]

4.3.1.3 CalDAG-GEF

Activation of integrin αIIbβ3 in platelets can be induced by both Ca^{2+} and DAG. Crittenden et al.[96] demonstrated that the activation of αIIbβ3 in platelets is mediated through the Rap1GEF CalDAG-GEFI. Knock-out of this GEF results in aberrant platelet aggregation, which is not due to differential expression of the αIIbβ3 integrins. Interestingly, whereas stimulation with low levels of PMA or thrombin did abolish integrin activation, stimulation with higher levels induced aggregation.[96] PMA stimulation results in the activation of both CalDAG-GEFI as well as PKC. PKC has been implicated in integrin activation.[97] Perhaps PKC is activated due to the higher concentrations of PMA or thrombin, inducing an alternate pathway then CalDAG-GEFI to induce integrin activation. A later report from the same group confirmed that CalDAG-GEFI and PKC are in parallel pathways, both resulting in αIIbβ3

integrin activation. Interestingly, both mediate their effect through Rap1 activation. CalDAG-GEFI induces rapid Rap1 activation, whereas PKC is responsible for sustained Rap1 activation.[97]

In primary T cells Ghandour et al.[98] reported to find an increased affinity of LFA-1 due to activation of the CalDAG-GEFI-Rap1 pathway by both PMA and the stromal cell-derived factor-1α (SDF-1α). Interestingly, they reported to see no effect on the activation of VLA-4. Rap1 has however been implicated in the activation of VLA-4 in Jurkat T cells.[29] This was established through the activation of the Rap1GEF C3G, indicating that the mode of activation of Rap1 determines which integrins are activated and thus what the outcome will be.

CalDAG-GEF expression in lymphocytes is either very low or absent. Early work by Katagiri and coworkers demonstrating the role of Rap1 in LFA-1 activation, indicated that whereas Rap1 did mediate the activation of LFA-1 by PMA in HL-60 cells, a promyeloid-like leukemia derived cell line, in Jurkat T cells the activation of LFA-1 by PMA was not mediated by Rap1.[28] This is in line with the expression profile of CalDAG-GEF, with its expression being high in most leukocytes except for lymphocytes.[96]

4.3.1.4 PDZ-GEF

The role of PDZ-GEF in integrin activation has not yet been fully elucidated. PDZ-GEFs homologue in *Drosophila*, *Dizzy*, has been shown to be necessary for macrophage migration in *Drosophila* embryo's. It may be that PDZ-GEF is an important Rap1GEF during embryogenesis. It was shown that *Dizzy* induces the activation of the βPS integrin through Rap1 activation.[99] Little investigation into the role of PDZ-GEF in integrin activation in human cell lines has been performed, although overexpression of PDZ-GEF in OvCar3 cells increases adhesion through the activation of Rap1.[23] Furthermore, in an attempt to determine the role for PDZ-GEF1 in AJs in HUVECs, Sakurai et al.[20] knocked down PDZ-GEF1. However, this knockdown resulted in complete loss of adherence, instead of the loss of cell-cell junctions alone, suggesting that PDZ-GEF1 plays a role in integrin-mediated adhesion.

4.3.2 GAPs

4.3.2.1 Rap1GAP

In primary CD3+ T cells overexpression of Rap1GAP inhibited the activation of LFA-1, due to its inhibitory effect on Rap1.[98] Similarly, overexpression of Rap1GAP in the human megakaryocytic cell line, DAMI, inhibited adhesion to fibronectin through the αIIbβ3 integrin.[100] In melanomas it was found that Rap1GAP is downregulated.[81] Normally Rap1 induces the activation of integrin αvβ3 in these cells.[101] The reduced levels of Rap1GAP results in aberrant focal adhesion formation, with absence of filamentous actin (F-actin) assembly and vinculin dispersed throughout the cytoplasm.[81] Similarly, Rap1GAP is downregulated in pancreatic cancer. The pancreatic cancer cell line MiaPaCa-2 shows high basal active Rap1 levels, correlating to the low Rap1GAP levels. F-actin assembly at the leading edge and focal adhesion kinase activation happens shortly after stimulation. Introducing Rap1GAP into these cells abolishes both of these processes of integrin activation.[102]

4.3.2.2 Spa-1

Tsukatmoto et al.[103] demonstrated that Spa-1 overexpression in HeLa cells and in the promyelocytic 32D cells resulted in decreased Rap1 activity and decreased adhesion. However, as they showed, this decreased adhesion was not due to altered integrin

expression.[103] It was later demonstrated in primary CD3+ T cells overexpression of Spa-1 abolished the activation of LFA-1 by Rap1.[98]

4.4 Cadherins

Cadherins are the main components of adherence junction, forming cell-cell contacts. Cadherins interact with proteins of neighboring cells and with intracellular proteins, thereby linking the cytoskeletons of neighboring cells and delivering and receiving signals. They are involved in morphogenesis, wound healing and cancer progression.[104]

Cadherins engage in homophilic trans-interactions and are dependent on extracellular Ca^{2+}. Cadherins are single-pass transmembrane glycoproteins. Their cytoplasmic domain is comprised of two separate domains, the juxta-membrane domain and the C-terminal tail. The juxta-membrane domain has a $p120^{ctn}$ -binding site and is involved in cadherin clustering, transport and endocytosis. Binding of $p120^{ctn}$ to cadherin stabilizes the plasma membrane localization of the cadherin. The C-terminal tail contains a β-catenin-binding domain and regulates binding to the actin cytoskeleton and is involved in outside-in signaling. This is accomplished through the formation of a complex of adaptor proteins including β-catenin, α-catenin, vinculin and α-actinin.[104, 105]

The most studied cadherins are E-cadherin and VE-cadherin, which form the major components of adherens junctions in epithelial and endothelial cells, respectively. Adherence junctions are regulated in two ways: plasma membrane levels of cadherins are regulated through vesicular sorting and contact stability is regulated through the linkage with the cytoskeleton.[104]

Nectins are cell surface proteins thought to be involved in the initial formation of cell-cell contacts. This initial cell-cell contact induces the recruitment of cadherins and other junctional adhesion molecules (JAMs) to these contact sites. Clustering of these cadherins and JAMs results in the further accumulation of cadherins and intercellular and intracellular components of adherence junctions, inducing a zipper-like formation of cadherins. [106]

Adherence junctions are dynamic complexes and show a high level of turnover, indicated by the tyrosine phosphorylation of β-catenin, the degradation of E-cadherin, regulated by the E3-ligase Hakai, and by the recycling of E-cadherin through endocytic and exocytic compartments. Stabilization of AJs is accomplished through diminished endocytosis of cadherins from the plasma membrane and by formation of cortical actin bundles, linked to the cadherins.[104]

4.5 Rap1 regulates cell-cell adhesion.

The first indication that Rap1 is implicated in cadherin regulation came from studies in *Drosophila*. Rap1 deficiency in cells in the wing resulted in the loss of the even distribution of DE-cadherin around the cell. Furthermore, cell-cell contacts were lost, allowing these cells of the wing to disperse between the surrounding wild-type epithelial cells.[107] These findings were later extrapolated to mammalian cells. Hogan et al.[31] reported a role for Rap1 in E-cadherin-based cell-cell contacts in human epithelial breast cancer (MCF7) cells. They demonstrated that new homophilic interactions between E-cadherins induced the recruitment of Rap1 to these adhesion sites, which in turn resulted in the recruitment of E-cadherin, suggesting a positive feedback loop. Similarly, in Madine-Darby canine kidney (MDCK) cells, expression of the constitutively active Rap1V12 results in the accumulation of E-cadherin at adherent junctions without effecting the expression level of E-cadherin,[108] whereas Asuri et al.[109] demonstrated the polarization of the Rap1 pool and its relocation towards newly forming cell junctions in MDCK cells.

As has been mentioned before, nectins are transmembrane proteins involved in the initial formation of AJs. Interestingly, the formation of intercellular homodimers induces outside-in signaling in which nectin induces the activation of Rap1 through Crk-C3G. This activation of Rap1 is necessary for the formation of E-cadherin based AJs in MDCK cells.[110] One way this may be accomplished is through the Rap1 effector afadin/AF6. Active Rap1 can bind afadin/AF6. Afidin/AF6 in turn stimulates the association between E-cadherin and $p120^{ctn}$, which inhibits the endocytosis of non-trans-interacting E-cadherin from the plasma membrane, thereby stabilizing and maintaining adherence junctions.[111]

As homophilic E-cadherin interactions are dependent on extracellular Ca^{2+}, ablation of Ca^{2+} disrupts these interactions. This disruption of cadherin-cadherin interactions also induces the recruitment of Rap1 to these sites[31], suggesting a rescue model in which disruption of cell-cell contacts recruits Rap1 to these sites and activation of Rap1 can then induce further recruitment of E-cadherin in order to restore cell-cell contacts. Indeed, Balzac and coworkers were able to demonstrate that the internalization of E-cadherin, as a consequence of cell-cell contact disruption, resulted in the activation of Rap1. This activation of Rap1 happened at Rab11-containing endosomes.[112] In OvCar3 cells it was shown that both overexpression of Rap1GAP1 or the inactive Rap1N17 resulted in reduced E-cadherin levels at cell-cell interaction sites, as well as reduced formation of new E-cadherin-E-cadherin interactions, indicating that Rap1 may be involved in both cell junction maintenance and formation, respectively.[108] It has been well established that the internalization and recycling of E-cadherin is an important process in the modulation and maintenance of cell-cell contacts. As Rap1 is activated by the internalization of E-cadherin and is necessary for the recruitment of E-cadherin to newly forming cell-cell contacts, it is plausible that Rap1 plays a major role in mediating this process.

It seems that whereas Rap1 is involved in the internalization and recruitment of E-cadherin to cell-cell contacts, most likely by controlling its recycling, it is not involved in the maintenance of mature cell-cell contacts. In fact, the formation of mature cell-cell contact results in the downregulation of Rap1 activity. Interestingly, formation of a Rap1-E-cadherin complex is inversely correlated with the formation E-cadherin-$p120^{ctn}$ complex.[112] $p120^{ctn}$ is involved in the stabilization of E-cadherin at the plasma membrane,[113] supporting the possible role for Rap1 in the internalization and recycling of E-cadherin.

Interestingly, Cdc42, a member of the Rho GTPase family, has previously been reported to be activated by new homophilic E-cadherin interactions and is involved in E-cadherin-based cell-cell contact maturation. Interestingly, Rap1GAP abolished the activation of Cdc42 suggesting that Cdc42 is a downstream effector of Rap1 in the regulation of cadherins.[31] This further supports the role of Rap1 in contact maturation.

Rap1 has also been found to be involved in the regulation of cell-cell contact in endothelial cells in which VE-cadherin is the main cadherin involved. In HUVEC cells, active Rap1 was found to be constitutively localized in the perinuclear region, as well as transiently at the plasma membrane during cell movement. Active Rap1 at the plasma membrane was found to be colocalized with $p120^{ctn}$, indicating that active Rap1 is located at AJs. As for E-cadherin, chelation of Ca^{2+}, and thus the disruption of intercellular VE-cadherin homophilic interactions, resulted in the activation of Rap1. Furthermore, initial engagement of VE-cadherins also resulted in the upregulation of active Rap1 and the activation of Rap1 was necessary for adequate adhesion to VE-cadherin-coated dishes, suggesting a positive feedback loop in which the initial engagement of VE-cadherin activated Rap1, resulting in further tightening of the cell-cell contact. It was shown that this was mediated through the binding of the scaffolding protein, MAGI-1. MAGI-1 binds VE-cadherin through binding with β-catenin. Furthermore, MAGI-1 binds the Rap1-GEF, PDZ-GEFI, which most likely is responsible for the activation of Rap1.[20]

Interestingly, it has been reported that activation of Ras inhibits cadherin-cadherin interactions through the activation of the MEK/ERK pathway. The effects of Rap1 on cell-cell junctions in OvCar3 cells was independent of the MEK/ERK pathway, indicating that this is an effect of Rap1 independent of Ras.[108]

4.6 The Rap1 activation pathway for cadherins.

4.6.1 GEFs

4.6.1.1 Epac

Endothelial cells lining blood vessels regulate endothelial barrier function through formation of AJs, TJs and formation of cortical actin bundles. cAMP is known to enhance endothelial barrier function, suggesting a potentiation of cell-cell contact formation and stabilization. Fukuhara et al.[114] demonstrated that this effect of cAMP was PKA-independent, as it was not affected by H89, suggesting a possible role for Epac. Indeed, constitutively active Epac increased VE-cadherin-dependent adhesion in HUVECs. Correspondingly, the Epac-specific cAMP-analogue 8CTP-2Me-cAMP also induced VE-cadherin dependent adhesion.[114, 115] As will be described, knock-down of PDZ-GEF2 in HUVECs renders newly forming AJ in a zipper-like morphology. Interestingly, stimulation of these HUVECs with 8CTP-2Me-cAMP transformed zipper-like cell-cell contacts into mature, sealed contacts, suggesting a role for Epac in AJ maturation.[116] Overexpression of Rap1GAPII abolished this effect, indicating that this effect was dependent on Rap1.[114] Furthermore, Epac-induced Rap1 activity also enhanced the stabilization of AJs through the formation of cortical actin bundles,[114, 115] however this effect of the Epac-Rap1 pathway was independent of its effects on cell-cell contacts through VE-cadherin.[115] cAMP was shown to tightly link VE-cadherin to the actin cytoskeleton, most likely through the Epac-Rap1 pathway.[114]

4.6.1.2 C3G

A prominent role for C3G in the regulation of E-cadherin was discovered when Hogan et al.[31] found a direct interaction between C3G and E-cadherin. C3G competes with β-catenin in binding E-cadherin as they share a common binding site. E-cadherins bind to the N-terminal domain of C3G, thereby activating the protein. Whereas there is barely any interaction between C3G and E-cadherin at mature cell-cell contacts, C3G binds to E-cadherin upon loss of intercellular cadherin-based cell-cell contacts.[31] Interestingly, internalization of E-cadherin results in the activation of Src, which is necessary for Rap1 activation by E-cadherin internalization,[112] further supporting the role of C3G in the activation of Rap1 by outside-in signaling of cadherins. As new cell-cell contacts establish, C3G interaction with E-cadherins is even further enhanced, followed by a gradual decrease as interactions start to mature. Interestingly, whereas the interaction of C3G with E-cadherin is not dependent on C3G recruitment, translocation of Rap1 toward adherens junctions is induced. Homophilic interactions of E-cadherin induces the recruitment of Rap1. This is not influenced by C3G, indicating that C3G is not necessary for the recruitment of Rap1 but it is necessary for the activation of Rap1. Activation of Rap1 by C3G at newly formed cell-cell contacts induces the recruitment of E-cadherin to these sites.[31] Correspondingly, overexpression of Rap1GAP inhibits this recruitment. The overexpression of Rap1GAP has no effect on mature cell-cell contacts, nor on the endocytosis of E-cadherin, suggesting that Rap1 is mainly involved in the maturation of new cell-cell contacts.[31]

4.6.1.3 PDZ-GEF

Rap1 is involved in the regulation of adherence junctions in endothelial cells through its actions on VE-cadherin. In order for VE-cadherin engagement to induce Rap1 activation, MAGI must be bound to the VE-cadherin-β-catenin complex. Interestingly MAGI also binds PDZ-GEFI in vascular endothelial cells,[20] suggesting that this may be the GEF responsible for the activation of Rap1 by VE-cadherin.

PDZ-GEF2 was demonstrated to be involved in the maturation of AJs. Knock-down of PDZ-GEF2 in lung carcinoma cell line A549 rendered cell-cell contacts in a zipper-like formation, indicating that PDZ-GEF2 and Rap1 are necessary for the maturation of E-cadherin-based AJs.[116] Interestingly, recruitment of E-cadherin to the junctions was not disrupted by the knock-down of PDZ-GEF2[116], suggesting distinct roles for the Rap1GEFs, as C3G is necessary for E-cadherin recruitment[31], whereas PDZ-GEF is not, but is involved in AJ maturation.[116] Similar effects of the knock-down of PDZ-GEF in HUVECs were found, indicating that the PDZ-GEF2-Rap1 has a similar effect on VE-cadherin. Correspondingly to other observations, knock-down of PDZ-GEF2 did not affect mature cell-cell contacts, supporting the role for Rap1 in junction formation, but not maintenance.[116] As for PDZ-GEF2, PDZ-GEF1 has also been demonstrated to be involved in AJ formation in epithelial cells. Similar to VE-cadherin, PDZ-GEF1 immunoprecipitates in complex with E-cadherin in a β-catenin-dependent manner in MDCK cells. Disruption of E-cadherin junctions by chelating Ca^{2+} results in activation of Rap1. This effect is diminished by the knockdown of PDZ-GEF1.[109]

4.6.1.4 Dock4

Dock4 is a GEF for Rap1, discovered due to its frequent mutation in human breast, ovarian and prostate cancers and in gliomas. Osteosarcoma cells lacking Dock4 do not form intercellular junctions and reach high saturation densities. Introduction of Dock4 into these cells induces cell junction formation, which is mediated through Rap1.[48]

4.6.2 GAPs

4.6.2.1 Rap1GAP

The maturation of adherence junctions in epithelial cells, accompanied by the downregulation of Rap1 activity, implies that the formation of mature cell-cell contacts recruits and/or induces the activation of GAPs for Rap1. Interestingly, Ohba et al.[117] reported to find active Rap1GAP located near the plasma membrane, allowing the possibility for it to be involved in the downregulation of Rap1 at cell-cell junctions. Overexpression of Rap1GAPII in HUVECs inhibited their adhesion to VE-cadherin coated dishes[114], suggesting the possibility for a role in cell-cell contacts, however no reports on the role of endogenous Rap1GAPs exist yet.

5. DISCUSSION

5.1 Coordination between integrins, cadherins and the Raf/MEK/ERK pathway.

Whether cells begin to proliferate depends on a variety of signals. Binding of growth factors (GFs) to growth factor receptors (GFRs; receptor tyrosine kinases, RTKs) can induce intracellular signalling pathways resulting in proliferation. However, signalling through GFR alone is not sufficient to induce proliferation. All adherent cells require adhesion to the extracellular matrix (ECM) for cell-cycle progression. Integrins can control GFR signalling through several mechanisms, including regulation of receptor expression, receptor localization and receptor modifications. Furthermore, integrins can influence the GF expression level itself and the control of GFR signalling can happen via shared downstream signalling molecules. Inversely, integrin signalling, either through its activation of GFRs or through direct activation of signalling pathways, is also insufficient for cell-cycle progression, indicating synergistic signalling of GFRs and integrins.[118] Indeed, there is crosstalk between GF-induced ERK phosphorylation and integrin-induced ERK phosphorylation. As mentioned, stimulation of ERK by GFs or integrins alone is not sufficient for cell-cycle progression. Stimulation by either mechanism induces transient activation of ERK, whereas sustained activation of ERK is necessary for the transcription of cyclin D1, which is necessary for cell-cycle progression. Simultaneous stimulation by GFRs and integrins induces such sustained activation of ERK.[56, 119] Conversely to stimulation of proliferation through cell-matrix adhesion, loss of contact inhibits cell growth.[118]

Integrins control cell-cycle progression through several parallel, but converging pathways, resulting in the expression of cyclin D1. In the absence of adhesion, inhibition of the pathways normally inducing proliferation, occurs through integrins. Integrins can both activate ERK as well as induce ERK translocation to the nucleus, a step necessary for cell-cycle induction. Integrins can induce ERK through a pathway involving Shc, Grb2, Sos, Ras and focal adhesion kinase (FAK). Interestingly, this pathway is also involved in GFR induced ERK activation. The activation of Shc by integrins can happen through the activation of focal adhesion kinase (FAK) or Src, both proteins induced by integrin ligation. Src can also induce FAK phosphorylation, further enhancing this process.[56] Besides the upregulation of cyclin D1, the activation of the ERK pathway by both growth factors and integrin signalling inhibits the expression of $p27^{kip1}$, another event necessary for cell-cycle progression.[120]
Although outside-in signalling through integrins and its effect on proliferation has been extensively demonstrated, the effect of outside-in signalling on proliferation through cadherins is starting to emerge. Whereas outside-in signalling through integrins generally induces a pro-proliferative signal, outside-in signalling through cadherins can induce both pro- and anti-proliferative signals, depending on the cadherin and the GFR involved.[121]
Cell-cell contacts are established as cells grow more dense. These cell-cell contacts inhibit proliferation in order to prevent unchecked growth. As for integrins, ligation of E-cadherin induces E-cadherin-dependent modulation of GFRs and thus modulation of signalling cascades. Only little research has focused on E-cadherin-E-cadherin complex formation and its effect on ERK activation. Stimulatory, inhibitory and no effect of E-cadherin on ERK has been reported.[122-124]

Interestingly, Rap1 is involved in the regulation of ERK and thus proliferation, regulation of anchorage through integrins and regulation of cell-cell contacts through cadherins. As these processes are closely interconnected, it would be interesting to determine whether Rap1 is at the crossroad of these processes. In order to determine this it is necessary to determine whether it is possible that the pathways in which Rap1 is involved, and which result in the different phenomena, could overlap. Many gaps still exist on the involvement of

Rap1 in ERK modulation. Furthermore, although the involvement of Rap1 in integrin activation and adherence junction formation has been firmly established, which Rap1GEFs and how and where in the process they are involved, has not been fully elucidated. In order to determine whether Rap1 links ERK activation or inhibition with adhesion, more detailed knowledge is needed on the activation pathways and the subcellular Rap1 pools involved. However, reports so far do suggest that several GEFs cannot be involved in both pathways, whereas other reports do allow for the linking of the pathways.

5.2 The subcellular pool of Rap1 determines the outcome.

Rap1 has been reported to be involved in the regulation of the Raf/MEK/ERK pathway. Both positive and negative regulation of the pathway has been reported, depending on the expression pattern of B-Raf. It seems that only in those cell types that express B-Raf, Rap1 is able to induce the Raf/MEK/ERK pathway. More precisely, Rap1 can activate the 95-kDa isoforms of B-Raf resulting in activation of MEK and ERK, whereas it does not activate the 62-kDa isoform.[22]

In contrast to the reports on Rap1 either inhibiting or activating the Raf/MEK/ERK pathway, Zwartkruis et al.[73] have reported to find no effect on the activation of ERK at all. They induced Rap1 activation by either elevating free Ca^{2+}, using a DAG analogue or by elevating cAMP levels, all second messengers known to activate Rap1. Although this did lead to the activation of Rap1, no effect was seen on ERK phosphorylation. In contrast, they did see an effect on the activation of Ral, another known target of Rap1 and Ras. Interestingly, this effect was only observed for the constitutively active RapV12 mutant or when Rap1 was activated through the RapGEF C3G. When trying to achieve these results through stimulation of the second messengers DAG or cAMP, no effect of Rap1 on the activation of Ral could be determined.[73] This suggests that the outcome of the activation of Rap1 depends on which GEF is involved, indicating that perhaps only the activation of a specific pool of Rap1 can interfere with Ras signalling towards the Raf/MEK/ERK pathway. Unfortunately, Zwartkruis et al. have not looked at the effects of the RapV12 mutant or the activation of Rap1 by C3G on ERK phosphorylation.[73] Research by Wang and colleagues supports the notion that depending on the Rap1GEF involved, a certain subcellular pool of Rap1 is activated, and that this determines what the outcome is. They demonstrated that activation of Rap1 by Epac did not result in the activation of the Raf/MEK/ERK pathway, whereas activation of Rap1 by C3G did result in the activation of the B-Raf/MEK/ERK pathway. They demonstrated that the Rap1 activated by Epac was a perinuclear pool, whereas B-Raf needs to be activated and located near the plasma membrane where it is further phosphorylated allowing it to induce MEK. In contrast, C3G activates a pool of Rap1 located at the plasma membrane.[36]

Thus, it seems that the outcome of Rap1 activation is not only dependent on the expression profile of its effectors, but also on which GEF is responsible for the activation of Rap1 and thus which subcellular pool of Rap1 is activated. Therefore, in order to determine whether Rap1 could be at the crossroad between cell adhesion, either through integrins or cadherins, and proliferation, it is necessary to determine which GEFs for Rap1 are involved in both phenomena and whether this activation takes place in the same subcellular region.

5.3 Linking the Raf/MEK/ERK pathway and adhesion.

In the OvCar3 cell line it was demonstrated that Rap1 was involved in both integrin activation and adherens junction formation. Price et al.[108] demonstrated that overexpression of the dominant negative Rap1 mutant or Rap1GAP1 reduced the level of E-cadherin at cell-cell contacts, whereas Rangarajan et al.[93] demonstrated that Rap1 induced the activation of $\alpha 3\beta 1$ integrins in an Epac-dependent manner. As has been demonstrated by Wang et al.[36], the

activation of a pool of Rap1 at the plasma membrane is necessary for the induction of the Raf/MEK/ERK pathway. As Epac is located toward the perinuclear region, it is unlikely that it can link the Raf/MEK/ERK pathway with adhesion. Indeed, several research groups have demonstrated that specific activation of Epac does not result in the activation of ERK.[36, 74] Thus, it is not plausible that the Epac/Rap1 pathway links adhesion to proliferation. Although a role for Epac in the formation of cell-cell contacts in OvCar3 cells has not been determined, Epac has been shown to be involved in the regulation of VE-cadherin-dependent adhesion in HUVECs.[114]

CalDAG-GEF, PDZ-GEF and C3G are all Rap1GEFs reported to be involved in both modulation of the Raf/MEK/ERK pathway and adhesion. In contrast to Epac, CalDAG-GEFI has been implicated in regulation of both the Raf/MEK/ERK pathway and integrins. However, determining whether CalDAG-GEF1 mediated Rap1 activation could link the Raf/MEK/ERK pathway and cell-matrix adhesion is speculative. CalDAG-GEFI has been reported to activate ERK through Rap1 in PC12 cells, which express B-Raf.[76] However, as has been explained before, mostly hematopoietic cell lines have been used as model for integrin activation and thus no information on the role of the CalDAG-GEFI/Rap1 pathway in cell-matrix adhesion in PC12 cells has been published. CalDAG-GEFI has been shown to be able to activate both LFA-1 in primary T-cells,[98] as well as αIIbβ3 integrins in platelets,[96, 97] by activating Rap1. However, in order to determine whether Rap1 can link the two pathways by activation through CalDAG-GEFI it has to be shown that CalDAG-GEFI is involved in both processes in the same cell type. Noteworthy is that PC12 cells are from neurologic origin which normally express N-cadherin. Rap1, however, was reported not to be involved in N-cadherin regulation.[31] Furthermore, whereas CalDAG-GEFI has been implicated in the activation of integrins in T-cells through Rap1, Rap1 has been reported to inhibit ERK activation in T-cells.[63] However, binding of active β1 integrins to collagen type 1 in T-cells results in the activation of the Ras/Raf-1/MEK/ERK pathway, resulting in survival.[125] It would be contradictory for Rap1 to simultaneously be involved in both integrin activation and the inhibition of ERK, as the integrin activation itself induces ERK phosphorylation. However, upon activation, CalDAG-GEFI translocates toward the plasma membrane,[43] supporting the possible role in both integrin regulation and Raf/MEK/ERK modulation. In contrast, there are no reports on the involvement of CalDAG-GEF in the regulation of cadherins.

Secondly, PDZ-GEF has been reported to be involved in the modulation of the Raf/MEK/ERK pathway, integrin activation and cadherin regulation. Liao and coworkers reported that the activation of Rap1 through PDZ-GEFI resulted in the activation of the B-Raf/MEK/ERK pathway. For this they transfected Cos7 cells with constructs containing PDZ-GEFI, Rap1 and B-Raf.[47] In contrast, loss-of-function mutations in *Drosophila* resulted in reduced activation of endogenous Rap1 and ERK.[78] However, whether this was due to direct stimulation of the Raf/MEK/ERK pathway by active Rap1 was not demonstrated. Furthermore, in *Drosophila* PDZ-GEF has been found to induce βPS integrins in macrophages[99] and is involved in AJ maturation by forming a complex with β-catenin and E- or VE-cadherin.[109, 116] Interestingly, disrupting E-cadherin based cell-cell contacts in MDCK cells, which originate from the kidney, induces the activation of Rap1. This is at least partially mediated through PDZ-GEFI.[109] Cell lines derived from kidneys express B-Raf, suggesting that Rap1 can induce the Raf/MEK/ERK pathway.[65, 126] A density-dependent inhibition of proliferation is mediated through cell-cell contacts. Therefore, it is not likely that the pool of PDZ-GEFI involved in AJ formation and maintenance, is responsible for activation of the Raf/MEK/ERK pathway as these are contradictory events. In contrast, it is possible that the Rap1 pool activated by PDZ-GEFI, resulting in integrin activation, is the same pool modulating the Raf/MEK/ERK pathway. If so, an interesting notion is that PDZ-GEFI itself is

an effector of Rap1, suggesting a positive feedback-loop. This positive feedback-loop may be necessary to increase the amount of active Rap1 to induce both pathways. However, it may well be so that the activation of integrins by active Rap1 is the cause of the increased ERK phosphorylation.
As mentioned before, when transfecting Cos7 cells with PDZ-GEFI, Rap1 and B-Raf, Liao et al.[47] reported PFZ-GEFI to be capable of activating the Raf/MEK/ERK pathway. However, they also reported that the activation of PDZ-GEFI induced its translocation to the perinuclear region. Wang et al.[36] have demonstrated that the pool of Rap1 located in this region is not capable of activating or inhibiting Raf. However, as PDZ-GEFI has also been shown to be located at integrins[20] and cadherins[20, 116], this may also suggest a new manner of crosstalk between integrins and cadherins, or a positive feedback-loop for cadherin or integrin activation.

The third Rap1GEF possibly linking the activation of the Raf/MEK/ERK pathway to adhesion through Rap1 is C3G. Rap1 has an inhibitory effect on ERK phosphorylation in NIH 3T3 cells. Indeed, Vossler et al.[59] reported that NIH 3T3 cells do not express B-Raf, supporting the inhibitory role of Rap1 activation on the Raf/MEK/ERK pathway. It was demonstrated by various groups that cAMP inhibits ERK phosphorylation in these cells.[3, 59, 127] In contrast, Enserink et al.[74] reported to see no effect of cAMP on ERK phosphorylation when stimulating NIH 3T3 with the Epac specific cAMP analogue, 8CTP-2Me-cAMP. However, cAMP can also activate Rap1 through PKA. PKA can phosphorylate Src, which activates Cbl resulting in the activation of Crk/C3G, thus eventually resulting in Rap1 activation. Indeed, Schmitt et al.[127] demonstrated that elevating the cAMP level in NIH 3T3 cells induces the activation of this pathway, resulting in the activation of Rap1, which then inhibits ERK phosphorylation. Interestingly, Takahashi et al.[95] demonstrated that stimulation of NIH 3T3 cells with PDGF induces the activation and translocation of the Crk/C3G complex and Rap1 to the leading edge. This results in the activation of β3 integrins.[95] As PDGF does not induce cAMP formation in the cell, Crk is most likely activated by binding with its SH2 domain to phosphorylated tyrosines on the activated PDGF receptor.
As the Rap1GEF C3G is involved in both the activation of ERK and integrin β3 through Rap1, it is possible that the activation of C3G can simultaneously induce proliferation and cell-matrix adhesion, linking the two processes. However, as the manner of activation of C3G differs, namely through cAMP and PKA or directly through the PDGF receptor, it is likely that a different subcellular pool of C3G is activated resulting in a separate subcellular pool of Rap1 to be activated. Furthermore, Zwartkruis et al.[73] demonstrated that the activation of Rap1 by PDGF in Rat-1 fibroblasts did not effect ERK activation by PDGF. This suggests that the C3G pool activated by cAMP, and resulting in the inhibition of ERK phosphorylation, is not the same pool responsible for the activation of Rap1 and β3 integrins through PDGF. It would be interesting to determine whether the Crk/C3G/Rap1 pathway induced by cAMP and G-coupled receptors are the same. If so, a possible cross point for the two pathways exists here.

Another report suggesting a role for the C3G/Rap1 pathway in linking proliferation and adhesion came from Buensuceso and coworkers.[128] As has been previously mentioned, integrin activation is necessary for proliferation and active integrins can induce the proliferation pathway Raf/MEK/ERK. Interestingly, Buensuceso et al.[128] demonstrated that CHO cells in suspension show a high level of active CrkII/C3G and Rap1, whereas the level of phosphorylated ERK is very low. The low levels of active ERK could possibly be the result of inhibition by active Rap1. Interestingly, transfecting CHO with B-Raf increases the level of active ERK in suspended cells. In contrast, when CHO cells start to adhere to fibronectin ERK phosphorylation levels start to rise, whereas simultaneously active CrkII/C3G and Rap1 levels decrease. However, this increase in ERK phosphorylation upon adhesion coincides with

Ras activation.[128] Thus whether the inverse relationship between Rap1 and ERK activity are a direct consequence of Rap1 interfering with the Raf/MEK/ERK pathway or whether it is an indirect effect due to the activation of integrins and consequently the induction of Ras, is unclear. However, these results indicate that a pool of Rap1 is activated in cells in suspension, possibly able to modulate the ERK pathway. In contrast, Takahashi et al.[71] found that when subconfluent, keratinocytes express both the 95-kDa and the 62-kDa isoforms of B-Raf. In these subconfluent circumstances Rap1 is capable of activating ERK. However, as cells reach confluency keratinocytes loose the expression of the 95-kDa isoforms of B-Raf, switching Rap1 from an activating to an inhibitory protein of ERK.[71] This suggests a whole new model as to how Rap1 is involved in contact-dependent inhibition of proliferation. The formation of cell-cell contacts, possibly through the actions of Rap1, result in the inhibition of expression of the 95-kDa B-Raf isoforms. This cell contact-dependent switch in B-Raf expression causes Rap1 to inhibit proliferation, thus inducing contact-dependent growth inhibition. Whether the Rap1 possibly involved in cell-cell contact formation and the Rap1 involved in ERK regulation are the same pool needs to be determined.

A third set of experiments pointing toward the involvement of Rap1 in both proliferation and adhesion was performed in thyroid cells. The RET/PTC oncogene induces uncontrolled proliferation when expressed in thyroid cells. De Falco et al.[65] demonstrated that RET/PTC activates the CrkL/C3G complex which in turn results in activated Rap1. This activation of Rap1 results in the activation of the Raf/MEK/ERK pathway. Furthermore, De Falco et al.[65] were able to demonstrate that this simultaneously resulted in the formation of stress fibers, a process involved in focal adhesion and AJ formation, suggesting that the activation of Rap1 through C3G simultaneously induces cell adhesion and proliferation.[65] Interestingly, Ribeiro-Neto and coworkers demonstrated in thyroid cell lines that elevation of cAMP induced Rap1-dependent G1/S-phase transition.[69] Interestingly, as described before, transition through the G1/S-phase occurs via stimulation of ERK. For this, sustained activation of ERK is necessary which is accomplished through simultaneous activation through GFs and integrins. It is therefore possible that the simultaneous regulation of ERK and stress fiber formation by the RET/PTC oncogene, mediated through the effects of Rap, induce cell cycle progression. On the other hand, it has been reported for several oncogenes that they can induce sustained ERK phosphorylation in an integrin-independent manner. This may well be true for RET/PTC.

C3G has already been proven to be the Rap1GEF in Fisher Rat Thyroid (FRT) cells, responsible for the crosstalk between cadherins and integrins. Upon formation of E-cadherin-E-cadherin interactions Rap1 activity is downregulated. Disruption of these E-cadherin intracellular homodimers and their endocytosis induces Rap1 activation. This activation of Rap1 results in activation of integrins.[112] It has been demonstrated that in thyroid cells the activation of Rap1 through C3G can induce ERK activation.[65, 69] Interestingly, the formation of AJs downregulated Rap1 activation,[112] suggesting the downregulation of ERK activity, which would be expected from AJ formation. Whether these effects are really mediated through the same pool of Rap1 is elusive. It would be of interest to fluorescently tag Rap1 to determine whether the pool of Rap1, activated through E-cadherin internalization, responsible for integrin activation and the Rap1 pool activated by C3G and responsible for ERK activity modulation are the same pool.

As mentioned before, AJs formation inhibits ERK phosphorylation and proliferation. This is to ensure that tissue overgrowth does not take place. However, if tissue degenerates or in the case of wound healing, one would want cells to start proliferating again. One can imagine that reduction of cell number results in the reduction of cell-cell contacts. Interestingly, disruption of cadherin-based AJs results in the activation of Rap1. Perhaps cell-cell contact disruption-induced Rap1 activation not only restores cell-cell contacts by

enhancing cell-cell contact formation, but also prevents tissue degeneration by enhancing proliferation. However, again it would be difficult to determine whether this is a direct effect of Rap1 on the Raf/MEK/ERK pathway. Internalization of E-cadherin results in the activation of integrins by activating Rap1.[112] The activation of integrins could also enhance proliferation.

5.4 Rap1 in tumorigenesis.

If Rap1 directly induces proliferation through the B-Raf/MEK/ERK pathway, it would be expected that Rap1 is a putative proto-oncogene. The downstream effector of Rap1, B-Raf, has indeed been found to be mutated in several cancers, resulting in the overactivation of ERK and proliferation.[120] Rap1 has been implicated in the progression of several types of tumors, however Rap1 alone has not been found to be sufficient to induce tumorigenesis. Rather it has been found to facilitate the formation of tumors by stimulating proliferation or has been found to be involved in tumor progression by enhancing extravasion.[101, 129] Whether the enhanced cell growth is mediated directly by the stimulation of ERK by Rap1, or indirectly by enhancing integrin activation or inhibiting apoptosis is unclear.

5.5 Conclusion

Incomplete information makes it difficult to determine whether Rap1 lays at the crossroad between adhesion and proliferation. In order to link the two processes, the same subcellular pool of Rap1 needs to be involved in several pathways. Therefore it is most likely that if Rap1 links the two, there should be one Rap1GEF responsible for the activation of Rap1, inducing in both processes simultaneously. The best candidates for this are C3G, PDZ-GEF and CalDAG-GEF. However, even though the results of Rap1 activation on integrin activity and cadherin formation are straightforward, the effect of Rap1 on the Raf/MEK/ERK pathway are less straightforward. Depending on the cellular context Rap1 may have a stimulatory or inhibitory effect on the pathway. In contrast, the coordination of the Raf/MEK/ERK pathway and integrins or cadherins are also quite straightforward. Whereas adhesion through integrins mostly correlates with activation of the Raf/MEK/ERK pathway, adhesion through cadherins mostly inhibits this pathway. If Rap1 simultaneously regulates the Raf/MEK/ERK pathway and integrin activity or AJs, this should mean that cells in which Rap1 activates integrins, Rap1 should also activate the Raf/MEK/ERK pathway. Similarly, cells in which Rap1 regulates AJs, Rap1 should inhibit the Raf/MEK/ERK pathway. This is not the case, as Rap1 already regulates and coordinates integrin activity and AJs in one cell type.[112] Furthermore, in several cell types Rap1 has been reported to inhibit ERK phosphorylation[3, 63], whereas it has also been reported to induce integrin activation.[29, 94, 95]

5.6 Future prospects

Although it seems unlikely that Rap1 coordinates cell adhesion with proliferation, further research may shed more light on the subject. Determining whether one signal, activating a pool of Rap1 located at the plasma membrane, and inducing both cell adhesion and regulating proliferation will answer the question whether Rap1 is at the crossroad of these events. However, for now research should focus on the role of Rap1 in cell adhesion and metastasis. Several groups have indicated that an increased level of Rap1 activity in a tumor cell can enhance the efficiency of extravasion.[101, 129] By increasing integrin activation, cells become more migratory, thereby enhancing the level of extravasion and metastasis.[101] By focusing on Rap1 as a therapeutic target, research may reveal a new method in combating

tumors that are metastasizing. However, even though Rap1 on it self does not seem to be enough for the development of new tumors, research has pointed out that in certain cell types the absence or the overactivation of Rap1 may well enhance tumorus growth.[3, 22] Thus, research on Rap1 as a therapeutic target for metastasizing tumours needs to focus on the interplay between using Rap1 to reduce extravasion and how this effects proliferation.

REFERENCE LIST

1. Noda,M. *et al.* Detection of genes with a potential for suppressing the transformed phenotype associated with activated ras genes. *Proc. Natl. Acad. Sci. U. S. A* **86**, 162-166 (1989).

2. Kitayama,H., Sugimoto,Y., Matsuzaki,T., Ikawa,Y., & Noda,M. A ras-related gene with transformation suppressor activity. *Cell* **56**, 77-84 (1989).

3. Schmitt,J.M. & Stork,P.J. Cyclic AMP-mediated inhibition of cell growth requires the small G protein Rap1. *Mol. Cell Biol.* **21**, 3671-3683 (2001).

4. Pizon,V., Lerosey,I., Chardin,P., & Tavitian,A. Nucleotide sequence of a human cDNA encoding a ras-related protein (rap1B). *Nucleic Acids Res.* **16**, 7719 (1988).

5. Pizon,V., Chardin,P., Lerosey,I., Olofsson,B., & Tavitian,A. Human cDNAs rap1 and rap2 homologous to the Drosophila gene Dras3 encode proteins closely related to ras in the 'effector' region. *Oncogene* **3**, 201-204 (1988).

6. Scrima,A., Thomas,C., Deaconescu,D., & Wittinghofer,A. The Rap-RapGAP complex: GTP hydrolysis without catalytic glutamine and arginine residues. *EMBO J.* **27**, 1145-1153 (2008).

7. Wittinghofer,A. & Pai,E.F. The structure of Ras protein: a model for a universal molecular switch. *Trends Biochem. Sci.* **16**, 382-387 (1991).

8. Hall,B.E., Yang,S.S., Boriack-Sjodin,P.A., Kuriyan,J., & Bar-Sagi,D. Structure-based mutagenesis reveals distinct functions for Ras switch 1 and switch 2 in Sos-catalyzed guanine nucleotide exchange. *J. Biol. Chem.* **276**, 27629-27637 (2001).

9. van den Berghe N., Cool,R.H., & Wittinghofer,A. Discriminatory residues in Ras and Rap for guanine nucleotide exchange factor recognition. *J. Biol. Chem.* **274**, 11078-11085 (1999).

10. Bokoch,G.M. & Quilliam,L.A. Guanine nucleotide binding properties of rap1 purified from human neutrophils. *Biochem. J.* **267**, 407-411 (1990).

11. Li,G. & Zhang,X.C. GTP hydrolysis mechanism of Ras-like GTPases. *J. Mol. Biol.* **340**, 921-932 (2004).

12. Daumke,O., Weyand,M., Chakrabarti,P.P., Vetter,I.R., & Wittinghofer,A. The GTPase-activating protein Rap1GAP uses a catalytic asparagine. *Nature* **429**, 197-201 (2004).

13. Chakrabarti,P.P. *et al.* Insight into catalysis of a unique GTPase reaction by a combined biochemical and FTIR approach. *J. Mol. Biol.* **367**, 983-995 (2007).

14. Pai,E.F. *et al.* Refined crystal structure of the triphosphate conformation of H-ras p21 at 1.35 A resolution: implications for the mechanism of GTP hydrolysis. *EMBO J.* **9**, 2351-2359 (1990).

15. Sacco,E. *et al.* The isolated catalytic hairpin of the Ras-specific guanine nucleotide exchange factor Cdc25Mm retains nucleotide dissociation activity but has impaired nucleotide exchange activity. *FEBS Lett.* **579**, 6851-6858 (2005).

16. Sacco,E. *et al.* Catalytic competence of the Ras-GEF domain of hSos1 requires intra-REM domain interactions mediated by phenylalanine 577. *FEBS Lett.* **580**, 6322-6328 (2006).

17. Wittinghofer,A. & Nassar,N. How Ras-related proteins talk to their effectors. *Trends Biochem. Sci.* **21**, 488-491 (1996).

18. Nassar,N. *et al.* Ras/Rap effector specificity determined by charge reversal. *Nat. Struct. Biol.* **3**, 723-729 (1996).

19. Beranger,F., Goud,B., Tavitian,A., & de Gunzburg J. Association of the Ras-antagonistic Rap1/Krev-1 proteins with the Golgi complex. *Proc. Natl. Acad. Sci. U. S. A* **88**, 1606-1610 (1991).

20. Sakurai,A. *et al.* MAGI-1 is required for Rap1 activation upon cell-cell contact and for enhancement of vascular endothelial cadherin-mediated cell adhesion. *Mol. Biol. Cell* **17**, 966-976 (2006).

21. Carey,K.D., Watson,R.T., Pessin,J.E., & Stork,P.J. The requirement of specific membrane domains for Raf-1 phosphorylation and activation. *J. Biol. Chem.* **278**, 3185-3196 (2003).

22. Fujita,T., Meguro,T., Fukuyama,R., Nakamuta,H., & Koida,M. New signaling pathway for parathyroid hormone and cyclic AMP action on extracellular-regulated kinase and cell proliferation in bone cells. Checkpoint of modulation by cyclic AMP. *J. Biol. Chem.* **277**, 22191-22200 (2002).

23. Enserink,J.M. *et al.* The cAMP-Epac-Rap1 pathway regulates cell spreading and cell adhesion to laminin-5 through the alpha3beta1 integrin but not the alpha6beta4 integrin. *J. Biol. Chem.* **279**, 44889-44896 (2004).

24. Arai,A., Nosaka,Y., Kohsaka,H., Miyasaka,N., & Miura,O. CrkL activates integrin-mediated hematopoietic cell adhesion through the guanine nucleotide exchange factor C3G. *Blood* **93**, 3713-3722 (1999).

25. Bertoni,A. *et al.* Relationships between Rap1b, affinity modulation of integrin alpha IIbbeta 3, and the actin cytoskeleton. *J. Biol. Chem.* **277**, 25715-25721 (2002).

26. Bos,J.L. Linking Rap to cell adhesion. *Curr. Opin. Cell Biol.* **17**, 123-128 (2005).

27. Caron,E., Self,A.J., & Hall,A. The GTPase Rap1 controls functional activation of macrophage integrin alphaMbeta2 by LPS and other inflammatory mediators. *Curr. Biol.* **10**, 974-978 (2000).

28. Katagiri,K. *et al.* Rap1 is a potent activation signal for leukocyte function-associated antigen 1 distinct from protein kinase C and phosphatidylinositol-3-OH kinase. *Mol. Cell Biol.* **20**, 1956-1969 (2000).

29. Reedquist,K.A. *et al.* The small GTPase, Rap1, mediates CD31-induced integrin adhesion. *J. Cell Biol.* **148**, 1151-1158 (2000).

30. Sebzda,E., Bracke,M., Tugal,T., Hogg,N., & Cantrell,D.A. Rap1A positively regulates T cells via integrin activation rather than inhibiting lymphocyte signaling. *Nat. Immunol.* **3**, 251-258 (2002).

31. Hogan,C. *et al.* Rap1 regulates the formation of E-cadherin-based cell-cell contacts. *Mol. Cell Biol.* **24**, 6690-6700 (2004).

32. Bos,J.L., de Rooij J., & Reedquist,K.A. Rap1 signalling: adhering to new models. *Nat. Rev. Mol. Cell Biol.* **2**, 369-377 (2001).

33. Radha,V., Rajanna,A., & Swarup,G. Phosphorylated guanine nucleotide exchange factor C3G, induced by pervanadate and Src family kinases localizes to the Golgi and subcortical actin cytoskeleton. *BMC. Cell Biol.* **5**, 31 (2004).

34. Feller,S.M. Crk family adaptors-signalling complex formation and biological roles. *Oncogene* **20**, 6348-6371 (2001).

35. Tanaka,S. *et al.* C3G, a guanine nucleotide-releasing protein expressed ubiquitously, binds to the Src homology 3 domains of CRK and GRB2/ASH proteins. *Proc. Natl. Acad. Sci. U. S. A* **91**, 3443-3447 (1994).

36. Wang,Z. *et al.* Rap1-mediated activation of extracellular signal-regulated kinases by cyclic AMP is dependent on the mode of Rap1 activation. *Mol. Cell Biol.* **26**, 2130-2145 (2006).

37. de Rooij J. *et al.* Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* **396**, 474-477 (1998).

38. Holz,G.G., Kang,G., Harbeck,M., Roe,M.W., & Chepurny,O.G. Cell physiology of cAMP sensor Epac. *J. Physiol* **577**, 5-15 (2006).

39. Kawasaki,H. *et al.* A Rap guanine nucleotide exchange factor enriched highly in the basal ganglia. *Proc. Natl. Acad. Sci. U. S. A* **95**, 13278-13283 (1998).

40. Stork,P.J. Does Rap1 deserve a bad Rap? *Trends Biochem. Sci.* **28**, 267-275 (2003).

41. Bos,J.L., de,R.J., & Reedquist,K.A. Rap1 signalling: adhering to new models. *Nat. Rev. Mol. Cell Biol.* **2**, 369-377 (2001).

42. Feller,S.M. Crk family adaptors-signalling complex formation and biological roles. *Oncogene* **20**, 6348-6371 (2001).

43. Clyde-Smith,J. *et al.* Characterization of RasGRP2, a plasma membrane-targeted, dual specificity Ras/Rap exchange factor. *J. Biol. Chem.* **275**, 32260-32267 (2000).

44. de Rooij J. *et al.* PDZ-GEF1, a guanine nucleotide exchange factor specific for Rap1 and Rap2. *J. Biol. Chem.* **274**, 38125-38130 (1999).

45. Ohtsuka,T. *et al.* nRap GEP: a novel neural GDP/GTP exchange protein for rap1 small G protein that interacts with synaptic scaffolding molecule (S-SCAM). *Biochem. Biophys. Res. Commun.* **265**, 38-44 (1999).

46. Gao,X. *et al.* Identification and characterization of RA-GEF-2, a Rap guanine nucleotide exchange factor that serves as a downstream target of M-Ras. *J. Biol. Chem.* **276**, 42219-42225 (2001).

47. Liao,Y. *et al.* RA-GEF-1, a guanine nucleotide exchange factor for Rap1, is activated by translocation induced by association with Rap1*GTP and enhances Rap1-dependent B-Raf activation. *J. Biol. Chem.* **276**, 28478-28483 (2001).

48. Yajnik,V. *et al.* DOCK4, a GTPase activator, is disrupted during tumorigenesis. *Cell* **112**, 673-684 (2003).

49. Rubinfeld,B. *et al.* Molecular cloning of a GTPase activating protein specific for the Krev-1 protein p21rap1. *Cell* **65**, 1033-1042 (1991).

50. Mochizuki,N. *et al.* Activation of the ERK/MAPK pathway by an isoform of rap1GAP associated with G alpha(i). *Nature* **400**, 891-894 (1999).

51. Kurachi,H. *et al.* Human SPA-1 gene product selectively expressed in lymphoid tissues is a specific GTPase-activating protein for Rap1 and Rap2. Segregate expression profiles from a rap1GAP gene product. *J. Biol. Chem.* **272**, 28081-28088 (1997).

52. Gao,Q., Srinivasan,S., Boyer,S.N., Wazer,D.E., & Band,V. The E6 oncoproteins of high-risk papillomaviruses bind to a novel putative GAP protein, E6TP1, and target it for degradation. *Mol. Cell Biol.* **19**, 733-744 (1999).

53. Singh,L. *et al.* The high-risk human papillomavirus type 16 E6 counters the GAP function of E6TP1 toward small Rap G proteins. *J. Virol.* **77**, 1614-1620 (2003).

54. McCubrey,J.A. *et al.* Targeting the RAF/MEK/ERK, PI3K/AKT and p53 pathways in hematopoietic drug resistance. *Adv. Enzyme Regul.* **47**, 64-103 (2007).

55. Peyssonnaux,C. & Eychene,A. The Raf/MEK/ERK pathway: new concepts of activation. *Biol. Cell* **93**, 53-62 (2001).

56. Yee,K.L., Weaver,V.M., & Hammer,D.A. Integrin-mediated signalling through the MAP-kinase pathway. *IET. Syst. Biol.* **2**, 8-15 (2008).

57. Cook,S.J., Rubinfeld,B., Albert,I., & McCormick,F. RapV12 antagonizes Ras-dependent activation of ERK1 and ERK2 by LPA and EGF in Rat-1 fibroblasts. *EMBO J.* **12**, 3475-3485 (1993).

58. Lin,Y., Mettling,C., & Chou,C. Rap1-suppressed tumorigenesis is concomitant with the interference in ras effector signaling. *FEBS Lett.* **467**, 184-188 (2000).

59. Vossler,M.R. *et al.* cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. *Cell* **89**, 73-82 (1997).

60. Okada,T., Masuda,T., Shinkai,M., Kariya,K., & Kataoka,T. Post-translational modification of H-Ras is required for activation of, but not for association with, B-Raf. *J. Biol. Chem.* **271**, 4671-4678 (1996).

61. Lelievre,V., Meunier,A.C., Caigneaux,E., Falcon,J., & Muller,J.M. Differential expression and function of PACAP and VIP receptors in four human colonic adenocarcinoma cell lines. *Cell Signal.* **10**, 13-26 (1998).

62. Alleaume,C., Eychene,A., Caigneaux,E., Muller,J.M., & Philippe,M. Vasoactive intestinal peptide stimulates proliferation in HT29 human colonic adenocarcinoma cells: concomitant activation of Ras/Rap1-B-Raf-ERK signalling pathway. *Neuropeptides* **37**, 98-104 (2003).

63. Ishida,D. *et al.* Antigen-driven T cell anergy and defective memory T cell response via deregulated Rap1 activation in SPA-1-deficient mice. *Proc. Natl. Acad. Sci. U. S. A* **100**, 10919-10924 (2003).

64. Altschuler,D.L. & Ribeiro-Neto,F. Mitogenic and oncogenic properties of the small G protein Rap1b. *Proc. Natl. Acad. Sci. U. S. A* **95**, 7475-7479 (1998).

65. De Falco,V. *et al.* RET/papillary thyroid carcinoma oncogenic signaling through the Rap1 small GTPase. *Cancer Res.* **67**, 381-390 (2007).

66. Jin,A. *et al.* BCR/ABL and IL-3 activate Rap1 to stimulate the B-Raf/MEK/Erk and Akt signaling pathways and to regulate proliferation, apoptosis, and adhesion. *Oncogene* **25**, 4332-4340 (2006).

67. Mizuchi,D. *et al.* BCR/ABL activates Rap1 and B-Raf to stimulate the MEK/Erk signaling pathway in hematopoietic cells. *Biochem. Biophys. Res. Commun.* **326**, 645-651 (2005).

68. Ribeiro-Neto,F., Urbani,J., Lemee,N., Lou,L., & Altschuler,D.L. On the mitogenic properties of Rap1b: cAMP-induced G(1)/S entry requires activated and phosphorylated Rap1b. *Proc. Natl. Acad. Sci. U. S. A* **99**, 5418-5423 (2002).

69. Ribeiro-Neto,F. *et al.* cAMP-dependent oncogenic action of Rap1b in the thyroid gland. *J. Biol. Chem.* **279**, 46868-46875 (2004).

70. Stork,P.J. & Dillon,T.J. Multiple roles of Rap1 in hematopoietic cells: complementary versus antagonistic functions. *Blood* **106**, 2952-2961 (2005).

71. Takahashi,H. *et al.* Cyclic AMP differentially regulates cell proliferation of normal human keratinocytes through ERK activation depending on the expression pattern of B-Raf. *Arch. Dermatol. Res.* **296**, 74-82 (2004).

72. Schmitt,J.M. & Stork,P.J. beta 2-adrenergic receptor activates extracellular signal-regulated kinases (ERKs) via the small G protein rap1 and the serine/threonine kinase B-Raf. *J. Biol. Chem.* **275**, 25342-25350 (2000).

73. Zwartkruis,F.J., Wolthuis,R.M., Nabben,N.M., Franke,B., & Bos,J.L. Extracellular signal-regulated activation of Rap1 fails to interfere in Ras effector signalling. *EMBO J.* **17**, 5905-5912 (1998).

74. Enserink,J.M. *et al.* A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. *Nat. Cell Biol.* **4**, 901-906 (2002).

75. Schmitt,J.M. & Stork,P.J. PKA phosphorylation of Src mediates cAMP's inhibition of cell growth via Rap1. *Mol. Cell* **9**, 85-94 (2002).

76. Guo,F.F., Kumahara,E., & Saffen,D. A CalDAG-GEFI/Rap1/B-Raf cassette couples M(1) muscarinic acetylcholine receptors to the activation of ERK1/2. *J. Biol. Chem.* **276**, 25568-25581 (2001).

77. Lin,A.L. *et al.* Distinct pathways of ERK activation by the muscarinic agonists pilocarpine and carbachol in a human salivary cell line. *Am. J. Physiol Cell Physiol* **294**, C1454-C1464 (2008).

78. Lee,J.H. *et al.* Drosophila PDZ-GEF, a guanine nucleotide exchange factor for Rap1 GTPase, reveals a novel upstream regulatory mechanism in the mitogen-activated protein kinase signaling pathway. *Mol. Cell Biol.* **22**, 7658-7666 (2002).

79. Okamoto,O.K., Carvalho,A.C., Marti,L.C., Vencio,R.Z., & Moreira-Filho,C.A. Common molecular pathways involved in human CD133+/CD34+ progenitor cell expansion and cancer. *Cancer Cell Int.* **7**, 11 (2007).

80. Yajnik,V. *et al.* DOCK4, a GTPase activator, is disrupted during tumorigenesis. *Cell* **112**, 673-684 (2003).

81. Zheng,H. *et al.* Down-regulation of Rap1GAP via promoter hypermethylation promotes melanoma cell proliferation, survival, and migration. *Cancer Res.* **69**, 449-457 (2009).

82. Mizuchi,D. *et al.* BCR/ABL activates Rap1 and B-Raf to stimulate the MEK/Erk signaling pathway in hematopoietic cells. *Biochem. Biophys. Res. Commun.* **326**, 645-651 (2005).

83. Ishida,D. *et al.* Myeloproliferative stem cell disorders by deregulated Rap1 activation in SPA-1-deficient mice. *Cancer Cell* **4**, 55-65 (2003).

84. Banno,A. & Ginsberg,M.H. Integrin activation. *Biochem. Soc. Trans.* **36**, 229-234 (2008).

85. Retta,S.F., Balzac,F., & Avolio,M. Rap1: a turnabout for the crosstalk between cadherins and integrins. *Eur. J. Cell Biol.* **85**, 283-293 (2006).

86. Wegener,K.L. & Campbell,I.D. Transmembrane and cytoplasmic domains in integrin activation and protein-protein interactions (review). *Mol. Membr. Biol.* **25**, 376-387 (2008).

87. Arai,A. *et al.* Rap1 is activated by erythropoietin or interleukin-3 and is involved in regulation of beta1 integrin-mediated hematopoietic cell adhesion. *J. Biol. Chem.* **276**, 10453-10462 (2001).

88. Noda,M. *et al.* Detection of genes with a potential for suppressing the transformed phenotype associated with activated ras genes. *Proc. Natl. Acad. Sci. U. S. A* **86**, 162-166 (1989).

89. Han,J. *et al.* Reconstructing and deconstructing agonist-induced activation of integrin alphaIIbbeta3. *Curr. Biol.* **16**, 1796-1806 (2006).

90. Lafuente,E.M. *et al.* RIAM, an Ena/VASP and Profilin ligand, interacts with Rap1-GTP and mediates Rap1-induced adhesion. *Dev. Cell* **7**, 585-595 (2004).

91. Katagiri,K., Maeda,A., Shimonaka,M., & Kinashi,T. RAPL, a Rap1-binding molecule that mediates Rap1-induced adhesion through spatial regulation of LFA-1. *Nat. Immunol.* **4**, 741-748 (2003).

92. Whittard,J.D. & Akiyama,S.K. Positive regulation of cell-cell and cell-substrate adhesion by protein kinase A. *J. Cell Sci.* **114**, 3265-3272 (2001).

93. Rangarajan,S. *et al.* Cyclic AMP induces integrin-mediated cell adhesion through Epac and Rap1 upon stimulation of the beta 2-adrenergic receptor. *J. Cell Biol.* **160**, 487-493 (2003).

94. Nolz,J.C. *et al.* The WAVE2 complex regulates T cell receptor signaling to integrins via Abl- and CrkL-C3G-mediated activation of Rap1. *J. Cell Biol.* **182**, 1231-1244 (2008).

95. Takahashi,M. *et al.* Sequential activation of Rap1 and Rac1 small G proteins by PDGF locally at leading edges of NIH3T3 cells. *Genes Cells* **13**, 549-569 (2008).

96. Crittenden,J.R. *et al.* CalDAG-GEFI integrates signaling for platelet aggregation and thrombus formation. *Nat. Med.* **10**, 982-986 (2004).

97. Cifuni,S.M., Wagner,D.D., & Bergmeier,W. CalDAG-GEFI and protein kinase C represent alternative pathways leading to activation of integrin alphaIIbbeta3 in platelets. *Blood* **112**, 1696-1703 (2008).

98. Ghandour,H., Cullere,X., Alvarez,A., Luscinskas,F.W., & Mayadas,T.N. Essential role for Rap1 GTPase and its guanine exchange factor CalDAG-GEFI in LFA-1 but not VLA-4 integrin mediated human T-cell adhesion. *Blood* **110**, 3682-3690 (2007).

99. Huelsmann,S., Hepper,C., Marchese,D., Knoll,C., & Reuter,R. The PDZ-GEF dizzy regulates cell shape of migrating macrophages via Rap1 and integrins in the Drosophila embryo. *Development* **133**, 2915-2924 (2006).

100. de Bruyn,K.M., Zwartkruis,F.J., de Rooij J., Akkerman,J.W., & Bos,J.L. The small GTPase Rap1 is activated by turbulence and is involved in integrin [alpha]IIb[beta]3-mediated cell adhesion in human megakaryocytes. *J. Biol. Chem.* **278**, 22412-22417 (2003).

101. Gao,L. *et al.* Ras-associated protein-1 regulates extracellular signal-regulated kinase activation and migration in melanoma cells: two processes important to melanoma tumorigenesis and metastasis. *Cancer Res.* **66**, 7880-7888 (2006).

102. Zhang,L. *et al.* Identification of a putative tumor suppressor gene Rap1GAP in pancreatic cancer. *Cancer Res.* **66**, 898-906 (2006).

103. Tsukamoto,N., Hattori,M., Yang,H., Bos,J.L., & Minato,N. Rap1 GTPase-activating protein SPA-1 negatively regulates cell adhesion. *J. Biol. Chem.* **274**, 18463-18469 (1999).

104. Gumbiner,B.M. Regulation of cadherin adhesive activity. *J. Cell Biol.* **148**, 399-404 (2000).

105. Kemler,R. From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. *Trends Genet.* **9**, 317-321 (1993).

106. Takai,Y., Miyoshi,J., Ikeda,W., & Ogita,H. Nectins and nectin-like molecules: roles in contact inhibition of cell movement and proliferation. *Nat. Rev. Mol. Cell Biol.* **9**, 603-615 (2008).

107. Knox,A.L. & Brown,N.H. Rap1 GTPase regulation of adherens junction positioning and cell adhesion. *Science* **295**, 1285-1288 (2002).

108. Price,L.S. *et al.* Rap1 regulates E-cadherin-mediated cell-cell adhesion. *J. Biol. Chem.* **279**, 35127-35132 (2004).

109. Asuri,S., Yan,J., Paranavitana,N.C., & Quilliam,L.A. E-cadherin dis-engagement activates the Rap1 GTPase. *J. Cell Biochem.* **105**, 1027-1037 (2008).

110. Fukuyama,T. *et al.* Involvement of the c-Src-Crk-C3G-Rap1 signaling in the nectin-induced activation of Cdc42 and formation of adherens junctions. *J. Biol. Chem.* **280**, 815-825 (2005).

111. Hoshino,T. *et al.* Regulation of E-cadherin endocytosis by nectin through afadin, Rap1, and p120ctn. *J. Biol. Chem.* **280**, 24095-24103 (2005).

112. Balzac,F. *et al.* E-cadherin endocytosis regulates the activity of Rap1: a traffic light GTPase at the crossroads between cadherin and integrin function. *J. Cell Sci.* **118**, 4765-4783 (2005).

113. Peifer,M. & Yap,A.S. Traffic control: p120-catenin acts as a gatekeeper to control the fate of classical cadherins in mammalian cells. *J. Cell Biol.* **163**, 437-440 (2003).

114. Fukuhara,S. *et al.* Cyclic AMP potentiates vascular endothelial cadherin-mediated cell-cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling pathway. *Mol. Cell Biol.* **25**, 136-146 (2005).

115. Kooistra,M.R., Corada,M., Dejana,E., & Bos,J.L. Epac1 regulates integrity of endothelial cell junctions through VE-cadherin. *FEBS Lett.* **579**, 4966-4972 (2005).

116. Dube,N. *et al.* The RapGEF PDZ-GEF2 is required for maturation of cell-cell junctions. *Cell Signal.* **20**, 1608-1615 (2008).

117. Ohba,Y., Kurokawa,K., & Matsuda,M. Mechanism of the spatio-temporal regulation of Ras and Rap1. *EMBO J.* **22**, 859-869 (2003).

118. Streuli,C.H. & Akhtar,N. Signal co-operation between integrins and other receptor systems. *Biochem. J.* **418**, 491-506 (2009).

119. Roovers,K., Davey,G., Zhu,X., Bottazzi,M.E., & Assoian,R.K. Alpha5beta1 integrin controls cyclin D1 expression by sustaining mitogen-activated protein kinase activity in growth factor-treated cells. *Mol. Biol. Cell* **10**, 3197-3204 (1999).

120. Bhatt,K.V. *et al.* Adhesion control of cyclin D1 and p27Kip1 levels is deregulated in melanoma cells through BRAF-MEK-ERK signaling. *Oncogene* **24**, 3459-3471 (2005).

121. Muller,E.J., Williamson,L., Kolly,C., & Suter,M.M. Outside-in signaling through integrins and cadherins: a central mechanism to control epidermal growth and differentiation? *J. Invest Dermatol.* **128**, 501-516 (2008).

122. Pece,S. & Gutkind,J.S. Signaling from E-cadherins to the MAPK pathway by the recruitment and activation of epidermal growth factor receptors upon cell-cell contact formation. *J. Biol. Chem.* **275**, 41227-41233 (2000).

123. Vivanco,I. & Sawyers,C.L. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat. Rev. Cancer* **2**, 489-501 (2002).

124. Perrais,M., Chen,X., Perez-Moreno,M., & Gumbiner,B.M. E-cadherin homophilic ligation inhibits cell growth and epidermal growth factor receptor signaling independently of other cell interactions. *Mol. Biol. Cell* **18**, 2013-2025 (2007).

125. Chetoui,N., Gendron,S., Chamoux,E., & Aoudjit,F. Collagen type I-mediated activation of ERK/MAP Kinase is dependent on Ras, Raf-1 and protein phosphatase 2A in Jurkat T cells. *Mol. Immunol.* **43**, 1687-1693 (2006).

126. Yoon,H.S., Ramachandiran,S., Chacko,M.A., Monks,T.J., & Lau,S.S. Tuberous sclerosis-2 tumor suppressor modulates ERK and B-Raf activity in transformed renal epithelial cells. *Am. J. Physiol Renal Physiol* **286**, F417-F424 (2004).

127. Schmitt,J.M. & Stork,P.J. PKA phosphorylation of Src mediates cAMP's inhibition of cell growth via Rap1. *Mol. Cell* **9**, 85-94 (2002).

128. Buensuceso,C.S. & O'Toole,T.E. The association of CRKII with C3G can be regulated by integrins and defines a novel means to regulate the mitogen-activated protein kinases. *J. Biol. Chem.* **275**, 13118-13125 (2000).

129. Crawford,N.P. *et al.* Germline polymorphisms in SIPA1 are associated with metastasis and other indicators of poor prognosis in breast cancer. *Breast Cancer Res.* **8**, R16 (2006).