Interfering with Rac1 activity in FRT thyroid epithelial cells impairs the expression of the polarized phenotype and of the E-cadherin function

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ABSTRACT

The acquisition of cell polarity, which includes the establishment of the tight junction barrier, polarized assembly of the cytoskeleton and appropriate organization of membrane traffic, requires external cues, that in epithelial cells are represented by the interaction of cells with their neighbors and with the extracellular matrix. The Rho family of small GTPases, regulate many biological processes including cell cycle progression, apoptosis, migration and intercellular adhesion.

We focused on the analysis of the role of Rac1 protein in the acquisition and maintenance of the polarized phenotype in the FRT rat thyroid epithelial cell line. In this work a novel experimental approach, i.e. the use of an inducible dominant-negative form of the Rac1 protein, ER-Rac1N17, was used to demonstrate the involvement of this small GTPase in the epithelial polarization process and to unravel its mechanism of action.

Oriented cell migration, transepithelial resistance acquisition, and formation of polarized cysts in suspension cultures were investigated. All these parameters are related to the polarized phenotype and were found to be affected after inhibition of Rac1 activity. To unravel the molecular mechanism by which Rac1 affected cell polarity, we investigated the establishment of E-cadherin-dependent cell-cell contacts, which is another key event in the process of epithelial polarization, by calcium switch assays. We determined the dynamics of subcellular localization of Rac1 and of E-cadherin molecules to understand if, and how, the two proteins were intimately related functionally. We conclude that Rac1 inhibition affects cell polarity by impairing E-cadherin recycling to the plasma membrane.
INTRODUCTION
1.1 Cell polarity

Polarization of cells is a fundamental process in biology, which reflects asymmetric organization of cellular components and structures. The establishment and maintenance of cell polarity involves many processes such as signaling cascades, membrane trafficking events and cytoskeleton dynamics, all of which need to be coordinated in a highly regulated manner. The polarity is observed in most differentiated cells including epithelial cells with apical-basal polarity, neurons in which signals propagate in one direction from dendrites to axons, and migrating cells. Other cells such as blood cells do not show any explicit polarity, but can express it when they interact with other cells or when they migrate. Cell polarity is involved in many processes including differentiation, proliferation and morphogenesis both in unicellular and multicellular organisms. In a wide range of elementary cellular processes, many constituents of the cell, such as plasma membrane proteins, organelles, and cytoskeleton components are organized asymmetrically within the cells. This asymmetrical pattern of organization is enhanced by cell differentiation processes resulting in dynamic cell compartments specialized in complex vectorial functions. The polarity can be defined in two different ways depending of the point of view from which we analyze the phenomenon: structural and functional polarity. In the first case, cell polarity is defined as the asymmetric distribution of proteins and membrane lipids, the oriented distribution of organelles and cytoskeleton elements. In the second, it consists in the property to perform tasks in an oriented manner, such as the transport of ions, the transfer of proteins and migration. These properties coexist in most polarized cells such as epithelial cells, in which the polarized phenotype is morphologically visible: 1) in the functional specializations of the apical surface, as invaginations (microvillus or cilia); 2) in the position of the Golgi into the apical zone; 3) in the accumulation of the products secreted into the apical cytoplasm; 4) in the specializations of the lateral surface (i.e. the presence of numerous cell junctions, which bind tightly cells to each other and make tissue compact and resistant to trauma). The polarized distribution of functions in polarized cells requires the coordinated interaction of three machineries that regulate the basic mechanisms of intracellular protein trafficking and distribution. First, intrinsic protein-sorting signals
and cellular decoding machineries regulate protein trafficking to plasma membrane domains; second, intracellular signaling complexes define the plasma membrane domains to which proteins are delivered; and third, proteins that are involved in cell–cell and cell–substrate adhesion orientate the three-dimensional distribution of intracellular signaling complexes and, accordingly, the direction of membrane traffic. The integration of these mechanisms into a complex and dynamic network is crucial for normal tissue function and is often defective in disease states. The establishment of the polarized phenotype occurs in several steps and generates reorganization both of the cytoskeleton and the cell surface. Isolated, dispersed cells are non-polarized, but when such cells are seeded in a culture dish, the initial events observed are attachment of the cells to the substratum and establishment of cell-cell contacts (Rodriguez-Boulan and Nelson 1989). The attachment of non-polarized single cells of epithelial origin to the substrate generates a signal to form an immature surface containing specific apical markers (Vega-Salas, Salas et al. 1987); basolateral membrane proteins are, at this stage, randomly distributed at the entire cell surface. The molecular mechanism of this process involves redistribution of membrane proteins via cytoskeleton rearrangements. Once acquired, the polarity is maintained through several mechanisms. The first is the formation of tight junctions, which, besides constituting a sealing element in the barrier function of epithelia, acts as a fence to prevent intermixing of membrane proteins and lipids in the apical and basolateral membranes (Gumbiner 1987). Second, membrane components can be maintained by anchorage to components of the sub membranous cytoskeleton (Nelson and Veshnock 1987), by interaction with extracellular matrix components (Parry, Cullen et al. 1987), or by bind with CAMs of neighbouring cells (McNeill, Ozawa et al. 1990) and by association with immobile glycolipids domains. In fact, many membrane proteins occur in microdomains both in apical and the basolateral plasma membrane.

1.2 The polarity in epithelial cells
The epithelial cells are contiguous, working closely together and are connected to each other by junctional complexes that make possible the realization of barriers with selective permeability. Epithelial sheets cover organs and body cavities, such as the lung, gut or skin. They thereby impart mechanical protection or mediate secretion, absorption and sensory functions. The epithelial cells are highly polarized. The
intrinsic polarity of epithelial cells is essential to their physiological function and is often disrupted in human disease. Deregulation of cell polarity can cause disorders and cancer. In disease states, such as cancer, epithelial cells lose polarity (through epithelial–mesenchymal transition (EMT), remove from multicellular interactions, migrate and then reintegrate into a second tissue, in which they undergo structural and functional reorganization to reside at the new site. The plasma membrane of polarized epithelial cells is divided into two distinct domains: the apical domain and basolateral domain (Simons and Fuller, 1985; Simons, 1993), which are morphologically, functionally and biochemically different (Rodriguez-Boulan, 2005). The apical region is defined as the area lying above the tight junctions and contains the apical membrane facing the lumen or the outer surface, it has, often, specialized structures such as eyelash or a brush border and is rich in sphingolipids (glycosphingolipids and sphingomyelin). The basolateral region is below the tight junctions and contains the basolateral membrane, which is in contact with adjacent cells and the underling ECM. Intracellular junctions, such as tight junctions and adherens junctions, separate Apical and basolateral domains. Tight junctions form a barrier for proteins and lipids in the membrane, thereby maintaining distinct compositions of apical and basolateral (Figure 1) plasma membrane domains. (Matter and Mellman, 1994; Mostov et al., 2000). The different composition of two domains is continuously supported by intracellular sorting mechanism which regulate the insertion of new proteins and degradation of old proteins in specific plasma membrane domains. This process requires two fundamental requirements: the presence of signals on protein sorting and recognition of these signals by a cellular machinery of "sorting" able to decipher them allowing, therefore, to selectively direct the newly synthesized protein to the different domains of the membrane plasma. Through a series of studies based on both a biochemical approach that of imaging in living cells, it was hypothesized that the sorting of proteins to the two compartments of the plasma membrane takes place in trans Golgi (TGN). (Matter and Mellman, 1994; Mostov et all., 2000). All proteins destined for the plasma membrane are synthesized in the endoplasmic reticulum and traveling together through the different tanks of the Golgi apparatus (Figure 2), where, thanks to the presence of specific signals of sorting, are incorporated into different vesicles and separately relayed to the apical domain and basolateral membrane (Wandinger-Ness et al., 1990; Rodriguez-Boulan and Powell, 1992; Keller et al., 2001; Kreitzer et al.,...
Basolateral sorting motifs, with some exceptions are found in their cytoplasmic tail and are composed of amino acid primary sequences, many of which fit the consensus sequence for binding to adaptor protein (AP) complexes. In contrast to basolateral sorting signals, apical targeting motifs were found throughout the length of membrane proteins: in ectodomain, the transmembrane and the cytoplasmic tail section. Sorting to the apical surface appears to be mediated by three main types of signals: 1) domains of N-and / or O-glycosylation, recognizable by cellular lectins (Fiedler and Simons, 1995; Yeaman et al., 1997; Naim et al., 1999; Rodriguez-Boulan and Gonzalez, 1999), 2) discrete signals contained in the cytoplasmic tail of transmembrane proteins in seven sections (Chuang and Sung, 1998; Sun et al., 1998), and 3) incorporation of apical proteins in lipid microdomains enriched in glycosphingolipids and cholesterol, called rafts, at the level of the Golgi (Kundu et al., 1996; Lin et al., 1998) (Figure. 3). In epithelial cells, the apical domain is particularly rich in glycolipids, cholesterol, H/K ATPase and ionic channels. The basolateral domain contains proteins involved in communication with adjacent cells. The thyrocytes, thyroid epithelial cells, are an excellent example of a polarized epithelial cell. In vivo, the thyrocytes are organized into follicles, structures spheroidal closed bounded by a monolayer of cells resting on basement membrane. The follicles represent the functional unit of the thyroid because they are able to perform the essential functions of the entire gland and to produce hormones (Cleats et al, 1986). Into the lumen of the follicle is secreted thyroglobulin and iodine are secreted, it, thanks to a peroxides in the apical membrane, binds to thyroglobulin and allows the formation of thyroid hormones. Iodide is transported across both the basolateral and the apical plasma membrane. The basolateral membrane contains two components essential for the execution of thyroid-specific functions: the TSH receptor and the iodide pump (Westermark, Westermark et al. 1986). At this surface the thyroid hormones are also released after their liberation from intracellular degradated thyroglobulin. The apical secretion of thyroglobulin, iodine direct transport from base to apical, the internalization of iodined thyroglobulin and secretion of thyroid hormones from the basolateral membrane are all expressions of the phenotype of these polarized epithelial cells.
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Fig. 1 Image of an epithelial cell. The epithelial plasma membrane is divided into two surfaces: an apical surface facing the lumen and a basolateral surface contacting adjacent cells and the extracellular matrix. The main role of the tight junctions is to prevent the mixing of proteins and lipids between the two surfaces.

Fig. 2 Transport from the Golgi apparatus. The proteins are sorted in the trans Golgi network thanks to the recognition of specific signals into vesicles and transported to their final destination.

1.3 Protein complexes involved in the acquisition of cell polarity

Cell polarization is achieved by the concerted actions of polarity proteins. These
molecules are conserved throughout evolution and can react to extrinsic or intrinsic polarity cues (for example, growth factor gradients or the microtubule cytoskeleton, respectively). By assembling multiprotein complexes, they induce downstream signaling to trigger the establishment of cellular asymmetry. There are three protein complexes responsible for the development and maintenance of apical-basolateral polarity: the complex PALS1-PATJ-Crumbs (CRB complex called), the complex Par3-aPKC PAR6 (called complex MLP) and the Scribble complex-DLG-LGL. Knockdown of either PALS1 or PATJ in epithelia causes a delay in tight junction formation (Straight et al. 2004; Shin et al. 2005). Cells deficient in Crb3 fail to form tight junctions and do not polarize properly (Karp et al. 2008; Whiteman et al. 2008). These three complexes have a different localization in cells. The first two are localized in the apical region of the membrane; the third is concentrated along lower side of the membrane. In some processes of polarization they cooperate in the induction of polarity, whereas in other systems can also act as antagonists

1.3.1 The PAR complex

In recent years, the PAR complex has emerged as a central player in the mechanisms that regulate cell polarity in the different types. (Wodarz A 2002; Kemphues K 2000). It was initially described in the nematode C. elegans and later in the fruit fly D. melanogaster and vertebrates. The PAR complex is composed of two scaffold proteins PAR3 and PAR6 and a serine threonine kinase aPKC (atypical protein kinase). This complex is evolutionally conserved from worms to vertebrates. In particular, PAR6 has a molecular weight of 37 kDa and contain three conserved domains that mediate the interactions with the other member of the complex. A Phox / Bem 1 (PB1) domain located at the N-terminal that binds to other PB1-domain-containing proteins including aPKC, the adjacent CRIB domain that binds to Cdc42 or Rac exclusively in their activated GTP-bound state. Lastly, PDZ domain that allows interaction to other proteins with PDZ PAR3 (Joberty, Petersen et al. 2000; Hung and Sheng 2002). PAR6 proteins do not include enzymatic domains and they function by bringing together several proteins at a specific localization such as tight junctions or the leading edge of a migrating cell etc. The exact function of PAR6 has not yet been elucidated, but since Garrard et al. (Garrard, Capaldo et al. 2003) established that Cdc42-GTP can induce a conformational change in PAR6B, Gao and Macara (Gao and Macara 2004) proposed
a functional model for PAR6, in which the N-terminal folds back and interacts with the CRIB-PDZ domain. Cdc42-GTP binding to PAR6 results in the unfolding of PAR6, thus exposing the PALS1 binding site (PDZ domain in PAR6). The key function of PAR6 should be to allow the interaction between aPKC and its downstream effectors such as PAR3 and LGL. (T. Yamanaka 2003). Phosphorylation of LGL will result in its detachment from the aPKC/PAR6 dimer, leading to the formation of another functional complex: PAR6/PAR3/aPKC (Yamanaka, Horikoshi et al. 2003). PAR3 has been extensively studied in epithelial cells. PAR3 proteins are, partially, located in cell-cell contact regions and colocalized with ZO1 at tight junctions. It contains three PDZ domains and the first of these interacts with PAR6. It seems likely that the starting point required for PAR3 to target the tight junctions is its ability to form homodimer via its N-terminal region and to bind to the junctional adhesion molecules (JAMs); this association seems to be required for the correct association of PAR3 at the apical side of the cell–cell contact region during the process of polarization (Mizuno, Suzuki et al. 2003). PAR3 may then be stabilized upon binding directly to JAM via its first PDZ domain, and these two proteins may then be co-distributed to the sites of cell–cell contact (Ebnet, Suzuki et al. 2001). Once PAR3 occupies this site, it can play the role of a scaffold in the recruitment of proteins involved in the formation of the junctions, such as PAR6 or aPKC. Many studies have in fact shown that overexpression or depletion of PAR3 in epithelial cells leads to the disruption of tight junctions. Moreover, beyond to the members of PAR complex, PAR3 interacts with and other proteins such as Tiam1 or LIMK2 and these interactions seem to be dependent on its phosphorylation state (Hurd et al, 2003, Wang et al, 2006). Unlike conventional PKCs, αPKC (75 kDa) is unique in having a PB1 domain in the N-terminal, which interacts with PAR6 (Bose et al, 2006; Assemat et al, 2007). Moreover it is the only members of the PAR complex showing catalytic activity thanks to catalytic domain at the C-terminal region conserved among PKC proteins. This domain is known to phosphorylate several proteins such as PAR3 and LGL. In MDCK epithelial cells, aPKCs localize with the other members of the PAR complex at tight junctions (Izumi, Hirose et al. 1998); As Cdc42-GTP binds to PAR6, it seems likely that Cdc42-GTP may form a complex with aPKC via the adaptor PAR6. The expression of Cdc42-GTP leads to the translocation of aPKC from the nucleus to the cytoplasm and cell periphery, where the complex will be involved in
tight junction formation. Cdc42 is activated upon E cadherin mediated cell–cell adhesion, resulting in phosphorylation and thus activation of aPKC, and this chain of events is crucial to tight junction formation.

1.3.2 The Crumbs complex

The Crumbs complex was identified in the epithelia of Drosophila and subsequently in vertebrates. Mammalian CRB are transmembrane proteins, whereas the other proteins present in this complex, PALS1 and PATJ, are cytoplasmic scaffolding proteins. CRB proteins have a transmembrane domain and a cytoplasmic domain very well conserved. In the cytoplasmic tails, CRB protein contains two motives: a FERM (band 4.1-ezrin-radixin-moesin) protein-binding domain consisting of 12 amino acids containing a GTY motif and a PDZ-binding domain consisting of ERLI residues. The FERM domain is a protein–protein interaction domain which exists in various proteins, many of which serve as adapters linking transmembrane proteins to the cortical actin cytoskeleton. In addition to playing a role in the formation of tight junctions, CRB is involved in the differentiation of the apical membrane (Fogg, Liu et al. 2005) and contributes to stabilizing apical cell junctions. PALS1 (77 kDa) is a scaffold protein that has multiple protein–protein interaction domains. It consists of two L27 domains, a PDZ domain, an SH3 domain, a hook domain and a GUK domain. PALS1 binds CRB and PATJ (M.H. Roh et al 2002) with its a PDZ domain and the first L27 domain respectively. Knockdown of PALS1 in MDCK cells leads to tight junction and polarity defects (Straight, et al, 2004) and to the mis-targeting of E-Cadherin to the cell membrane (Wang, et al, 2007). The PATJ L27 domain present at the N-terminal is followed by up to ten PDZ domains. The 6th and 8th PDZ modules of PATJ interact directly with ZO3 and Claudin1, respectively, via the PDZ-binding domain present at the C-terminal ends of these proteins. The fact that over expression or down regulation of PATJ in epithelial cells disrupts the tight junction-specific localization of ZO1, ZO3 and Occludin, suggests that PATJ might be involved in stabilizing tight junctions (Lemmers, Medina et al. 2002; Michel, Arsanto et al. 2005).

1.3.2 The Scribble complex

The Scribble complex comprises three proteins SCRIBBLE, Lgl (Lethal giant larvae) and Dlg (Discs large) which are all thought to behave as scaffold proteins and regulate
protein-protein interactions (Humbert, P. et al 2003; Bilder, D 2004) SCRIBBLE and DLG co-localize to the lateral membrane and similarly Lgl is predominantly localized basolaterally in epithelial cells. The exact nature of the physical interactions between SCRIB, LGL and DLG has not yet been clearly defined. SCRIB (175 kDa) is a large cytoplasmic multidomain protein that plays many roles in flies and mammals. It has 16 LRR at its N-terminal, followed by 2 LAP-specific domains (LAPSD), a linker region, 4 PDZ domains and a C-terminal lacking any identifiable motives. Navarro et al have established that the LRR repeats occurring in SCRIB determine its ability to target the basolateral epithelial membranes. SCRIB binds directly to the C-terminal motif of ZO2 via its PDZ domains 3 and 4, the SCRIB/ZO2 interaction probably takes place at the cell junctions before ZO2 is segregated in the tight junctions of the apical membrane (Metais, Navarro et al. 2005). Suppression of SCRIB expression in MDCK cells causes a delay in tight junction assembly and affects the epithelial morphology. Mammalian DLG shows three PDZ domains, a SH3 domain, a hook domain and a GUK domain. The formation of a polarized epithelial cell layer with functional tight junctions requires spatio-temporal coordination of the activity of the polarity complexes that that regulate the establishment and maintenance of the apical polarity in the cell. Nevertheless their localizations are different (CRB and PAR complexes are restricted to the apical region of the lateral membrane whereas SCRIB complex is concentrated along the lateral membrane. E-cadherin/E-cadherin interactions in the cell–cell adhesion region trigger Cdc42 GTP activation (Kim 2000) and the phosphorylation of aPKC, which in turn phosphorylates LGL. Phosphorylated LGL dissociates from PAR6/aPKC dimer and distributes to the lateral membrane, where it could interact with DLG and SCRIB (Plant, Fawcett et al. 2003), aPKC is then able to interact with and phosphorylate PAR3, allowing the formation of the active PAR complex at the apical junctions. A direct connection therefore exists between the activity of the basolateral complex containing LGL and the active apical PAR complex. aPKC is required for the stable localization of PAR3, and PAR3 phosphorylated at S827 residue accumulates at tight junctions (Nagai-Tamai, Mizuno et al. 2002; Suzuki, Ishiyama et al. 2002). Once it has been phosphorylated at the S827 residue, PAR3 therefore dissociates from aPKC and this protein is able to bind directly to the CRB cytoplasmic tail that contains two threonine residues (T6 and T9) in an evolutionarily conserved region, which are potential targets for aPKC
phosphorylation. CRB binds to PAR6 directly or via PALS1, to promote the differentiation of the premature junctional structure into mature epithelial structures. SCRIB complex restricts the localization of CRB and PAR complexes to the apical region of epithelial cells, where they may act together to regulate tight junctions formation (Hurd, Gao et al. 2003; Lemmers, Michel et al. 2004) (figure 3)

Fig. 3 Conserved proteins of the partitioning defective (PAR), Scribble and Crumbs complexes guide the establishment of cell polarity in various organism. In polarized mammalian epithelial cells, the PAR3 and Crumbs-3 complexes localize predominantly to tight junctions, whereas components of the Scribble complex show basolateral localization. PAR-3, PAR-6 and aPKC are ubiquitously expressed and function in various cell polarization processes. The kinase activity of aPKC is required for a functional PAR complex, and aPKC-mediated phosphorylation of target proteins is a key event of downstream polarity signalling. The Crumbs complex comprises the transmembrane protein Crumbs and the cytoplasmic scaffolding molecules PALS1 and PATJ. PATJ is thought to connect the Crumbs-3 complex with structural tight-junction proteins in polarized epithelial cells. In vertebrates, Scribble binds directly to Lgl and indirectly to Dlg. Several molecular interactions between the three complexes have been identified. Mutual exclusion of the Scribble complex and the apical junctional complexes controls apico–basal polarity, and aPKC-mediated phosphorylation of LGL2 and PAR1. (taken from Sandra Iden and John G. Collard 2008).

1. 4 Cell-cell junctions and cell polarity.
The integrity of the epithelial cell layer(s) that protects multicellular organisms from the external environment is maintained through the formation of several intercellular junctional complexes including tight junctions (TJ), adherens junctions (AJs), and desmosomes, whereas gap junctions provide for intercellular communication. Another important factor is adhesion to a basement membrane composed of extra-cellular matrix proteins. The transmembrane proteins constituting these junctions are linked to
components of the cytoskeleton, thereby establishing connections to other cell-cell and cell-substratum adhesion sites. In addition, a growing number of cytoplasmic scaffolding molecules associated with these junctions are involved in regulating such diverse processes as transcription, cell proliferation, cell polarity, and the formation of a regulated diffusion barrier. TJs and AJs are differentially distributed along the intercellular cleft. TJs form an apical rim, whereas AJs are localized basolaterally below TJs. TJs and AJs are highly regulated dynamic structures, whose pivotal regulatory partners, among many other regulators, include Rho family GTPases, which control both the actin cytoskeleton and the integrity of intercellular junctions.

**Tight junctions (TJ)** are localized at the most apical part of epithelial cells, and form a network of close contacts between membranes of adjacent cells. TJs control the paracellular transport of ions, water, solutes and cells, and in addition they constitute a fence separating apical and basolateral membrane proteins. TJs are formed of two types of membrane proteins, occludin and claudins, which are associated with cytoplasmic proteins, linking TJs to the actin cytoskeleton. The tight junctions are composed of a network of sealing filaments. Each sealing strand is composed of a long line of transmembrane adhesion molecules immersed in each of the two interacting membranes. The extracellular domains of these proteins are united directly with one another to occlude the intercellular space.

**Occludin** is a protein of 65 kDa contain four transmembrane domains and two extracellular loops. The C-terminal domain, localized in the cytoplasm, directly binds to ZO-1 (zonula occludens), which in turn associates with the apical actin. This region is rich in phosphorylation sites (tyrosine, serine, and threonine) which can be modified by kinases or phosphatases. Non-phosphorylated occludin is distributed on basolateral membrane and in cytoplasmic vesicles, whereas phosphorylated occludin is localized in TJs, leading to a decreased paracellular permeability.

**Claudins**, similar to occludin, has four transmembrane domains with two extracellular loops and a C-terminal cytoplasmic domain, which binds through PDZ binding motifs to ZO proteins, to PATJ). Claudins recruit occludin to tight junctions. They are the major components of TJ strands and have a determinant role in the barrier function. They form paracellular channels, which are selective for ions through their first extracellular loop (Harstock and Nelson, 2008).
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**ZO (Zonula occludens)** proteins (ZO-1; ZO-2; ZO-3) are scaffolding proteins linking TJ proteins to the actin cytoskeleton, and also linking TJs to AJs. The ZO family proteins belong to the family of guanylate kinases (MAGUKs) and are composed of three domains: a PDZ domain that allows ZO proteins binding to claudins, an SH3 domain responsible for binding to α-catenin and guanylate kinase (GUK) domain, which lacks catalytic activity, but interaction with other proteins, such as occludins (Gonzalez-Mariscal, Betanzos et al. 2000). ZO Proteins colocalize with E-cadherin in primordial junctions in not polarized cells but successively they concentrate in tight junctions by interacting with occludins and claudins, thus allowing the maturation of this adhesions.

**Junctional adhesion molecules (JAM)** (32 kDa) contain a transmembrane segment, an extracellular domain encompassing two immunoglobulin-like subdomains, and a short cytoplasmic tail. JAMs function as cell–cell adhesion molecules through their extracellular domains, which are capable of homophilic interaction and can form heterophilic associations with various ligands such as integrins. In addition, JAMs associate with intracellular partners such as ZO-1 and the protease-activated receptor PAR-3 (Figure 4).

**Adherens junctions.** A key event in epithelial polarization is the establishment of E-Cadherin–dependent cell-cell contact. AJs result from the complex association of multiple components and play a pivotal role in the initiation of intercellular contacts between neighboring cells and in stabilizing adhesion. Moreover, AJs are multifunctional structures involved in the control of the actin cytoskeleton, focal adhesion, intracellular signaling, and transcription regulation. The core of the Adherens junction includes interactions among transmembrane glycoproteins of the classical cadherin superfamily, such as E-cadherin, and the catenin family members include p120-catenin, β-catenin, and α-catenin. Together, these proteins control the formation, maintenance and function of adherens junctions.
E-cadherin Cadherins (120 kDa) contain only one transmembrane segment and five extracellular repeat domains, which form Ca\(^{++}\)-dependent homophilic interactions with cadherins from the same or adjacent cells. Other members of this family are N-(neural), P (placental), and R(retina), VE (vascular endothelia)-cadherin (Gooding JM, 2004). Cadherins have five characteristic extracellular cadherin (EC) repeat domains. These domains form trans-cadherin interactions between neighboring cells and initiate weak cell-cell adhesion. Binding of Ca\(^{2+}\) to each EC domain is required for the correct conformational organization of the cadherin extracellular domain and allows cadherin to arrange into a rigid and organized structure that is resistant to proteolysis. The cytoplasmic domain of E-cadherin can be divided in two portions: the carboxi terminal “catenin binding domains (CBD) for β-catenin and the juxtamembrane domain that binds p120-catenin and and Hakai, an E3-ubiquitin ligase. These domain allows to cadherin to bind proteins that regulate E-cadherin endocytosis, recycling and
degradation, intracellular signaling and gene transcription, and local control of the actin cytoskeleton (Halbleib JM, 2006). E-cadherin has an important role in cell–cell adhesion as well as in the stabilization of epithelial cell phenotype. Decreased cadherin function induces disassembly of cell–cell adhesion and disturbs cadherin-mediated signaling, while leading to de-differentiation from an epithelial to a mesenchymal phenotype, as well as to an increased cell migration characteristic of invasive tumor cells (E. Lozano, 2003)

**Catenins** mediate the interplay between a cadherin complex and the actin cytoskeleton, and also govern several signaling pathways that control morphogenesis and tissue homeostasis. The catenin family comprises p120-catenin, β-catenin and α-catenin.

**p120-catenin (120 kDa)**—p120-catenin was first identified as a substrate for Src-tyrosine receptor kinase and later defined as a member of the catenin family based on sequence homology to an armadillo domain of β-catenin. p120 binds to the juxta-membrane domain of E-cadherin and stabilize E-cadherin at the plasma membrane during the formation of cell-cell contacts. Phosphorylated p120 binds to E-cadherin with high affinity and thus prevents its endocytosis and degradation, or it increases the recycling of internalized E-cadherin to membranes. One possible mechanism of targeting cadherin for degradation involves Hakai, an E3-ubiquitin ligase, which binds E-cadherin in a Src-dependent manner. Expression of Hakai increased both the ubiquitination and rate of E-cadherin endocytosis. In addition, p120 modulates the actin cytoskeleton through inactivation of RhoA and activation of Rac and Cdc42.

**β-catenin (88 kDa)**—β-catenin is characterized by 13 repeats of a characteristic armadillo domain of ~42 amino acids that form triple α-helix. Beta-catenin binds the C-terminal cytoplasmic domain of E-Cadherin when phosphorylated on three serine by CKII and GSK-3β. In contrast, tyrosine phosphorylation of β-catenin by Src, Abl, or EGF receptor at Y489 or Y654 disrupts binding to Cadherin. It is proposed that the E-cadherin/β-catenin interaction occurs in the endoplasmic reticulum (ER) and is required for cadherin exit from the ER (Chen YT, 1999). Normally cytosolic levels of β-catenin are low due to rapid targeting of excess β-catenin to the proteosome (Aberle H, 1997). β-Catenin also binds to α-catenin in a mutual exclusive manner.

**α-catenin (120 kDa)**—α-Catenin contains three vinculin homology domains and differs considerably in sequence from the other catenins. α-catenin is the link between...
the cadherin/beta-catenin complex and the actin cytoskeleton. It exists both as monomers and dimers. Recently, it has been shown that monomeric α-catenin binds to β-catenin and not to actin, and inversely dimeric α-catenin associates with actin filaments but not with β-catenin and appears to compete binding of the Arp2/3 complex. Indeed actin filaments bound to α-catenin can no longer associate with the Arp2–3 complex, thereby suppresses actin polymerization (Drees F, 2005). This allosteric switch between monomeric and dimeric states appears to be the molecular explanation for the lack of simultaneous binding of α-catenin to both β-catenin and actin filaments. Moreover, α-catenin binds to other actin-binding proteins: ZO-1, a linker between AJ and TJ structures (Figure 5).

**Fig. 5** a) Cadherin, a calcium-dependent adhesion molecule, is linked to bundles of actin filaments through β-catenin and α-catenin. Cadherins can dimerize in cis and trans, thereby forming rigid adhesions. b) the E-cadherin molecule is composed of repeated extracellular cadherin (EC) domains (or cadherin repeats), which mediate calcium-dependent homophilic interactions; a transmembrane domain (TMD); a juxtamembrane domain (JMD); and a distal -catenin-binding domain (D-b-D). (taken by Masaki Fukata & Kozo Kaibuchi 2001)

Extra-cellular matrix adhesion. Epithelial cells polarize in response to cell–cell and cell–matrix adhesion. There has been much recent progress in understanding the general polarizing machinery of epithelia, but is unclear how this machinery is controlled by the extracellular environment. Epithelial cells interact with the ECM via integrins receptors. Integrins are crucially important because they are the main receptor proteins that cells use to both bind to and respond to the extracellular matrix. Integrins are single-pass transmembrane receptors that bind several proteins of ECM such as collagen laminin and fibronectin (Figure 6). Its cytoplasmatic domain interacts with many proteins, including talin, to assemble large plaques called focal adhesions.
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(Critchley, D. R. 2000) that represent the centers where many tyrosine kinases recruit to allow cells to exert traction forces on their substrates. Cell extracellular matrix adhesion did not appear to be essential for epithelial polarity but it determines the orientation of polarity. Indeed, cells that are allowed to form epithelial cysts in the absence of integrins have their polarity axis inverted (Nitsch and Wollman, 1980; Ojakiann and Schwimmer, 1994). Integrins do not possess enzymatic activity, however, activation and/or ligand binding induces integrin clustering that leads to the recruitment of multiple signalling molecules and actin filaments (Hynes 2002). Integrin-mediated adhesion provides signals that control cell motility, proliferation, survival, differentiation, and gene expression (Giancotti and Ruoslahti, 1999). In addition, integrin signals are frequently required for coupling growth factor receptors to downstream effectors. Recent work has focused on the activation of signalling by the small GTPase Rac1 in response to integrin-mediated cell adhesion to the extracellular matrix (ECM). These small GTP-binding proteins in turn regulate cell adhesion and changes in cell morphology by triggering dynamic changes in the actin cytoskeleton. Integrin activation of Rac1 and Cdc42 signaling induces the formation of lamellapodia and filopodia, which are necessary for cell spreading. Activation of Rac by integrins upon fibronectin binding induces GTP loading, similar to the activation triggered by growth factor receptors; but distinct from growth factor regulation, integrins also target Rac to specific plasma membrane microdomains, where Rac can interact with its downstream effector molecule PAK to induce signalling (Del Pozo 2004; Guan 2004). Binding between Rac and the lipid bilayer is regulated by RhoGDI, which keeps Rac soluble in the cytoplasm by shielding the isoprenoid moiety (Hoffman, Nassar et al. 2000). RhoGDI binds Rac in the cytosol to prevent both membrane and effector binding. The hypothesis is that integrins would locally increase the affinity of the plasma membrane for Rac, favouring RhoGDI displacement and allowing Rac effector binding (Del Pozo, Kiosses et al. 2002) in the vicinity of focal adhesions.
Fig. 6 The integrin family is composed of 24 αβ heterodimeric members. An integrin molecule is composed of two noncovalently associated transmembrane glycoprotein subunits called α and β. By binding to a matrix protein outside the cell and to the actin cytoskeleton (via the anchor proteins indicated) inside the cell the protein serves as a transmembrane linker. The α and β subunits are held together by noncovalent bonds. The α subunit is made initially as a single 140,000-dalton polypeptide chain, which is then cleaved into one small transmembrane domain and one large extracellular domain that contains four divalent-cation-binding sites; the two domains remain held together by a disulfide bond. The extracellular part of the β subunit contains a single divalent-cation-binding site, as well as a repeating cysteine-rich region, where intrachain disulfide bonding occurs.

1.5 Epithelial apico–basal cell polarity formation.

Formation of apico–basal polarity has been studied intensively in mammalian epithelial cells, and many proteins have been identified. The maturation of primordial adhesions to linear apico–basal polarized cell–cell contacts with discrete adherens and tight junctions requires a gradual and sequential mechanism, consisting of various stages and achieved by the cooperation of the three complexes that regulate cell polarity: the apical PAR3 and Crumbs-3 complexes and the basolateral Scribble with the proteins involved in cell-cell contacts. The establishment of epithelial cell–cell contacts and the subsequent apico–basal polarization requires E-cadherin-mediated cell–cell adhesion. The first event is given by the formation of lamellipodia and
filopodia of adjacent cells that assemble to primordial adhesions. These structures allow two neighbour cells to move and come into contact with each other. Following this step nectin–afadin are the first complexes, which assemble at the primary cell–cell contacts. The nectin–afadin complex is involved in Ca++-independent intercellular adhesion and plays a role in the organization of AJs and TJs. Nectin contains a single trans-membrane domain, an extracellular region containing three immunoglobulin-like domains, and a cytoplasmic extension. Nectin binds to the cytoplasmic C-terminus of afadin through a PDZ domain, and afadin binds actin filament. Nectin–afadin form dimers and nectin molecules from neighboring cells associate with each other through at least the first immunoglobulin-like domain. Subsequently they recruit E-cadherin, that expand the lateral surface and allow the concentration of all the proteins that will form the tight junctions in this domain. These primordial junctions fuse and mature in AJs along the basolateral side of neighboring cells. Finally, at the apical side the JAM proteins assemble and allow the recruitment of claudins, occludins, thereby stabilizing cell-cell junctions.

RhoGTPases plays a role in the formation of AJs. They control the formation and maintenance of cadherin-mediated intercellular junctions. The homophilic interaction of E-cadherin molecules results in a local activation of Rac1 and Cdc42 and inhibition of RhoA. Rac1 seems to have a major role in controlling AJs. The presence of active Rac1 and its downstream effectors was demonstrated, in the early contact zone between cell-cell, suggesting that activation of Rac1 promotes the early stages of adhesion (Price, Leng et al 1998). How Rac1 is activated at cell–cell junctions have been identified in some cell types: it has been reported that E-cadherin-mediated cell–cell adhesions stimulate phosphatidylinositol 3-kinase (PI3K) activity in MDCKII cells. Moreover, PI3K has been shown to interact with E-cadherin (Pece et al. 1999) and β-catenin (Espada et al. 1999). Because PI3K is thought to function upstream of Rac1, these observations indicate the possible involvement of PI3K in E-cadherin-dependent Rac1 activation. Activation of Rac1 through PI3K by E-cadherin-mediated cell–cell adhesions seems to require at least two steps: (1) Rac1 recruitment to sites of cell–cell contacts, and (2) Rac1 activation by a GEF. It has been suggested that Tiam1 is the GEF recruited in the sites of primordial adhesions through its interaction with phospholipids produced by active phosphatidylinositol-3-kinase (PI-3 kinase). The polarity complex, Par3-Par6-aPKC, interacts with the plasma membrane through the
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binding of Par-3 with JAM-A protein, (Itoh, Sasaki et al. 2001). Par3 directly interacts with Tiam1 that activates Rac1. Binding of Rac1-GTP to PAR6 allows PAR6 to activate aPKC. This results in a translocation of occludins, claudins and JAM in the apical membrane with the subsequent formation of tight junctions. In conclusion, the activation of Rac1 mediated by Tiam1 is required for the formation of tight junctions and the activation of these proteins occurs upstream from the activation of the complex polarity Par. The polarity complexes function to determine the site of the tight junction in the process of cell polarization, indeed, CRB and PATJ play an important role in the correct localization of tight junction proteins at the apical side of adherens junctions (Figure 8).

Fig 7 Apico–basal polarization. The first step is the formation of the first cell–cell contacts (primordial junctions), which occur following the formation of lamellipodia and filopodia, in the second step nectins are recruited, leading to the formation of the first type of cell–cell junctions (primordial junctions). These junctions are then stabilized by recruiting the E-cadherin structural proteins, those that form adheren junctions (AJs) and tight junctions (TJs) and signalling proteins, including α-catenin, β-catenin, afadin and ZO1, are recruited to immature cell–cell contacts. Transmembrane proteins of the junctional adhesion molecule (JAM) and nectin family are implicated in localizing PAR complex to primordial adhesions. Rho GTPases are activated downstream of cadherin clustering. Through association with PAR3, TIA1 couples E-cadherin dependent RAC1 activation to activation aPKC, thereby inducing phosphorylation of downstream targets and subsequent polarization and maturation into fully polarized epithelial (taken by Sandra Iden, 2008)

1.6 RHO GTPases
Approximately one percent of the human genome encodes proteins that regulate or are regulated by direct interaction with members of the Rho family of small GTPases. Through a series of complex biochemical networks, these highly conserved molecular switches, control some of the most fundamental processes of cell biology common to
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all eukaryotes, such as morphogenesis, polarity, movement, and cell division. Rho GTPases are members of the Ras superfamily of monomeric 20–30 kDa GTP-binding proteins. More than 100 small G-proteins have been identified in eukaryotes from yeast to human. The members of this superfamily are structurally classified into at least five families: the Ras, Rho, Rab, Sar1/Arf, and Ran families (reviewed by Takaj et al. 2001). Among these, the best characterized members are Rho, Rac and Cdc42. According to the structures of small G-proteins, they have two interconvertible forms: GDP-bound inactive and GTP-bound active forms. An upstream signal stimulates the dissociation of GDP from the GDP-bound form, which is followed by the binding of GTP, eventually leading to the conformational change of the downstream effector-binding region so that this region interacts with the downstream effectors(s). The rate-limiting step of the GDP/GTP exchange reaction is the dissociation of GDP from the GDP-bound form. This reaction is extremely slow and therefore stimulated by a regulator, guanine nucleotide exchange factor (GEF) of which activity is often regulated by an upstream signal. GEP first interacts with the GDP-bound form and releases bound GDP to form a binary complex of a small G-protein and GEF. Then, GEF in this complex is replaced by GTP to form the GTP-bound form. Have been identified 30 guanosine nucleotide exchange factors (GEFs). They can be divided into 2 large families. One group is defined by the presence of two characteristic domains, which account for the catalytic activity. These GEFs contain a catalytic Dbl homology (DH) domain, which is almost invariably followed by a pleckstrin-homology (PH) domain. The PH domain interacts with phospholipids, which may activate the catalytic DH domain of GEFs and localise them to the plasma membrane (Mertens, Roovers et al. 2003; Rossman, Der et al. 2005). The second group of GEFs for Rho GTPases consists of proteins related to Dock180 (dedicator of cytokinesis 180). These proteins contain a Dock-homology region-2 (DHR2 or CZH2) domain, which makes these proteins catalytically active (Brugnera, Haney et al. 2002; Meller, Merlot et al. 2005). Besides promoting the exchange of nucleotides, GEFs contain various additional domains and are able to influence and determine the signalling route downstream of Rho GTPases by direct binding to different effector molecules, or to serve as scaffold proteins that associate with components of downstream effector signalling pathways (Mertens, Roovers et al. 2003; Rossman, Der et al. 2005). The GTP-bound form is converted by the action of the intrinsic GTPase activity to the GDP-bound form,
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which then releases the bound downstream effectors. The GTPase activity of each small G-protein is relatively very slow and is stimulated by GTPase-activating proteins (GAPs), which increase the intrinsic rate of GTP hydrolysis. About 60 GAPs have been identified (Moon and Zheng 2003; Tcherkezian and Lamarche-Vane 2007) all characterized by an arginine residue, called arginine finger, which interact with the active site of small GTPase (Bos et al 2007). Most GAPs are specific for each member or subfamily of small G-proteins (M.S. Boguski, F. McCormick 1993), but some GAPs, such as p190, a GAP active on Rho/Rac/Cdc42 proteins, show wider substrate specificity (J. Settleman 1992). In this way, one cycle of activation and inactivation is achieved and small G proteins serve as molecular switches that transduce an upstream signal to a downstream effector. The GDP/GTP exchange reactions of Rho/Rac/Cdc42 and Rab proteins are furthermore regulated by another type of regulator: Rho GDP dissociation inhibitors (GDI) (T. Sasaki, 1990; T. Ueda 1990). These proteins inhibit both the basal and GEF-stimulated dissociation of GDP from the GDP-bound form and keeps the small G-protein in the GDP-bound form. The binding of GDI to a Rho GTPase occurs through an immunoglobulin-like C-terminal domain, and that a hydrophobic pocket in this domain can accommodate the geranyl-geranyl lipid, whilst a flexible N-terminal domain inhibits GDP GTP exchange. Rho GTPases are able to interact with membranes via a post-translational C-terminal geranylgeranyl lipid modification. So Rho GDI proteins are involved not only in the regulation of their activation but also in their translocation between the cytosol and the membrane (T. Sasaki 1998; Y. Takai 1995). Furthermore, GDIs are able to interact with active, GTP-bound GTPases, preventing hydrolysis and interaction with downstream effectors. Association with GDIs thus keeps Rho GTPases in the cytoplasm, inactive or unable to signal towards downstream effectors (Robbe, Otto-Bruc et al. 2003). Phosphorylation of Rho GDIs may lead to dissociation of the complex and allow Rho GTPases to translocate from the cytoplasm to the plasma membrane, where they can be activated by GEFs and bind effectors. Specific signals mediated by integrins or other proteins, promote the dissociation of GDI from the Rho GTPase protein. The activation state of all Rho GTPases is dependent on the balance of the activities of their regulators (GEFs, GAPs and GDIs). Thus, the local amount of GTP-bound protein and the time during which the protein is active determines the downstream signalling at specific sites in cells. With a few exceptions, Rho family members have a
N-terminal portion that interacts with trinucleotides GTP and GDP, followed by the switch I and switch II regions essential for binding to effector proteins and a C-terminal sequence that ends with a CAAX motif (Wherlock and Mellor 2002). Post-translational modifications of Rho GTPases at the C-terminus, such as prenylation (farnesylation or geranylgeranylation) or palmitoylation, determine their intracellular localization (Figure. 8).

![Regulation of small G-protein activity](image)

**Fig. 8 Regulation of small G-protein activity:** GDP-bound inactive GTPases are localized mainly in the cytosol, maintained thereby GDIs masking the C-terminal tail required for plasma membrane localization. Guanine nucleotide exchange factors (GEFs) release guanosine diphosphate (GDP) from Rho GTPases promoting the binding of guanosine triphosphate (GTP) and activation of Rho GTPases. GDP dissociation inhibitor (GDI) inhibits the dissociation of GDP from Rho GTPases and thus prevents association of GDP-GTPase to cell membrane. GTPase activating proteins (GAPs) stimulate the intrinsic GTPase activity of Rho GTPases and convert GTP-bound form of Rho GTPases to inactive GDP-GTPases. In resting cells Rho GTPases exist mostly in GDP-bound form and in complexes with Rho GDI in the cytosol. The GTP-bound form of Rho GTPases is associated with cell membranes. (Saskia, Ellenbroek et al. 2007).

### 1.7 Functions of Rho GTPase

Multiple small G-proteins form a signal cascade and thereby transduce their signals to the downstream effectors. Given the involvement of Rho GTPases in such a wide variety of important cellular processes, it is not surprising that 30 or more potential effectors for Rho, Rac and Cdc42 have been identified. These proteins interact specifically with the GTP-bound form of the GTPase. The conformational differences between the GTP and GDP-bound forms are restricted to switch regions I and II. Effector proteins, therefore, had to utilize these differences to discriminate between
the GTP- and GDP-bound forms. Numerous point mutations have been introduced into Switch I of Rho, Rac and Cdc42 the effector region. The most common mechanism of effector activation by Rho GTPases appears to be the disruption of intramolecular auto-inhibitory interactions to expose functional domains within their effector protein (L.Bishop and A. Hall 2000; Fig. 9)

**Fig. 9** General model for activation of effector proteins

1. Binding of RhoGTPase to effector relieves an auto-inhibitory intramolecular interaction
2. The effector remains active until GTP hydrolysis takes place.
3. Alternatively a modification of the effector may maintain activity even after dissociation of the GTPase.

### 1.7.1 Regulation of the actin cytoskeleton

Reorganization of the actin cytoskeleton plays crucial roles in many cellular functions such as cell shape change, cell motility, cell adhesion, and cytokinesis. The actin cytoskeleton is composed of actin filaments and many specialized actin-binding proteins (Small JV 1994; Zigmond SH 1996). Filamentous actin is generally organized into a number of discrete structures: 1) actin stress fibers: bundles of actin filaments that traverse the cell and are linked to the extracellular matrix through focal adhesions; 2) lamellipodia: thin protrusive actin sheets that dominate the edges of cultured fibroblasts and many migrating cells; membrane ruffles observed at the leading edge of the cell result from lamellipodia that lift up off the substratum and fold backward; and 3) filopodia: fingerlike protrusions that contain a tight bundle of long actin filaments in the direction of the protrusion. For the most part, this regulation of
actin polymerization is orchestrated by Rho/Rac/Cdc42 proteins. Rho proteins regulate stress fiber formation while Rac proteins regulate ruffling and lamellipodium formation, and Cdc42 regulates filopodium formation (Figure 11).

These actin dynamics are regulated by coordinated activation of different signalling pathways downstream of the small GTPases. RhoA can interact with its effector ROCK protein, which can subsequently activate myosin light chain kinase, leading to activation of myosin (by phosphorylation) and increasing contractility and formation of stress fibres. Furthermore, RhoA can stimulate actin polymerization via its effectors mDia1 and mDia2 proteins, which catalyze F-actin assembly in filopodia and lamellae (Hotulainen and Lappalainen 2006; Gupton, Eisenmann et al. 2007). Most recently, N-WASP, a ubiquitously expressed Cdc42-interacting protein and the Arp2/3 complex, has been shown to participate in the downstream cascade of Cdc42 for the Cdc42-induced actin polymerization. N-WASP has a pleckstrin homology (PH) domain that binds PIP2, and a Cdc42/Rac interactive binding (CRIB) domain. The binding both of GTP-Cdc42 and PIP2 to N-WASP activate N-WASP by stabilizing the active conformation of this molecule. The C-terminus of N-WASP thereby binds the Arp2/3 complex, that generates new barbed ends stimulating its ability to nucleate actin polymerization. Therefore, the interaction of N-WASP with the Arp2/3 complex
directly connects the Cdc42-mediated signal transduction pathway to the stimulation of actin polymerization. Several other potential effectors of Cdc42 and Rac proteins have also been identified. Among them, the family of serine/threonine protein kinases known as PAKs could be the most likely candidate. Thus, it is likely that PAK proteins are involved in mediating the effect of Cdc42/Rac proteins on the cytoskeleton. In addition, POR1 (partner of Rac) a 34-kDa protein, interacts specifically with GTP-Rac proteins, and is implicated to mediating Rac protein-dependent membrane ruffling. Another protein with a potential role in cytoskeletal organization is IQGAP. IQGAP interacts with both GTP-Rac1 and GTP-Cdc42 and localizes to membrane ruffles. IQGAP has been shown to be localized in cell–cell adhesion sites. It has been suggested that activated Cdc42 or GTP-Rac1 block the ability of IQGAP to inhibit assembly of a cadherin-catenin complex promoting adherens junctions formation (S. Kuroda, 1998).

1.7.2. Regulation of the microtubule cytoskeleton

Similar to actin filaments, microtubules have an intrinsic polarity, with a minus end and a dynamic plus end, usually at the cell periphery. The intracellular organization of microtubules makes a major contribution to cell polarity and to the distribution of intracellular organelles, such as the Golgi and mitotic spindle. Small Rho GTPases are important regulators of the microtubule cytoskeleton, via regulation of activity of several downstream effector proteins. Microtubules play a major role in defining cell shape and polarity through the specific interaction of their plus ends with proteins at the cell cortex. This plus end capture of microtubules has been attributed to a number of plus end–binding proteins, whose activities are influenced by Rho GTPases. CLIP-170, for example, can simultaneously bind to microtubules and to the scaffold protein IQGAP, a Rac/Cdc42 effector that is enriched at the leading edge of migrating cells. Expression of constitutively active Rac or Cdc42 enhances the ability of CLIP-170 to bind to IQGAP, thereby promoting plus end capture (M. Fukata et al., 2002). Interestingly, mDia is a downstream effector protein of RhoA, which is involved in both the regulation of the actin cytoskeleton as well as the microtubule cytoskeleton. Both Rac1 and Cdc42 can influence microtubule stability also by mediating PAK signalling to stathmin, an important microtubule destabilizing protein (Daub, Gevaert
et al. 2001). RhoA can promote the formation of stable and aligned microtubules via signalling through mDia (Palazzo, Cook et al. 2001; Yamana, Arakawa et al. 2006).

1. 7. 3 Rho GTPases and cytoskeleton-dependent processes

Dynamic rearrangements of the cytoskeleton and cell adhesion are required for various cellular processes, such as shape changes, migration, and cytokinesis and cell polarity. Reorganization of actin filaments and cell-substratum contacts is believed to be involved in cell motility. Membrane ruffling is observed at the leading edge of motile cells and is also thought to be essential for cell motility. A force arising from actin polymerization appears to drive lamellipodia protrusion. This process is thought to be regulated by Rac. A force derived from myosin II triggered by MLC phosphorylation, in the area of membrane ruffling and in the posterior region of motile cells may also contribute to the cell movement (K.Kaibuchi 1999). Adhesive structures such as tight junctions (TJs), adherens junctions (AJs) and desmosomes are linked to the cytoskeleton and determine epithelial morphology and functionality and therefore play an essential role in the maintenance of tissue architecture (Halbleib and Nelson 2006). Rho GTPases have been shown to regulate the formation and maintenance of these adhesive structures (AJs and TJs) (Mertens, Pegtel et al. 2006). Interestingly, Rho GTPase signalling can contribute not only to stabilisation but also to disassembly of AJs leading to EMT. RhoA signalling via mDia and subsequent actin polymerisation is required for formation and maintenance of AJs, whereas RhoA signalling through ROCK results in disruption of AJs caused by increased contractility (Sahai and Marshall 2002). Various studies have suggested that Rho GTPase signalling is required for the regulation of membrane-trafficking processes such as exocytosis, endocytosis and phagocytosis, processes which are dependent on actin and microtubule dynamics and essential for establishment of asymmetrical distribution of proteins in polarized cells (Symons and Rusk 2003).

1.8 Rac1

Rac with Rho and Cdc42 is one of the most extensively studied members of Rho GTPases family and it was initially discovered as Ras-related C3 botulinum toxin substrate 1 in 1989. There are three distinct Rac isoforms encoded by different genes: Rac1-2 and 3, which share between 89-93% amino acid sequence identify (Didsbury
et al 1989; Wherlock and Mellor, 2002). Rac1 and Rac3 are ubiquitously expressed and therefore regulate a wide variety of cellular processes, while Rac2 is predominantly expressed in cells of the hematopoietic lineage. Rac can be found in the cell in two forms: an active form GTP-binding ligand and an inactive GDP-bound form. In response to extracellular signals, interconversion between these two states is made by the GEF that convert Rac in an active form, while the GAP report it in an inactive state (Van Aelst and D'Souza-Schorey 1997; Etienne-Manneville and Hall 2002). Thus it acts as molecular switches cycling between an active GTP-bound and an inactive GDP-bound form, Rac in the cytoplasm is sequestered by GDI that bind and mask its group prenilico responsible for its localization to the plasma membrane. The dissociation of GDI allows Rac to translocate to the membrane and be functionally active. It has been shown that the signal transduced by integrins promotes the dissociation of the complex Rho GDI-Rac and the recruitment of Rac on the plasma membrane. Like most other GTPases, these proteins adopt different conformations depending on the bound nucleotide, the main differences lying in the conformation of two short and flexible loop structures designated as the switch I and switch II region.

1.8.1 Rac1: effectors and functions

Rac1 has been shown to play a fundamental role in a wide variety of cellular processes, including actin cytoskeletal reorganization, cell transformation, the induction of DNA synthesis, superoxide production, axonal guidance and cell migration. Rac1 is implicated in reorganization of the actin cytoskeleton, specifically lamellipodia formation, which is thought to contribute to cell movement (Ridley, Paterson et al. 1992). Rac1 was shown to reside at the leading edge of migrating cells, and microtubule growth can activate Rac1 to promote lamellipodial protrusion. The actin-rich lamellae formed at the leading edge in fibroblasts are similar to the membrane dynamics at developing cell-cell contacts in epithelial cells, where actin is recruited to physically strengthen adherens junctions (Vasioukhin, Bauer et al. 2000; Ehrlich, Hansen et al. 2002). The main effectors of Rac involved in this process are: PAK (p21-activated kinase), PI4-P5K, WAVE (family verprolin-homologous protein) belonging to the family of proteins WASP, IQGAP and SRA1 (Rac1 associated protein) (Jaffe and Hall, 2005). Each of these effectors contributes to the
reorganization of the actin cytoskeleton through different mechanisms. IQGAP and SRA1 directly interact with actin filaments in vitro. IQGAP connects actin filaments with microtubule stabilizing it, while SRA1 forms a complex with WAVE, which activates Arp 2/3 complex. PI4-P5K directly interacts with Rac-GTP by determining the increase in the levels of PIP2, with consequent release of the proteins involved in the mechanism of capping” of actin. TIAM1 is the main GEF that regulates actin polymerization Rac-mediated. The actual model proposed is that TIAM1 binds ARP 2/3 and following this link, TIAM1 actives Rac. After this activation Rac1 binds WAVE complex and promotes actin filaments nucleation through ARP 2/3 complex with subsequent lamellipodia formation. Finally, another effector of Rac1 is a serine threonine kinase PAK, located at the level of the ruffle membrane. PAK1 binds Rac1 in aGTP-dependent manner, potently stimulating PAK kinase activity and leading to cytoskeletal dynamics, adhesion, and transcription. PAK, activated by Rac1, induces the phosphorylation of proteins involved in the reorganization of the actin cytoskeleton promoting the lamellipodia formation (Bishop and Hall, 2000). Rac1 has been shown to mediate actin polymerization in other cell types including stimulated blood platelets, lymphocytes, mast cells, and endothelial cells. The actin polymerization machinery is another mediator between Rac1 activation and E-cadherin-mediated cell–cell adhesion. Recruitment and activation of Rac1 and Cdc42 by homophilic E-cadherin ligations can induce actin polymerization through the Arp2/3 complex and WAVEs or WASP. Together with other actin-binding proteins such as cortactin, polymerized actin filaments reinforce cadherin-mediated adhesions (Gates and Peifer 2005; Scott and Yap 2006). Another mechanism of AJ control by Rac1 includes its binds to IQGAP1. IQGAP1 is localized to sites of cell-cell contact in epithelial cells and it negatively regulates E-cadherin-mediated cell-cell adhesion by interacting with b-catenin. In this way it induces the dissociation of a-catenin from b-catenin. Rac1 positively regulate cadherin-mediated cell-cell adhesion by inhibiting the interaction of IQGAP1 with b-catenin. Rac in the GTP-bound form interaction with IQGAP1 and thus prevent the association of IQGAP1 with β-catenin, which results in the stabilization of cadherin–catenin complex. In the GDP-bound form Rac do not interact with IQGAP1, which associates with β-catenin, thereby displacing α-catenin from its binding to β-catenin. This leads to a dissociation of α-catenin linked to actin filaments from the cadherin–catenin complex, conferring a weak adhesive
activity (J. Noritake, 2004). Based on these studies E-cadherin exists in a dynamic equilibrium between the E-cadherin-β-catenin-α-catenin complex and the E-cadherin-α-catenin-IQGAP1 complex at sites of cell-cell contacts. When the amounts of activated Rac1 increases, Rac1 binds IQGAP1, thereby inhibiting its bind to β-catenin and IQGAP1 cannot dissociate α-catenin from the cadherin-catenin complex leading to strong adhesions. By contrast, when the amounts of inactivated Rac1 increases, IQGAP1 is freed from Rac1 and interacts with β-catenin to dissociate β-catenin from the cadherin-α-catenin-complex, resulting in weak adhesions (Figure 11). Because actin cytoskeletal dynamics are intimately linked to vesicular trafficking, it is not surprising that Rac1 has been implicated in this process. Rac1 is also involved in one or more steps of the phagocytosis process by mediating localized polymerization of actin at the membrane to promote the internalization of attached particles and microorganisms.

Rac1 is implicated in reactive oxygen species (ROS) generation in primary cells via NADPH oxidase via Nox and it can regulate different functions such as transcription factor activation, proliferation, transformation, apoptosis. In addition to its effects on the actin cytoskeleton, Rac1 signaling can affect cell growth through a variety of mechanisms. Rac1 signals through PAK to activate c-Jun Nterminal kinase (JNK), placing Rac1 between Ha-Ras and MEKK in a signaling cascade from growth factor receptors and v-Src to JNK activation Rac1 has also been shown to influence nuclear signaling through its effectors MLK2/3, which have been shown to activate the JNK pathway (Teramoto, Coso et al. 1996). Rac1 signaling can be important for cellular
transformation via modulation of anti-apoptotic and cell cycle machineries. Rac1 positively regulates transcription at NFkB transcription factor-dependent promoters and facilitates phosphatidylinositol-3 kinase (PI3K)-dependent activation of AKT ser/thr kinase, thereby permitting the survival of transformed cells (Perona, Montaner et al. 1997). Rac1 can also influence transformation through regulation of cyclin D1, a cell cycle protein that is frequently overexpressed in cancer (Westwick, Lambert et al. 1997; Joyce, Bouzahzah et al. 1999) (Figure 12).

**Fig. 12 Rac1 signaling model.** Rac1 is a signal transducer and receive information via activated GEFs from a several extracellular stimuli such as receptor kinases, G protein-coupled receptors, and integrins. The GTP-bound Rac1 adopts an active conformation capable of binding effector molecules such as IQGAP, IRSp53/WAVE, PAK, MLK2/3, and p67phox. These effectors regulate many cellular functions, such as cytoskeleton remodeling, microtubule stability, gene transcription, and superoxide production (this picture was taken from Bosco, Mulloy et al. 2009).
AIM OF THE PROJECT
2 AIM OF THE PROJECT

The aim of this project is to analyze the role of the small Rho GTPase Rac1 in the acquisition of the polarized phenotype in epithelial cells. The small GTPases Rac1 is a member of the family of Rho GTPases, monomeric proteins of 20-30 kDa, which belong to the superfamily of the Ras GTP-binding proteins. They act in the cell as molecular switches cycling between a GTP-bound active state and a GDP-bound inactive state. Two classes of regulators control the nucleotide state: GEFs (guanine nucleotide exchange factors), which promote the exchange of GDP for GTP, and GAPS (GTPase-activating proteins) that enhance the intrinsic GTPase activity of Rho GTPases. Only in the GTP-bound state these proteins are able to bind effectors proteins and transduce signals from a large variety of membrane receptors, including adhesion receptors (such as integrins), and G-protein coupled receptors, that in turn have been implicated in many cellular process such as gene transcription, vesicle trafficking and cytoskeleton assembly. Moreover, it was recently shown that Rac1 and its effectors are key regulators of microfilament and microtubule dynamics and, consequently, are crucially involved in polarity signalling.

We used as a model system FRT cells (an epithelial cell line derived from Fisher rat thyroid), because they are highly polarized and have been well characterized in our laboratory. In particular, I worked to develop a system of inducible inactivation of Rac1 in order to study the effects that follow the activation of this protein. My first aim has been to stably transfect FRT cells with a cDNA encoding a dominant negative form of Rac1 fused to the ligand-binding domain of the estrogen receptor ER (ER-Rac1N17).

The chimeric protein ER-Rac1N17 contains a threonine to asparagines substitution at residue 17 which abolishes the protein’s affinity for GTP and reduces the affinity for GDP. For this reason ER-Rac1N17 is always in a nucleotide free state or in its inactive, GDP-bound state. As a consequence, it might bind strongly Rac1GEFs, competing with endogenous Rac1 and inhibiting it. Rac1N17-Rac1GEFs complex is a non productive complex unable to generate a downstream response (L.Bishop and A.Hall). The activation status of the chimeric protein ER-Rac1N17 can be perfectly manipulated through the use of 4-OH-tamoxifen but several minutes are required to see the effect. The binding of the ligand to the estrogen receptor induces the
translocation of the chimeric protein ER-RacN17 to the membrane where it can
interact with its many effectors.

In my thesis project I tested the hypothesis that a reduced Rac1 activity caused
defects in the polarization of the FRT cell line.

To this aim, we analyzed the effects of the ER-Rac1N17 protein, activated upon 4-
OH-tamoxifen addition, on some parameters related to the epithelial polarized
phenotype: 1. the ability to heal a wound in a confluent monolayer by directional
migration; 2. the acquisition of trans-epithelial resistance (TER) by confluent
monolayers grown on filters; 3. cell aggregation and formation of polarized cysts in
suspension culture.

Cell migration of individual cells or groups of adherent cells is a polarized process
because migrating cells exhibit a front rear polarized morphology. It has been shown
that directional migration of many cell types requires coordinated crosstalk between
Rho GTPases and polarity proteins. To study the effects of the ER-Rac1N17 on cells
migration we performed the wound healing test on confluent monolayers in plastic
dishes.

Rac1 might play a role in the acquisition and maintenance of cell polarity by
regulating the formation of junctionals complexes. The turnover of junctionals
complexes anchoring adjacent epithelial cells and their integrity is crucial for the
acquisition of the polarized phenotype. Tight junctions are responsible of for the
establishment of a selective epithelial barrier and are essential to avoid the mixing of
membrane lipids and proteins between the apical and the basolateral domains. We
hypothesized that Rac1 plays a role in regulating the formation and integrity of
junctionals complexes. This process can be monitored by measuring the level of TER,
which is the resistance that the epithelial monolayer opposes to the passage of ions and
represents an index of the degree of polarization of the cell monolayer. When
epithelial cells are grown on top of filters in bicameral systems they undergo
sequential stages of functional maturation, that lead to junction establishment and
reinforcement.

A characteristic morphogenetic event that can be observed when FRT epithelial cells
are grown as aggregates in suspension culture is aggregate compaction and their
evolution into polarized three-dimensional structures, named cysts (or inverted
follicles). The ability to form cysts is linked to the acquisition of a polarized
AIM OF THE PROJECT

phenotype: cells are connected by adherens and tight junctions, segregate membrane proteins in distinct plasma-membrane domains, and vectorially pump fluid inside the follicular lumen. We decided to investigate the effect of Rac1 inhibition on the morphogenetic ability of FRT cells to develop these polarized cysts.

To unravel the molecular mechanism by which Rac1 affects the acquisition and maintenance of cell polarity, we investigated the establishment of E-cadherin-dependent cell-cell contacts, which is another key event in the process of epithelial polarization. We determined the dynamics of subcellular localization of Rac1 and of E-cadherin molecules to understand if, and how, both proteins are intimately related functionally. We performed several experiments to prove that Rac1 inhibition impairs E-cadherin ability to form functional contacts.

Overall, in this work a novel experimental approach, i.e. the use of an inducible dominant-negative form of the Rac1 protein, has been used to demonstrate the involvement of this small GTPase in the epithelial polarization process and to unravel its mechanism of action.
RESULTS
RESULTS

3.1 Goals of the project and model systems

Epithelial polarity is a complex phenomenon directed by external cues, such as cell-cell and cell-matrix interactions, and addressed to the acquisition of a unique cytoarchitecture that concerns the specialized organization of organelles, plasma membrane, and cytoskeleton.

Rac1 belongs to the Rho GTPases family and through its effectors regulates microfilament and microtubule dynamics (ref review). Since epithelial polarization leads to drastic changes in the cytoskeleton organization (Mays et al, 1994; Bacallao & MacNeill, 2009; Weisz and Rodriguez-Boulan, 2009; Datta et al, 2011) it has been hypothesized that Rac1 could play a crucial role in this process. Indeed, several findings showed that the establishment of the initial zone of cell-cell contacts requires the local activation of Rac (Ehrlich et al, 2002; Hoshino et al, 2004; Lambert et al; 2002) which, driving the formation of actin-based protusions (Ridley 2006), can promote the formation of new E-cadherin-based contacts between neighboring cells. In addition, Rac can also stimulate the activity of PI3K and the Cdc42- and Arp2/3-mediated actin nucleation, as well as the recruitment of cortactin (Vasioukinin et al, 2000; Ehrlich et al, 2002), all of which may help to promote an increase of the zone of cell-cell contacts. Together with Cdc42, Rac appears also to contribute to the assembly of functional tight junctions (Chen and Macara 2005; Mertens et l, 2005). Although all these findings support a critical role of Rac in epithelial polarization, it is not clear how and at which steps Rac regulates this process.

The present project was mainly focused on the analysis of the role of the Rac1 protein in the acquisition and maintenance of the polarized phenotype in epithelial cells. One of the potential ways to study the function of a protein is to block its activity within cells. The use of dominant-negative mutants can be a simple tool to achieve this task. These mutated proteins interfere with the function of their normal cellular counterparts
RESULTS

or with proteins that interact with them. To study whether and how Rac1 is implicated in the acquisition of a polarized phenotype we decided to use its dominant negative form (Rac1N17). A model of how the dominant negative RasN17 functions has been previously proposed by Feing (Feing LA, 1999) and Bishop and Hall have speculated that it was the same for Rac1N17 (Bishop L and Hall A, 2000).

Rac1N17 protein contains a threonine to asparagine substitution at residue 17. As a consequence it has a higher affinity for GDP than for GTP and fails to bind downstream effectors in vivo. The threonine residue at position 17 binds magnesium ions (Mg^{2+}) that will be located in the nucleotide-binding pocket of both GTP- and GDP-bound Rac1. Rac1N17 exhibits reduced Mg^{2+} binding (Paduch et al, 2001; ma la dimostrazione formale è solo per Ras, lo lasciamo lo stesso?). Proper binding of Mg^{2+} may be necessary to Rac1 in order to reach an active conformation, because the effector domain also binds this tethered Mg^{2+} when Rac1 switches to the active state. However, Rac1N17 has another important property: it binds more tightly to Rac1GEFs than wild-type Rac1. Indeed, GDP-bound Rac1 binds to Rac1GEFs with low affinity. This binding promotes the release of bound GDP, leading to a high affinity transient binary complex between nucleotide-free Rac1 and the RacGEF. This complex would be very stable in cells except for the fact that nucleotides have a higher affinity for nucleotide-free Rac1 than does RacGEF. As GTP is present at higher concentrations than GDP, GTP quickly binds nucleotide-free Rac1 and displaces the RacGEF. Rac1N17 binds more strongly to RacGEFs than does normal Rac, for two reasons. First, Rac1N17 shows severely reduced affinities for nucleotides, thus guanine nucleotides are less likely to displace RacGEFs from these mutants in the cells. Second, the nucleotide-free form of the mutant has higher affinity for RacGEFs than does wild-type Rac. Rac1N17/ Rac1GEFs complex is a non-productive complex unable to generate a downstream response.

As a model cellular system we used FRT cells, an epithelial cell line derived from Fischer rat thyroid, that are well polarized both morphologically and functionally (Nitsch L et al., 1985) and have been well characterized in our laboratory (Nitsch, et al, 1985, Zurzolo et al, 1991, 1994, 1996). FRT cells are metabolically dedifferentiated, but still express the thyroid-specific transcription factor Pax8 (Mascia...

In agreement with previously data showing that Rac1 regulates cell proliferation through a variety of mechanisms (Bosco et al, 2009) and that Rat 2 fibroblasts expressing Rac1N17 are arrested in G2/M (Moore et al, 1997), we found that the expression of Rac1N17 in FRT cells impairs their growth (Mascia, personal communications). Thus, we were not able to obtain FRT cell clones stably expressing Rac1N17 and, therefore, we concluded that cells cannot tolerate a prolonged expression of inhibitory Rac1 mutant. For this reason we generated an inducible chimeric protein (ER-Rac1N17; Fig. 1A) by fusing Rac1N17 downstream of the ligand-binding domain (ER) of the murine mutant estrogen receptor (G525R; Littlewood TD, 1995). This mutant no longer binds natural estrogens (which are normally present in the serum of the culture medium), possess no TAF-2 transactivation activity, and it is responsive only to activation by the synthetic steroid 4-OH-tamoxifen (Littlewood TD, 1995), therefore overcoming all possible drawbacks related to the use of hormone-binding domain as a switch (Figure 1).

**Fig. 1** Schematic drawing of inducible (ER-Rac1N17) and non-inducible (Rac1N17) dominant-negative form of Rac1. **a)** ER-Rac1N17 contains the hormone binding domain of mutated murine estrogen receptor (which is responsive only to 4-OH-Tamoxifen [4-OHT]) fused to the mutant form of Rac1, Rac1N17. Upstream of ER domain there is the AU1 tag which consists of a small peptide of six amino acids (DTYRYI) **b)** Rac1N17 construct bears a FLAG tag at the N-terminus.
3.2 Transfection of ER-Rac1N17 in FRT cells and analysis of its expression

My first aim has been to stably transfect FRT cells with the cDNA encoding the chimeric protein ER-Rac1N17 under the control of the cytomegalovirus promoter. After neomycin selection we isolated many resistant clones: a lot of them resulted completely negative, other ones displayed low percentage of positive cells. Totally, it was rather difficult to obtain pure positive clones possibly because the overexpression of Rac1N17, although inducible, could display a residual activity not compatible with cell survival. Nevertheless, by cloning dilution we were able to obtain several clones expressing the chimeric protein at different detectable levels and almost in all cells as assessed both by western blot analysis and by indirect immunofluorescence assays (Fig. 2a, b). To discriminate between the transfected chimeric protein and endogenous Rac1 we used an antibody against the AU1 tag (Fig. 1a), a peptide of 6 aminoacids (DTYRYI) derived from the protein of the bovine papilloma virus 1 (BPV1) or an antibody against the ER domain itself. As shown in Fig. 2a, ER-Rac1N17 migrated in the gel according to its molecular weight (57kDa). Moreover the clones displayed a good fluorescent signal (Fig. 2b).

To better understand how we could manipulate the system, we performed kinetics of stimulation with 4-OH-tamoxifen. To this aim, cells were grown on glass slides for two days and incubated with 4-OH-tamoxifen (100 nM in the culture medium) for various times: 10’, 30’, 1 h, 5 h, 10h e 24h. At intervals, cells were fixed and stained with anti-AU1 antibodies for immunofluorescence. ER-Rac1N17 was diffusely distributed in the cytosol in the absence of 4-OH-tamoxifen. A period of 30 minutes incubation with 4-OH-tamoxifen was sufficient for a detectable fraction of the ER-Rac1N17 protein to reach the plasma membrane and by 1 hour the protein localized on the plasma membrane of all cells, at sites of cell-cell contacts. Hence, ER domain prevents Rac1N17 mutant to move to the cell surface; conformational changes, due to 4-OH-tamoxifen binding, lead to its translocation. Taken together these data indicate that the activation status of ER-Rac1N17 can be manipulated through the use of 4-OH-tamoxifen and that the activation occurs within minutes.
**RESULTS**

Fig. 2 a) Western blot analysis of FRT cells stably expressing ER-Rac1N17. Cells were grown on dishes to confluency and lysed in RIPA buffer. Proteins were separated by SDS-PAGE and revealed by using a specific antibody against AU1 tag. b) Immunofluorescence assay of FRT cells stably transfected with ER-Rac1N17. Cells were treated with 4-OH-tamoxifen for 1 hour, fixed with 4% paraformaldehyde for 20', permeabilized with 0.3% TX100 for 5' and stained with the AU1 antibody. Note that ER-RacN17 is expressed at different detectable levels in the clones.
RESULTS

Fig 3. Kinetics of induction of ER-Rac1N17 with 4-OH-tamoxifen. FRT cells expressing ER-Rac1N17 were stimulated for various times with 100nM 4-OH-tamoxifen (10’, 30’, 1h, 5h, 10h, 24h) and then fixed and analyzed by immunofluorescence (a). Note that after 30’, in almost all cells, the majority of ER-Rac1N17 is localized in the plasma membrane at cell-cell contacts. Alternatively cells were lysed and samples separated in SDS-PAGE and revealed with an anti-ER antibody (b). Quantification of three independent experiments is shown. Bars, SD.
Note that there is a gradual increase of protein levels over time. c) Cells were incubated 24 and 48 hours with 4-OH-tamoxifen and then treated as in b. Note that expression levels of the chimeric protein are higher in presence of tamoxifen.

Moreover we observed that, upon 4-OH-tamoxifen binding, the chimeric protein was mostly localized on the plasma membrane like the endogenous Rac1 (Fig. 3a). Indeed, in confluent wild-type FRT cells Rac1 was present both in the cytoplasm, either as diffuse or in small clusters, and on the plasma membrane, at sites of cell-cell contacts. Similarly, in non-confluent cells (where cells form colonies of different size) Rac1 was localized at the cell surface in lamellipodia and membrane ruffles, which are peripheral membrane structures related to the migratory activity of the cells, and eventually end up with fusion of the colonies. Furthermore the localization of ER-RacN17 was comparable to that of non-inducible dominant-negative form of Rac1 (Flag-Rac1N17; Fig. 3b), thus indicating that the fusion with ER-domain does not alter the behavior of the mutant protein. These data also indicate that Rac1 recruitment to the plasma membrane occurs independently of its nucleotide-bound and active state as previously described for inhibitory mutants of Ras (Feig, 1999). All these results suggest that the chimeric protein is a good tool to study the role of Rac1 in the polarization process.

Fig 4. a) Immunofluorescence of Rac1 protein in FRT cells. Confluent and non-confluent (at the stage of small colonies) FRT cells were fixed and stained with anti-Rac1 antibody. Note that Rac1 is localized both on the plasma membrane and in the cytoplasm. b) Immunofluorescence of Flag-Rac1N17. After fixation, cells were stained with an anti-FLAG antibody and revealed with a FITC-conjugated secondary antibody.
RESULTS

Interestingly, by investigating the expression of ER-RacN17 upon tamoxifen incubation (Fig. 3b, c) we found that an increase of protein levels occurred over time as it was strongly evident after 24 and 48 hours of treatment (Fig. 3c). This could imply that the binding with tamoxifen stabilizes the protein preventing its degradation as it has previously proposed for an inducible, dominant-negative mutant of Ras (De Menna et al. 2012) and suggests an additional mechanism by which the ER-domain could act.

The change in the distribution ER-Rac1N17 in the cells after 4-OH-tamoxifen stimulation was also examined by a membrane/cytosol fractionation assay. The relative amount of ER-Rac1N17 that was associated to membrane fractions and to cytosolic fractions, in controls and in 4-OH-tamoxifen stimulated cells, was examined. In this experiments FRT cells stably transfected with ER-Rac1N17, grown on dishes for 48 hours, were stimulated with 4-OH-tamoxifen for 1 hour. In good agreement with immunofluorescence data, in the absence of 4-OH-tamoxifen the protein was distributed mainly in the cytosolic fraction while, after 1 hour of tamoxifen treatment, about 90% of the total protein was found in the membrane fraction (Fig. 5).

Fig. 5  Membrane/cytosol fractionation assay. FRT cells stably expressing FRT-ER-Rac1N17, grown in dishes to confluency, were incubated for 1h with 4-OHT and, then, homogenized with a cell scraper in hypotonic buffer. After nuclei sedimentation, samples were centrifuged at 33,000 rpm for 30 min at 4°C in order to separate cytosolic fraction (supernatant) from membrane fraction (pellet). The latter was resuspended and solubilized in 2X Laemmli buffer. The samples were then subjected to western blot analysis loading on the gels the same amount of the two fractions. As controls the distribution of E-cadherin and GAPDH (membrane and cytosolic protein, respectively) were analyzed. Note that upon 4-OHT incubation ER-Rac1N17 translocates from cytoplasm (Cyt) to the membrane fraction (M).
We then asked whether the effects of 4-OH-tamoxifen stimulation would be maintained. To this aim, 4-OH-tamoxifen was added to the cultures for 1 hour and several washings with culture medium were made to completely remove 4-OH tamoxifen. Cells were fixed and stained with the anti-AU1 antibody either 24 or 48 hours after further culture in normal culture medium. It turned out that both after 24 and 48 hours ER-Rac1N17 was still localized on the plasma membrane, at sites of cell-cell contacts. There are two possible explanations for this results. The first is that we cannot really remove 4-OH-tamoxifen and that it has a half-life sufficiently long to continue to activate ER-Rac1N17. The second is that ER-Rac1N17, once it is activated, remains on plasma membrane for long time and has a long half-life. To distinguish between these two hypotheses it would be useful to analyze the half-life of the chimeric protein and compare it to the endogenous one.

Fig. 6) Analysis of the effects of tamoxifen removal on ER-Rac1N17 distribution. ER-Rac1N17 expressing cells were treated 1h with 4-OHT. Then, 4-OHT was removed by several washings with culture medium and cells were fixed after 24h and 48h, and ER-Rac1N17 distribution was analyzed by immunofluorescence assay. Note that upon tamoxifen removal in a high percentage of the cells ER-Rac1N17 is still present on plasma membrane.

3.3 Consequences of the expression of the dominant-negative form of Rac1 on the endogenous Rac1 protein

As previously found for other GTPases (Feig et al 1999), it is expected that inhibitory mutants should act by interfering with the activity of the wild-type protein. Hence, we investigated whether and how the expression of ER-Rac1N17 has an effect on endogenous Rac1 (Fig. 7). We found that Rac1 is expressed at comparable levels both in absence and in presence of 4-OH-tamoxifen (Fig. 7a), indicating that the stable expression of the mutant does not affect the synthesis and/or the turnover of endogenous Rac1. Moreover ER-Rac1N17 expression does
RESULTS

a) Example of Western Blot (WB) showing the expression of α-Rac1 over different time points:

- Ctr
- 10'
- 30'
- 1h
- 5h
- 10h
- Ctr
- 24h

b) Example of Immunofluorescence images showing the localization of ER-Rac1N17, Rac1, and the merge:

- Ctr
- 1h
- 5h
- 10h
- 24h
RESULTS

c) Analysis of the effects of the expression of dominant-negative Rac1 in FRT cells. (a) Amounts of Rac1 in FRT-ER-Rac1N17 cells. FRT cells stably expressing FRT-ER-Rac1N17, grown on dishes or on coverslips up to confluency, were incubated with 4-OHT for the indicated times and subjected to western blotting (a) or immunofluorescence assay (b). Briefly, at the end of each time point, cells were lysed with Ripa Buffer and, after SDS-PAGE separation, proteins were revealed using an anti-Rac1 antibody. Alternatively cells were fixed and permeabilized with methanol/Triton X100 and stained with specific antibodies against ER and Rac1 (which reveals both ER-RacN17 and endogenous Rac1). Note that the expression of dominant-negative mutant does not affect either the expression levels of Rac1 or its localization (compare the signal of Rac1 in transfected and untrasfected cells). (c) Upon 4-OHT incubation (1h) cells were lysed and fractionated as described in Fig. 5. The samples were then subjected to western blot analysis. Note that in absence and presence of 4-OHT Rac1 is distributed both in cytosol (Cyt) and in membrane fraction (right panel) as in wild-type cells (left panel). (d) The activity of Rac1 is measured through a GTPase pull down assay by using glutathione beads coupled to Cdc42/Rac1 interactive binding region (CRIB). After precipitation, samples were revealed by immunoblotting with an anti-Rac1 antibody. 1/10 of lysates before GST pull down were loaded on the gel and revealed with anti Rac antibody (totals). A decrease of Rac1 activity was observed after 3 hours of 4-OHT incubation.

Fig. 7 Analysis of the effects of the expression of dominant-negative Rac1 in FRT cells. (a) Amounts of Rac1 in FRT-ER-Rac1N17 cells. FRT cells stably expressing FRT-ER-Rac1N17, grown on dishes or on coverslips up to confluency, were incubated with 4-OHT for the indicated times and subjected to western blotting (a) or immunofluorescence assay (b). Briefly, at the end of each time point, cells were lysed with Ripa Buffer and, after SDS-PAGE separation, proteins were revealed using an anti-Rac1 antibody. Alternatively cells were fixed and permeabilized with methanol/Triton X100 and stained with specific antibodies against ER and Rac1 (which reveals both ER-RacN17 and endogenous Rac1). Note that the expression of dominant-negative mutant does not affect either the expression levels of Rac1 or its localization (compare the signal of Rac1 in transfected and untrasfected cells). (c) Upon 4-OHT incubation (1h) cells were lysed and fractionated as described in Fig. 5. The samples were then subjected to western blot analysis. Note that in absence and presence of 4-OHT Rac1 is distributed both in cytosol (Cyt) and in membrane fraction (right panel) as in wild-type cells (left panel). (d) The activity of Rac1 is measured through a GTPase pull down assay by using glutathione beads coupled to Cdc42/Rac1 interactive binding region (CRIB). After precipitation, samples were revealed by immunoblotting with an anti-Rac1 antibody. 1/10 of lysates before GST pull down were loaded on the gel and revealed with anti Rac antibody (totals). A decrease of Rac1 activity was observed after 3 hours of 4-OHT incubation.

alter the localization of endogenous Rac1 (Fig. 7b), which, as in wt FRT cells (Fig. 4a,b), is distributed both on the cytoplasm and on the plasma membrane. Consistently, we found that both in absence and in presence of 4-OH-tamoxifen Rac1 resulted in cytosol and membrane fraction (Fig. 7c, right panel) in a ratio similar to that observed
in wild-type cells (Fig. 7c, left panel). By GFT-CRIB pull down assay we measured the activity of Rac1 in controls and after induction of the mutant expression. We found that the amount of active Rac1 decreased in the cells after 3 hours of 4-OH-tamoxifen stimulation (Fig. 7d), indicating that the expression of a dominant negative of Rac1 interferes with the activity of the wild-type endogenous protein. Moreover these results further confirm that the ER-Rac1N17 mutant represents a good experimental tool.

### 3.4 Analysis of the role of Rac1 in the acquisition of cell polarity

In order to investigate the role of Rac1 in the acquisition of the polarized phenotype we examined the effects of the ER-Rac1N17 induction on parameters related to cell polarization such as directional migration, trans-epithelial resistance (TER) acquisition by confluent monolayers grown on filters, cell aggregation and formation of polarized cysts in suspension culture.

#### 3.4.1 The dominant-negative Rac1 reduced the migratory ability of cells.

Cell migration is a multistep process involving changes in the cytoskeleton, cell-substrate adhesions and the extracellular matrix component. Cell migration is generally initiated in response to extracellular stimuli, like diffusible factors, signals on neighboring cells and/or signals from the extracellular matrix. The idea that Rho family GTPases could regulate cell migration is well established and is derived, on one side, from the observation that they mediate the formation of specific actin containing structures such as lamellipodia (Etienne-Manneville and Hall, 2002) and, on the other side, because they regulate several other processes relevant to cell migration, including cell-substrate adhesion, cell-cell adhesion, protein secretion and vesicle trafficking. The epithelial cells do not migrate as single cells but rather migrate as sheets or clusters. Upon scratching of an in vitro cell monolayer, the synchronized movement of the cell sheet is induced. As with single cells, the migrating sheet detects the direction of migration and polarizes with the protrusive activity constrained to the
results

Interaction with neighbors can provide additional directional cues to cells in the monolayer. We therefore decided to analyze whether Rac1 inhibition affected cell migration. To this aim we performed wound-healing assays, which are simple, low-cost and well-developed methods to study directional cell migration in vitro based on three main steps: i) creation of "wound" in a cell monolayer with a micropipet tip; ii) capturing of images at the beginning, and at regular intervals, until the wound is closed; iii) measuring the distance between the two sides of the wound over time, thus determining the ability to heal the wound. In our experiments cells were grown to confluency and, 24 hours after plating, they were shifted to a serum free medium for the rest of the experiment in order to prevent cell proliferation. In this way, the repair of the wound can be attributed exclusively to the cell migratory capability and not to their replication. We performed the assay with or without 4-OH-tamoxifen, which was added to the culture medium immediately after making the wound. We monitored the repair of the wound by observing the cells with phase contrast microscopy and taking pictures at different times. We found that wild-type FRT cells completely repaired the wound within 48 hours both in the presence or absence of 4-OH-tamoxifen indicating that tamoxifen, per se, does not influence the migration of FRT cells. In FRT cells stably expressing ER-Rac1N17 there was a significant delay in the healing of the wound upon 4-OH-tamoxifen-treatment in contrast to untreated cells that behaved as wild-type cells (Fig. 8b). These results indicate that the over-expression of a dominant-negative form of Rac1 significantly delays the closure of the wound do to a slower cells migration that is likely do to Rac1 inactivation.
RESULTS

FRT WT

a) 

Ctr | 4-OHT

0h

24h

48h

![Cell images](image)

![Bar graph](image)
**RESULTS**

**Fig. 8**

Evaluation of the effect of ER-Rac1N17 expression

**FRT-ER-Rac1N17**

<table>
<thead>
<tr>
<th>Ctr</th>
<th>4-OHT</th>
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<tr>
<td>0h</td>
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<tr>
<td>24h</td>
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<tr>
<td>48h</td>
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![Graph showing wound closure percentage over time](image)

- FRT-ERRac1N17 w/o 4-OHT
- FRT-ERRac1N17 + 4-OHT
RESULTS

Fig. 8 Evaluation of the effect of ER-Rac1N17 expression on the migratory properties of FRT cells. FRT (a) or FRT-ER-Rac1N17 (b) cells were seeded on plastic dishes to confluency and 24h after plating a scratch on the monolayer was performed with a tip. The healing of the wound was monitored over time in the absence (Ctr) or in the presence of 4-OHT. Pictures were taken by phase contrast microscopy immediately after the scratch (Time 0) and 24h and 48h later. Representative fields of the scratch are shown for each time point. Pixel densities in the wound areas was measured using the Cell software (Olympus Biosystem Gmb) and expressed as percentage of wound aperture (normalized respect to the initial size of the wound posed as 100%) vs time. Error bars, mean ± SD. Rac1 inhibition by the activation of ER-Rac1N17 reduces the migratory ability of FRT cells.

3.4.2 The inhibition of Rac1 impairs the acquisition of transepithelial resistance (TER).

Transepithelial resistance (TER) measurements have become universally established as the most convenient and non-destructive method to evaluate and monitor the polarization process in epithelial cells. The confluency and tightness of the cellular monolayers is quickly determined by the increase in TER values. This event correlates with tight junction formation and the corresponding decrease in tight junction paracellular permeability. TER is the measure of the resistance that the epithelial monolayer opposes to the passage of ions and it increases during the days in culture up to reaching a plateau. FRT cells can reach quite high TER values (Zurzolo et al, 1991) if compared to other commonly used epithelial cell lines (i.e. MDCK, Caco2). TER was measured in FRT ER-Rac1N17 cell monolayers grown on transwell filters (support of election for polarized growth), in the absence or presence of 4-OH-Tamoxifen treatment, by utilizing the Millicel-ERS resistance monitoring apparatus. In a first set of experiments 4-OH-Tamoxifen was added 24 hours after plating, when cells were not completely polarized yet. First, it was verified that tamoxifen did not influence the acquisition of TER in FRT cells (Fig. 9a -grey curve). We found that the tamoxifen-activation of ER-Rac1N17 prevented the achievement of high TER values and, 24 hours after the addition of 4-OH-tamoxifen, the TER dropped to very low levels (Fig. 9b). In contrast, in untreated cells (Fig. 9b, black curve) TER increased over time and, four days after plating, reached very high, plateau values similar to wild-type FRT cells (Fig. 9a). These results indicate that the inactivation of Rac1 impairs the expression of high TER values. Moreover, to better understand the role of Rac1 in the acquisition of TER we repeated this experiment measuring the TER at early time-points (Fig. 9c). We found
that 5 hours after 4-OHT incubation the TER started to decrease and it progressively fell down until it reached a value similar to the empty filter, at 24 hours (Fig. 9c). This correlates with the fact that the activity of Rac1 initiates to decrease 3 hours after tamoxifen incubation (Fig. 7d) and, beside, suggests that it exists a threshold value, in terms of number of inactive Rac1 molecules, which must be exceeded to impair this cell function.
Fig 9. Rac1 inhibition affects TER acquisition in cells grown on filters in bicameral systems. (a-c) FRTwt and ER-Rac1N17 cells were grown on transwell filters and, 24h after plating, 4-OHT was added. The experiments were made both in presence (grey lines) and absence (black lines) of 4-OHT. (d) 4-OHT was added at the moment of seeding the cells and TER was measured after 24 hours. Note that the expression of the dominant negative form of Rac1 prevents the achievement of high TER values.

Since cell monolayers oppose resistance to ion passage already at 24-hours after seeding (Fig. 9A-C), in order to test whether Rac1 plays a role in the first steps of acquisition of TER (with respect to its maintenance), tamoxifen was added directly at the time of plating and TER was measured thereafter. In this condition the cells were unable to achieve a significant TER value compared to controls, that did not show any impairment in TER acquisition. This result suggests that prolonged Rac1 inhibition
affects the ability of cells to acquire transepithelial resistance and, therefore, to polarize.

3.4.3 The inhibition of Rac1 affected polarized cyst formation

Most epithelial organs consist of monolayers of cells that adhere to each other through cell–cell junctions. These monolayers are arranged in follicular or tubular structures that enclose a central lumen and are surrounded by a basement membrane. Individual epithelial cells establish the apical domain next to the lumen, indicating a link between cellular polarity and tissue structure. The characteristic morphogenetic event that can be observed when FRT epithelial cells are grown as aggregates in suspension culture, is that the aggregates undergo compaction and then evolve into polarized three-dimensional structures, indicated as cysts (or inverted follicles) (Tacchetti et al, 1991). The ability to form cysts is linked to the acquisition of a polarized phenotype: cells are connected by tight junctions, segregate membrane proteins in distinct plasma-membrane domains, and vectorially pump liquid inside the follicular lumen. Rac1 is known to be necessary to orient epithelial polarity throughout the polarization process and it has been shown to promote tubulocystic structures in the MDCK cellular system (O’Brien et al, 2001). To examine the role of Rac1 during cyst morphogenesis we plated cells expressing ER-Rac1N17 in suspension on agarose-coated dishes with or without 4-OH-tamoxifen and we monitored the cells over time. As shown in Fig 10, FRT cells formed cysts already at 24 hours after plating. Cists were stable and could be kept in suspension culture for long time (Fig 10). We found that FRT cells stably expressing ER-RacN17 behaved similar to wild-type cells when cultured in the absence of tamoxifen, while upon 4-OHT stimulation, cells aggregated, but this aggregates did not develop into polarized cysts (Fig. 10). These phenomena were more evident at 48h.
RESULTS

Fig. 10) Rac1 inhibition affects polarized cysts formation. wt FRT or FRT stably transfected with ER-Rac1N17 were plated in suspension on agarose coated dishes in absence or presence of 4-OHT. The cultures were monitored at intervals for the presence of cysts and pictures at different times were acquired by phase-contrast microscopy. Note that in FRT cells, both in absence and in presence of 4-OHT, cysts form at 24 hours and they are stable structures. In contrast, the 4-OHT induction of dominant negative form of Rac1 impairs the formation of cysts.

The above results clearly show that the expression a dominant-negative form of Rac1 affects directional migration, acquisition of TER, cyst formation, all parameters related to cell polarization. Thus, altogether these data clearly indicate that Rac1 plays a critical role in the acquisition of the polarized phenotype. Next aim is to understand how Rac1 regulates cell polarization and by which molecular mechanisms.

Cells are attached to one another at their lateral membranes. The lateral membrane is characterized by a series of junctions, including adherens junctions, which perform multiple functions, including initiation and stabilization of cell-cell adhesion, and tight junctions, which separate the basolateral surface from the apical surface. Adherens junctions (AJ) consist of the transmembrane protein E-cadherin, and intracellular components, p120-catenin, α-catenin and β-catenin. Tight junctions (TJ) consist of the transmembrane proteins occludin and claudin, and the cytoplasmic scaffolding proteins ZO-1, 2, and 3. The formation of junctional complex anchoring adjacent epithelial cells and their integrity is crucial for the acquisition and maintenance of
RESULTS

polarity. TJ are responsible of selective epithelial barrier and are essential to avoid mixing lipids and proteins between apical and basolateral domains.

The cell-cell adhesion mediated by E-cadherin of adjacent cells is the first step of polarization process, which is abolished upon addition of antibodies anti-cadherin. Recent evidence suggests that Rac1 has a role in regulating the formation and integrity of these complexes, although the molecular details are unknown.

The over expression of a dominant negative Rac1 mutant (Rac1N17) results in the dislocation of AJ components and F-actin from preformed adhesion in MDCK cells and in primary keratinocytes cells. It has been shown that Rac1 interacts with complex Par3/Par6/αPKC through its GEF, Tiam1, participating in the mechanism of formation of tight junctions.

Formation of the Adherens junction leads to assembly of the tight junction, but E-Cadherin is not required to maintain tight junction.

3.5 Rac1-GFP and E-cadherin co-localized on the plasma membrane at regions of cell-cell contacts

E-cadherin is the major adhesion receptor present in the membrane of epithelial cells and plays a fundamental role in establishing and maintaining homotypic cell-cell adhesion between cells, thereby contributing to the organization of tissues (Tackeichi et al, 1988). Control of cadherin localization at the membrane is crucial for the determination of local adhesive properties. Cell-cell junctions are permanently formed, renewed or disassembled in order to respond to the different metabolic cell requirements. Therefore, cell-cell junctions have to be plastic and dynamic, and this implies a continous remodeling of their membrane organization.

We analyzed the distribution of E-cadherin in FRT cells. As expected, E-cadherin was mainly localized on the plasma membrane predominantly at cell-cell contacts (Fig. 11). A significative amount of the protein is also distributed in the perinuclear region, in a compartment with morphological features of the Golgi complex, as it is likely for a secretory proteins. In order to understand the relationship between E-cadherin and Rac1 we investigated the distribution of E-cadherin in FRT cells stably transfected with the DNA encoding the chimeric protein Rac1-GFP. Confocal microscope images showed that Rac1-GFP, similarly to endogenous Rac1, localized predominantly on the
RESULTS

lateral domain of the cells. Interestingly, we found that, at steady state, Rac1-GFP co-localized with E-cadherin at cell-cell contacts, as evidenced by the overlapping of green and red signal (Fig. 11), indicating that Rac1 and cadherin are in the same compartments. Moreover this suggests that Rac1 can control the E-cadherin activity.

3.6 Rac1 inhibition affects E-cadherin function

Effect of Rac1 mutant on E-cadherin distribution.

To understand how Rac1 could modulate the activity of E-cadherin we analyzed the effects of Rac1 inhibition on the E-cadherin plasma membrane localization and dynamics. To this aim we performed immunofluorescence assays on FRT-ER-Rac1N17 cells grown both on transwell filters (Fig. 12a) and on glass cover slips (Fig. 12b). After 24 and 48 hours of 4-OH-tamoxifen treatment we found that Rac1 inhibition led to different signal morphology in E-cadherin distribution. While control cells exhibited the characteristic localization of E-cadherin prevalently on cell contact sites, treated cells displayed an irregular signal and a strong reduction of fluorescence signal (more evident after 48h of treatment, Fig. 12), indicating that the amount of surface cadherin was reduced. This is consistent with previous observations showing that frequently there is a decrease of the surface expression of E-cadherin in epithelial-mesenchymal transition as in the progression of many metastatic carcinomas (Thiery et al, 2002). This findings, together with the fact that the total levels of E-cadherin were
unchanged (Fig. 12 c), suggests that inhibition of Rac1 could affect E-cadherin redistribution.
RESULTS

Fig. 12) Effect of Rac1 inhibition on the distribution of E-cadherin. FRT-ER-Rac1N17 monolayers, grown on transwell filters (a) or on 12-mm diameter glass cover slips (b), were stimulated with 4-OHT for the indicated times and then fixed and stained with anti-E-cadherin antibody. Images were acquired by confocal microscopy by using the same settings (laser power, gain, etc) and the xy images shown were taken in the comparable Z-plane. Alternatively, cells were lysed in RIPA buffer and samples, after SDS-PAGE separation, were immunoblotted and analysed for E-cadherin (c).

In both culture conditions Rac1 inactivation alters the E-cadherin distribution and overall, after 48h of induction, there is a great reduction of E-cadherin signal at cell-cell contacts. In contrast the total amount of E-cadherin is unchanged.

Effect of Rac1 mutant on detergent solubility of E-cadherin.

In epithelial cells with mature cell-cell junctions, a significant fraction of TJ and AJ proteins partitions into a TX-100-insoluble pool that may reflect binding with the cytoskeleton or protein oligomerization (Wong et al 1997). Since inactivation of Rac1 enhances paracellular permeability (Fig. 10) and induced E-cadherin redistribution (Fig. 11), we analyzed the TX-100 solubility profiles of this protein.

In untreated cells the amount of E-cadherin predominantly partitioned to the TX-100 insoluble pool (Fig. 13). The induction of Rac1N17 induces a shift into the TX-100-soluble pool as also further emphasized by densitometric estimation of the ratio of TX-100-insoluble to -soluble pool (Fig. 13). These results indicate that a significant fraction of TX100-insoluble E-cadherin is bound with the actin cytoskeleton and is influenced by Rac1 inactivation, which possibly could cause a decrease in cell-cell adhesion. On the other hands, these data suggest that active Rac1 is required to ensure functional adherens junctions by controlling the E-cadherin dynamics.
**Fig. 13. Triton X-100 (TX-100) solubility profile of E-cadherin in FRT-cells expressing Rac1 mutants.** FRT monolayers were induced to express mutant GTPases for 24 h. Cells were incubated at 4°C with extraction buffer containing 1% TX-100. A: TX-100-insoluble (I) and -soluble (S) fractions were analyzed by SDS-PAGE and immunoblotted for E-cadherin. Quantification of 3 independent experiments is shown, ±SD. Note that Rac1 inhibition results in an increase of E-cadherin soluble to TX-100 extraction.

**3.7 Rac1 activation is crucial for the early steps of epithelial cell polarization**

The above results indicate that Rac1 inactivation affects the distribution of E-cadherin as its binding to cytoskeleton implying that Rac1 modulates the functions of E-cadherin and, consequently, of adherens junctions. However our analysis is performed at steady-state and, although adherens junctions are dynamically renewed in mature adhesions, we can underestimate the multifaceted role of Rac1 in the acquisition of polarized phenotype. Hence, to better understand the role of Rac1 in cell polarization, we investigated the effects of Rac1 inactivation during the early phases of this process. To this aim we performed calcium switch experiments, which represent a simple
method to transiently and reversibly impair cell-cell adhesion (Gonzalez-Mariscal et al., 1985; Pece et al., 1999). Cells were plated on transwell filters and, 24 hours after seeding, were incubated for 1 hour with EGTA, a Ca\(^{2+}\) chelator, in serum free media. As previously found in MDCK cells (Gonzalez-Mariscal et al., 1985), this treatment led to a drastic drop of TER (Fig. 13a) also in FRT cells, indicating that adhesion junctions were disassembled. Indeed as shown in Fig. 13b, cells lost contact with neighboring cells and they became rounded. Moreover, E-cadherin, which was localized at sites of cell-cell contact in cells cultured in normal Ca\(^{2+}\) levels (Fig. 13b, left), translocated from sites of cell-cell contacts to the cytosol and became diffusely distributed in FRT cells treated with EGTA (Fig. 14b, middle). After the restoration of Ca\(^{2+}\), cell-cell contacts were re-established (Fig. 14b, right), TER was recovered to values comparable to control cells (Fig. 14a) and E-cadherin could again be detected at sites of cell-cell contact (Fig. 14b, left); by 5 hours after Ca\(^{2+}\) restoration the rescue was complete. FRT-ER-RacN17 cells behave as wild-type FRT cells upon Ca-switch and restoration (Fig. 14c,d). By contrast, 4-OH-tamoxifen induction of ER-Rac1N17 cells during the Ca\(^{2+}\) restoration impaired the recovery of TER (Fig. 13c), which remained at values comparable to that of empty filters (\(\approx 120\) ohm). Although the cells formed new cell contacts, the amount of E-cadherin that re-localized at these sites was drastically reduced (Fig. 14d,e).

FRT wt

![Graph](image_url)
b) Anti-E-Cad

<table>
<thead>
<tr>
<th>CTR</th>
<th>EGTA</th>
<th>+ Ca²⁺</th>
</tr>
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c) FRT ER-RACN17

![Graph showing changes in TER (ohm) over time for different conditions: CTR, EGTA, and + Ca²⁺.](image)
RESULTS

d) 

CTR

EGTA

Ca^{2+} W/O
4-OHT

Ca^{2+} +
4-OHT

e) 

![Intensity graph](chart.png)
RESULTS

Fig. 14 Effects of Rac1 inactivation upon Ca\textsuperscript{2+} switch assay. FRT cells (a-b) and FRT-ER-Rac1N17 cells (c-e) were incubated with EGTA for 1h to disrupt cell-cell adhesion and then cells were incubated in culture medium containing calcium. TER was measured after EGTA treatment and after Ca- restoration for the indicated times (a, c). Alternatively cells were fixed and stained with an anti-E-cadherin antibody followed by a TRITC-conjugated secondary antibody and with anti-ER antibody followed by a FITC-conjugated secondary antibody (b, d). Quantization of fluorescence of E-cadherin signal was performed by using Zeiss software in two independent experiments (e). Error bars, mean ± SD. Note that Rac1 inactivation impairs the recovery of TER upon Ca-restoration and results in a reduced recruitment of E-cadherin at cell-cell contacts.

Interestingly, by combining fractionation assays with the calcium switch experiment (Fig. 15), we found that upon Ca\textsuperscript{2+} restoration endogenous Rac1 partitioned prevalently into the membrane fractions (with a ratio higher than at steady-state; compare with Fig. 7c), suggesting that it was strongly recruited to the plasma membrane. This is in agreement with previous data showing that Rac1, together with E-cadherin, becomes relocalized at cell-cell contacts after Ca-restoration (Nagakawa et al 2001). By contrast, upon 4-OH-tamoxifen stimulation, endogenous Rac1 failed to be enriched in the membrane fraction (Fig. 15), indicating that the induction of dominant-negative mutant prevents its membrane recruitment. Moreover, these results suggest that Rac1 is crucial to recruit E-cadherin to cell-cell contact sites during the first steps of adhesion junction formation.

Altogether these data indicate that the activation of Rac1 is crucial during the first steps of cell polarization. Consistently with data obtained at steady-state (Fig. 12), it is clear that the expression of a dominant negative mutant affects E-cadherin distribution and dynamics. This, in turn, could be critical for the homeostasis of adherens junctions.

Fig.15) Membrane/cytosol fractionation assay upon calcium-switch. FRT-ER-Rac1N17 cells grown on filter were treated for 1h with EGTA, 24h after plating. After Ca-restoration (5 hours) cells were homogenized and centrifuged to separate cytosol and membrane fractions as described in Fig 5. Samples were immunoblotted with specific antibodies against ER or Rac1. Upon 4-OH-tamoxifen stimulation, endogenous Rac1 failed to be enriched in membrane fraction. In contrast, as shown in Fig. 5, the
dominant-negative mutant is purified mainly in the membrane fraction in the presence of 4-OH-tamoxifen.

3.8 Rac1 inhibition affects E-cadherin traffic and membrane organization

On the basis of the above data it appears clear that active Rac1 is required for E-cadherin organization and dynamics, both essential to the maintenance of epithelial integrity during tissue homeostasis and remodeling (Baum et al, 2011). E-cadherin dynamics is mediated through several mechanisms such as diffusion, trafficking in function of the biological system. In mature junctions, membrane E-cadherin is quickly renewed by endocytosis in many cell type (De beco et al, 2012).

Thus, we asked how Rac1 inhibition impairs E-cadherin distribution/organization. By biochemical assays of endocytosis, we found that a chemical inhibitor of Rac1 activity affected the kinetics of E-cadherin internalization (data not shown). In particular, while in control cells we detected the maximum rate of E-cadherin endocytosis after 30 min, there was a shift upon Rac1 inhibition, suggesting that E-cadherin was accumulated in the cells. We are currently repeating these experiments in FRT ER-RacN17 cells and preliminary results seems to confirm those obtained by chemical inhibition.

E-vadherin was constitutively internalized into early endosomes and recycled back to the plasma membrane of polarized cells (Le et al, 1999). It is likely that Rac1 inhibition prevented its normal recycling. This could be a crucial step for the acquisition and/or maintenance of the polarized phenotype. To test this hypothesis we performed calcium switch assay in the presence of cycloheximide, which was added two hours before EGTA treatment (the time necessary to empty Golgi apparatus from E-cadherin signal). We found that after Ca-restoration E-cadherin was relocated to the plasma membrane also in presence of cycloheximide, indicating that the E-cadherin derived from the intracellular pool which formed during junction disruption (Fig. 16).

In contrast, upon Rac1N17 mutant induction, a low amount of E-cadherin was re-localized to the surface (Fig.16), supporting the hypothesis that Rac1 inactivation blocks E-cadherin recycling.
RESULTS

Fig. 16 Calcium switch assay in presence of cycloheximide. FRT-ER-Rac1N17 cells were subjected to calcium switch and restore as aforementioned. Two hours before EGTA treatment, cells were incubated with cycloheximide (CHX) to block protein synthesis and during Ca2+ chelation and restoration. After fixation, cells were stained with anti E-cadherin antibody followed by a FITC-conjugated secondary antibody. Serial confocal sections were collected from top to bottom of cell monolayers. E-cadherin recruited to plasma membrane after restoration derived from intracellular pool.

Moreover, it is plausible that E-cadherin accumulated intracellularly upon ER-Rac1N17 activation were degraded. We observed no change in the total amount of E-cadherin assayed by western blotting in untreated and tamoxifen-treated cells after Ca2+ chelation and restoration (Fig. 17). Interestingly, upon ER-Rac1N17 activation, we detected an increase of degradation products (Fig. 17, middle gel). Indeed we found an increase of two specific bands, 35 KDa and 20KDa (Palacios F. 2005), revealed by using an antibody directed against the cytoplasmatic domain of E-cadherin.
RESULTS

**Fig. 17. Rac1N17 leads to E-cadherin degradation.** FRT-ER-Rac1N17 cells, grown as a monolayer on plastics were subjected to calcium switch assay as aforementioned. Cells, were lysed in RIPA buffer and examined for E-cadherin degradation by resolving proteins by SDS-PAGE. Samples were immunoblotted with anti-E-cadherin antibody. The molecular weight of the degradation fragments are indicated (35 KDa; 20KDa). We used tubulin as normalization.

In order to investigate whether Rac1 inactivation could influence the membrane organization of E-cadherin, we combined Triton X-100 extraction assays with the calcium switch experiments. As at steady-state, upon Ca2+ chelation and restoration in untreated cells E-cadherin predominantly partitioned to the TX-100 insoluble pool (Fig. 18). By contrast, the dominant-negative induction prevented E-cadherin insolubility to detergent extraction (Fig. 18) and accumulation in Triton X-100-soluble fraction. These data provide strong evidence that Rac1 inactivation affects the membrane organization of E-cadherin that possibly, as consequence, is not anymore functional.
RESULTS

Fig. 18) **Triton X100 extraction assay after calcium-switch** Cells, grown as monolayer in plastic dishes, were subjected to calcium-switch both in presence and absence of tamoxifen. Samples were extracted in Triton X100 and separated in soluble and insoluble fractions as aforementioned. Immunoblot analysis showed a change in the TX-100-insoluble (I) profiles of E-Cadherin when Rac1 was inhibited.

Finally, we demonstrated by Co-IP assay that Rac1 and E-cadherin did not physically interact with each other, indicating that Rac1 regulates E-cadherin functions through its effectors.

Fig 19) **Rac1 and E-cadherin co-immunoprecipitation assays.** Cells expressing ER-Rac1N17 were lysed in RIPA Buffer and incubated overnight with anti-Rac1 or anti-E-caderin. Immunoprecipitates were collected using protein beads and resolved by SDS-PAGE. The proteins were probed with anti E-cadherin or anti-Rac1 antibodies.

3.10 **Rac1 affects the maintenance of polarized phenotype in epithelial cells**
Once we demonstrated that Rac1 is involved in the acquisition of polarized phenotype we asked whether it might have a role in the maintenance of cell polarity. To this aim we tested the effects of Rac1 inhibition on the integrity of polarized monolayers and preformed cysts. To this aim, the tamoxifen induction was performed when cells, after 5 days of culture, reached the maximum polarization state (as evident by the fact that TER is at plateau) or, alternatively, when cells, grown on coated agarose dishes, formed functional cysts (Fig.20 and 21). As shown in Fug 20, TER dropped upon 24h with tamoxifen, whilst in control cells TER remained to plateau values (∼ 2000 ohm). Consistently, dominant negative induction collapsed the cysts (Fig. 21), indicating that Rac1 inactivation also affects the maintenance of polarized phenotype. Thus, these data suggest that Rac1 is implicated both in the acquisition and maintenance of cell polarity. While Rac1 seems regulate the cell polarization modulating the E-cadherin and, in turn, the adherens junctions functions, it could directly control the integrity of tight junctions or through E-cadherin mediated cell-cell adhesion. We are currently investigating these aspects.

Fig. 20 Activation of ER-Rac1N17 by 4-OHT affect TER maintenance. FRT cells stably expressing ER-Rac1N17 were grown in polarized condition on transwell filters for five days and then 4-OHT was added. The experiments were made both in presence (red line) and absence (black line) of 4-OHT. TER measurements were conducted every 24 h. High values of transepithelial resistance (TER), generated in absence of 4-OHT and this, correlate with the acquisition of a full polarized phenotypes of cells. ER-Rac1N17 activation by 4-OHT led to TER dropped.
Fig. 21 Activation of ER-Rac1N17 by 4-OHT affect cysts maintenance. FRT-ER-Rac1N17 cells were plated in suspension on agarose coated and 24 hours from plating when cysts are formed 4-OHT was added. The experiments were made both in presence and absence of 4-OHT. When we inactivated Rac1 cysts collapsed.
DISCUSSION
5.1 FRT cells like a cellular model to study cell polarity

As a model system we used FRT cells, an epithelial cell line, which was derived from rat thyroid. FRT cells are well polarized both morphologically and functionally but do not express tissue-specific properties (Nitsch, et al, 1985, Zurzolo et al, 1994, 1996). They are metabolically dedifferentiated, but still express the thyroid-specific transcription factor Pax8 (Mascia A et al. 1997). They are connected by a continuous belt of tight junctions (Zurzolo et al, 1996). When FRT cells are cultured to confluency as monolayers on filters, they become polarized and develop very high transepithelial resistance (TER). In cultures on plastic dishes, FRT cells have the ability to form domes that correspond to domains of the epithelial layer where cells detach from the plastic, due to transepithelial transport of ions and water, and accumulation of fluid underneath the cell layer (Nitsch et al. 1985; Garbi et al. 1996). In suspension culture they form cysts in which a polarized monolayer of cells has the apical surface facing the outside.

5.2 Aspects investigated and results obtained

Epithelial cells have a polarized organization of the plasma membrane, cytoskeleton and cytoplasmatic organelles. Loss of polarity may lead to human diseases and it is a crucial step in the progression of cancer. The major cause of mortality in cancer patients is metastasis. In order to metastasize cancer cells must acquire mutations that disrupt epithelial structure, resulting in an epithelial-mesenchymal transition (EMT). Because the metastasis is the primary cause of death, it is important to understand the molecular mechanisms by which epithelial polarity is established and maintained.

The acquisition of cell polarity requires external cues, including interaction of cells with their neighbours and with the extracellular matrix, and involves the coordinated action of several proteins able to decrypt and translate these external signals. Downstream of cell-cell adhesion, several pathways act to establish and maintain a polarized cellular architecture. The Rho small G protein family, consisting of the Rho, Rac and Cdc42 subfamilies, regulates many biological processes including cell cycle
progression, apoptosis, migration and intercellular adhesion. We focused here on the analysis of the role of Rac1 protein in the acquisition and maintenance of the polarized phenotype in the FRT rat thyroid epithelial cell line. Our goal was to understand how signals transduced via Rac1 plays a role in these processes. To this aim, we used an inducible dominant-negative form of Rac1, ER-RacN17, which was stably expressed in FRT cells. Several aspects, including directional migration, TER acquisition, cell aggregation and formation of polarized cysts, were investigated.

In this thesis we demonstrated that ER-Rac1N17 activation upon 4-OH-tamoxifen treatment reduced the wound healing efficiency in a scratch test, interfered with the acquisition of transepithelial resistance by confluent monolayers on filters, and impaired cyst formation by cells in suspension culture. We investigated the molecular mechanisms by which these processes occur. Epithelial cells establish stable contacts between individuals cells, and between individual cells and the extracellular-matrix, in order to maintain a fully polarized state. Cell-cell and cell-matrix adhesion processes are mediated by specific cell adhesion molecules (CAMs). The principal CAM of epithelial cells is E-cadherin which is organized in adherens junctions. Cell-cell adhesion mediated by E-cadherin is a critical step in the polarization process.

We decided to demonstrate here that Rac1 regulates the early phases of cell-cell adhesion formation. To this aim firstly we analyzed the distribution of E-cadherin and Rac1 in FRT cells. We found co-localization between Rac1 and E-cadherin at sites of cell-cell contact, suggesting that Rac1 concentrated at the plasma membrane, in association with the adherens junctions. Strikingly, we found a progressive loss of E-cadherin from the plasma membrane upon inhibition of Rac1 by the ER-Rac1N17 activation with 4-OH-tamoxifen.

To examine the effects that the loss of Rac1 activity has on the formation of cell–cell contacts, calcium switch experiments were performed. We found that E-cadherin traslocates to the cytosol during disruption of cell-cell adhesion by Ca2+ chelation with EGTA and reappeared at the sites of cell adhesion after Ca2+ restoration. Activation of ER-Rac1N17 by 4-OH-tamoxifen during Ca2+ restoration led to a high decrease of the protein at the sites of cell-cell contacts, although it did not completely abolish the reappearance of E-cadherin on the plasma membrane. How does Rac1 inhibition correlate with changes in E-cadherin engagement? In agreement with the results we obtained by immunofluorescence microscopy, the detergent solubility assay
(Triton X-100 extraction assay) during calcium switch experiments indicated that the amount of the cytoskeleton-associated E-cadherin decreased upon ER-Rac1N17 activation by 4-OH-tamoxifen, suggesting that Rac1 is important to maintain functional E-cadherin (i.e. attached to actin filaments, in adherens junction) on the plasma membrane. To better understand the effect of ER-Rac1N17 during adherens junctions de novo formation, we combined calcium switch experiments with the membrane/cytosolic fractionation assay. We found that during Ca^{2+} readdition in the absence of 4-OH-tamoxifen, endogenous Rac1 mainly partitioned in the membrane fraction while in the presence of 4-OH-tamoxifen it significantly shifted to the cytosolic fraction suggesting that ER-Rac1N17, when activated by 4-OHT treatment, partially displaces the endogenous Rac1 from the membrane. These results also suggest that Rac1 localization to the plasma membrane is important to recruit E-cadherin to cell-cell contact sites during the first steps of cell-adhesion. Adherens junctions depend on the rapid turnover of E-cadherin; it can be speculated that Rac1 regulates this process. Calcium switch assays performed in the presence of cycloheximide indicated that the E-cadherin which reached the plasma membrane after calcium readdition derived from the preexisting pool and not from de novo synthesis. E-cadherin translocated into the cell and formed intracellular pools as a consequence of junction disruption. We think that ER-Rac1N17 activation upon tamoxifen might affect E-cadherin recycling.

5.3 Use of dominant-inhibitory mutant of Rac1

One of the most powerful ways to study the function of a protein is to specifically block its activity within cells. We generated a novel inducible system to suppress RAC1 activation and to study its consequences during the acquisition of the polarized phenotype. We fused a dominant-negative form of Rac1, Rac1N17, downstream of the ligand-binding domain (ER) of the murine mutant estrogens receptor, which is responsive to activation by 4-OH-tamoxifen only. Usually, dominant-inhibitory mutants interfere with the function of their normal cellular counterparts or with proteins that interact with them. The Rac1N17 protein contains an asparagine
substitution that abolishes the protein affinity for GTP and reduces the affinity for GDP. As a consequence of that, the mutant protein fails to bind downstream targets in the cell because it cannot bind GTP in vivo. When expressed in cells, it binds to RacGEFs more strongly than does normal Rac and forms 'dead-end' complexes.

Among the first results we obtained is the demonstration, by immunofluorescence assay, that upon tamoxifen incubation a significant amount of ER-Rac1N17 moved from the cytosol to the plasma membrane, at sites of cell-contacts. This suggested that Rac1 recruitment to membranes occurs independently of its nucleotide-bound and active state. As indicated in the results, this effect is observed within an hour. Once stimulated, ER-Rac1N17 protein remains stably associated to the membrane.

Crystallographic and NMR analysis of Rac1 have revealed that the conformational difference between the GTP and GDP-bound form are restricted in two regions named Switch I and Switch II. Switch I and Switch II regions, with β1, β2 and β3 regions, form a pocket in which GEF binds thus promoting the activation of Rac1 (Vetter IR 2001). However, little is known about the construct we used. We speculate that the ER domain masks this binding site for GEFs. After binding to 4-OH tamoxifen, the protein is subjected to a conformational change, the GEF domain becomes accessible and, possibly, dissociation from RhoGDIs occurs. RhoGDIs produce both negative and positive actions: they inhibit the intrinsic GDP/GTP exchange of Rac1, favoring the maintenance of GTPase in the inactive conformation. In addition, they use a deep hydrophobic cavity to trap the Rac1 prenyl group, thus keeping the GTPase away from the membrane (Xosè R. Bustelo 2012). We hypothesize that ER-Rac1N17 traslocates to the membrane to binds to GEFs that are located there (Angeliki Malliri, 2004).

Our results indicate that the chimeric protein ER-Rac1N17 behaves like the endogenous protein and that its activation state can be perfectly manipulated through the use of tamoxifen. Standard protocols to measure the Rac1GTPase activity after incubation with 4-OH-tamoxifen were performed showing that the ER-Rac1N17 chimera is able to inhibit the activity of endogenous Rac1. Therefore, the expression of this chimeric protein is an excellent tool to study the role of Rac1 in the processes of polarization.
**Effect of ER-Rac1N17 activation on the acquisition of the polarized phenotype in FRT cells**

**Polarized migration.** The cytoskeletal rearrangements caused by the activation of RhoGTPases play a key role in cell motility that requires the coordinated and polarized fulfilment of several processes. Cell migration is the net effect of Cdc42 and Rac1 mediated expansion of cellular protrusions in the direction of migration (the front of the cell) and RhoA driven retraction of membrane at the trailing edge (the rear of the cell) (Iden and Collard, 2008). Cells that migrate have a front-rear-polarized morphology with asymmetric distribution of surface receptor, adhesion proteins and cytoskeletal components. Recent study in fibroblasts indicated that RhoGTPases collaborate with polarity proteins to control directional cell migration. Components of the PAR, Scribble and Crumbs complexes localize to the leading edge and regulate front-rear polarization, and apparently crosstalk directly to Rho GTPases through the modulation of their GEFs (Iden and Collard, 2008). Some effectors of Rac1 have been found to specifically mediate cell motility such as PAK, that is a protein kinase playing an important role in the cytoskeletal-mediated changes affecting cell motility, or IQGAP and Par6 that promote motility through disruption of the normal organization of neighbouring cells. More evidence for the roles of Rac1 in cell motility comes from fibroblasts deficient for the tumour suppressor gene Pten. These cells are more motile and contain higher levels of Rac1GTP than wt cells. The motile behaviour of these cells can be suppressed by dominant-negative mutant forms of Rac1 (Liliental, J. 2000). Ridley et al have proposed that Rac1 is necessary for the HGF-induced motility in MDCK cells. FRT cells, as other types of epithelial cells, move as groups of adherent cells, as possibly do epithelial cells in vivo during tissue morphogenesis. The observations we made by wound healing assay indicated that when ER-Rac1N17 is activated in FRT cells, they manifest a reduced migratory activity and a delay in closing the wound, in agreement with the bulk of evidences that Rho GTPases regulate cell motility.

**Abnormal cell migration is a characteristic of malignant cancer cells and is one component of metastatic process, the principal clinical problem in cancer. RhoGTPases are most likely to contribute to cancer cell migration and invasion.**
Expression of Rho family proteins is deregulated in some tumors and correlates with progression of disease (Sahai and Marshall, 2002). Recently Sanz-Moreno and all (Sanz-Moreno et al, 2008) show that mesenchymal-type movement in melanoma cells is driven by activation of Rac1 through a complex containing NEDD9, a recently identified melanoma metastasis gene, and DOCK3, a Rac1-GEF. Once activated, Rac signals through WAVE2 directing mesenchymal movement. Moreover, the over-expressed of rac1 has been found in various tumors and accumulating evidence indicate that Rac1-dependent cell signaling is important for malignant transformation (Gomez PT 2005) as in the progression of testicular (Kamai T 2004) gastric (Pan Y, 2004), and breast cancer (Schnelzer A). To understand the mechanisms by which cells migrate might be of help to develop specific drugs that interfere with the metastatic process. Rho GTPases might involve in all stages during cancer progression. They can affect tumor cells through modulation of gene transcription, cell division and survival, intracellular transport of signaling molecules or modifying the interaction of cancer cells with surrounding stromal cells. To analysis of how Rho GTPases work in cells and contribute to tumors is very complex but at the same time promising for potential future therapeutical intervention.

Acquisition of transepithelial resistance (TER). To understand the role of Rac1 in the process of cell polarization, we analyzed the effect of its inhibition on the acquisition of TER, that represents an index of the degree of polarization of the cell monolayer. FRT cells can reach quite high TER values if compared to other commonly used epithelial cell lines such as MDCK and Caco2. The observations made on TER acquisition indicated that the incubation of FRT-ERac1N17 cells with 4-OH tamoxifen impairs the increase of transepithelial resistance, and a significant decrease of TER values is shown within 24h after the addition of 4-OH tamoxifen by monolayers that had already acquired a high TER. This decrease persists over time and is virtually irreversible. Indeed, even if we performed several washings to eliminate 4-OH tamoxifen from the culture, the TER did not increase but maintained low values, comparable to those of an empty filter. When 4-OH tamoxifen was added at the plating time, the monolayers were unable to achieve a significant TER value. These data indicate that the inhibition of Rac1 affects the ability of cells to acquire TER and, therefore, to reach their final mature polarized phenotype. Using MDCK
cells that expressed constitutively-active or dominant-negative forms of Rho family GTPases, it was previously demonstrated that Rho family proteins are involved in the early development of epithelial cell polarity and in the maintenance of the tight junction (Jou TS, 1998). Rac1 inhibition might interfere with the machinery and/or with the formation of stable junctionals complexes at the plasma membrane.

**Formation of tree-dimensional structures in suspension culture.** Rat thyroid epithelial cells from fresh tissue, grown in suspension culture, are capable to reorganize into follicle-like structures (Nitsch and Wollman PNAS 1980) that express most, if not all, properties of thyroid follicles in vivo (Tacchetti et al. 1986). Under similar culture conditions, in medium with high concentration of serum and in the absence of a surrounding, adhesive gel (such as collagen), the thyrocytes reorganize into cystic structures with polarity inverted with respect to follicles, the apical domain facing the outside (the culture medium) (Nitsch and Wollman, JCB 1980). FRT cells in suspension culture are also capable of forming cystic structure, whose polarity can be manipulated by the presence of different types of gels (Garbi et al, 1987). An advance in the study of cytogenesis derived from the analysis of a similar in vitro model system in which collagen gel cultures of MDCK epithelial cells were used. Indeed, Wang and coll. reported that MDCK cells, just like FRT cells, form cysts with inverted polarity with the apical pole facing the free cellular surface of the cyst periphery when grown in suspension culture (Wang et al. 1990). The role of RhoGTPases in the three-dimensional organization of these epithelial cysts was studied in a report demonstrating that the expression of dominant-negative Rac1 caused an inversion of cyst polarity due to abnormal basolateral laminin assembly (Lucy Erin O’Brien 2001). Other groups demonstrated that Rac1 plays a role in the reorientation of polarity. MDCK inside-out cysts expressing a dominant-negative form of Rac1 failed to re-orient polarity when embedded in collagen, suggesting that Rac1 plays a critical role in signaling cues from the ECM to orient the cells. Rac1 might control extracellular matrix-induced reorientation of apico–basal polarity in three-dimensional cultures of epithelial cysts in a PI3K- and aPKC-dependent manner (Liu et al. 2007).

To examine the role of Rac1 during cyst morphogenesis we used FRT cell lines that expressed the conditional dominant-negative mutant ER-Rac1N17. The results
DISCUSSION

obtained suggested that Rac1 activity is necessary to promote formation of polarized structures. Treatment with 4-OH tamoxifen, in fact, inhibited the formation of polarized structures. The mechanism by which this occurs remains to be established. On question is whether tight junction formation is perturbed under this condition. Many studies suggest that the activity of the Rho family GTPases must be carefully balanced to preserve tight junctions and adherens junctions integrity (Mertens 2006)

5.5 Rac1 inhibition and polarized traffic of E-cadherin

The establishment of epithelial cell–cell contacts and the subsequent apico–basal polarization requires E-cadherin-mediated cell–cell adhesion. Several studies indicate that Rho family GTPases controls cadherin-mediated cell–cell adhesions. During the very early phases of generation of polarity, Cdc42 and Rac-1 are activated by the initiation of cell contacts formed by trans-interactions of nectins and cadherins (Fukuhara A, and al. 2003; Kukuhara T. and al. 2004). Indeed, E-cadherin-mediated cell interactions result in the rapid activation of Cdc42 in MCF-7 epithelial cells (Kim et al. 2000) and Rac1 in MDCK cells (Yamada and Nelson, 2007). Homophilic ligations of E-cadherin has been shown to recruit and to activate Rac1 at adhesion sites in MDCKII cells (Betson et al. 2002; Braga et al. 1997; Nakagawa et al. 2001; Noren et al. 2001). How Rho family GTPases control E-cadherin-mediated intercellular adhesions remains an interesting question. It has been reported that the maturation of primordial adhesions to linear apico–basal polarized cell–cell contacts with discrete adherens and tight junctions requires the stimulation of aPKC by crosstalk between Rho GTPases and the PAR complex. Binding of Rac1–GTP to PAR6 releases an intrinsic ability of PAR6 to activate aPKC, the interaction between TIAM1 and PAR3 couples RAC1 activation and loss of TIAM1 impairs the establishment of functional tight junctions in keratinocytes. Braga and colleagues (Braga et al. 1997) found that the Rho family GTPases affect the formation of cell–cell junctions. When dominant-negative Rac1 (Rac1N17) is microinjected into keratinocytes, cadherin accumulation is inhibited at sites of cell-cell contacts. Subsequent studies from Takaishi and colleagues (1997) revealed that overexpression of constitutively active Rac1 (Rac1V12) in MDCKII cells promotes
basal accumulation of E-cadherin, beta-catenin, and actin filaments at sites of cell-cell contacts, whereas overexpression of Rac1N17 reduces their accumulation. We examined E-cadherin localization following Rac1 inhibition. By immunofluorescence assay we demonstrated a decreased level of plasma membrane E-cadherin following long-term incubation with 4-OH tamoxifen and hypothesized that this effect of inhibition of Rac1 on E-cadherin membrane localization could represent the molecular basis of the defect in cell polarization we evidenced by monitoring the TER. We investigated the role of Rac1 on the localization of E-cadherin by the calcium-switch assay, during the early steps of cell-cell contact formation. We demonstrated that GFP-Rac1 colocalized with E-cadherin at sites of cell-cell contacts and translocated to the cytosol during disruption of E-cadherin mediated cell-cell adhesion by calcium chelation. Upon re-establishment of cell-cell adhesion Rac1 relocalized, together with E-cadherin, at sites of cell-cell contacts. When we performed the calcium switch assay in the presence of 4-OH tamoxifen, we found a strong reduction of the signal due to membrane-cadherin compared to control cells w/o 4-OH tamoxifen. Calcium-switch assays performed in the presence of cycloheximide demonstrated that the E-cadherin which reached the plasma membrane after calcium readdition derived from preexisting pool where it possibly relocalized after junction disruption. A similar decrease of E-cadherin on plasma membrane was found, in fact, after incubation with cycloheximide. These data suggested that Rac1 could regulate the rapid turnover of E-cadherin to and from the lateral surface. Indeed, to maintain the dynamics of epithelial monolayer, E-cadherin is rapidly removed from plasma membrane and then subsequently recycled back to the cell surface to reform new cell-cell contacts. Wang and collaborators (Wang et al. 2005) showed that both Rac1 and Cdc42 are involved in the polarized trafficking of E-cadherin in MDCK kidney cells, determining efficient post-Golgi sorting of E-cadherin and its delivery to the lateral cell surface. Reduced surface staining of E-cadherin have been described in polarized MDCK cells after the knock-down of Rac-1 using small interfering RNA (Noritake et al. 2004). The data obtained in the FRT system of polarized cells and presented here are consistent with these observations.

Rac1 activation was shown to inhibit endocytosis of E-cadherin and to stabilize the protein on the plasma membrane. An important role has been proposed for the Rac/Cdc42-IQGAP1 system in the dynamic organization and maintenance of the E-
cadherin-based adherens junctions (Izumi et al., 2004; ). IQGAP1 is known to negatively regulate E-cadherin mediated cell-cell adhesion by interacting with beta-catenin and thereby stabilizing cadherin-mediated cell-cell contacts. It has been suggested that activated GTP-Rac1 blocks the ability of IQGAP to inhibit assembly of a cadherin-catenin complex promoting adherens junction formation (S. Kuroda, 1998). We suppose that when ER-Rac1N17, upon activation with 4-OH tamoxifen, promotes an increase in the amount of inactivate Rac1, IQGAP is free from Rac1 and dissociates beta-catenin from the cadherin-catenin complex, destabilizing the adherens junctions. To support this hypothesis we analyzed, by detergent solubility assay (Triton X100 extraction assay), the amount of cytoskeleton-associated E-cadherin during calcium-switch experiments and we demonstrated a significant decrease upon ER-Rac1N17 activation by 4-OH-tamoxifen. Possibly Rac1 is important to build-up functional adherens junctions.

It has been reported that E-cadherin-mediated cell-cell adhesions stimulate phosphatidylinositol 3-kinase (PI3K) activity in MDCKII cells (Pece et al. 1999). Moreover, PI3K has been shown to interact with E-cadherin (Pece et al. 1999). Since PI3K is thought to function upstream of Rac1 (Kotani et al. 1994), these observations indicate the possible involvement of PI3K in E-cadherin-dependent Rac1 activation. Activation of Rac1 through PI3K by E-cadherin-mediated cell-cell adhesions seems to require at least two steps: (1) Rac1 recruitment to sites of cell-cell contacts, and (2) Rac1 activation by a GEF that responds to PI3K products. Consistently, Rac1 rapidly accumulates at the sites of E-cadherin engagement (1 – 3 min), and this accumulation is independent on Rac1 GTP binding/hydrolysis (Perez et al. 2008). Considering that Tiam1 is localized at sites of cell-cell contacts and functions downstream of PI3K, Tiam1 appears to act as a Rac1 GEF that functions downstream of E-cadherin engagement. Another possible mechanism might be the capacity of ER-Rac1N17 to sequester on plasma membrane Rac1GEF TIAM1 preventing PI3K pathway activation.

Moreover, a molecular and functional link between Rac1 and Rab7 has been proposed since active Rac1, via its effectors Armus, is able to locally inactivate Rab7 and to facilitate E-cadherin degradation in lysosomes (Frasa et al., 2010). Preliminary results on FRT thyroid epithelial cells are in good agreement with these data since the inhibition of Rac1 promotes E-cadherin internalization and increase of E-cadherin
degradation products. Overall, these results represent a strong evidence for a predominant function of Rac1 in the formation of epithelial apico–basal polarization.

5.6 Rac1 inactivation affects the maintenance of a polarized phenotype in FRT epithelial cells.

Apical-basal polarity of epithelial cells is maintained through TJs, which function as intercellular barriers that regulate paracellular permeability by segregating the plasma membrane into two compartments. Rac and Cdc42 regulate TJ functions in epithelial cells. Indeed, it has been demonstrated that constitutively active mutant of Rac alters the TJ organization in MDCK cells (Tzuu-Shuh Jou 1998), while a dominant negative mutant of Rac also causes a redistribution of claudin-1 and -2 in addition to JAM-1 (Popoff MR, 2009). The Par polarity complex, which consists of Par3, Par6 and atypical protein kinase C (aPKC), localize to primordial TJs and has been shown to be required for their maturation from primordial AJs in epithelial cells (Macara, 2004). Cadherin transduces signal to activate Rac1, which result in the local activation of the Par polarity complex stimulating the assembly of TJs. Recently, two different studies in epidermal keratinocytes showed that the Rac1-specific GEF Tiam1 acts upstream of Par polarity complex during this process. Indeed, Tiam1 directly associates with Par 3 controlling TJs assembly. Tiam1-deficient cells form normal primordial adhesions but they impaired in TJ maturation. Reconstitution of Rac1 activity by expression of exogenous Tiam1 or dominant-active Rac1V12, but not by a Tiam1 mutant unable to activate Rac1, fully restores TJs biogenesis in Tiam1-deficient cells (Mertens A.E., 2005). Here we demonstrated that Rac1 inactivation leads to disruption of polarized structures (Figs. 20, 21). Indeed the inhibitory mutant of Rac1 induced the collapse of cysts as well as the integrity of cell monolayers as shown by the drastic drop of TER. Our data are consistent with recent findings showing that the inactivation of Rac1 leads to the dispersion of apical markers in cyst structures (Yagi S., 2011) implying that Rac1 can regulate the TJ functions. However, the same authors found that Rac1 suppression exclusively at the apical membrane is required for the maintenance of cyst structure, suggesting that the activity of Rac1 might be spatial and temporal regulated: Rac1 activity is homogenous at very early stages of cystogenesis,
whereas at the late stages its activity is higher at the lateral than at the apical membrane (Yagi S., 2011). Further studies will be necessary to better understand how and by which molecular mechanisms Rac1 could contribute to maintain the polarized phenotype.
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5.1 Cell culture

For the experiments have been used epithelial cells of rat thyroid Fischer (FRT). Cells were cultured in Petri dishes from 100 to 35 mm of diameter and were kept in an incubator at 37°C in a humidified atmosphere containing 5% CO2 and 95% air. FRT cells were maintained in F12 Coon’s medium supplemented with 5% Fetal Bovine Serum FBS (GIBCO). Culture medium was changed every three days. Growth was monitored daily by phase contrast microscopy. When the cells reached confluence were washed once with a solution of trypsin 0.3%, glucose 0.1%. 2 mM EGTA in PBS pH 7.3 (7.13 mM KCl, 1.47 mM KH2PO4, 137 mM NaCl, Na2 HPO4 7 H2O 8.6). Incubated with the same trypsin solution for 10-15 minutes at 37°C and then resuspended in the medium culture and centrifuged at 1000 rpm for 4 minutes. The pellet was resuspended again in F12 Coon’s medium containing 5% Fetal Bovine Serum, and the cells were plated in Petri dishes.

5.2 Plasmids and transfection

FRT cells were stably transfected with the plasmid encoding the chimeric protein ER-Rac1N17 consists of a fragment (inserted into Bgl II/EcoRI sites) encoding amino acids 281-599 of a tamoxifen-responsive mutant of the murine estrogen receptor ERTM fused to a fragment (inserted into Eco-Not I sites) encoding the protein Rac1N17 cloned into pCEFL AU1 expression vector under the CMV promter. The construct is in frame with the leader sequence and the tag, which is represented by a small peptide of six amino acids (DTYRYI) that is recognized by specific antibodies. Rac1N17 protein is in frame with ER. Plasmid contains the internal resistance to neomycin for selection in eukaryotic cells. Without 4-OH-tamoxifen the protein is inactive. 4-OH tamoxifen (100 nM) interacts with the hormone-binding domain, and it induced rapid and prolonged protein activation. For stable transfections experiments FRT cells were
plated at 20% confluency in 100-mm dishes and transfected with 4 µg/dish of plasmid encoding the chimeric protein ER-Rac1N17.

![Diagram of pCEFL AU1-ER](image)

**Fig.1 Schematic view of pCEFL AU1 ER.** It contains a sequence of mutated murine estrogen receptor responsive to 4-OH-tamoxifen, it is fused to a fragment encoding the protein Rac1 N17 cloned into the vector pCEFL AU1 under the CMV promoter. Plasmid contains the internal resistance to neomycin for selection in eukaryotic cells. To obtain stable transfectants, the cells were co-transfected with 4 µg of the plasmid containing AU1-ER-Rac1N17 cDNA. Transfection was performed with lipofectin. Neomycin resistant clones were selected in Coon’s modified Ham’s F12 medium containing 5% FBS and (?) µg/ml of neomycin.

FRT parental cells were also stably transfected with the plasmid expressing pFLAG Rac1N17 or EGFP-Rac1

### 5.3 Immunofluorescence

The immunofluorescence assay allows localizing a protein of interest within a biological sample by recognition of its epitope by a specific antibody. The primary antibody is then revealed by another antibody which may be conjugated with a tracer. The tracers used in this assay are fluorochromes, molecules which affected by a light of a determinate wavelength, emitted light of higher wavelength. If the tracer is directly bound to the specific antibody against the protein of interest we speak of direct immunofluorescence, if it is bound to a secondary antibody directed against the primary antibody we talk about of indirect immunofluorescence. Immunofluorescence studies were performed on cells seeded into 12-mm diameter glass cover slips or on top of filters in bicameral systems in medium containing 5% Fetal Bovine Serum for several time. A secondary antibody labeled with fluorochromes is used to recognize a
primary antibody against protein of interest. Cells were fixed for 20 minutes in PBS containing 4% paraformaldehyde (Sigma-St. Luis, MO-USA), washed two times for 5 minutes in NH4Cl 50mM, permeabilized with PBS containing Triton X-100 0,3% for 5-7 minutes and successively washed three time with PBS. Alternatively, in indirect immunofluorescence assay on polarized cells they were fixed and permeabilized with methanol- acetone 1:1 solution for 2 minute at -20° C and cells were washed three times with PBS. After incubation for 20 minutes in a solution containing 1% BSA (bovine serum albumin) in PBS, cells were incubated 1h in humidified atmosphere with the primary antibody at room temperature and after 3 washing with PBS/BSA 0,5% they were incubated in humidified atmosphere at room temperature with the appropriate secondary antibodies conjugated to TRITC (rhodamine tetrametilata) or fluorescein (FITC) diluted 1:50 in PBS. After final washes with PBS, the cover slips were mounted on a microscope slide using a 50% solution of glycerol in PBS and examined with a confocal laser scanner microscope Zeiss 510LSM. The λ of the Argon ion laser was set at 488nm that of the HeNe laser was set at 543nm. Fluorescence emission was reveled by BP 505-530 band pass filter for Alexa Fluor 488 and by BP 560-LP band pass filter for Alexa Fluor 543. Double staining immunofluorescence images were acquired simultaneously in the green and red channels at a resolution of 1024x1024 pixels.

5.4 Antibodies and reagents

The following antibodies were used: the mouse mAb anti-AU1 that recognizes a six amino acid epitope (DTYRYI) in the AU1 ER-RAC1 (N17) protein (MMS-130R, Covance). Because of the small size of the epitope, it is unlikely to alter the activity of the cloned sequence.; the rabbit polyclonal ERα Antibody (MC-20):sc542 (Santa Cruz) against a peptide mapping at the C-terminus of ERα of mouse origin; the mouse mAb anti-Rac1 (clone 23A8) against recombinant protein containing the full length Rac1 (Upstate); mouse mAb anti E-Cadherin against amino acid 735-883 in the C-terminal domain of human E-Cadherin (BD, Transduction Laboratories); Secondary antibodies for indirect immunofluorescence conjugated to TRITC (rhodamine tetrametilata) or fluorescein (FITC) have been used correctly.
Secondary antibodies for Immunoblotting anti-mouse and anti-rabbit conjugated with horseradish peroxidase (Amersham Pharmacia-Buckinghamshire, UK). To inhibit endogenous Rac1 the specific inhibitor, NSC23766 (Calbiochem) at a dose of 100-150 μM was used. NSC23766 is a cell-permeable pyrimidine compound that specifically inhibits Rac1 GDP/GTP exchange activity by interfering with the interaction between Rac1 and Rac specific GEFs, Trio and Tiam1 (Gao et al., 2004). To activate the AU1 ER-RAC1(N17) protein 4-OH-Tamoxifen (Sigma Aldrich) was used. It is an active metabolite of tamoxifen which binds estrogen receptors (ER) and estrogen-related receptors (ERR) with estrogenic and anti-estrogenic effects. Cell culture reagents were purchased from Gibco Laboratories (Grand Island, NY).

5.5 Immunoblotting

Cells were seeded to confluence in 100 mm diameter dishes or on filters. Cell culture dishes were placed on ice and were washed two times with ice-cold phosphate-buffered saline (PBS) (KCl 13.7 mM, KH2PO4 1.47mM NaCl 137mM, Na2 HPO4 7 H2O 8.06). Cells were lysated with RIPA buffer [150 mM sodium chloride, 50 mM Tris-HCl, pH 7.4, 1 mM ethylenediamine tetra acetic acid (EDTA), 1 mM phenylmethylsulfonyl fluorite (PMSF), 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% sodium dodecylsulfate (SDS), 5 μg/ml aprotinin and 5 μg/ml leupeptin] for 5 min at 4°C by shaking. The lysated was cleared by centrifugation at 3000 rpm for 5 min. Protein concentration was determined with the Bio-Rad protein assay. The lysated was boiled for 5 min in 1x SDS sample buffer (50 mM Tris-HCl pH 6.8, 12.5% glycerol, 1% sodium dodecylsulfate, 0.01% bromophenol blue) containing 5% betamercaptoethanol. Proteins were separated by SDS-PAGE, transferred on Cellulosenitrate, membrane (Schleider and Schuell, Keene, NH) 2h at 350 mA constant. The nitrocellulose was blocked 1h by incubation with TTBS (50 mM Tris, pH 7.9, 150 mM NaCl and 0.05% Tween 20) 5% milk at room temperature and successively immunoblotted with the primary antibody diluted in TTBS 5% milk. The antibodies against the proteins of interest were all used diluted 1:1000 or 1:3000 and incubated one hour at room temperature. The nitrocellulose was washed five times (5 min each) in TTBS, and the appropriate peroxidase-conjugated anti-rabbit IgG or anti-
mouse IgG, were used. Then, the membrane was washed again four times (5 min each) in TTBS. The blots were developed with the ECL system (Supersignal West Pico, Celbio, PIERCE, Rockford, IL, USA).

5.6 Immunoprecipitation

FRT expressing ER-Rac1N17 protein were growth in 100-mm dishes or on filters in bicameral systems for 24 or 48h; after they were washed twice with ice-cold phosphate-buffered saline (PBS) and were lysed 5 minutes on ice with RIPA buffer. Cells were collected by scraping in eppendorf. The lysates were incubated over night at 4 °C with anti-Rac1(4µg) or anti-E-Cadherin(2µg) antibodies. The immunocomplex were incubated with protein A Sepharose beads for 2 h at 4°C and , then, were centrifugated at 14000 rpm at 4°C for 2 min in a refrigerated centrifuge. The supernatant was removed and used as control, the pellet was resuspended and solubilized in leamly 2X. Immunocomplex was subjected to SDS-PAGE followed by immunoblotting with anti-Rac1 or anti-E-Cadherin antibodies

5.7 Bicameral culture system and TER measurements

Acquisition and the maintenance of cell polarity by epithelial cells in monolayer culture might be monitored by measuring the transepithelial resistance (TER), which is generated when cells are grown on top of filters in bicameral systems. Filters are composed of some inert materials as polycarbonate and nitrocellulose and glued on rings in plastic, which are placed in conventional culture wells. Filters have pores of 0.4 μm and a diameter of 24 mm. This system allows creating two compartments above and below the filter, and culture medium is added to both compartments of the system. The development of this technique has made it possible to study in detail mechanism governing epithelial polarity, occluding barrier function and vectorial transport in epithelial cells. Since FRT cells are derived from thyroid follicular epithelium the cell layer on the filter can be considered to represent the wall of a follicle which has been opened and laid on top of the filter with the luminal surface
facing the culture medium in the upper (apical) chamber compartment, and the basal membrane in close contact with the filter surface.

![Diagram](image)

**Fig. 2** drawing of the bicameral system. The cells were seeded on the filter with the apical membrane in the upper compartment and the basal membrane in close contact with the filter surface.

It is important to seed cells on the top of the filter and allow the cells to grow to confluence and form a continuous growth-arrested monolayer. In general, higher is the seeding density and more rapidly are reached both confluence and maximal TER values. This event correlates with tight junction’s formation and great decrease in tight junction paracellular permeability. It is known that epithelial cell lines from different sources are characterized by different maximal TER values. In all experiments presented in this thesis commercial bicameral chambers were used, (TranswellTM, Costar Corp., Cambridge, MA/USA). A layer of polarized, confluent cells can generates a barrier between the two compartments, apical and basolateral that they separate and under these conditions, it is resistant to the passage of current applied through electrodes connected to a voltmeter (TER, transepithelial resistance). The TER was measured by placing an electrode in the apical compartment and another electrode in the basolateral compartment. The electrical potential difference is about of 3 mV and was measured the resistance that the monolayer opposed to the passage of electric current by another pair of electrodes connected to a voltmeter. TER of cell monolayers was measured with the aid of the Millicel-ERS resistance monitoring apparatus (Millipore), which directly provides the values of the resistance in ohms.
TER values are expressed as Ohms. In this report TER measurements were performed in order to define the degree of polarization of the cells when endogenous Rac1 activity was experimentally inhibited by dominant negative ER-Rac (N17) protein induction. This system allows creating two compartments above and below the filter and the culture medium was added to both compartments.

### 5.8 Suspension cultures

To performed suspension cultures, confluent monolayers of FRT cell line were trypsinized and the single cell suspension (2x10^2) was plated on regular 35-mm tissue culture dishes previously coated by a thin layer of 1% agarose (Sigma) in H2O to prevent cell attachment to the dish. Agarose was heated for 30 min at 100°C. While still hot, 1 ml of the solution was added to each plastic tissue culture dish and allowed to sit for about 10 sec. The agarose solution was then aspirated and the dishes were left at room temperature until the remaining thin layer of agarose solidified. Dishes were washed with medium before use. In this condition FRTwt cells and ER-Rac1N17 expressing cells first aggregate e successively they form polarized three-dimensional structures, known as cysts, formed by a single layer of tightly connected cells delimiting a central cavity (lumen) that could be seen by phase contrast microscopy. Most lumens were surrounded by a single layer of cells, but in some places additional cells were evident. The normal polarity of thyroid epithelial cells reverses when follicles are in suspension culture. During the polarity reversal, the cells remain attached to their neighbors. The surface features characteristic of the region of the cell next to the lumen (tight junction and microvilli) appear on the cell surface next to the medium and the surface features characteristic of the region of the cell next to the medium appear in the inside of the cyst. The polarity inversion involves changes first in the surface features of the epithelial cell and then in the position of cytoplasm organelles. Our experiments were performed both in presence and in absence of tamoxifene in order to analyze the effect of Rac1 inhibition on formation of this polarized structure.
5.9 Wound healing assay

The wound healing assay allows studying cell migration. It is simple, inexpensive, and one of the earliest developed methods to study directional cell migration in vitro. This method mimics cell migration during wound healing in vivo. The basic steps involve creating a “wound” in a cell monolayer, capturing the images at the beginning and at regular intervals during cell migration to close the wound, and comparing the images to quantify the migration rate of the cells. It is important that all the cultures are confluent at the start of the experiment. Therefore cells were grown to confluence in F12 Coon’s medium containing 5% FBS medium and then were maintained in serum free medium for 24h. The monolayers were scratched with a sterile, disposable 1000 ml plastic pipette tip, the cells were washed 3 times and monolayers were photographed at different time intervals after the initial scratch to monitor the velocity of cell migration. Photographs were taken at 10 X magnification using phase-contrast microscopy immediately after wound incision and 24h and 48h later. Pixel densities in the wound areas was measured using the Cell software (Olympus Biosystem Gmb) and expressed as percentage of wound aperture where 100 % is the value obtained at Time 0. In this thesis the wound healing test was adopted to monitor the migratory activity in cells expressing the dominant negative form of Rac1. Experiments were performed with or without 4-OH tamoxifen.

5.10 Expression and purification of recombinant GST-PAK-CD fusion proteins

E. coli BL21 cells transformed with the GST-fusion constructs is grown for 16-18 h in bacterial dishes with LB/agar/ with ampicillin (100 μg/ml) at 37°C. One colony was picked and was grown in 3 ml of LB/ampicillin for 6-8h. Then, 10 μl of bacterial suspension were diluted in 200 ml of LB/ampicillin and let to grown for 16-18h and successively in 2L of the same solution for about 1h to permit to bacteria to achieve
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the appropriate optical density value (about 600), in fact bacteria scatter light in proportion to their numbers and this is an important factor to induce recombinant proteins expression. Expression of recombinant protein is induced by addition of IPTG 1 mM and further incubation in Luria broth (LB)/ampicillin at 37°C for 3h. Cells are harvested by centrifugation (30 min at 3000 rpm), resuspended in 40 mL bacterial lysis buffer (TRITON X100 10%, EDTA 1mM, Aprotinin 40μg/ml, 3mM PMSF), and then sonicated (3×30 s, 50% cycle, mark 4). Cell lysates are centrifuged at 4°C for 45 min at 4000 rpm, and the supernatant is incubated with glutathione-coupled Sepharose 4B beads (Amersham) for 1 h at 4°C (in a ratio of 1ml of 50% bead slurry per 20mL of supernatant). Protein bound to the beads is washed 3 times in cell lysis buffer. The Amount of bound fusion protein is estimated by comparing to bovine serum albumin (BSA) standards resolved in parallel on a 12% reducing polyacrylamide gel, and afterwards stained with Coomassie blue. Protein bound to the beads was conserved at -20°C.

5.11 GTPase activity assays. GST-Pull-Down

To evaluate the rate of Rac1 activity in several experimental situations it was used a GST-Pull-Down assay. This assays is based on the use of a chimeric protein consisting of the glutathione-binding moiety of glutathione-S-transferase (GST) fused to part of an effectors molecule which binds to the GTPase in its GTP-bound form. The complex of fusion protein and GTPase is then isolated from a cell lysated by immobilization (“pull down”) of the GST moiety on a Sepharose substrate to which glutathione has been adsorbed. Finally, following elution from the glutathione Sepharose, the captured GTPbound GTPase is detected by Western blotting. For the Rac activity assays, the CRIB domain of the kinase PAK was fused to GST (GST-PAK-CD). An equivalent number of cells (3x106) were analyzed for each different sample. Cell-culture dishes were placed on ice, and cells were washed with ice-cold phosphate-buffered saline (PBS) 2 times, then, cell-lysis buffer, RIPA buffer (see SDS PAGE and Western Blot) was added in the dishes (500 μL–1 mL). A cell scraper were used to harvest cell lysates, and they were incubated with lysis buffer, in tubes eppendorf, for 15 min at
4°C in shaking and were then centrifuged for 5 min at 3000rpm at 4°C. Protein concentration was determined in supernatant with the Bio-Rad protein assay. Aliquots was taken from the supernatant (100 μg of total protein) and were incubated with bacterially produced GST-PAK1-CD protein bound to glutathione-coupled Sepharose at 4°C for 30. The beads and proteins bound to the fusion protein were washed 3 times in an excess of cold cell-lysis buffer, eluted in Laemmli sample buffer, boiled for 5 min, and then analyzed for SDS-PAGE and Western blotting using mouse mAb anti-Rac1 diluted 1:1000 (UpstateBiotechnoloy). ECL detection followed the incubation with a HRP-conjugated secondary antibody (diluted 1.1000) (Figure 13).

**Fig. 13** Outline of the experimental scheme used to isolate Rac1 in his active GTP-bound state. Cell lysated is mixed with GST-fusion protein attached to glutathione-bearing Sepharose beads. Centrifugation of the Sepharose beads is followed by washing and elution of the captured active-Rac in sample-loading buffer. Protein is then resolved by SDS-PAGE and Western Blot.

### 5.12 Precipitation of proteins with trichloroacetic acid (TCA).

To experimental samples TCA at 15% of concentration was added. Then, samples were incubated on ice in the cold room for 2 hours or alternatively over night at 4°C; then they were centrifuged at 14,000 rpm at 4 ° C for 30 minutes; successively they
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were washed three times with 0.5 ml of cold acetone and centrifuged again for 2 minutes. Supernatant was aspirated and the residue acetone was evaporated under a stream of air; the pellet was resuspended with 2X Laemmli sample buffer in agitation on the thermo mixer at 33 °C for 30 minutes; the pH is neutralized with vapors of ammoniac.

5.13 Calcium switch assay

FRT wt and FRT ER-Rac1N17 expressing cells were seeded as monolayers into dishes or on filters and cultured for 24 or 48h. E-cadherin mediated cell-cell contacts were disrupted by treatment with EGTA to a final concentration of 4Mm for 1h at 37°C. Therefore, intracellular contacts were allowed to re-establish in the presence of fresh calcium-containing medium for 5h at 37°C. In this report this assay was performed to study the effect of Rac1 inhibition on the early phases of cell-cell contacts formation to study its rule in the acquisition of cell polarity. For this reason the readdition of calcium to the cells was performed both in presence and in absence of 4-OH-tamoxifen. At 5h after calcium restoration cells were lysed on ice and assayed for extraction Triton X-100 assay or membrane/cytosolic fractionation assay. To determinate whether de novo protein synthesis or E-cadherin of recycling was recruited to plasma membrane upon calcium switch assay we performed calcium switch assay in presence of cycloheximide to block protein synthesis. Cells were incubated with medium without serum containing 150 uM cycloheximide at 37°C for 2h before switching cells to low-calcium medium, time necessary to empty Golgi apparatus from E-Cadherin signal.

5.14 Extraction in Triton X-100.

Confluent monolayers of FRTwt and FRT-ER-Rac1N17 cells were grown on 100 mm diameter dishes or on filters in bicameral systems for 24 or 24h. Then, cells were washed two times with ice-cold phosphate-buffered saline (PBS) and were incubated 20’ on ice with 500 µl of extraction buffer (150 mM NaCl, 25 mM Hepes pH 7.5; 1%
**Materials and Methods**

Triton-X100 and protease inhibitors. Cells lysates were centrifuged at 14000 rpm in Eppendorf 5417R centrifuge for 2 min. at 4 °C to separate the soluble proteins from the insoluble to the detergent Triton X-100. The supernatants represented the TX-100-soluble fraction (S) and were collected in new tubes eppendorf and subjected to low-speed centrifugation to remove cell debris. The pellets, which represent the TX-100-insoluble fraction (I), were solubilized in 100 uL of solubilization buffer (50 mM Tris-HCl pH 8.8, 5 mM EDTA and 1% SDS) and were shacked in term mixer at 30°C. When the pellet was solved was boiled at 100°C for 3’. Subsequently, pellets were resuspended using an insulin syringe fitted on Aug 22Ga until was completely solved (10 times) and then was diluted with 400 μl of extraction buffer. To both TX-100-soluble fraction and TX-100-insoluble fraction trichloroacetic acid was added. Equal volumes of each fraction were analyzed by SDS-PAGE and Immunoblotting for E-Cadherin protein.

5.15 Membrane-cytosolic fractionation assay

FRT expressing ER-Rac1N17 protein were growth in 60-mm dishes or on filters in bicameral systems for 24 or 48h and then were treated 1h with tamoxifen; after they were washed twice with ice-cold phosphate-buffered saline (PBS) and were lysed 5’ on ice with 1ml of hypotonic buffer (7.4 10 Mm TRIS Ph, 1,5 MgCl2 mM, 5 mM KCl, 1 mM DTT, 0,2 mM Na2VO3 1 mM phenylmethylsulfonyl fluorite (PMSF) 1μg/ml aprotinin and 1μg/ml leupeptin). Cells were collected by scraping and cell lysates were homogenized 15 times in the Douncer Homogenizer. Homogenates were centrifuged at 3000 rpm for 5 min to pellet nuclei and intact cells, and supernatants were then centrifugated at 33000 rpm at 4°C for 30 min in a refrigerated ultracentrifuge (TL100) to sediment plasma membranes. The cytosol-containing supernatant was removed and total protein was precipitate with TCA. Pellet, which rappresented memfrane fraction was resuspended and solubilized in leamly 2X. Membrane and cytosol fractions were then assayed for equal volumes were analyzed by Western blotting. The membrane fractions were verified to be free of contaminating cytosol by immunoblotting for the cytosolic marker GDPH.
Treatment with 1h of Tamoxifen is sufficient to transfer a detectable fraction of the ER-Rac1N17 protein from cytoplasm to the membrane.
6. REFERENCE


REFERENCE


Kamai T, Yamanishi T, Shirataki H, Takagi K, Asami H, Ito Y, Yoshida K: Overexpression of RhoA, Rac1, and Cdc42 GTPases is associated with


Nagai-Tamai, Mizuno et al. 2002


Tzuu-Shuh Jou*, Eveline E. Schneeberger‡, and W. James Nelson* Structural and Functional Regulation of Tight Junctions by RhoA and Rac1 Small GTPase Published July 13, 1998 // JCB vol. 142 no. 1 101-115 The Rockefeller University Press, doi: 10.1083/jcb.142.1.101


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