

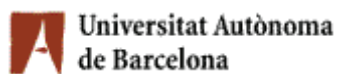
p120-catenin and Rac1:
key players in canonical Wnt signaling

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ABBREVIATIONS

AJ	Adherens Junctions
CBD	Catenin Binding Domain
JMD	Juxtamembrane Domain
EC	Extracellular Domain
GDI	Guanine nucleotide Dissociation Inhibitor
GEF	Guanine nucleotide Exchange Factor
GAP	GTPase Activating Protein
St	Standard
Wt	Wild-type
WB	Western Blot
CpG	Cytosine and Guanine nucleotides connected by phosphodiester bond
PAGE	PolyAcrylamide Gel Electrophoresis
shRNA	short hairpin RiboNucleotide Acid
GST	Glutathione S Transferase
GFP	Green Fluorescent Protein
GDP	Guanosine Diphosphate
GTP	Guanosine Triphosphate

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INTRODUCTION

I. INTRODUCTION

To form multicellular organisms the cells must be somehow bound together and eukaryotes have evolved in different ways to satisfy this need. Of all the ways in which animal cells are structured together into multicellular tissues, the epithelial arrangement is perhaps the most fundamentally important. The epithelial sheet has much the same significance for the evolution of complex multicellular organisms that the cell membrane has for the evolution of complex single cells.

Extracellular contacts between epithelial cells define the bounded (basolateral) and free (apical) cell surface (1). An important property of epithelial cells is their assembly into a physical and ion- and size-selective barrier separating tissues. Intercellular junctions, such as adherens and tight junctions, play a crucial role in the formation and maintenance of epithelial barriers. Desmosomes form the third structure of this complex (Figure 1).

Tight junctions are the most apical structure of the apical complex demarcating the border between apical and basolateral membrane domains that act as a primary barrier to the diffusion of solutes through the intercellular space. They are formed by transmembrane molecules (Occludin, Claudins and Junctional Adhesion Molecules, JAMs) that associate with cytoplasmic proteins (Zonula Occludens, ZO-1, ZO-2 and ZO-3). Adherens junctions are positioned immediately below tight junctions and characterized by joining the actin filaments of neighboring cells together, and desmosomes are even stronger connections that join the intermediate filaments of neighboring cells. Adherens junctions and desmosomes are calcium-dependent cell-cell adhesion complexes both composed of transmembrane proteins of the cadherin superfamily (Figure 1).

This specific structure, as well as different cell shapes from other tissues, is formed to specialize cell function to provide barriers that regulate ionic homeostasis between different biological compartments. Moreover, a more recently identified function of

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intercellular junctions is that they provide the cell not only with structural integrity but also function as landmarks, spatially confining signaling molecules and serving as docking sites for vesicles (2).

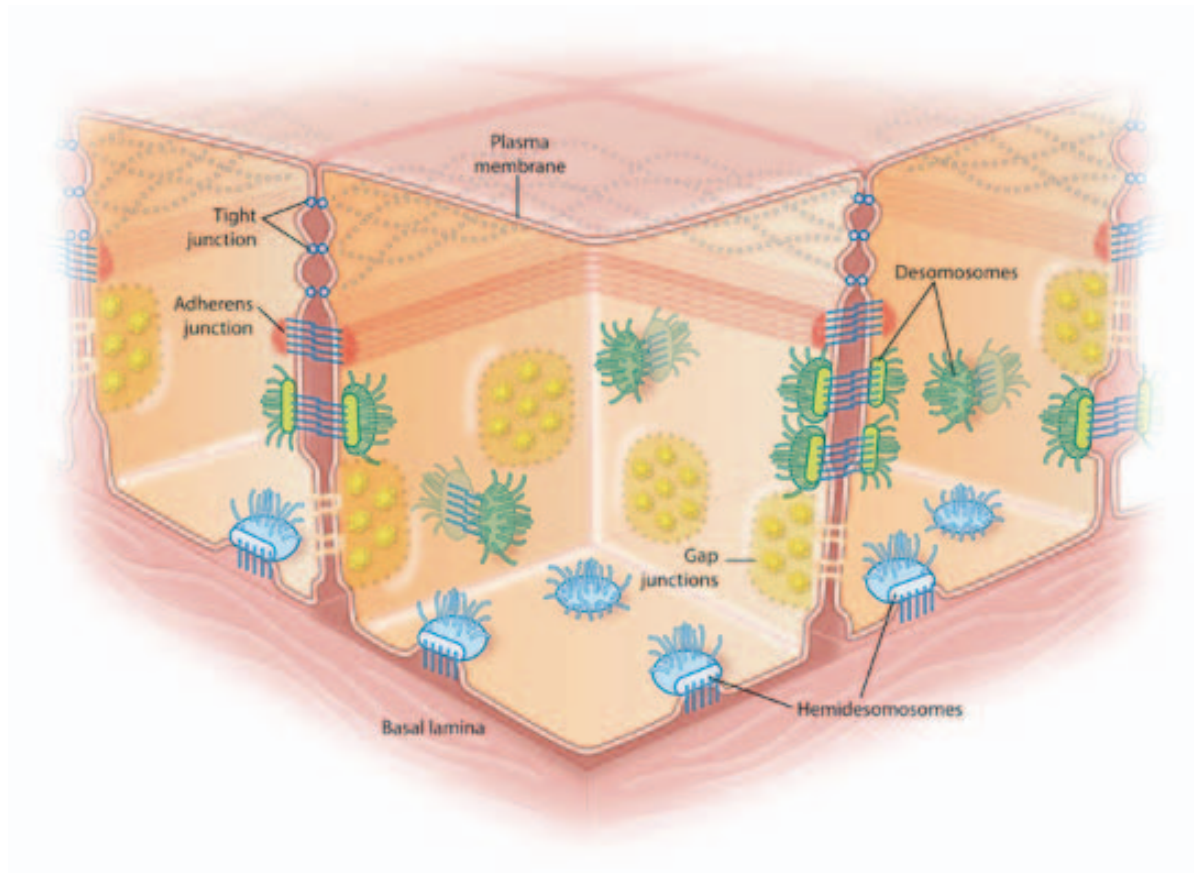


Figure 1. Different types and localization of cell junctions in an epithelial tissue.
(2010 Nature Education)

1. ADHERENS JUNCTIONS

Adherens junctions (AJs) are crucial for the initiation and maintenance of intercellular adhesion in a wide variety of tissues and cell populations (3). Although their ultrastructure suggests that adherens and tight junctions form stable structures, it is now obvious that they are highly dynamic complexes even in fully polarized epithelia. Strength is needed to maintain stable cell associations under external stress such as tissue morphogenesis or body movement. Dynamic changes in cell adhesion are needed to resolve and establish new cell contacts during developmental cell movements, tissue renewal and wound repair (4, 5). In addition, disassembly of cell–

cell contacts occurs during normal development and in metastasis of transformed cells. All of these processes require close coordination of cadherin-mediated adhesive contacts with changes in the underlying cytoskeleton. How do adhesive contacts meet the dual challenge of maintaining tissue architecture and facilitating cell movement? In the adherens junction, the cytoplasmic complex of cadherins with catenins and elements of the actin cytoskeleton serve this essential role.

1.1 E-CADHERIN

Cadherin adhesion molecules are core adherens junctions components (6). Cadherins constitute a large family of cell surface proteins, many of which participate in Ca^{2+} -dependent cell adhesion that plays a fundamental role in the formation of solid tissues (3, 7, 8). Many events in the development of multicellular assemblies are associated with changes in cadherin expression (7, 9). Expression of particular cadherins often correlates with formation of different tissue structures, and in mature tissues different cell assemblies are often demarcated by particular cadherins (10). Conversely, down-regulation or loss of cadherins correlates with an increased metastatic potential of the affected cells that arises from the loss of their adhesive properties (3, 11).

The best understood cadherins are the “classical” cadherins of vertebrates. Classical cadherins have been shown to mediate adhesive interactions at the intercellular junction structures and they are closely related to desmosomal cadherins (12, 13), which mediate adhesion at desmosomes. Desmosomal cadherins differ from classical cadherins in portions of their cytoplasmic regions, which enable their interaction with the intermediate filament system (14).

Classical cadherins are constituted by an amino-terminal extracellular region or ectodomain (EC) composed of five repeats followed by a single transmembrane domain and a carboxy-terminal intracellular region. Interactions between ectodomains on apposed cells mediate specific cell-cell contacts, whereas the intracellular region functionally links cadherins to the underlying cytoskeleton (Figure 2) (15).

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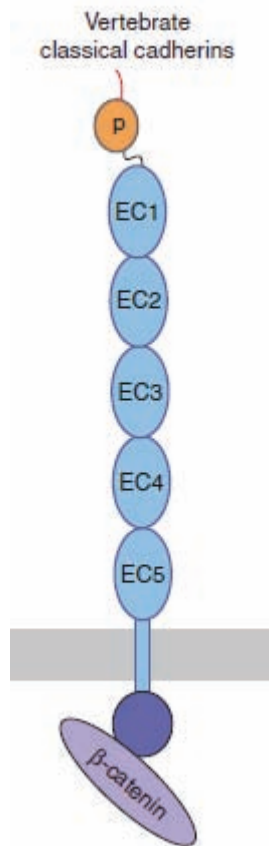


Figure 2. Vertebrate classical cadherins. Classical cadherins contain a prodomain (P) immediately following the signal sequence, which is removed by proteolysis. Mature classical and desmosomal cadherins have ectodomains composed of five extracellular cadherin (EC) repeats, a single transmembrane region, and a cytoplasmic domain that interacts with catenins. (Shapiro et al, Cold Spring Harb Perspect Biol 2009).

Their extracellular domains interact homophilically with cadherins on adjacent cells (16), meaning that cadherin molecules of one subtype bind to other molecules of the same subtype but not to cadherins of a different subtype. However the early notion that cadherin binding preferences are exclusively homophilic is not entirely correct. Some *in vitro* experiments revealed substantial cross-binding among different cadherin subtypes, including between N- and E-cadherins (17-20).

The vertebrate classical cadherins are subdivided into two closely related families, the type I and type II cadherins. At least 19 classical cadherins are conserved in vertebrate genomes: 6 type I family members and 13 type II cadherins (21). Type I cadherins include the founding cadherin superfamily member, E-cadherin, and have broad distributions in tissues (22). Type II cadherins include vascular endothelial cadherin (VE-cadherin), which is found in endothelial adherens junctions, and show a more refined pattern of expression, notably within the developing nervous system (9, 23).

The classic cadherins were originally named for the tissue in which they are most prominently expressed, but it has become clear that these expression patterns are not

exclusive, and most cadherins can be expressed in many different tissues. E-cadherin (epithelial cadherin) is expressed primarily in epithelial cells.

Cell adhesion by cadherins depends on the presence of Ca^{2+} . Crystal structures of ectodomain regions containing multiple EC domains show that the connections between successive domains are rigidified by Ca^{2+} coordination (24, 25). Three Ca^{2+} are coordinated from the base of one domain, the top of the next and the linker region between them. These Ca^{2+} binding sites are among the most highly conserved sequence features of cadherins across all species (21, 26)(Figure 3).

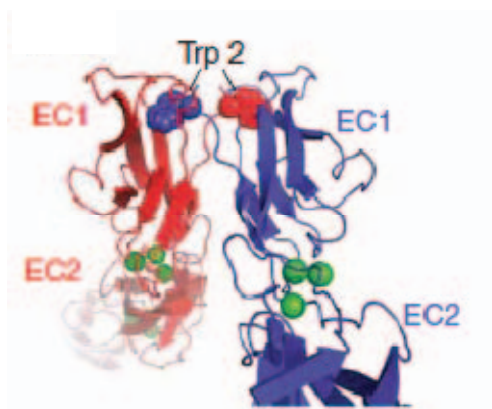


Figure 3. Cadherin extracellular domain structure. Close-up rotated view of the strand exchanged dimer EC1, highlighting the conserved “anchor” residue Trp 2. Bound calcium ions are shown as green spheres. (Shapiro and Weis, Cold Spring Harb Perspect Biol 2009)

The intracellular domains of classic cadherins mediate a re-organization of the actin cytoskeleton and promote intracellular signaling through interaction with the catenins (27-29).

β -catenin binds directly to the carboxyterminal catenin binding domain (CBD), while p120-catenin interacts directly with the so-called juxtamembrane domain (JMD) (30-32) (Figure 4). β -catenin interacts further with α -catenin, which increases cell adhesion linking to the actin cytoskeleton. As mentioned before, catenins have other roles in cadherin-mediated adhesion in addition to the direct physical linkage of cadherins to the actin cytoskeleton at the AJs. I will explain them further on.

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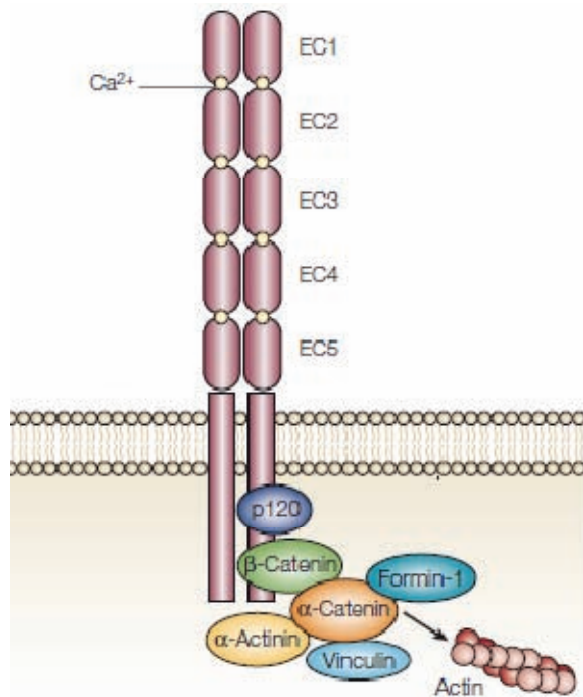


Figure 4. The cadherin-catenin protein complex. Classic cadherins form a core protein complex that consists of a parallel (*cis*) cadherin dimer and the catenin polypeptides.

The core catenin complex consists of p120ctn, bound to the juxtamembrane region, and β -catenin, bound to the distal region, which in turn binds α -catenin. In a less well understood way, α -catenin binds to actin and actin binding proteins, such as vinculin, α -actinin or formin-1 (Gumbiner BM, Nature 2005).

The cytoplasmic region of classical cadherins, roughly 150 amino acids long, is the most highly conserved portion of these proteins (21) and is unstructured in the absence of a binding partner (33). The high level of sequence identity in the cadherin cytoplasmic region suggests that all classical cadherins interact with the catenins in this fashion.

1.2 P120-CATENIN

p120-catenin (p120ctn) is the prototypic member of a subfamily of armadillo repeat domain proteins involved in intercellular adhesion. It was initially isolated in a screen for Src tyrosine kinase substrates and subsequently shown to interact with classical cadherins (34).

A characteristic feature of the p120ctn is its ability to bind cadherins (35-38) at the juxtamembrane site of the cytoplasmic tail of the cadherin molecule (30, 31). Mechanistic models proposed suggest that the low affinity of the interaction observed between p120ctn and E-cadherin is functionally relevant in cells, where p120ctn shuttles between a bound and cytoplasmic state (39).

The discovery of the transcription factor Kaiso as a novel p120ctn binding partner (40) provided the first tangible evidence that p120ctn might have additional roles in the nucleus (41).

Initially designated p120ctnCAS (for cadherin-associated Src substrate), p120ctn was renamed p120ctn (catenin; (42)) to avoid confusion with a different Src substrate, p130CAS. As shown in Figure 5, p120ctn is an 85-115 kDa protein that is composed by an N-terminal domain, a central domain with 10 Armadillo repeats and a C-terminal tail. It is through the Armadillo domain that p120ctn interacts with the cytoplasmatic tail of cadherins and with Kaiso. The N-terminal domain (also termed the phosphorylation domain and regulatory domain) is important for signal integration from tyrosine and serine kinases and binds nonreceptor tyrosine kinases such as Fer, Fyn and Yes (43) and the tyrosine phosphatases PTP μ and SHP-1 (3, 42, 44). This region also contains the majority of serines and threonines sites from the protein.



Figure 5. p120ctn structural features. Red targets show tyrosine residues phosphorylated *in vivo* by Src and green targets show serine and threonine residues phosphorylated *in vivo* (Alemà S and Salvatore AM, *Biochim Biophys Acta*, 2007)

In addition, it is now evident that most cells types express multiple isoforms of p120ctn, which are generated by alternative splicing of a single gene (35, 38, 45, 46). N-terminal splicing events results in the expression of p120ctn isoform type 1, 2, 3 or 4 (Figure 6). Furthermore, alternative splicing of the C-terminal ends leads to use of exon A, exon B, both exons A and B, or none of them. On rare occasions, p120ctn contains a sequence encoded by and additional exon (exon C), which is inserted within Armadillo repeat 6.

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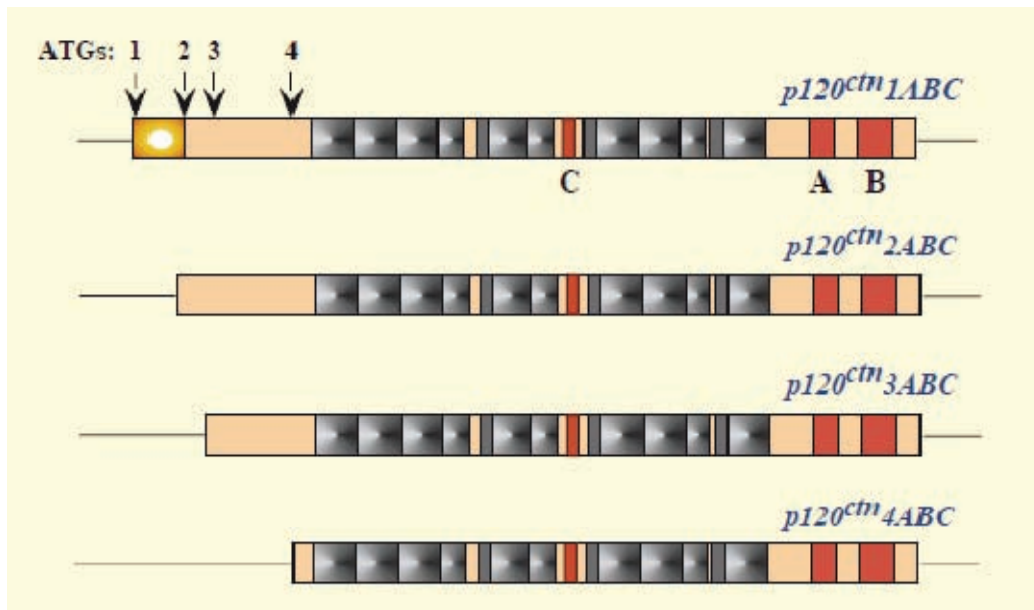


Figure 6. p120ctn nomenclature and alternative splicing. Armadillo repeats are depicted by gray boxes interrupted by short loops in repeats 4, 6 and 9. Four N-terminal ATG start generate p120ctn isoforms 1, 2, 3 and 4. p120ctn isoform 1 contains a putative coiled-coil domain (yellow box), which is absent in the rest of the isoforms. Additional alternative splicing results in alternative usage of exons in the C-terminal end. Exons A and B are often used, whereas exon C is used rarely (Anastasiadis PZ and Reynolds AB, J Cell Sci 2000).

The existence of cell-type-specific expression patterns implies functional differences between the p120ctn isoforms. For example, macrophages and fibroblasts make N-cadherin and preferentially express p120ctn 1A isoforms, whereas epithelial cells make E-cadherin and preferentially express smaller isoforms, such as p120ctn 3A. Alternative splicing usually affects only N- and C-terminal domains, so the Armadillo domain is left intact and free to interact with cadherins. Other members of the p120ctn family are also expressed as multiple isoforms. The regulated expression of multiple p120ctn family members and their isoforms might provide an important mechanism for fine-tuning the activities of the various cadherins in different cell types.

Members of the p120ctn family share a characteristic structural organization of the Armadillo domain, which suggests an ancient evolutionary relationship. However, two groups have emerged that differ both in the degree of similarity that they share with p120ctn, and in their subcellular localizations. The first group includes ARVCF (Armadillo repeat gene deleted in Velo-Cardio-Facial syndrome), δ -catenin (neural plakophilin-related Armadillo protein) and p0071. These proteins share more than 45% identity with p120ctn in their Armadillo domains. The second group, the plakophilins,

has approximately 30% identity to p120ctn in their Armadillo domains. Unlike the first group, which interact with cadherins through their Armadillo domains, plakophilins localize to desmosomes through interactions mediated by their N-terminal domains.

As mentioned before adherens junctions are dynamic, even in a normal polarized epithelial cell from a tissue are highly dynamic. Cadherin-based adhesive structures are major sites for tyrosine kinase signaling being enriched in tyrosine-phosphorylated proteins and a variety of nonreceptor and receptor tyrosine kinases (RTK), as well as protein tyrosine phosphatases (PTP) (3, 42, 44). The phosphorylation of specific junctional components could thus provide efficient means to orchestrate the turnover of adherens junctions. Tyrosine phosphorylation of adherens junction's components is a tightly controlled regulatory event: for instance, an increase in tyrosine phosphorylation of catenins has been reported in nascent adhesive contacts (47) while low levels of tyrosine phosphorylated junctional proteins are observed in confluent cells (48).

This phosphorylation scenario changes upon exposure of epithelial cells to oncogenic tyrosine kinases or to growth factors capable of triggering signaling cascades that lead to cell migration. For instance, β -catenin and p120ctn are highly tyrosine phosphorylated in Src transformed cells (49-51) and in response to epidermal growth factor (EGF) (52-54), hepatocyte growth factor (HGF) (53, 55) and vascular endothelial growth factor (VEGF) (56). Although tyrosine phosphorylation of adherens junction's components is generally associated with disruption of cell-cell adhesion, the relationship between direct phosphorylation of the catenins and defects in cadherin function has remained a poorly understood issue. Conversely, there are also evidences that phosphorylation in tyrosines of catenins increase their affinity for cadherins, as it is the case for p120ctn phosphorylated by Src and its affinity for E-cadherin (43, 57, 58).

1.3 β -CATENIN, α -CATENIN AND PLAKOGLOBIN

Structurally, β -catenin is a so-called armadillo-repeat protein, named after its homologue in *Drosophila melanogaster*, Armadillo. The Armadillo repeats serves as a

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binding site for most of its numerous binding partners. The N and C-terminal are regulatory domains that control its degradation, binding and signaling activities. β -catenin is also an intracellular signal transduction molecule that mediates signaling in the Wnt growth factor pathway (59). I will explain in detail later on.

Plakoglobin, also known as a γ -catenin, is an armadillo-repeat protein that is similar to β -catenin and that can bind to the catenin binding domain (CBD) of cadherins. It is highly enriched in desmosomes where it helps to mediate a link to the intermediate filament cytoskeleton, but can also be bound at adherens junctions. It does not seem to have the same Wnt-pathway signaling function as β -catenin, but could be involved in other signaling pathways.

α -catenin is a cytoskeletal protein that generates extensive flattening between neighbouring cell surfaces (60). It binds to actin and several other actin-binding proteins, as well as to the N-terminal region of β -catenin (it does not directly interact with cadherins). It binds to signaling proteins, such as Formin-1 (Figure 4), which regulate the actin cytoskeleton. It also seems to have a signaling role that regulates cell proliferation. β -catenin interacts with either α -catenin, which increases cell adhesion, or with IQGAP, which prevents cytoskeletal association of the complex (61). In contrast, p120ctn does not bind α -catenin or IQGAP (36, 61).

2. GTPASES

Rho guanosine triphosphates (GTPases) belong to the Ras superfamily. The Ras superfamily of GTPases is particularly interesting to cell biologists, as its members have turned out to be master regulators of many aspects of cell behavior. These small, monomeric GTPases, which number over 150 in mammals, fall into five major groups: Ras, Rho, Rab, Arf and Ran (Figure 7).

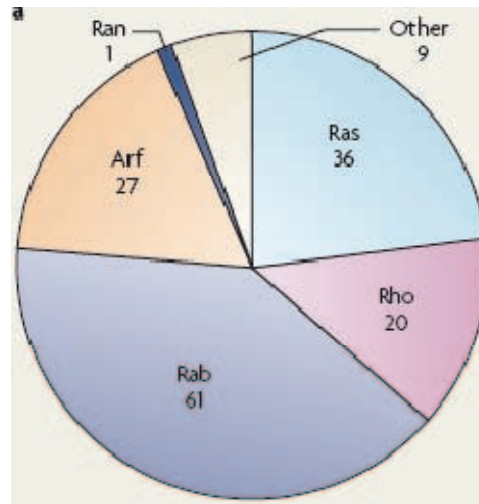


Figure 7. The human Ras superfamily. It is comprised of 154 members, which are divided into five major branches on the basis of sequence and functional similarities (Vigil D et al, Nature 2010)

Rho GTPases are a family of small proteins (21-25 kDa) that share structural homology and function as molecular switches in a variety of signaling pathways following stimulation of cell surface receptors. So far, 22 human members of the Rho family have been identified but the best characterized molecules are RhoA, Rac1 and Cdc42.

Typically Rho proteins are 190-250 residues long and consist only of the GTPase domain and short C-terminal extensions. Within their GTPase domain, they share approximately 30% amino acid identity with the Ras proteins and 40-95% identity within the family. All members contain the sequence motifs characteristic of all GTP-binding proteins and bind to GDP and GTP with high affinity (62, 63).

In addition, the majority of members undergo C-terminal post-translational modification by isoprenoid lipids (64). Together with other C-terminal modifications or

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sequences, isoprenoid addition facilitates their subcellular location and association with specific membranes or organelles. These lipid modifications are mainly palmitoylation or prenylations, being farnesylation and geranylation the most frequent post-translation modifications (65). Two factors concur to determine specific Rho GTPase function: tissue specificity of GTPase effectors and distinct intracellular localizations of Rho GTPases, due to the different lipid modifications (66).

The activity of Rho GTPases is determined by two different states: a GTP-bound active state and an inactive GDP-bound state. Cycling between the two states is controlled by three classes of molecules: Guanine nucleotide exchange factors (GEFs), which promote Rho proteins activity by catalyzing the exchange of GDP for GTP (67); GTPase activating proteins (GAPs), which promote inactivation of Rho proteins by stimulation of GTP hydrolysis; and Guanine nucleotide dissociation inhibitors (GDIs), which bind to GTPases and keep them in an inactive state form or prevent active GTPases from signaling towards their downstream targets (68). In the active GTP-bound state, Rho GTPases bind to downstream effectors and transduce signals from extracellular stimuli through intracellular signaling pathways to regulate cellular processes (Figure 8). GEFs can function on several Rho GTPases and in turn Rho GTPases can be activated by multiple GEFs (67).

As mentioned before, Rac1, RhoA and Cdc42 are the most studied components of the family for being the most highly conserved GTPases:

The Rac-related subfamily includes Rac1 (and its splice variant Rac1b), Rac2 and Rac3. (65). Several Rac-related proteins, sharing more than 80% identity, stimulate the formation of lamellipodia and membrane ruffles, presumably through interaction with the WAVE complex (69). The splice variant Rac1b contains an additional C-terminal 19-residue insert and is constitutively active due to an increased intrinsic guanine nucleotide exchange rate, decreased intrinsic GTPase activity, its inability to interact with GDIs and enhanced association with the plasma membrane (70, 71).

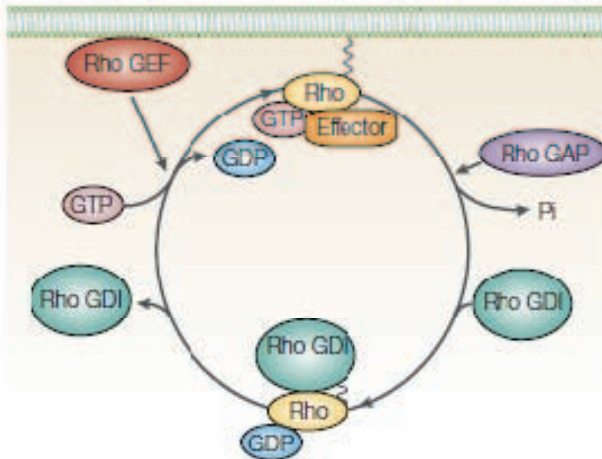


Figure 8. Regulation of Rho-family GTPases activity. Regulatory proteins tightly modulate this cycle: Guanine nucleotide-exchange factors (GEFs) activate Rho GTPases by promoting the release of GDP and the binding of GTP. GTPase-activating proteins (GAPs) inactivate Rho GTPases by increasing the intrinsic GTPase activity. Guanine nucleotide-dissociation inhibitors (GDIs) bind to C-terminal prenyl groups on some Rho proteins, sequestering them in the cytoplasm away from their regulators and targets (Fukata M and Kaibuchi K, Nat Rev Mol Cell Biol. 2001)

Rac1 is the best-studied member of this family and is ubiquitously expressed, whereas Rac2 is expressed only in hematopoietic cells, where it seems to have specialized functions (72). Rac3, highly expressed in brain, is strongly localized to the membranes where it appears to be hyperactive (73). Rac1-knockout mice are embryonic lethal and show a range of defects in germ-layer formation (74).

Animals have 3 Rho isoforms, RhoA, RhoB, and RhoC, sharing 85% aminoacids sequence identity (66, 75). Despite their similarity, both modulators (GEFs and GAPs) and downstream effectors show favored interaction with single Rho isoforms, and the three proteins play differential roles in cells.

The most fundamental and best-studied function of the Rho GTPases is regulation of actin cytoskeleton organization (76). The actin cytoskeleton has a pivotal role in processes such as maintenance of cell shape, cell motility, immunological response and cell division. The cytoskeleton is a dynamic structure that undergoes reorganization in response to extracellular stimulation initiated by activation of cell surface receptors. Although almost all Rho proteins play an important role in organizing the actin filament system, most of the information available on the function of Rho proteins stems from studies of Rac1, RhoA and Cdc42. Each of these appears to have a distinct role with regard to the actin cytoskeleton: Rac1 regulates the formation of lamellipodia and membrane ruffles (77), RhoA regulates the assembly of focal

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adhesions complexes and contractile myosin filaments (78), and Cdc42 regulates the formation of filopodia (79).

The cytoskeleton rearrangements triggered by Rho GTPases have a key function in cell motility, a cycle consisting of four ordered processes: membrane protrusion at the leading edge, adhesion of the protrusions to the substrate, translocation of the cell body and retraction of the trailing edge. Together, these processes result in coordinate and polarized movements of the cell (80, 81). Thus, during cell movement, Rac1 and Cdc42 stimulate formation of protrusions at the leading edge and RhoA induces retraction at the trailing edge. Unexpectedly, recent results show that RhoA can be also activated at the front of the cell during cell movements (82, 83). This coordinated reorganization permits cells to move toward a target.

Rho GTPases have also been demonstrated to mediate formation and maintenance of adhesion structures, such as tight and adherens junctions (TJ and AJ, respectively) and also contribute to AJ disassembly, leading to epithelial-to mesenchymal transition (EMT) (84, 85).

Numerous reports indicate that AJs assembly is preceded by the localized induction of filopodia and/or lamellipodia and that these protrusive structures drive intimate membrane contact between adjacent cells (86-88). The formation of localized protrusions may be initiated by early cadherin interactions that activate Rac1, and perhaps Cdc42, through the ability of cadherin complexes to bind α -catenin and reorganize the cytoskeleton, which induces IQGAP dissociation from β -catenin and actin (89). However, an alternative possibility has emerged from work on a relatively new family of cell-cell adhesion proteins, the nectins. These Ca^{2+} -independent, immunoglobulin-like trans-membrane proteins form homo- and heteromolecular interactions, which suggest that this is required for subsequent cadherin-based junctional assembly (90, 91). Nectin-nectin interactions activate both Cdc42 and Rac1, and this could provide the localized protrusive activity that facilitates subsequent cadherin-based adhesion (92). Unlike Rac1 and Cdc42, RhoA activity is thought to be required for an earlier step in junction formation that is the cadherin clustering at sites of cell-cell contact (93, 94).

In apparent contradiction, a number of studies have implicated Rho GTPases in the disassembly of cell–cell contacts and in tumor progression (95). A clear connection can be established between Rho proteins over-expression and a large variety of human tumors (96, 97). Rho GTPases have been reported to contribute to most steps of cancer initiation and progression including the acquisition of unlimited proliferation potential, survival and evasion from apoptosis, angiogenesis, tissue invasion and the establishment of metastasis (Figure 9).

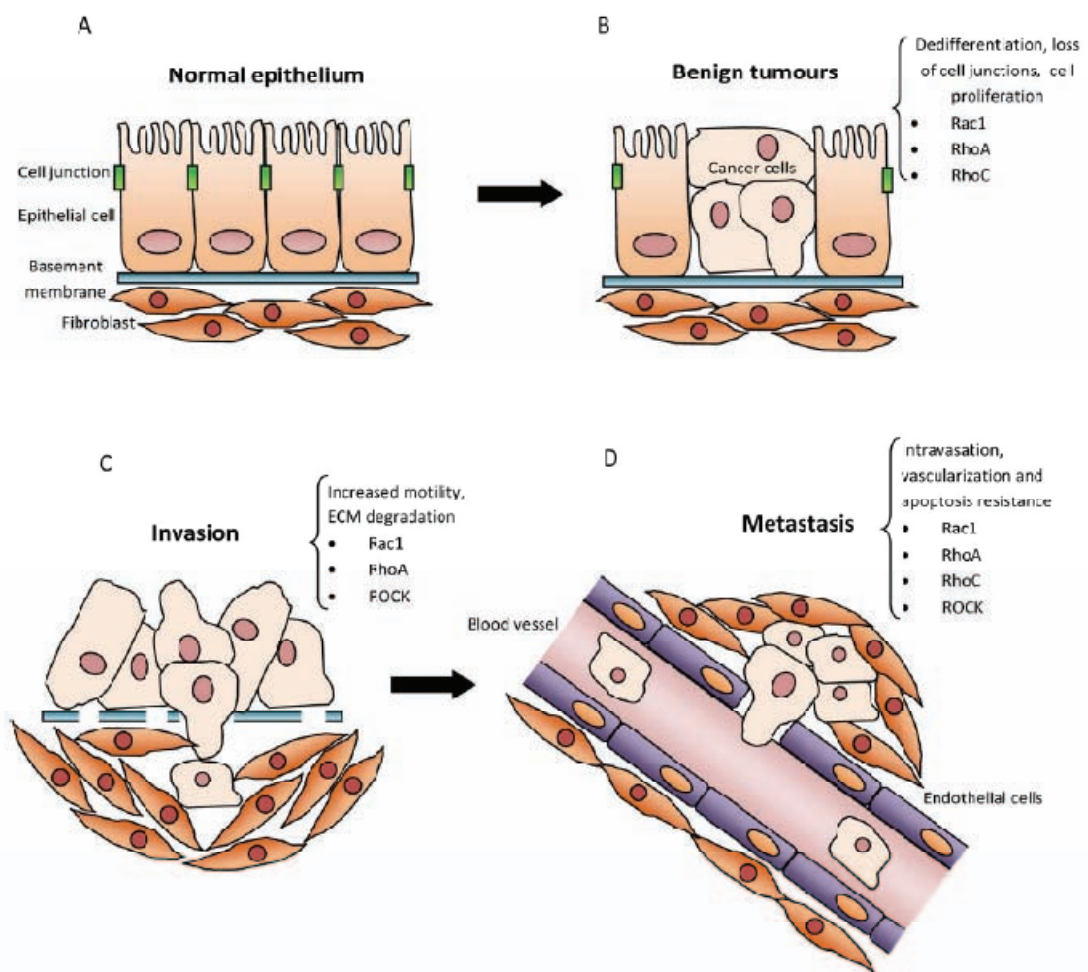


Figure 9. Involvement of Rho proteins at different stages of tumor progression. A) Normal epithelial cell polarity. B) Benign tumors: once the tumor is initiated, Rho proteins can contribute to tumor development by stimulating differentiation, growth and loss of cell polarity. C) Locally invasive tumors: Rho proteins contribute to tumor development by altering cell-cell and cell-matrix adhesion. D) Metastasis to distant site: Rho proteins are necessary to cross endothelial cell layers and promote expression of angiogenic factors, leading to an increase in vascularization of the tumor. (Parri M and Chiarugi P, Cell Commun Signal. 2010).

Primary tumors generally arise as a consequence of multiple mutations and epigenetic changes affecting key genes that ultimately affect proliferation and survival.

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Unexpectedly, to date, no mutations have been found in Rho proteins. Only one member of the Rho family of small GTPases (RhoH) has been reported to be genetically altered in non-Hodgkin's lymphomas and multiple myeloma. Since mutations in Rho proteins have not been found, deregulation of Rho GTPase signaling could occur at the level of expression or activation of Rho GTPases and also at the level of expression or activation of their regulators or downstream effectors (98).

Although Rho GTPases clearly participates in cell migration, the question remains of how to reconcile its role in migration with the "strengthening" effect on cell-cell contacts. Because Rho proteins are involved in two seemingly opposing activities, which are cell-cell junction assembly and cell migration, it is understandable that its effects are greatly influenced by environmental factors and cell type.

2.1 GUANINE NUCLEOTIDE EXCHANGE FACTORS (GEFs)

The Guanine nucleotide exchange factors (GEF) family is really large, consisting of over 70 proteins mainly belonging to the Dbl or the Dock families (75, 99).

Most of the recognized GEFs contain two characteristic domains: the Dbl-Homology (DH) domain, which accounts for the catalytic activity, and an adjacent, C-terminal, Pleckstrin-Homology (PH) domain. PH domains are responsible for binding phospholipids, leading to localization of Rho GEFs to the plasma membranes. In addition, PH domains regulate GEF activity through allosteric mechanisms (67) and function as docking sites for proteins that associate with signaling cascades regulated by Rho GTPases. GEFs contain many additional domains and influence the signaling pathway downstream of Rho proteins.

Many members of the Rho GEF family exist in an inactive or partially active state before stimulation. The basal state of those proteins may be regulated by one or more mechanisms, such as interaction between DH and PH domains, interaction of another regulatory domain with DH or PH domain, oligomerization between DH domains or direct protein-protein interactions (100).

The Vav family of proteins consists of three highly conserved mammalian members: Vav1, Vav2 and Vav3. While Vav1 is expressed exclusively in hematopoietic cells (101), Vav2 and Vav3 have much broader expression patterns (102, 103), although Vav3 expresses highest levels in hematopoietic cells and brain (104). In addition to these mammalian members, two Vav protein homologues were isolated in invertebrates (*Drosophila* (105) and *Caenorhabditis elegans* (106)) and fragments of Vav-like proteins are found in *X.laevis*, fish (*D.reiro*, *I.punctatus*) and worms (*S.stercoralis*).

In addition to the DH and PH domains common to most Rho GEFs, Vav proteins are composed of multiple structural domains enabling their involvement in numerous signaling cascades. These domains include: a calponin-homology (CH) domain, which functions as an actin binding domain in other proteins; an acidic motif (Ac) that contains three tyrosine phosphorylation sites (one of them, Y174 that interacts with the DH domain to prevent access by GTPases); a zing finger domain (ZF), and two SH3 (Src homology 3) domains flanking a single SH2 region (Figure 10).

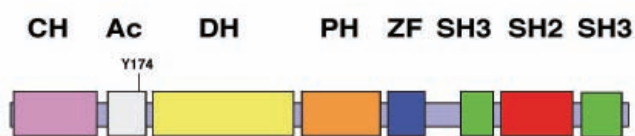


Figure 10. Structure of mammalian Vav proteins. The structural domains are described in the text. The inhibitory tyrosine residue involved in Vav inactivation (Y174) is shown. (Bustelo XR, Front Biosci 2002)

Vav proteins are unique in encoding a DH-PH cassette followed by a ZF and a SH3-SH3-SH3 cassette in the same molecule. This protein structure enables them to participate in multiple signaling pathways. Thus, in addition to their major function as GEFs for Rho GTPases, Vav proteins also act in many processes as adaptor proteins. Moreover, Vav proteins are the only known GEFs that contain a SH2 domain and conserved tyrosine motifs. This unique structure enables regulation of the catalytic activity of Vav proteins by tyrosine phosphorylation (107, 108)(Figure 11). However, there are evidences that other structural or energetic mechanisms can also cooperate to autoinhibit and activate Vav (109).

Several GEFs appear to be highly specific toward a single GTPase and other GEFs activate several GTPases, for instance Vav activates Cdc42, Rac1 and RhoA (110).

Introduction

Thus far, no constitutively activated mutants of Rho GTPases have been found to be associated with cancer; all associations with cancer have involved over-expression of Rho GTPases (100). This makes inhibition of GEFs a very promising strategy for combating human cancers involving Rho GTPases.

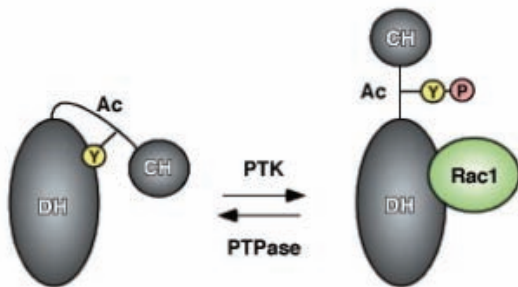


Figure 11. Schematic representation of the mechanism of activation of Vav by tyrosine phosphorylation. The phosphorylation of Y174 induces the release of the N-terminal domain (CH) from the GTPase binding site (DH). (Bustelo XR, Front Biosci 2002)

2.2. REGULATION OF RHO GTPASES BY P120CTN

Ectopic expression of p120ctn results in a variety of morphological effects, depending on the cell type and the level of p120ctn over-expression. In fibroblasts, high level over-expression causes a “branching” phenotype (58, 111, 112). Less pronounced branching effects, and increased formation of lamellipodia, are typically observed in epithelial cell types. The branching phenotype is also observed when p120ctn is over-expressed in cadherin-deficient cells (111). These effects occur as a consequence of a perturbation of actin stress fibers, structures which are dependent on RhoA activity. Similarly, Rac1 and Cdc42 are activated when p120ctn is over-expressed (113).

When over-expressed in normal cells, p120ctn rapidly saturates available cadherin-binding sites, and then accumulates in the cytoplasm. Interestingly, the effects of ectopically-expressed p120ctn are blocked by expression of various cadherin constructs that sequester the p120ctn excess (111, 113, 114). These data supported a model where both the branching morphology and p120ctn effects on focal adhesions and cell contractility are caused by cadherin-uncoupled, cytoplasmic p120ctn. The model was further supported by the low affinity of p120ctn for cadherin binding (relative to β -catenin), which suggests that its ability to shuttle between cadherin-bound and cytoplasmic pools may be a key feature of its action. According to this

model, the ability of p120ctn to affect Rho GTPases and cause morphological changes is blocked by cadherin binding.

Moreover, it is now apparent that the induction of morphological changes and regulation of Rho GTPases are shared properties of several p120ctn family members, including δ -catenin, p0071 and plakophilin 2 (115). Interestingly, *Xenopus* ARVCF induces branching (116), unlike human ARVCF which failed to induce morphological changes (117).

The mechanism by which p120ctn affects Rho GTPases is still unclear. A major issue is whether p120ctn inhibits RhoA and activates Rac1 and Cdc42 via the same or separate mechanisms. It is already known that RhoA directly interacts with the N-terminal domain of p120ctn and that this interaction inhibits the intrinsic GDP/GTP exchange activity of RhoA in a Guanine Nucleotide Inhibitor (GDI) manner (111, 118). Moreover, this p120ctn-RhoA binding is fine-tuned regulated by specific tyrosine phosphorylations in the N-terminal regulatory domain of p120ctn, as well as the p120ctn-E-cadherin binding. Moreover, binding of E-cadherin and RhoA to p120ctn can occur simultaneously. Binding of p120ctn to E-cadherin might induce a conformational change that would prevent the GDI activity or, alternatively, recruit a GEF responsible for the activation of RhoA, as it has been proposed (58, 111).

A different mechanism was suggested for Rac1 activation through Vav2 (Rho GEF for Rac1) interaction with p120ctn, which could account for the ability of p120ctn to activate Rac1 and Cdc42 (113). There are also some evidences that Rac1 activity can inhibit RhoA in some cells (119), but the mechanism by which p120ctn affects Rho GTPases and the possibility of a reciprocal balance between RhoA and Rac1/Cdc42 remains unclear.

Moreover, p120ctn and Rho GTPases play essential roles in the regulation of intercellular adhesion. They are both involved in regulation of cadherin clustering and stabilization, reorganization of the cytoskeleton and cadherin-mediated signaling (120, 121). How all these seemingly different effects relate to each other is largely unknown.

Introduction

Figure 12 provides a hypothetical model for the function of p120ctn at intercellular junctions and depicts potential mechanisms that could promote cadherin clustering and cell adhesion.

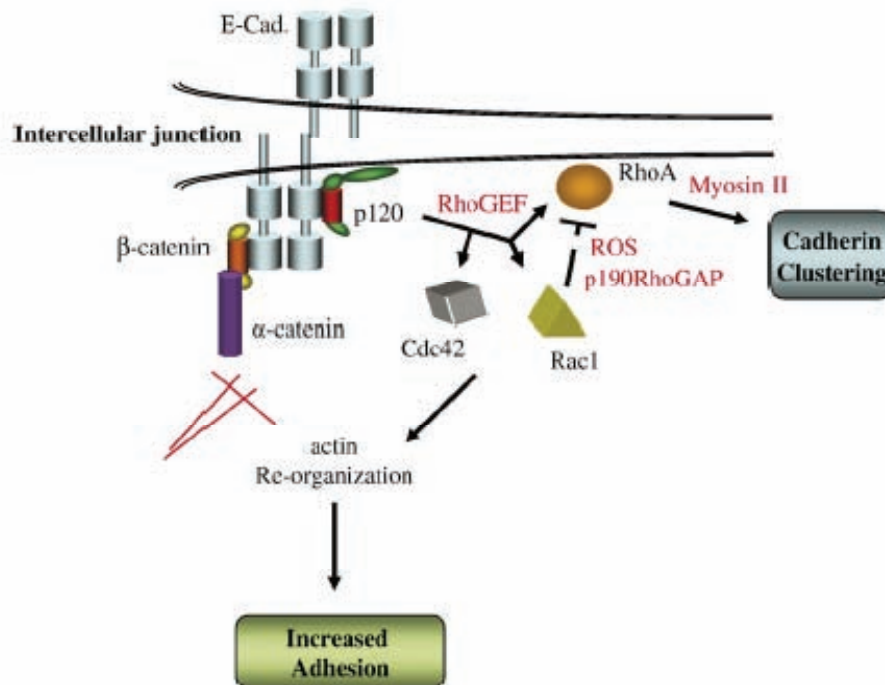


Figure 12 . Model of p120ctn and Rho GTPases functions at cell-cell junctions. p120ctn association promotes the membrane stabilization of cadherins. Upon cadherin homophilic ligation, p120ctn mediates the activation of Rho GTPases, via its interaction with Rho GEFs. The activation of RhoA promotes myosin II-dependent clustering of cadherin complexes. The activation of Rac1 and Cdc42 promote the re-organization of the actin cytoskeleton at nascent cell–cell contacts, maturation of intercellular junctions, and increased cell–cell adhesion. RhoA may be detrimental to cell adhesion after its initial role in clustering cadherin complexes, as it can oppose the actin re-organization induced by Rac1 and Cdc42. Therefore, its activity is downregulated at later time points via a Rac1-p190RhoGAP-mediated pathway. (Anastasiadis P.Z. *Biochim Biophys Acta*, 2007).

Similarly, several studies have indicated that Rho GTPases play important roles in many stages of vesicular trafficking (reviewed in (122, 123)) and in turn p120ctn binding to the juxtamembrane domain (JMD) of cadherins promotes cadherin stability preventing its endocytosis (reviewed in (124, 125)). Taken together and given the effects of p120ctn on both Rac1 and RhoA activities, it is also possible that p120ctn-mediated regulation of Rho GTPase activities at cell-cell junctions affects cadherin endocytosis.

Finally, regarding motility, p120ctn promotes actin polymerization by activating Rac1 and Cdc42, resulting in increased cell-cell adhesion if E-cadherin is present, or

alternatively, cellular protrusion and cell motility through the formation of lamellipodia and filopodia. Inhibition of RhoA by p120ctn may again have either positive or negative effects on cell motility, by promoting the reorganization of actin structures in the leading edge and presumably de-adhesion at the cell's trailing end, or by reducing contractility over the cell body, respectively (revised in (98)).

3. WNT SIGNALING

Wnt signaling is one of the fundamental mechanisms that direct cell proliferation, migration, cell polarity, and cell fate determination during embryonic development and tissue homeostasis while in the adult, primarily through the effects on stem cell proliferation and differentiation (126, 127). As a result, mutations in the Wnt pathway are often linked to human birth defects, cancer, and other diseases (128).

The canonical Wnt pathway controls the stability of β -catenin (Figure 13). Under basal conditions, cytoplasmic β -catenin is constantly degraded by the action of the Axin complex, which is composed of the scaffolding protein Axin, the tumor suppressor *adenomatous polyposis coli* gene product (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3). CK1 and GSK3 sequentially phosphorylate the N-terminal region of β -catenin, resulting in β -catenin recognition by β -Trcp, an E3 ubiquitin ligase subunit, and subsequent β -catenin ubiquitination and proteasomal degradation (129). This continual elimination of β -catenin prevents β -catenin from reaching the nucleus and Wnt target genes expression is repressed by the DNA-bound T cell factor/lymphoid enhancer factor (TCF/LEF) family of proteins, (Figure 13). Wnt/ β -catenin pathway is activated when a Wnt ligand binds Frizzled receptor (seven-pass transmembrane receptor) and its co-receptor LRP5/6 (low-density lipoprotein receptor-related protein 5 or 6). The formation of the complex Wnt-Fz-LRP5/6, together with the recruitment of the scaffolding protein Dishevelled (Dvl), results in LRP5/6 phosphorylation and the recruitment of the Axin complex to the receptors. This event leads to inhibition of the Axin-mediated β -catenin phosphorylation and thereby to the stabilization of β -catenin, which accumulates and translocates to the nucleus to interact with TCF/LEF transcription factors and activates Wnt target gene expression (Figure 13).

Introduction

Much less well understood are non-canonical Wnt signaling pathways, which are characterized as being TCF/ β -catenin transcription-independent.

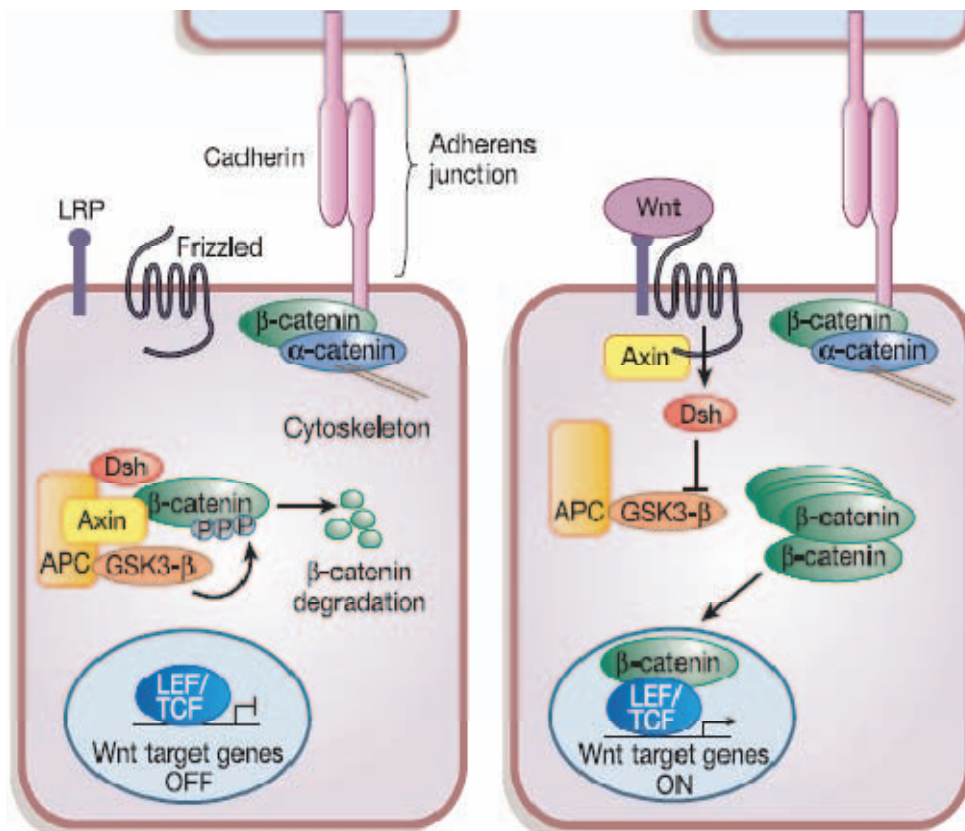


Figure 13. Overview of canonical Wnt pathway. In the absence of Wnt ligand (left panel), β -catenin free in the cytoplasm interacts with the degradation complex and is targeted to proteasomal degradation. Wnt target genes are repressed by repression factors. In the presence of Wnt ligand (right panel), LRP5/6 and its co-receptor interacts and Dvl is recruited. This leads to the phosphorylation of the LRP5/6 and Axin recruitment to the receptor complex. Therefore, Axin-mediated phosphorylation/degradation of β -catenin is inhibited and β -catenin accumulates in the cytoplasm, where it translocates to the nucleus and acts as a coactivator for TCF to activate Wnt-responsive genes. (Reya T and Clevers H, Nature 2005).

The Planar Cell Polarity (PCP) (or non-canonical) pathway promotes polarization within the plane of a tissue that means that cells organize themselves in a particular orientation with respect to a common body axis. This allows processes such as hair growth or cell division to occur in a spatially coordinated way throughout the tissue. Convergent extension (CE) movements (characteristic from tissues undergoing extensive morphogenesis such as vertebrate gastrulation) also involve Wnt signaling components acting in a non-canonical context (130). For PCP pathway, Wnt signaling is transduced through Fz independent of LRP5/6. This pathway through dishevelled mediates cytoskeletal changes through RhoA and Rac1. In addition, Wnt proteins can

also activate other non-canonical signaling pathways, including calcium-calmodulin-dependent kinase 2 (CaMK2, Wnt/Ca²⁺ pathway), protein kinase C (PKC) (131) and protein kinase A (PKA) (132) (Figure 14).

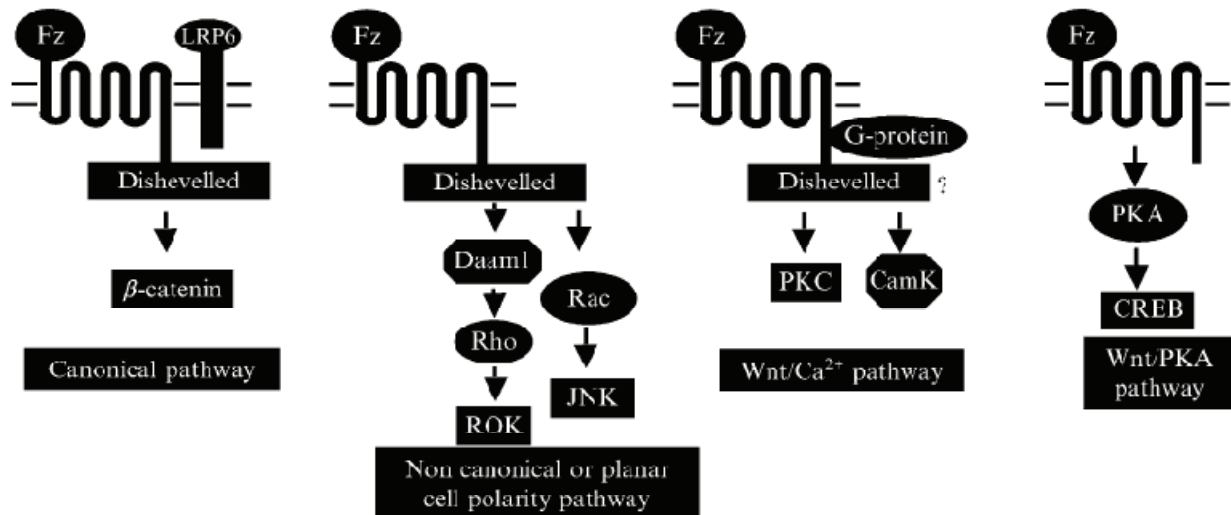


Figure 14. A schematic representation of the several known Wnt signal transduction cascades. For the non-canonical or Planar Cell Polarity (PCP) pathways, Wnt signaling is transduced through FZ (independent of LRP5/6). Trimeric G proteins (like GTPases) may be involved in both β -catenin and PCP pathways. For the Wnt/Ca²⁺ pathway there is disagreement about the involvement of disheveled. The Wnt/PKA pathway stimulates PKA activation and it is also not known whether disheveled plays a role in it. (Habas R and He X, Methods Enzymol, 2006)

3.1 WNT LIGANDS

Wnts are conserved in all metazoan animals. There are now 19 distinct family members known to be encoded in the human genome. Specific Wnt ligands like Wnt4, Wnt5a and Wnt11 appear to activate non-canonical rather than canonical pathways, which are activated by Wnt3, Wnt5, Wnt7 and others. However, it has been argued that receptor expression pattern may, in fact, be more relevant than the ligand in the choice of downstream signaling (126). Wnt ligands are cysteine-rich proteins of approximately 350-400 amino acids that contain an N-terminal signal peptide for secretion. Murin Wnt3a (Wnt ligand used to activate canonical pathway in this project) was the first purified and biochemically characterized Wnt protein (133). Wnt3a is efficiently secreted from the cell in contrast to most other Wnt proteins. Wnt proteins are glycosylated and lipid modified in the Endoplasmic Reticulum (ER) and transported from the Golgi to the plasma membrane for secretion (134).

3.2 WNT RECEPTORS

Two distinct receptor families are critical for Wnt/ β -catenin signaling: the Fz seven-pass transmembrane receptor (126) and LRP5/6 (135). The mammalian genome codifies for 10 Fz genes, most of which have variable capacities to activate β -catenin signaling and show functional redundancy (126). Between the 2 LRPs, LRP6 plays a more dominant role and is essential for embryogenesis, whereas LRP5 is dispensable for embryogenesis but critical for bone homeostasis. However, LRP5 and LRP6 are also partially redundant. It is noteworthy that Wnt3a palmitoylation (at cysteine 77) is important for binding to both Fz and LRP6, explaining the importance of this lipid modification (136). A particular Wnt may activate β -catenin and/or non-canonical pathways depending on the receptor complement (137). Fz function is involved in β -catenin and non-canonical pathways whereas the interaction with the co-receptor LRP5/6 is a specific requirement for the canonical pathway (135). Some evidences suggest that LRP5/6 antagonizes non-canonical Wnt signaling *in vivo*, possibly via competition for Wnt ligands (138). Other Wnt receptors exist such as Ryk and ROR2, which are not required for, but in some cases may antagonize, Wnt/ β -catenin signaling (137).

3.3 WNT AND GTPASES

Rho GTPases have been described to be key mediators of Wnt signaling pathway, most notably in the non-canonical Wnt pathway that involve cell polarization and migration, but more recently also in the canonical Wnt pathway. We are going to focus on the implication of GTPases in the canonical pathway as this is the context of the project and results shown further on.

There are still many missing links in canonical pathway but importantly there is not many knowledge about the mechanism by which β -catenin is translocated to the nucleus upon Wnt stimulation. We hope this work can contribute to elucidate this question, as we think this could be an important target for regulating the pathway in abnormal situations in the future.

It appears that Rac1 has an important role in this. On one hand, it has been described that in a colon cancer cell line, where the canonical Wnt signaling is constitutively activated, β -catenin/TCF transcription is inhibited by a dominant negative Rac1 and potentiated by a constitutively active Rac1 (139). On the other hand, the most recent paper to address this issue propose that Rac1-mediated activation of JNK2 leads to phosphorylation of β -catenin on two specific sites promoting nuclear localization (140). Another study showed that β -catenin co-immunoprecipitates with Tiam1 (GEF for Rac1) in Wnt stimulated cells and that Rac1 synergizes with β -catenin to increase the activity of the TCF/LEF-dependent transcription. This synergy was dependent on the nuclear presence of Rac1 (139). Finally, DOCK4 (specific GEF for Rac1) has been shown to be a part of the β -catenin degradation complex but it is not known whether it is related to the proposed role of Rac1 in the nuclear translocation of β -catenin (141). In the following Figure 15 we show the different Rac1 implications that had been described so far in the canonical Wnt signaling.

Introduction

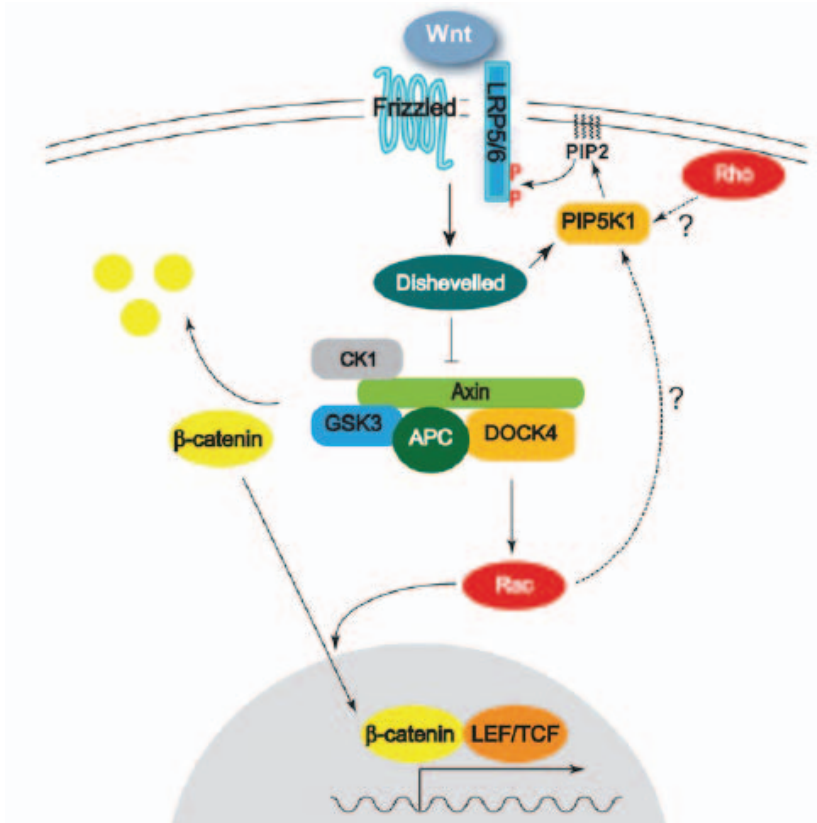


Figure 15. Rho GTPases signaling in the canonical Wnt pathway. Both Rac1 and RhoA have been implicated in regulating the enzyme PIP5K1, responsible for PIP2 biosynthesis. However, if they do so during canonical Wnt signaling is not known. PIP2-dependent phosphorylation of LRP5/6 is important for receptor aggregation and enhanced Wnt signaling downstream from Dishevelled. Moreover, Rac1 participates in Wnt signaling by promoting nuclear accumulation of β -catenin. A guanine nucleotide exchange factor, DOCK4, is found associated with the destruction complex, though it has not been directly implicated in nuclear import. (Schlessinger K, Hall A and Tolwinski N *Genes Dev*, 2009)

Concluding, the favored hypothesis for Rac1 function seems to be a role in β -catenin nuclear import, but we should consider alternative hypothesis such as promoting tighter binding to DNA complexes or impairing the export of β -catenin.

Conversely, not much is known about the mechanism that would lead to Rac1 activation upon canonical Wnt signaling. So far there is a mechanism described that involves the LRP5/6, Dvl, a heterotrimeric G protein and PI3K (140), but we will propose further on another mechanism PI3K-independent by which Rac1 is activated upon Wnt stimuli.

3.4 FUNCTIONAL REGULATION OF WNT SIGNALING PATHWAY

The intestinal tract consists of the small intestine (duodenum, jejunum and ileum) and the large intestine or colon. The epithelium of the small intestine is ordered into villi and crypts of Lieberkühn. Differentiated cells occupy the villi (Figure 16). Another type of differentiated cells, the Paneth cells, resides at the bottom of crypts and secretes antimicrobial agents (142). Proliferative stem and precursor cells occupy the bottom two-thirds of crypts, whereas differentiated cells constitute the surface epithelium and top third of the crypts (Figure 16).

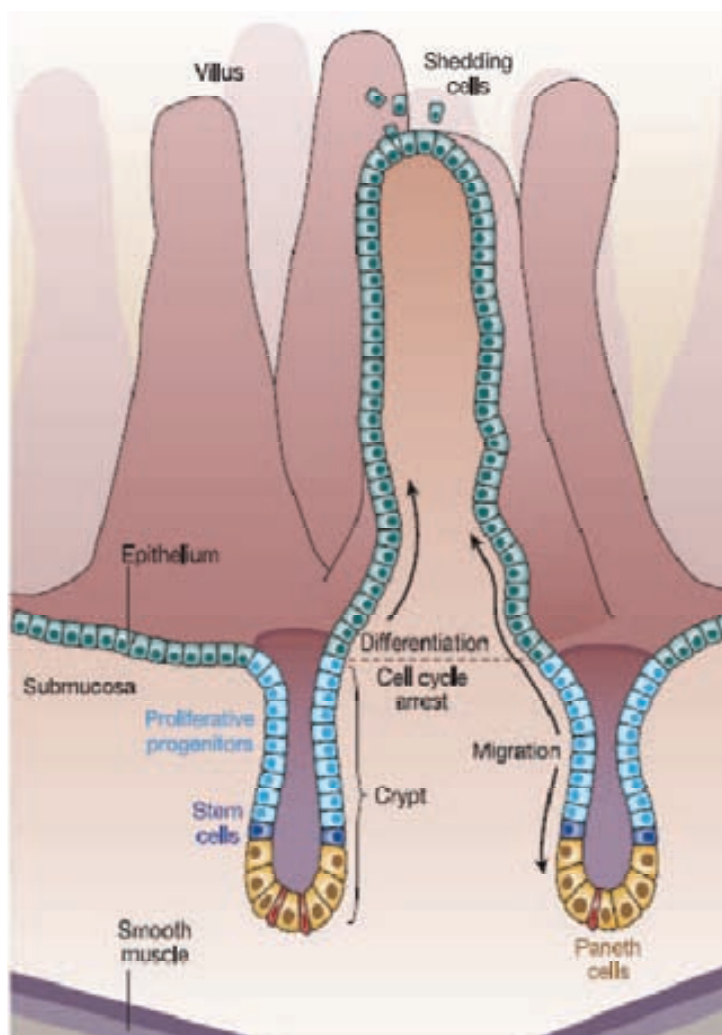


Figure 16. Tissue anatomy of the adult small intestine. Putative stem cells (dark blue) reside immediately above the Paneth cells (yellow) near the crypt bottom. Proliferating progenitors cells (light blue) constitute the rest of the crypt and the differentiated cells (green) populate the villi. (Reya T and Clevers H, Nature 2005)

The estimated number of stem cells is between one and six per crypt. The stem cells cycle slowly, continuously producing rapidly proliferating cells capable of differentiating towards all lineages. They not only self-renew throughout life but also regenerate the epithelium after injury. Progenitor cells undergo cell cycle arrest and

Introduction

start expressing differentiation markers when they reach the top of the crypt. The differentiated cells continue their migration on the villus stretching along the crypt-villus axis. Although crypts are monoclonal, each villus receives cells from multiple crypts and is therefore polyclonal (143).

Recent evidences indicate that Wnt signaling is the dominant force in controlling cell fate along the crypt-villus axis and is required for maintenance of the crypt progenitor phenotype. Nuclear β -catenin, the hallmark of Wnt signaling, is observed along the crypts of the intestine (144).

The Wnt signaling gradient also controls expression of the EphB/ephrinB sorting receptors and ligands (144). The resulting EphB/ephrinB gradients allow the establishment of crypt-villus boundaries as well as the positioning of Paneth cells at the crypt bottom.

The APC gene was among the first tumor suppressors to be cloned. A germline APC mutation is the genetic cause of a hereditary cancer syndrome termed Familial Adenomatous Polyposis (FAP) (145). FAP patients inherit one defective APC allele and as a consequence develop large numbers of colon adenomas, or polyps, in early adulthood. Inevitably, some of these polyps progress into malignant adenocarcinoma accompanied by mutations in additional oncogenes or tumor suppressor genes, such as K-Ras, p53 and Smad4. Loss of APC also occurs in most sporadic colorectal cancers (146), implying that the absence of functional APC transforms epithelial cells through aberrant activation of the Wnt pathway. Mutational inactivation of APC leads to the inappropriate stabilization of β -catenin (147). Several EphB genes are initially upregulated as Wnt/Tcf4 target genes in early adenomas, but their expression is lost upon cancer progression (148), apparently as the result of a selection process. In some cases of colorectal cancer in which APC is not mutated, the scaffolding protein axin 2 is mutant (149), or activating (oncogenic) point mutations in β -catenin remove its N-terminal Ser/Thr destruction motif (150).

Once the Wnt cascade is mutationally activated, the adenoma cells maintain their progenitor status indefinitely. This allows the adenomas to persist for many years, providing wide opportunity for the acquisition of further mutations.

Because Wnt signaling can act to maintain stem cells as well as cancer cells, the ability to modulate Wnt pathway either positively or negatively may be of therapeutic relevance. Thus, controlled activation of Wnt signaling may allow enhancing stem cell and progenitor cell activity when regeneration of the tissue is needed. In contrast, inhibiting the pathway may be very effective when cell renewal that drives to cancer is uncontrolled.

3.5 ADHERENS JUNCTIONS COMPONENTS MEET WNT SIGNALING

Members from our lab have recently described an important cooperation between canonical Wnt signaling pathway and adherens junctions components. These results unveil a crucial role for p120ctn in the regulation of β -catenin transcriptional activity different from β -catenin stability (151-153).

Upon binding of canonical Wnt ligands to Fz receptors, the co-receptor LRP5/6 is recruited and a ternary complex Fz/Wnt/LRP5 is formed. As we have reported (151, 153), LRP5/6 is constitutively bound to CK1 ϵ . This association is not direct but it is mediated by E-cadherin and p120ctn (see Figure 17A). In non-stimulated cells, p120ctn-bound CK1 ϵ is inactive. Upon Wnt3a stimulation, the complex containing LRP5/6, E-cadherin, p120ctn and CK1 ϵ rapidly associates with Fz, concomitantly with the activation of this protein kinase (Figure 17B). Both CK1 ϵ and p120ctn are required for Dvl-2 phosphorylation and binding to Fz/LRP5/6 complex (Figure 17C). Besides, Dvl-2 phosphorylation stimulates the interaction of Axin with the complex (Figure 17D). Furthermore, CK1 α (Axin is permanently bound to constitutive active CK1 α) phosphorylates E-cadherin in a residue yet to be identified, and p120ctn at Serines 268 and 269 (Figure 17E). These modifications disrupt the interaction of E-cadherin with LRP5/6 and of p120ctn with E-cadherin, respectively, and release p120ctn-bound CK1 ϵ from the signaling complex (Figure 17E). The release of p120ctn/CK1 ϵ has two different consequences: it allows p120ctn to bind to the transcriptional repressor Kaiso and therefore derepress its target genes (152) and it also promotes the cessation of the initial Wnt input, since coupling of CK1 ϵ to the signalosome is required for Dvl-2 phosphorylation.

Introduction

This thesis project presented further on, suggests another possible role for this E-cadherin-released p120ctn upon Wnt signaling, involving the regulation of Rac1 GTPase. These previous results described by our group have been crucial to build up together an interesting research project where p120ctn has a pivotal role in the context of canonical Wnt signaling.

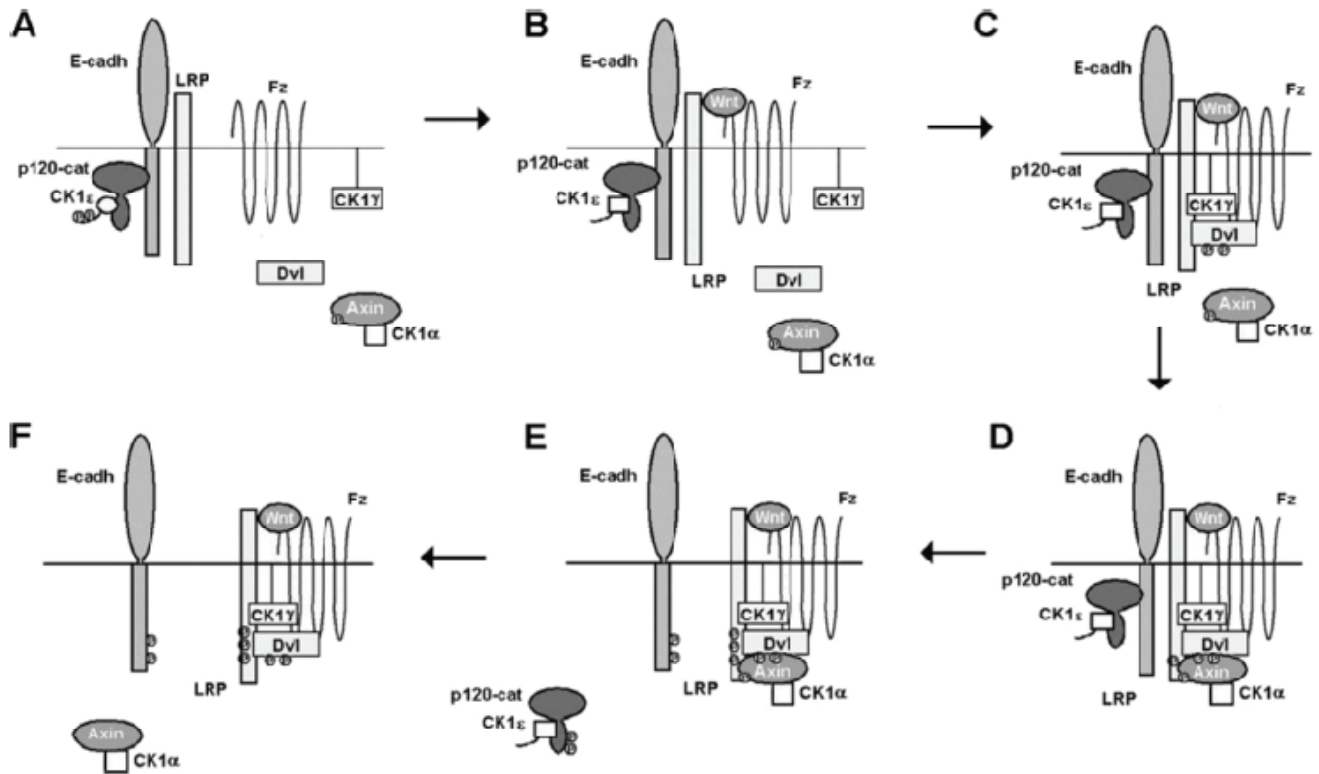


Figure 17. Proposed model for the involvement of p120ctn in canonical Wnt pathway. Different steps are described in the text (del Valle et al, Mol Cell Biol, 2011).

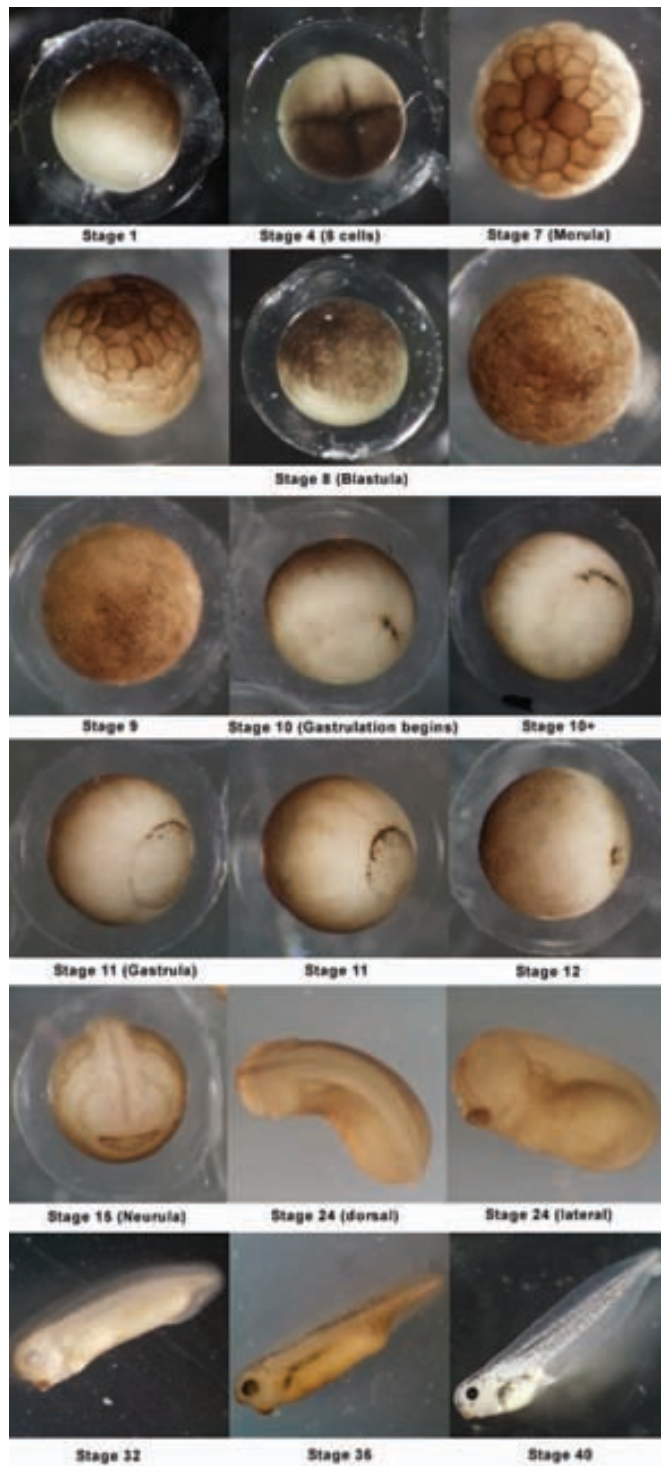


Figure 18. *Xenopus* embryo staging, based on morphologic criteria. (Cebra-Thomas 2003)

4. XENOPUS LAEVIS

AND DEVELOPMENT

It is well-established the extensive involvement of Wnt pathway in embryogenesis and adult tissue homeostasis. Although not genetically tractable, *Xenopus* has provided much insight into the mechanisms of Wnt signaling in development.

Early development of *Xenopus* is the best understood model system for tissue- and stage-specific Wnt signaling (154, 155). Wnt/ β -catenin signaling mediates three separate responses during the early developmental stages leading to gastrulation (see *Xenopus* developmental stages in Figure 18). First, from cleavage stage to early blastula (stages 3-8)

maternal Wnt/ β -catenin signaling establishes the dorsal axis of the embryo. Second, during later blastula stages (8.5-9.5), Wnt/ β -catenin signaling is active all around the equatorial region, and is required for mesoderm induction (154). Third, zygotic Wnt/ β -catenin signaling promotes ventral and lateral, but restricts dorsal, mesoderm development (155). This Wnt/ β -catenin signaling activity is best analysed during gastrulation by the expression of ventro-lateral mesoderm marker *Xpo*, and the

Introduction

dorsolateral mesoderm marker *XmyoD*. Nuclear β -catenin is present all around the equatorial region during blastula stages (156), so the question arises in how gene expression is tissue- and stage- specifically regulated downstream of Wnt/ β -catenin signaling.

Xenopus is a distinct vertebrate system that offers a rapid external development, large embryos that facilitate microinjections and the isolation of explants for phenotypic analyses and the ability to test mechanistic hypothesis using rescues assays.

OBJECTIVES

II. OBJECTIVES

It is well established that p120ctn is a pleiotropic protein that has different roles in the cell. On one hand, it is able to maintain and regulate adherens junctions through its binding to E-cadherin. Apart from being bound to E-cadherin, it can regulate Rho GTPases activity or bind to the transcriptional repressor Kaiso.

Our group has recently found that p120ctn also plays essential roles in the early responses to canonical Wnt signaling, since it provides the platform for the interaction of CK1 ϵ with the Wnt receptor complex. Moreover, at longer times, Wnt3a promotes the CK1 α -catalyzed phosphorylation of p120ctn at Ser268-269 decreasing p120ctn interaction with E-cadherin and thus releasing the catenin from the receptor complex. This E-cadherin-unbound p120ctn plays additional roles in Wnt signaling since it interacts with Kaiso, preventing the inhibition by this factor of the β -catenin/Tcf-4 complex.

In this context, we wanted to go further on the role of p120ctn in regulating Rac1 activity, trying to characterize its interaction and modulation of its activity by biochemical approaches. We next extended the *in vitro* approaches to a more physiological context, studying the role that the E-cadherin-unbound p120ctn pool exerts in the stimulation of Rac1 activity in the Wnt response and its subsequent effects in the β -catenin translocation to the nucleus.

RESULTS

III. RESULTS

1. RAC1 BINDS P120-CATENIN

It is known from other works and from previous results from our lab (58, 157) that p120ctn is able to directly bind RhoA GTPase and to act as a GDI, repressing its activity to exchange GDP for GTP. Regarding other Rho GTPases, it has also been described the ability of p120ctn to activate Rac1 involving its GEF Vav2 (113). This was the context that led us to explore the ability of p120ctn to directly regulate the activity of Rac1.

We first wanted to elucidate if Rac1 was also capable to directly bind p120ctn. As shown in Figures 2 and 3, Rac1-p120ctn interaction was detected *in vitro* by binding assays performed with purified recombinant proteins. We used different p120ctn deletion mutants that we had in the lab to map the site of interaction between Rac1 and p120ctn (see diagram of the different domains of p120ctn in Figure 1). We observed that, with the exception of the 1-96 fragment, the rest of the fragments directly interacted with Rac1. Moreover, the full-length protein isoform 1 (amino acids 1-911) had higher affinity for Rac1 than isoform 3 (amino acids 102-911). These results identified two different binding elements placed within amino acids 1-234 and 350-911, capable of associating with Rac1. These results also indicate that both N-terminal and C-terminal tails of the protein seem to be important for the correct Rac1 binding to p120ctn. We hypothesize that a correct folding of the terminal tails interacting with the central Armadillo domain of p120ctn are necessary for a complete Rac1 binding to p120ctn, as it has also been shown to occur with other armadillo-containing proteins like β -catenin or Plakoglobin (158).

Results

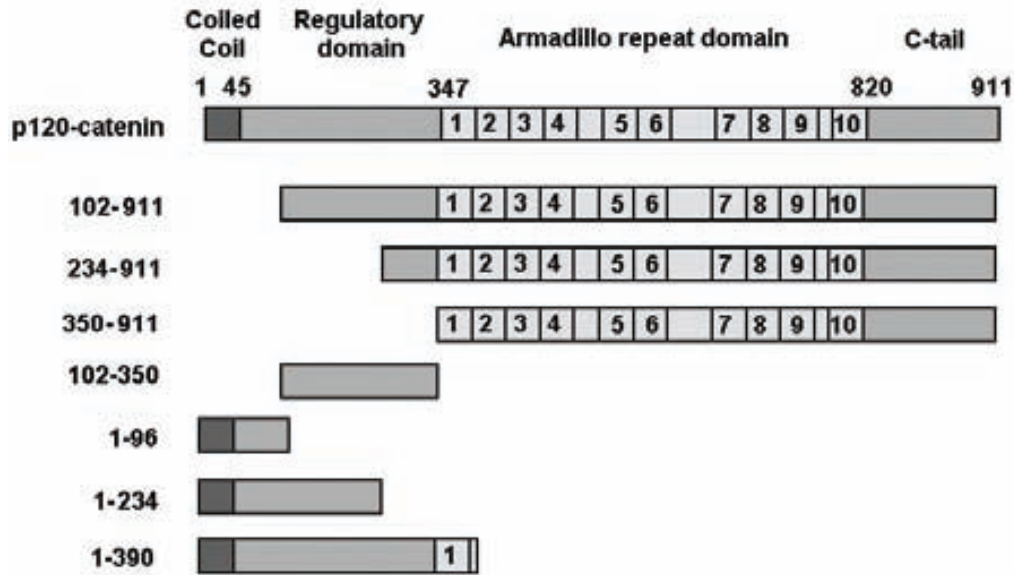


Figure 1. Diagram of the different domains of p120ctn. The length of the p120ctn deletion mutants used in this work is shown.

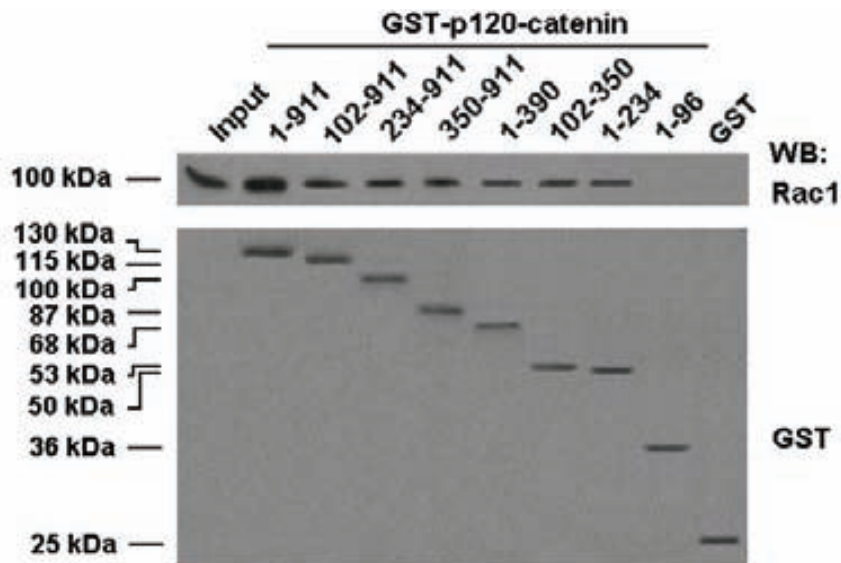


Figure 2. p120ctn directly binds Rac1. GST-p120ctn wt isoforms 1 and 3 and the deletion mutants shown in Figure 1 (2 pmol) were incubated with Rac1 (5 pmol). Protein complexes were affinity purified and analyzed with anti-Rac1 and anti-GST mAbs. Internal reference standard (0.5 pmol) for Rac1 was included (St).

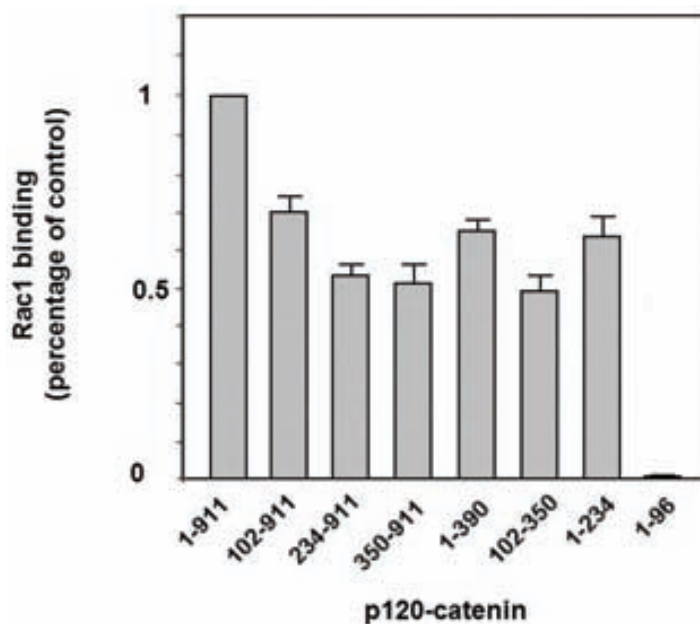


Figure 3. Diagram of Rac1 binding to different p120ctn deletion mutants. Autoradiograms from five different experiments performed in triplicate were quantified and the mean \pm s.d. was obtained for each condition. Each of the deletion mutants value is presented relative to that obtained with p120ctn 1-911 construct.

Differently seen with RhoA (58), p120ctn show the same affinity for Rac1 bound to GDP, GTP or nucleotide unloaded, as presented in Figure 4.

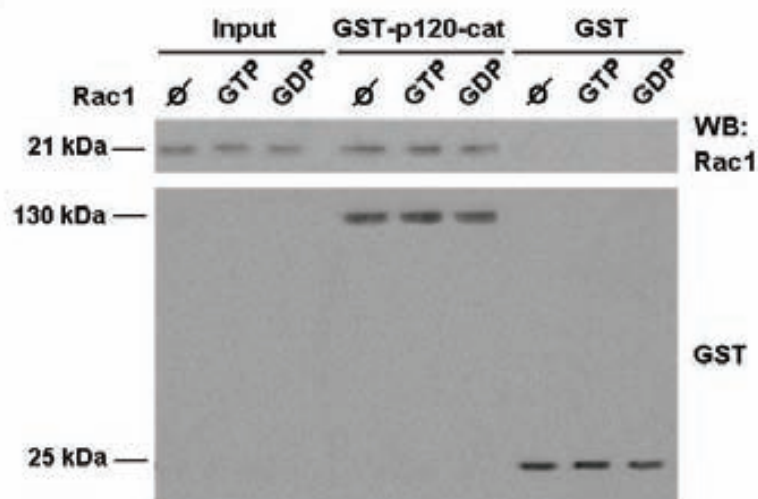


Figure 4. Rac1-p120ctn association is not different for nucleotide unloaded, GDP or GTP-bound Rac1. Recombinant Rac1 (25 pmol) was loaded with GTP or GDP (30 μ M) and incubated with GST-p120-catenin (5 pmol). Protein complexes were affinity purified with glutathione-Sepharose and analyzed by SDS-polyacrylamide gel electrophoresis and Western Blot with anti-Rac1 and anti-GST mAbs. Rac1 (1 pmol) was included as an internal reference (St).

Results

In addition, Rac1 did not interfere with p120ctn association to E-cadherin in *in vitro* experiments: neither an E-cadherin fragment corresponding to the cytosolic domain (cytoE-cadh) competed with Rac1 binding to GST-p120ctn (Figure 5A) nor recombinant Rac1 affected the association of cytoE-cadherin to the same fusion protein (Figure 5B). Each experiment was performed with isoforms 1 and 3 of p120ctn (amino acids 1-911 and 102-911, respectively).

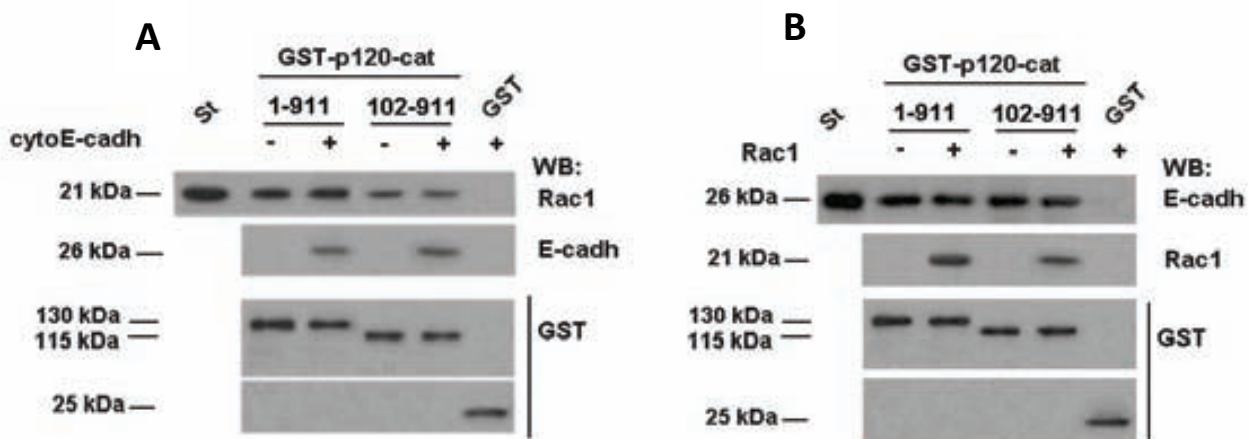


Figure 5. Rac1 did not interfere with p120ctn association with E-cadherin *in vitro*. GST-p120ctn wt isoform 1 or 3 (1.5 pmol) were incubated with recombinant Rac1 (A) or cyto-Ecadherin (B) (3 pmol). When indicated, GST-p120ctn was preincubated with a 10-fold excess of cyto-E-cadherin (A) or Rac 1 (B) (15 pmol). Protein complexes were affinity purified and analyzed with anti-E-cadherin, anti-Rac1 and anti-GST. An internal reference standard was included for Rac1 (0.5 pmol) (A) and cyto-Ecadherin (B).

Another member of the p120ctn family, ARVCF, also bound Rac1 (Figure 6). As for p120ctn, an ARVCF fragment comprising the first N-terminal 382 amino acids interacted with Rac1, although the binding was lower than with the full-length protein. In addition, both ARVCF constructs and p120ctn also directly bound to Vav2 (Figure 6), a GEF for Rac1. I will show the characterization of this interaction further on.

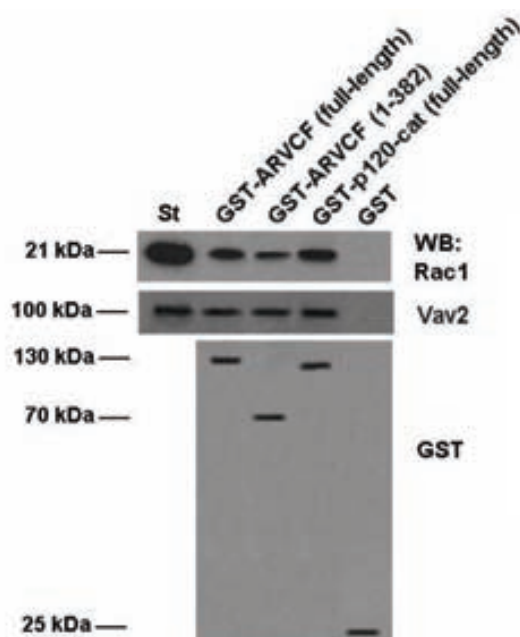


Figure 6. Rac1 directly interacts with ARVCF. GST-p120ctn wt isoform 1, GST-ARVCF wt and GST-ARVCF (1-382) (2 pmol) were incubated with Rac1 (5 pmol). Protein complexes were affinity purified and analyzed by Western Blot with anti-Rac1 and anti-GST mAbs. Rac1 (2 pmol) was included as an internal reference standard (St).

2. p120CTN IS NECESSARY FOR RAC1 ACTIVATION

2.1 p120CTN OVER-EXPRESSION ACTIVATES RAC1

In order to proof if p120ctn was able to regulate Rac1 activity, we co-expressed a Rac1 wild-type construct with either p120ctn isoform 1 or an empty vector in epithelial SW-480 cells. Active Rac1 was pulled-down by a PBD domain (p21 Binding Domain) from PAK protein (p21-activated kinase) fused to GST. This PBD domain specifically interacts with active Rac1 in the cell. As shown in Figure 7, higher levels of Rac1 activity were purified when p120ctn isoform 1 was over-expressed.

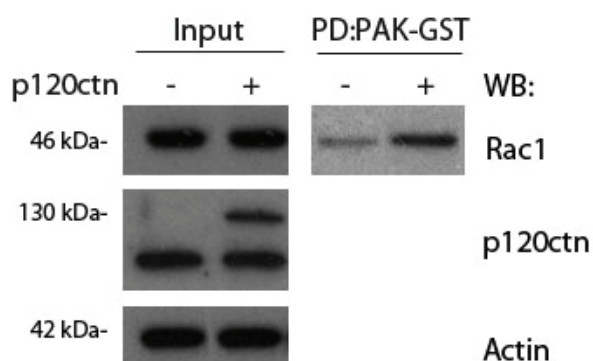


Figure 7. p120ctn overexpression activates Rac1. SW-480 cells were transfected with either p120ctn-GFP isoform 1 construct or the empty vector phrGFP and with Rac1-GFP. PAK pull-down assays were performed in the different conditions and proteins were analyzed by Western Blot.

Results

We have used several different cellular systems for the project but mainly we have worked with epithelial tumoral SW-480 colon cells that are mutated in APC and therefore deficient in β -catenin degradation and HEK-293 cells, APC wild-type and widely used for over-expressing proteins as they are very easily transfectable.

As shown in Figure 8 and similar to SW-480 cells, endogenous Rac1 was also activated upon over-expression of p120ctn isoform 1 in HEK-2993.

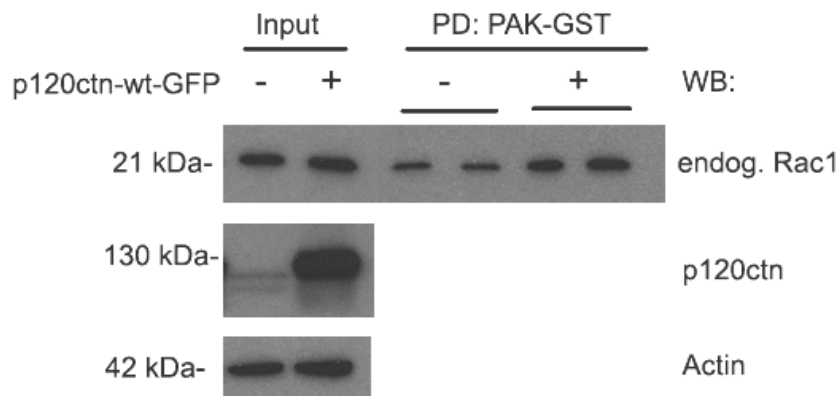


Figure 8. p120ctn overexpression activates Rac1. HEK-293 cells were transfected with either p120ctn-GFP isoform 1 construct or the empty vector p120ctn. PAK pull-down assays were performed in the different conditions and proteins were analyzed by Western Blot.

2.2 P120CTN DEPLETION INACTIVATES RAC1

To further demonstrate the direct implication of p120ctn in Rac1 activation, we proceeded to look at the effects of depleting cells from p120ctn. SW-480 cells that were infected with a shRNA specific for p120ctn showed a decrease in active Rac1 compared with cells infected with a control shRNA (Figure 9).

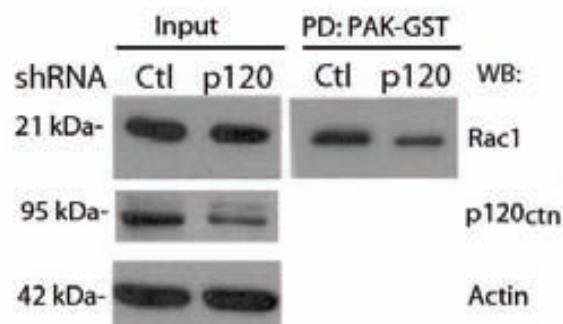


Figure 9. Rac1 activity decreases in p120ctn-depleted cells. A PAK pull-down assay was performed in SW-480 cells stably expressing control or shRNA specific for p120ctn. Active Rac1 was detected from the extracts by Western Blot.

SW-480 cells Control (shCtl) or shRNA for p120ctn (shp120ctn) protein levels were also analyzed. Rac1 levels did not change upon p120ctn-depletion (Figure 10). As expected, E-cadherin levels were down-regulated in p120ctn-depleted cells, since p120ctn stabilizes E-cadherin at the membrane (124, 125, 159, 160).

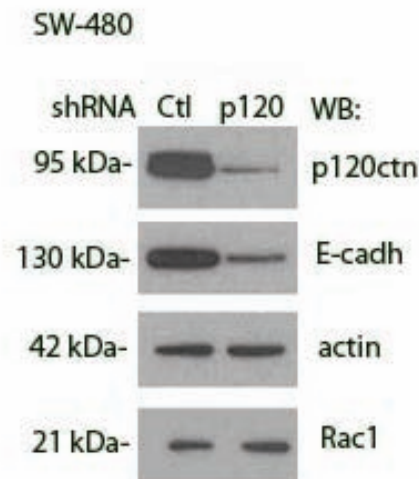


Figure 10. Rac1 total levels are not affected in p120ctn-depleted cells. Total cell extracts (1% SDS) were analyzed in SW-480 cells infected with control or specific shRNA for p120ctn. Proteins were analyzed by Western Blot.

In addition, and as shown for Rac1 (Figure 9), we wanted to analyze the levels of active RhoA in p120ctn-depleted cells. We pulled-down active RhoA with Rhotekin recombinant protein (well-known RhoA effector) fused to GST. It is widely known the opposite state of activation of Rac1 and RhoA in the cell as well as the RhoGDI effects of p120ctn on RhoA (58, 111). So as expected, RhoA activity was up-regulated in p120ctn-depleted cells, assuming that the GDI activity provided by p120ctn should be lower (Figure 11).

Results

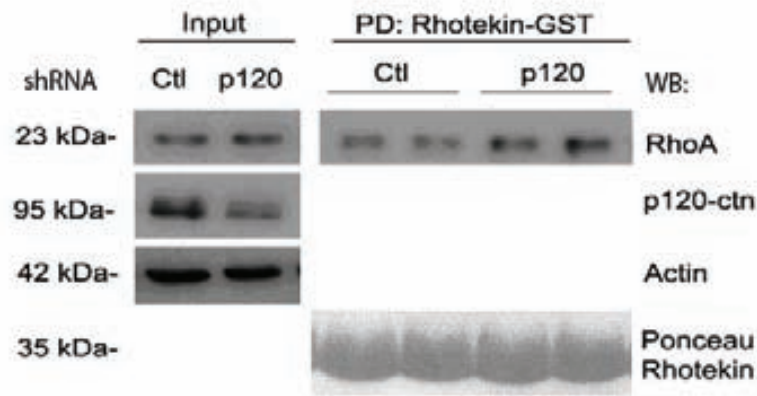


Figure 11. RhoA activity increases in p120ctn-depleted cells. Rhotekin pull-down assay was performed in SW-480 cells stably expressing control or shRNA specific for p120ctn. Active RhoA was detected from the extracts by Western Blot.

2.3 MOTILITY IS DECREASED IN P120CTN-DEPLETED CELLS

Rac1 is an important mediator of cell migration. It acts in the cell periphery leading to membrane protrusion, and it is essential for the migration of all cells examined so far (66, 161).

For this reason, motility should be affected in p120ctn-depleted cells, since they presented decreased Rac1 activity levels. As shown in Figure 12, p120ctn-depleted cells presented a slower wound recovery rate and they even did not finish closing the wound at 72h, while control cells perfectly did.

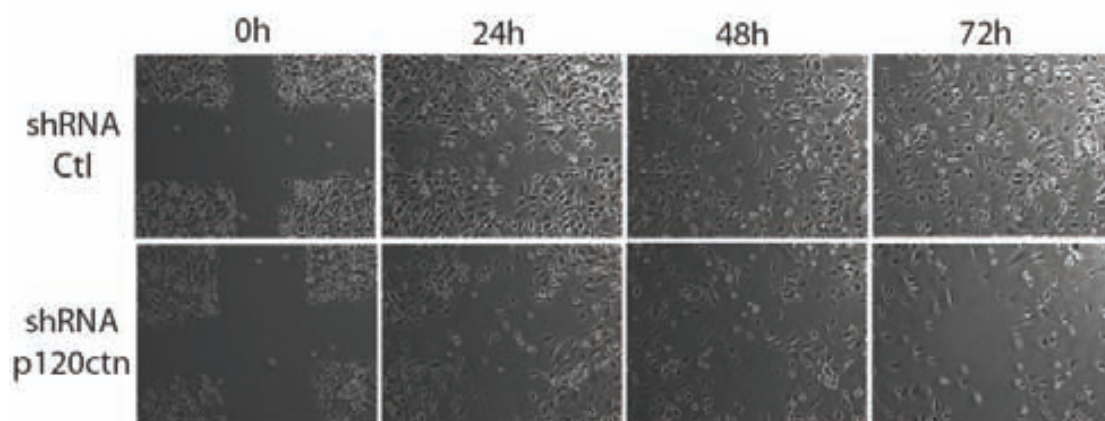


Figure 12. Motility is decreased in p120ctn-depleted cells. A wound healing assay was performed in SW-480 cells stably expressing control or shRNA specific for p120ctn. Cells were seeded in a plate where a wound was performed with a plastic pipette. Cells were captured until the wound was completely recovered.

3. VAV2 IS NECESSARY FOR RAC1 ACTIVATION THROUGH P120CTN

3.1 P120CTN DEPLETION PREVENTS VAV2/RAC1 INTERACTION

Vav2 is a Rac1 activator widely expressed in epithelial cells (162). We were focused on finding the Guanosine nucleotide Exchange Factor (GEF) responsible for p120ctn-mediated Rac1 activation mechanism and Vav2 seemed to be a good candidate, as we found significant changes between control and p120ctn-depleted cells. We detected Vav2 association with Rac1 by coimmunoprecipitation (coIP) and we observed a significant decrease in this association in p120ctn-depleted cells (Figure 13). This suggested that p120ctn was probably mediating the association between Rac1 and Vav2 favoring its interaction and that Vav2 could be the GEF involved in the p120ctn-mediated Rac1 activation.

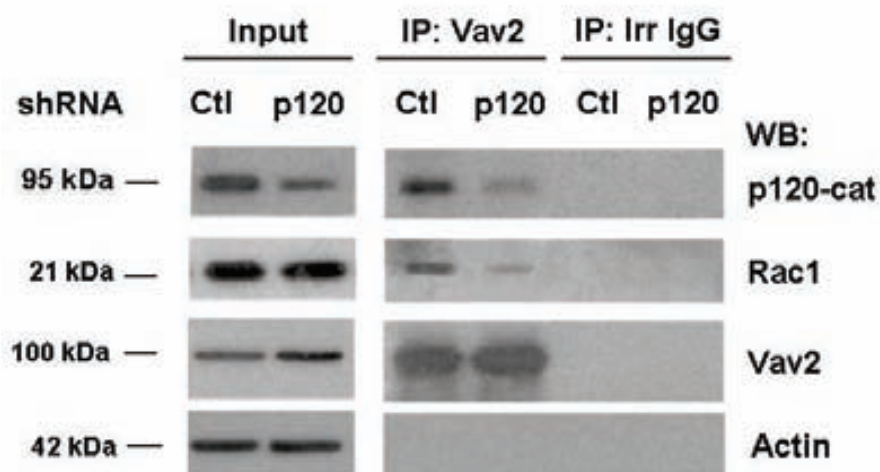


Figure 13. Vav2-Rac1 association decreases in p120ctn-depleted cells. Vav2 was immunoprecipitated from control and p120ctn-depleted SW-480 cells. Protein complexes were analyzed by Western Blot with anti-p120ctn, anti-Rac1 and anti-Vav2.

Results

3.2 P120CTN BINDS VAV2

We went further and we demonstrated a direct association between p120ctn and Vav2. We took advantage of the different deletion mutants of p120ctn mentioned before and we performed *in vitro* binding assays with the recombinant proteins to map the site of interaction of Vav2 in p120ctn. As shown in Figures 14 and 15, Vav2 did not interact with the 350-911 and the 1-234 fragments of p120ctn (Figure 14 lane 5 and 7, respectively) while it interacted with the rest of them. We concluded that, distinct from Rac1, Vav2 interacts with the N-terminal tail of p120ctn, within the amino acids 234-350.

As observed for Rac1, p120ctn isoform 1 (amino acids 1-911) bound Vav2 better than p120 isoform 3 (amino-acids 102-911). Similar results were obtained when Vav2 was pulled-down by GST-p120ctn from cell extracts (not shown).

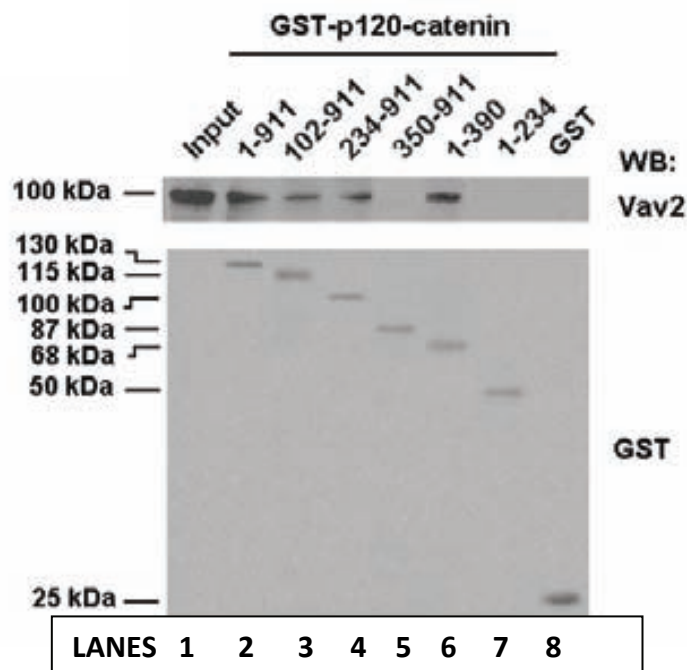


Figure 14. p120ctn directly binds Vav2. GST-p120ctn deletion mutants (2 pmol) were incubated with recombinant Vav2 (5 pmol). Protein complexes were affinity purified and analyzed with anti-Rac1 and anti-GST mAbs. Internal reference standard (0.5 pmol) for Rac1 was included (St).

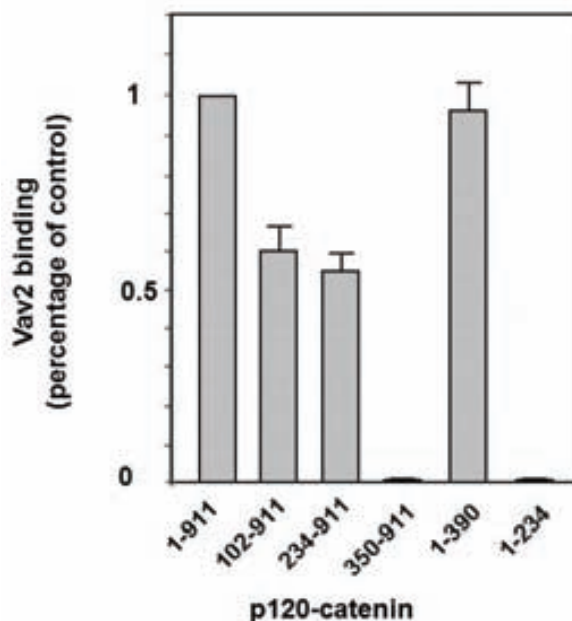


Figure 15. Diagram of Vav2 binding to different p120catn deletion mutants Autoradiograms from five different experiments performed in triplicate were quantified and the mean \pm s.d. was obtained for each condition. Each of the deletion mutants value is presented relative to that obtained with p120catn 1-911 construct.

Additionally, we looked for possible competition between p120catn association with Vav2 and binding to Rac1 or E-cadherin. Neither Rac1 nor cytoE-cadh prevented the interaction of Vav2 with p120catn (Figure 16).

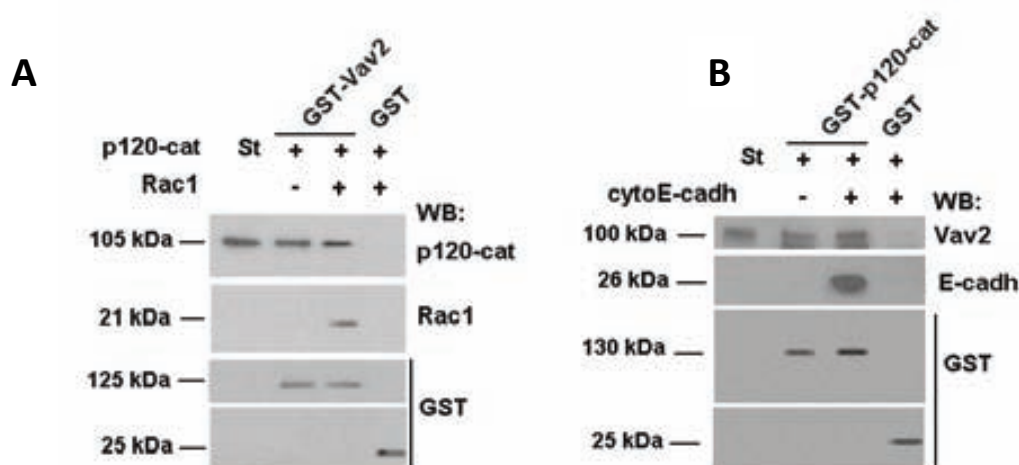


Figure 16. Rac1 and E-cadherin did not interfere with p120catn association with Vav2 *in vitro*. (A) GST-Vav2 or GST (2 pmol) were incubated with recombinant p120catn isoform 1 (5 pmol). When indicated the assay was performed in the presence of a 2-fold molecular excess of Rac1 (10 pmol). Protein complexes were affinity purified with Glutathione-Sepharose and analyzed by Western Blot with anti-Rac1, anti-Vav2 and anti-GST mAbs. p120catn (0.5 pmol) was included as an internal reference standard (St). (B) GST-p120catn isoform 1 or GST (1.5 pmol) were incubated with recombinant Vav2 (3 pmol) in the presence of 10-fold molecular excess of cyto-E-cadherin (15 pmol) when indicated. Protein complexes were affinity purified and analyzed by Western Blot with the indicated mAbs. Vav2 (0.3 pmol) was included as an internal reference standard (St).

4. RAC1 AND VAV2 ASSOCIATION WITH P120CTN IS REGULATED BY TYROSINE AND SERINE PHOSPHORYLATION

The N-terminal regulatory domain of p120ctn (amino acids 1-347) is phosphorylated by several tyrosine kinases. Among these, Src and Fyn modify tyrosines 217 and 112, respectively (43, 57) whereas the serine kinase CK1 phosphorylates serines 268 and 269 and others (151) (Figure 17).



Figure 17. Diagram of p120ctn. Tyrosine and serine residues phosphorylatable in the N-terminal domain are shown.

In the case of E-cadherin, phosphorylation of p120ctn by Src increases the *in vitro* association of these two proteins (57) and phosphorylation by CK1 inhibits p120ctn-E-cadherin association, both *in vitro* and *in vivo* (151). Another residue specifically phosphorylated by Fyn (Tyr112) has been described to be a key regulator of the p120ctn-RhoA interaction, both *in vitro* and *in vivo* (58). Taking together, phosphorylation by tyrosine and serine kinases in the N-terminal regulatory region of p120ctn regulates the interaction with its cofactors.

For this reason we wanted to verify if p120ctn phosphorylation by these kinases affected the binding to the new factors found to associate with p120ctn (Vav2 and Rac1). As shown in Figure 18, *in vitro* CK1 phosphorylation increased the binding of both proteins to p120ctn (lanes 1 and 2); conversely, phosphorylation by Src and Fyn down-regulated it (lane 1 and 3). This decrease was also evident when p120ctn was simultaneously phosphorylated by CK1 and Src/Fyn (lane 4). As a control, E-cadherin association was regulated as expected: down-regulated by CK1 and increased by Src and Fyn (lanes 1, 2 and 3).

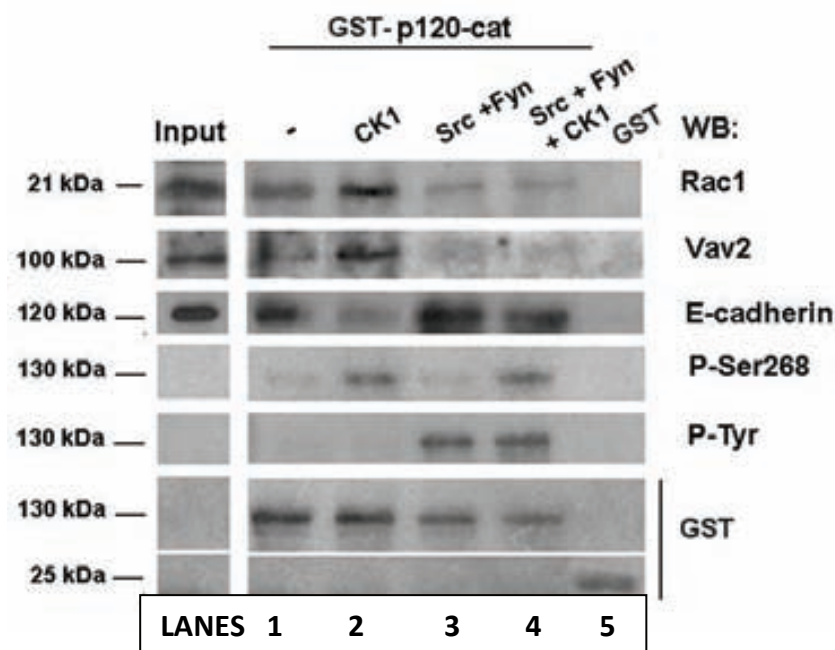


Figure 18. CK1 and Src/Fyn phosphorylations differently affect p120ctn/Rac1/Vav2 binding. GST-p120ctn was phosphorylated by CK1, Src and Fyn when indicated and a pull-down assay was performed incubating 7 pmols of GST-p120ctn or GST with cell extracts from SW-480 cells. Protein complexes were affinity purified and analyzed by Western Blot. 4 μ g of SW-480 lysate were included as an internal reference (Input).

The relevance of the different modified amino acids in p120ctn interaction with Rac1 and Vav2 was investigated. A p120ctn mutant mimicking phosphorylated Ser268 and Ser269 (S268-269DD), which inhibits p120ctn/E-cadherin interaction, behaved similarly than the wild-type p120ctn in Rac1 (Figure 19, lane 5 and 6, and Figure 21) and Vav2 binding (Figure 20, lane 2 and 5, and Figure 21), indicating that the CK1 effects on Vav2 and Rac1 binding are mediated by modification of other p120ctn residues.

Conversely, two Tyr-Glu p120ctn mutants (mimicking phosphorylation in tyrosines), Y112E and Y217E affected p120ctn interaction with Rac1 and Vav2. Y112E p120ctn mutant slightly decreased p120ctn association with Rac1 (Figure 19, lane 5 and 7, and Figure 21), whereas remarkably inhibited its binding to Vav2 (Figure 20, lane 2 and 3, and Figure 21); Y217E p120ctn mutant showed the same interaction with Vav2 as the wild-type protein (Figure 20, lane 2 and 4, and Figure 21), although it did not associate with Rac1 (Figure 19, lane 5 and 8, and Figure 21). As a control, Y112F or Y217F (Tyr-Phe) p120ctn mutants presented a similar binding to both proteins compared to the wild-type form (not shown). Therefore, these results indicated that Rac1 and Vav2

Results

binding to p120ctn show a different response to CK1 and Src/Fyn phosphorylation and that the binding of both proteins to p120ctn are differently affected by modification of specific p120ctn tyrosine residues.

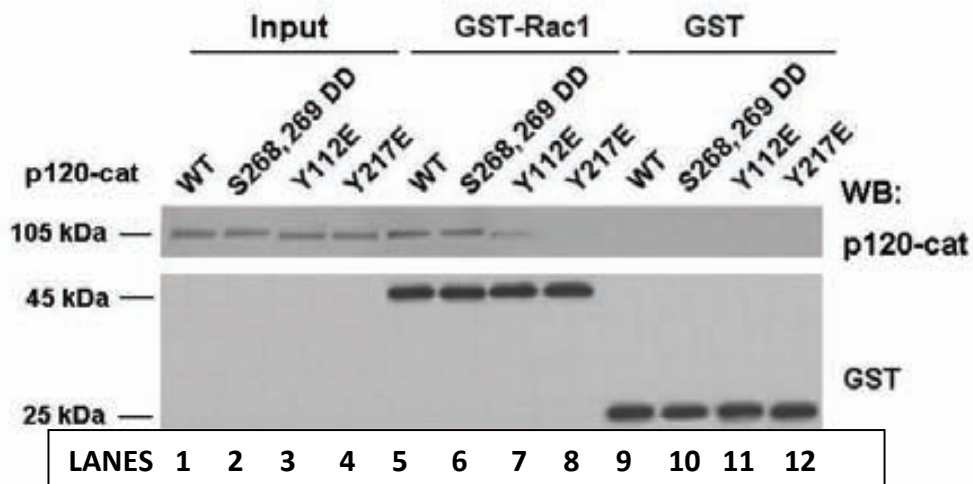


Figure 19. Rac1 differential binding to p120ctn phosphorylation point mutants. GST-Rac1 or GST (5 pmol) were incubated with recombinant isoforms 1 of p120ctn wt and p120ctn mutants Y112E and p120ctn Y217E (1 pmol), two Tyr-Glu mutants. Protein complexes were affinity purified and analyzed by Western Blot with anti-p120ctn and anti-GST mAbs. Internal reference standards (0.2 pmol) for p120ctn wt and point mutants were included (St).

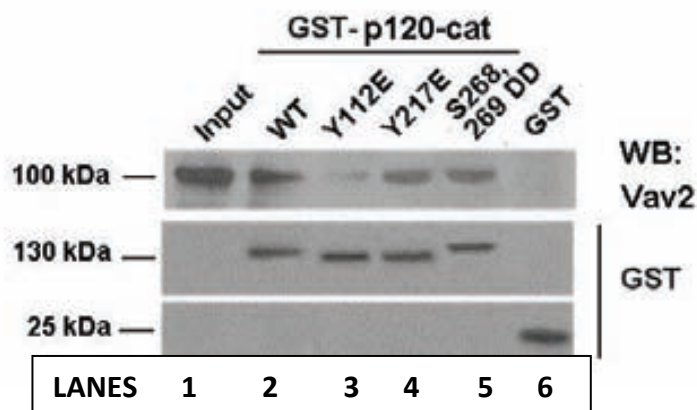


Figure 20. Vav2 differential binding to p120ctn phosphorylation point mutants. The isoform 1 of GST-p120ctn wt and three specific point mutants (Y112E, Y217E and S268S269DD) (5 pmol), were incubated with HEK-293 cell lysates. Protein complexes were affinity purified and analyzed by Western Blot with anti-Vav2 and anti-GST mAbs. 4 μ g of HEK-293 lysate were included as an internal reference (Input).

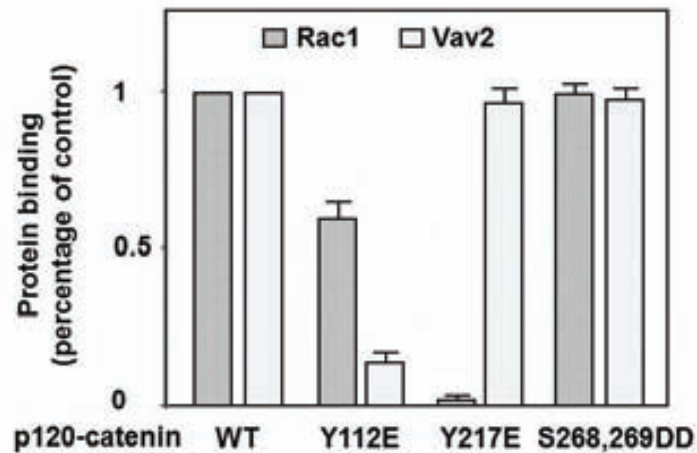


Figure 21. Diagram of Rac1 and Vav2 binding to different p120ctn phosphorylation point mutants. Autoradiograms from five different experiments performed in triplicate were quantified and the mean \pm s.d. was obtained for each condition. Each of the mutants value is presented relative to that obtained with p120-catenin WT construct.

Done before in our lab (151), recombinant p120ctn phosphorylated by CK1 was analyzed by Mass Spectrometry and several serines (Ser268-269 among them) were identified to be phosphorylated in the protein, mainly in the N-terminal part of the protein (Figure 22).

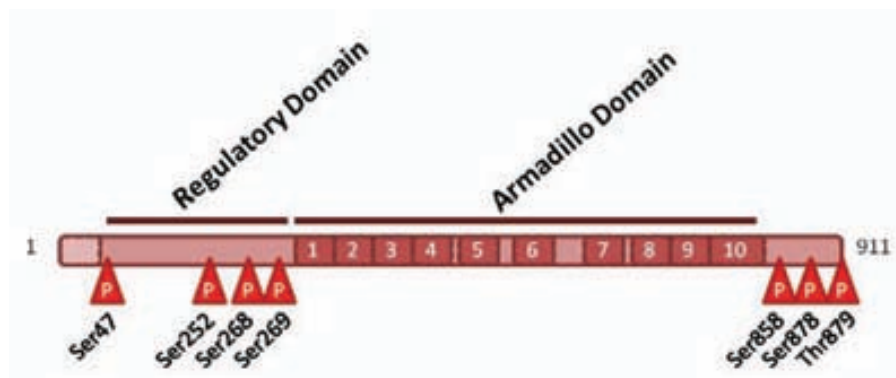


Figure 22. Diagram of p120ctn residues phosphorylated by CK1 (146). Two residues (S53 and S610) were discarded for not being conserved among different species.

Trying to find the phosphorylation site responsible for up-regulating p120ctn association with Vav2 and Rac1, we performed a binding assay with recombinant proteins using Rac1 and a p120ctn deletion mutant from amino acids 347 to 911 (armadillo-end) phosphorylated by CK1. As shown in Figure 23B, p120ctn (347-911) modified by CK1 did not increase association with Rac1 whereas p120ctn (1-911) did

Results

(Figure 23A), indicating that the residues phosphorylated by CK1 in the 347-911 domain are also not responsible for the regulation of p120ctn/Rac1 binding. This result limited our search to N-terminal residues (but not Ser268-269) to be the ones involved in this up-regulation.

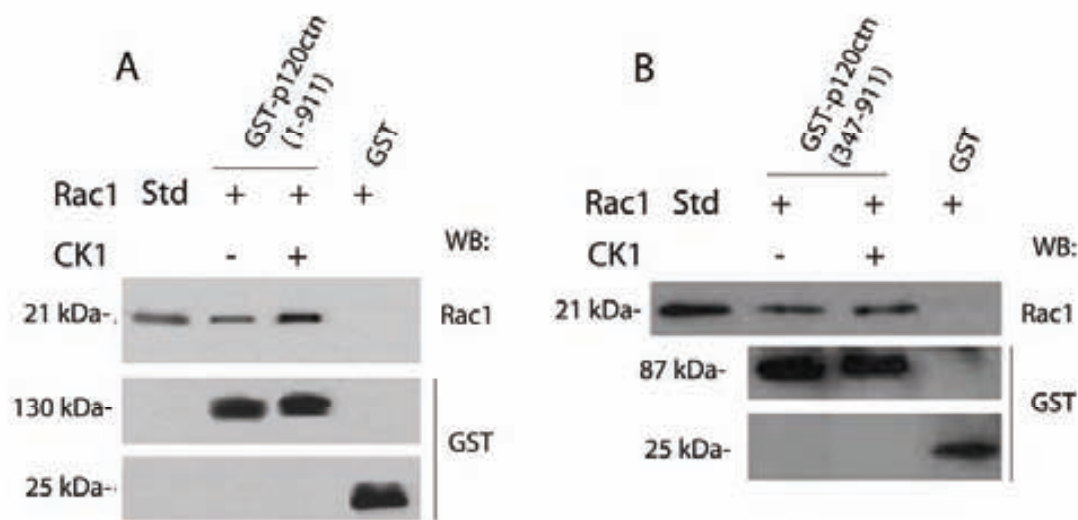


Figure 23. p120ctn (1-911) *in vitro* phosphorylated by CK1 increases association with Rac1 whereas p120ctn (347-911) did not. GST-p120ctn (1-911) (A) or GST-p120ctn (347-911) (B) (1 pmol) were phosphorylated by CK1 and incubated with recombinant Rac1 (5 pmols). Internal reference standard (0.5 pmol) for Rac1 was included.

We did a parallel experiment with Vav2 using a different p120ctn construct, since the fragment 347-911 did not bind Vav2 (see Figures 14 and 15). Instead, isoforms 1 and 3 (1-911 and 102-911, respectively) were phosphorylated and, as shown in Figure 24, Vav2 presented a higher affinity for p120ctn in both phosphorylated isoforms. From this result we can assume that serine 47 from the very N-terminal part of p120ctn is not responsible for the up-regulation (as isoform 3 does not contain the residue and we obtained CK1-mediated up-regulation anyway).

Taking together, we suggest that a putative residue that could be affecting p120ctn/Vav2 and p120ctn/Rac1 binding after CK1 phosphorylation could be the Serine 252. Unfortunately, we did not go further in this issue. This suggestion makes sense since this residue lies within the p120ctn interaction domain described before for Rac1 and Vav2.

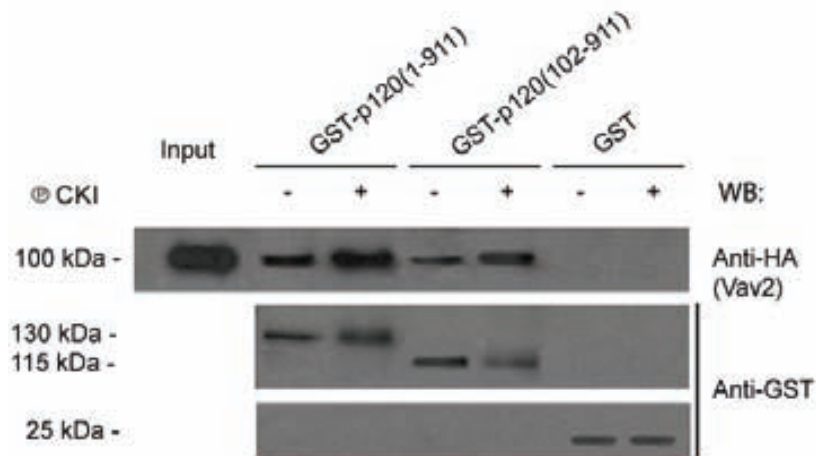


Figure 24. p120ctn isoforms 1 and 3 phosphorylated by CK1 interact with higher affinity with Rac1. HEK-293 cells lysate was incubated with either GST-p120ctn isoform 1, isoform 3 or GST (2 pmols), phosphorylated with CK1 kinase domain when indicated. Protein complexes were affinity purified and analyzed by Western Blot with anti-Vav2 and anti-GST mAbs. 4 μ g of HEK-293 lysate were included as an internal reference (Input).

5. OVER-EXPRESSION OF P120CTN MUTANTS DIFFERENTLY

ACTIVATE RAC1

Different p120ctn mutants fused to GFP were transfected into SW-480 and HEK-293 cells. Both the full-length p120ctn (isoform 1) and the shorter isoform 3 (amino acids 102-911) increased Rac1 activity, as detected by pull-down assays with GST-PAK (Figures 25 and 26). Although they were similarly expressed, p120ctn 1 (amino acids 1-911) more effectively activated Rac1, particularly in HEK-293 cells (Figure 26, data not shown). Other point or deletion mutants were not able to activate Rac1 in these assays. Thus, 1-234 or 350-911 fragments, capable to bind Rac1 with low affinity but not Vav2, did not stimulate Rac1 activity. Moreover, Y112E, partially defective in Rac1 association and totally unable to interact with Vav2, or Y217E, capable to associate to Vav2 but not to Rac1, were not able to activate Rac1 in this assay, suggesting that the interaction of p120ctn with both proteins is required for p120ctn to stimulate Rac1 activity.

Results

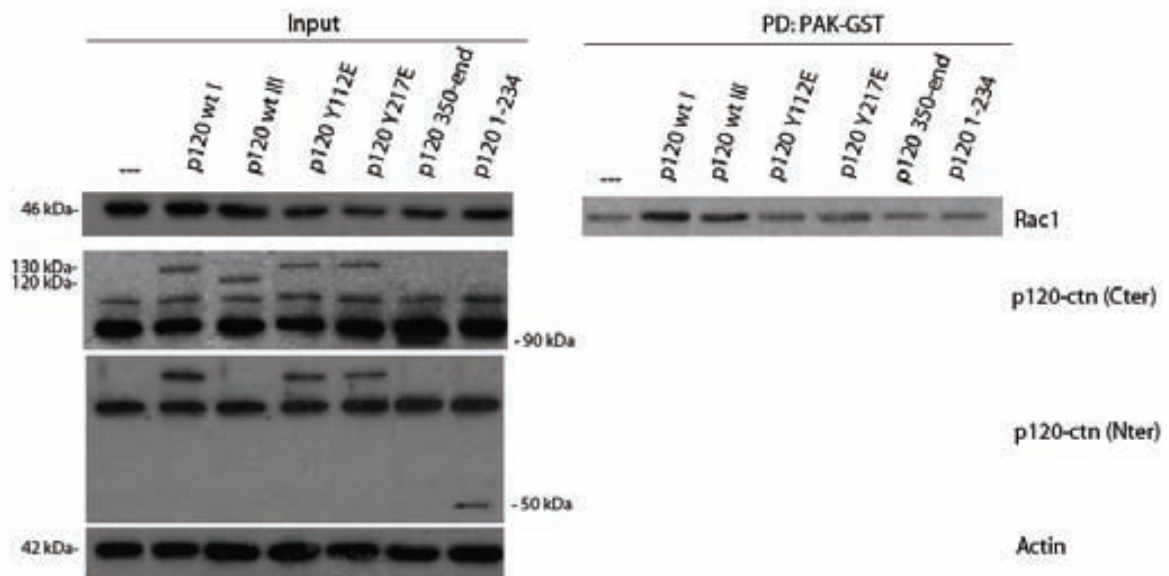


Figure 25. p120ctn point and deletion mutants differently activate Rac1. GST-PAK pull-down assays were performed in SW-480 cell extracts over-expressing either p120ctn-GFP wt isoforms 1 or 3, the isoform 1 of p120ctn-GFP point mutants Y112E or Y217E, p120ctn-GFP deletion mutants (350-911) or (1-234) or the empty vector phrGFP. Active Rac1 was detected by Western Blot.

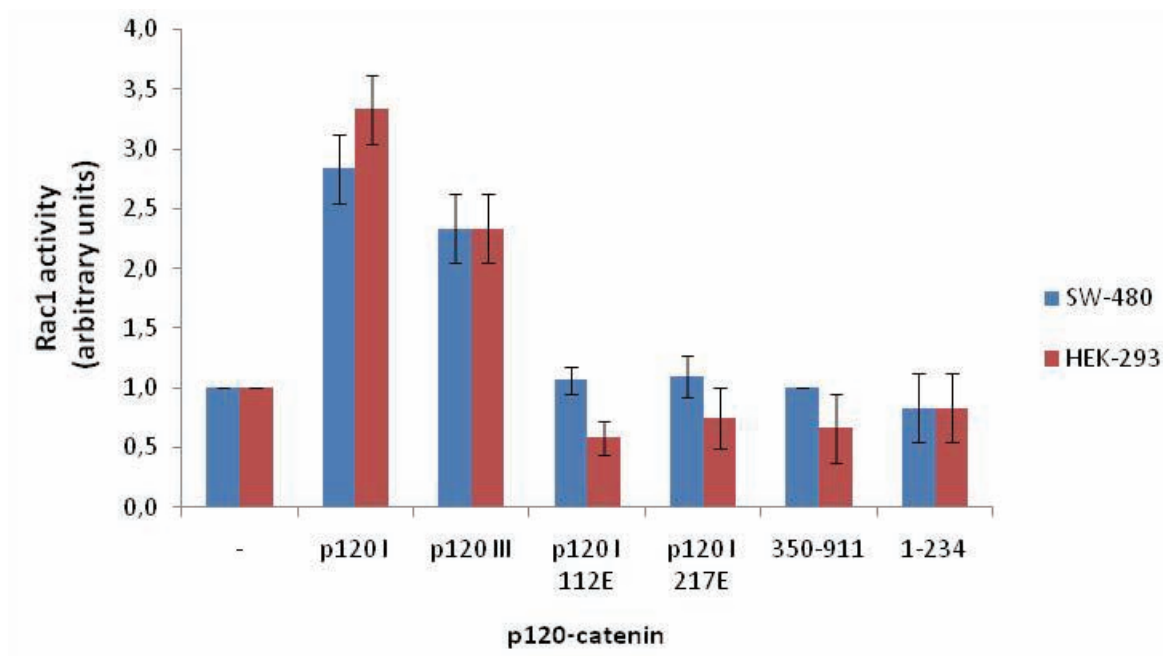


Figure 26. Diagram of different p120ctn mutants capability of activating Rac1. The autoradiograms from five different experiments performed in triplicate in SW-480 and HEK-293 cells were quantified and the mean \pm s.d. was obtained for each condition. Each value is presented relative to that obtained in non-stimulated cells.

Apart from the deletion and the tyrosine point mutants, we also wanted to analyze the serine point mutants when over-expressed in cells. As mentioned before, previous data from our group (151) demonstrated that CK1 phosphorylation disrupted p120ctn/E-cadherin interaction and the residues involved in this down-regulation were the ones stated before: Ser268-269.

It is important to remark that transient over-expression of any p120ctn construct in cells leads to an increase in the cytosolic pool of p120ctn, as there is not enough E-cadherin available to interact with all the p120ctn molecules. As already pointed out in the introduction, p120ctn has a clear differentiated role depending on its localization, stabilizing adherens junctions when bound to E-cadherin or regulating GTPases when E-cadherin unbound (among others). In order to differentiate the E-cadherin-bound from the E-cadherin-unbound p120ctn effects we can co-express p120ctn with an E-cadherin construct or an empty vector, as a control.

In this work, these two different p120ctn pools were simulated taking advantage of two point mutants that differently bind E-cadherin (S268-269DD and S268-269AA). Fluorescent images of cells over-expressing p120ctn mutants co-expressed or not with an E-cadherin construct are presented in Figure 27. The presence of E-cadherin clearly changed p120ctn localization redirecting it to the membrane. As expected, we also observed a different localization of the two p120ctn mutants S268-269DD/AA when co-expressed with E-cadherin: mutant S268-269AA (higher affinity for E-cadherin) localized at the membrane whereas mutant S268-269DD (mimicks CK1 phosphorylation, low affinity for E-cadherin) had a more diffuse localization.

Next, we performed a PAK pull-down assay in the same cells and transfecting the same constructs. As expected, the S268-269DD mutant more efficiently activated Rac1 than the S268-269AA (lanes 4 and 5), and the difference was more evident when constructs were co-expressed with E-cadherin (lanes 2 and 3) (Figure 28).

These results reinforce the idea that E-cadherin-unbound p120ctn is the one involved in Rac1 activation.

Results

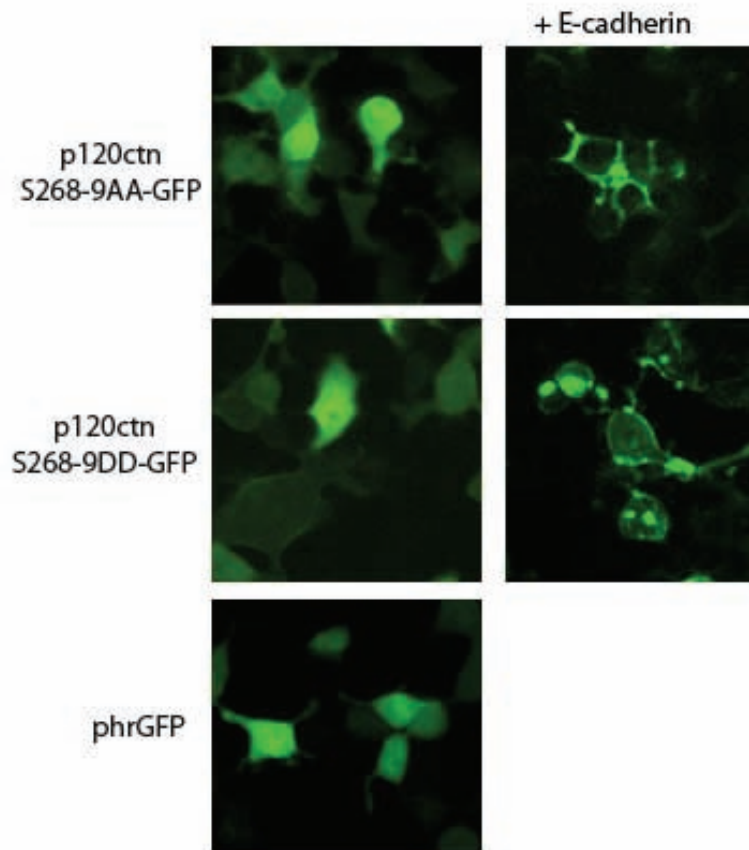


Figure 27. Different localization of p120ctn-GFP constructs (mutants S268269DD/AA). Constructs were transiently over-expressed in HEK-293 co-expressing E-cadherin or the corresponding empty vector. GFP was visualized in Fluorescence Microscope.

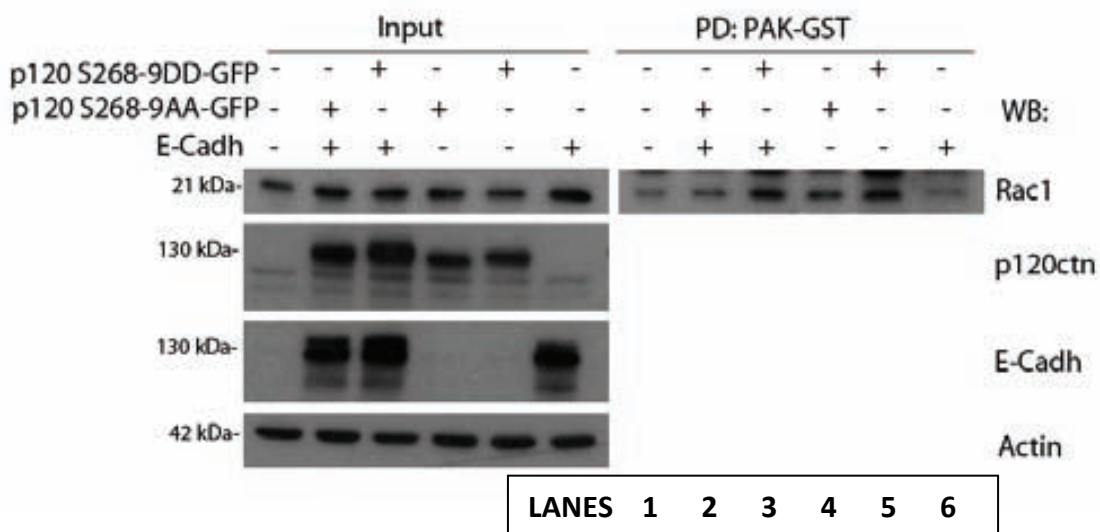


Figure 28. Different p120ctn S268-269DD/AA mutants capability to activate Rac1. GST-PAK pull-down assays were performed in HEK-293 cell extracts over-expressing p120ctn mutants (mutants S268269DD/AA) and co-expressing E-cadherin or the corresponding empty vector when indicated. Active Rac1 was detected by Western Blot.

6. p120CTN IS REQUIRED FOR RAC1 ACTIVATION BY WNT3A

p120ctn has been recently shown to be an essential participant in canonical Wnt signaling. It is required for the very early steps of activation providing the platform for the interaction of CK1 ϵ with the Wnt-receptor complex (151, 153) and also in later events being released from E-cadherin and controlling the bimodal activity of Kaiso transcriptional factor (152).

Rac1 had been mainly related with non-canonical Wnt pathways that involve polarized cell shape changes and migrations but also more recently with the canonical pathway leading to β -catenin-dependent transcription (163).

We knew so far that p120ctn was strongly implicated in Rac1 activation, p120/Rac1 association was up-regulated by p120ctn phosphorylation by CK1 (a kinase activated upon Wnt stimulation) and that p120ctn phosphorylated by CK1 (with low affinity for E-cadherin) had a stronger effect on Rac1 activity. For all these reasons and with the aim of finding a more physiological signaling pathway for our molecular model, we started to investigate the possible role of p120ctn in Wnt-mediated Rac1 activation.

6.1 RAC1 IS ACTIVATED IN WNT3A-STIMULATED CELLS

We have analyzed Rac1 stimulation by Wnt3a using two cellular systems: HEK-293 and SW-480 that although mutated in APC and therefore deficient in β -catenin degradation, are totally competent for other signals triggered by this stimulus (151-153). Upon Wnt3a addition, Rac1 became stimulated in both cell lines (Figures 29 and 30). Up-regulation in Rac1 activity was transient; it was maximal 2 hours after the addition of the stimulus and returned to the basal levels by 15 hours (Figures 29 and 30). No significant differences were detected between the two cell lines (Figure 30).

Results

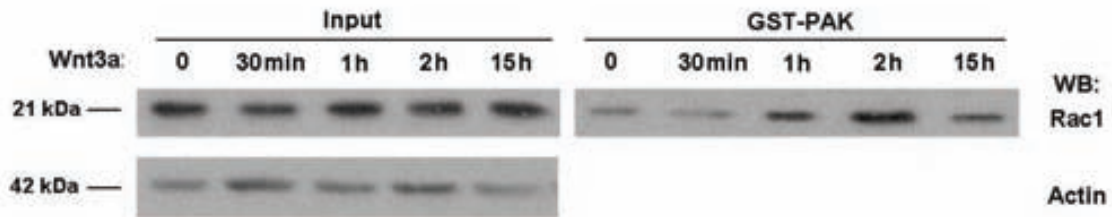


Figure 29. Time course for active Rac1 after Wnt stimulation. SW-480 cells treated with control or Wnt3a-conditioned medium for the indicated time were lysed and active Rac1 was precipitated using GST-PAK pull-down assay. Active Rac1 was detected by Western Blot.

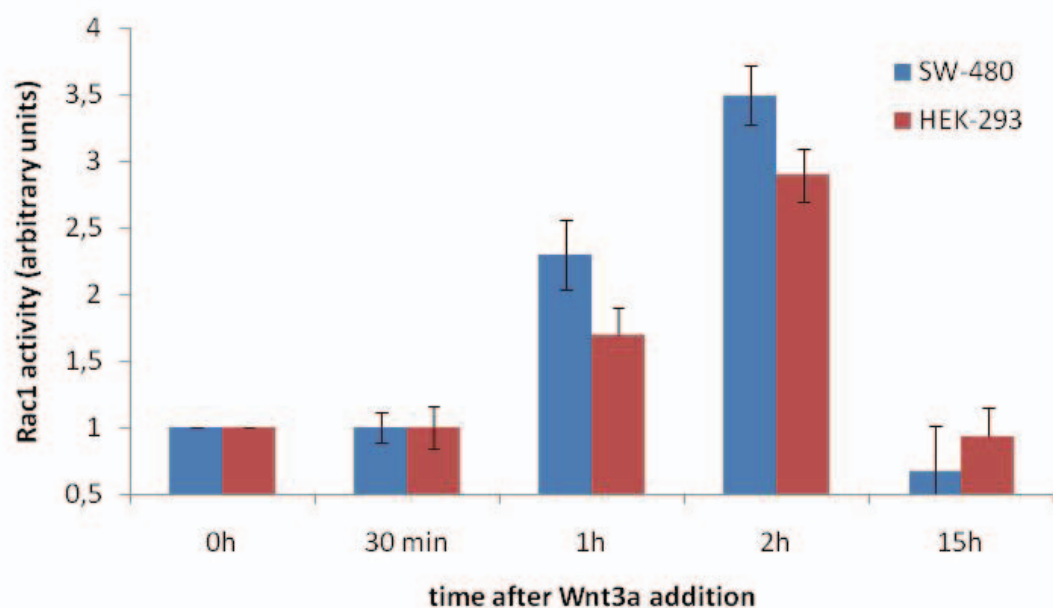


Figure 30. Diagram of the time course for active Rac1 after Wnt stimulation. The autoradiograms from five different experiments performed in triplicate as in Figure 29 in SW-480 and HEK-293 cells were quantified and the mean \pm s.d. was obtained for each condition. Each value is presented relative to that obtained in non-stimulated cells.

A part from these two cell lines, we also efficiently stimulated Rac1 activity upon 2 hours addition of Wnt3a-conditioned media in HT-29 cells, another colon carcinoma cell line mutant in APC (Figure 31).

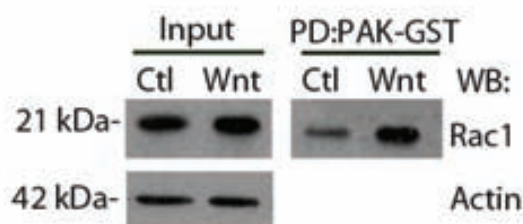


Figure 31. Rac1 activation upon Wnt stimulation. HT-29 cells treated with control or Wnt3a-conditioned medium for 2 hours were lysed and active Rac1 was precipitated using GST-PAK pull-down assay. Active Rac1 was detected by Western Blot.

We tested if RhoA activity was also affected by Wnt3a but after performing several times the Rhotekin pull-down assay we could not detect any changes (Figure 32).

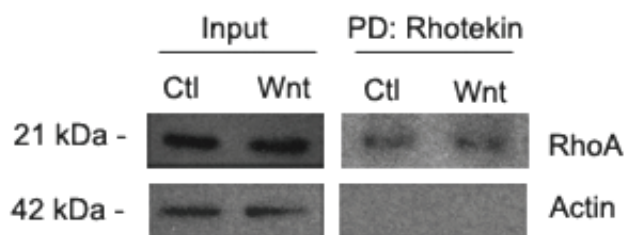


Figure 32. RhoA activity does not change upon Wnt stimulation. SW-480 cells treated with control or Wnt3a-conditioned medium for 2 hours were lysed and active Rac1 was precipitated using GST-Rhotekin pull-down assay. Active RhoA was detected by Western Blot.

6.2 CELLS DEPLETED OF P120CTN OR VAV2 INHIBIT WNT-INDUCED ACTIVATION OF RAC1

We performed PAK pull-down assays in p120ctn or Vav2-depleted SW-480 or HEK-293 cells after stimulation of 2 hours with Wnt3a-conditioned media. Rac1 activity was not sensitive to Wnt3a treatment neither in p120ctn-depleted cells nor in Vav2-depleted cells, contrary to what occurs in control cells (Figures 33 and 34). Moreover, and as seen before for p120ctn-depleted cells (Figure 9), Rac1 activity was lower in p120ctn or Vav2-depleted cells than in control ones (Figures 33 and 34).

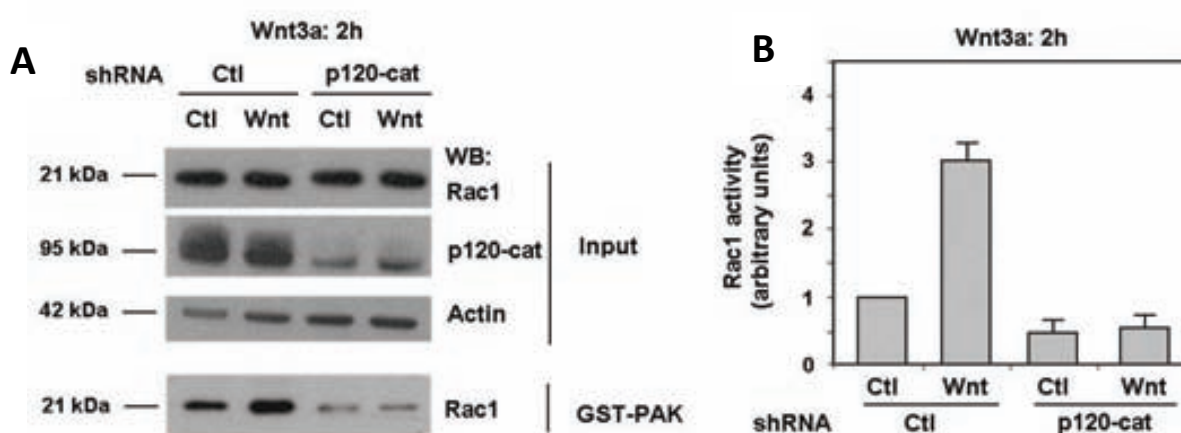


Figure 33. Rac1 activation by Wnt is dependent on p120ctn. (A) A PAK pull-down assay was performed in SW-480 cells stably expressing control or shRNA specific for p120ctn. Active Rac1 was detected from the extracts by Western Blot. (B) Autoradiograms from five different experiments performed in triplicate were quantified and the mean \pm s.d. was obtained for each condition. Each value is presented relative to that obtained in non-depleted cells treated with control medium.

Results

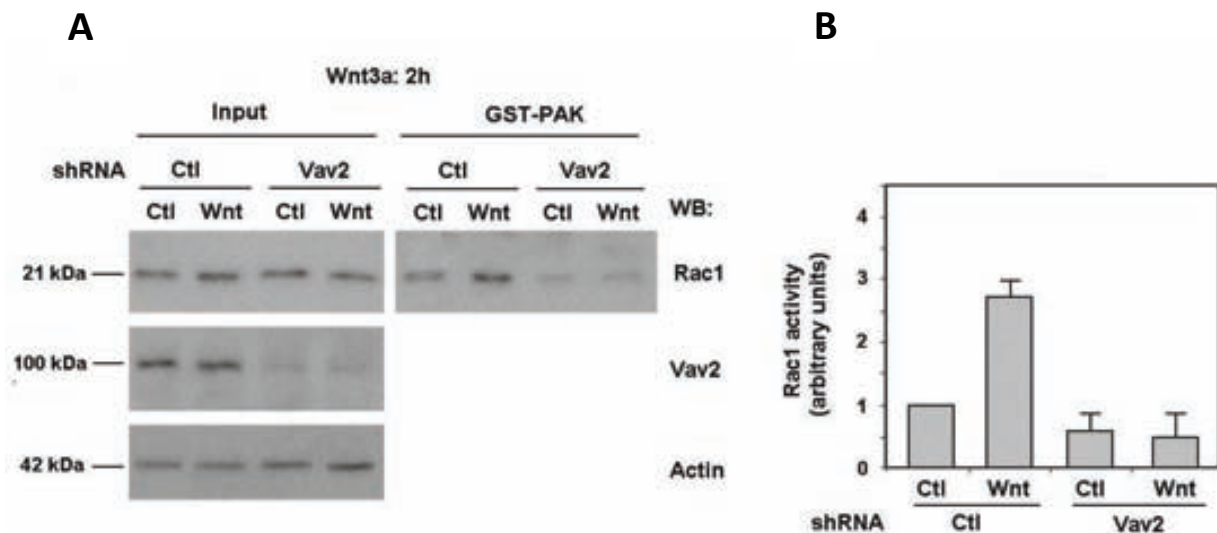


Figure 34. Rac1 activation by Wnt is dependent on Vav2. (A) A PAK pull-down assay was performed in SW-480 cells stably expressing control or shRNA specific for Vav2. Active Rac1 was detected from the extracts by Western Blot. (B) Autoradiograms from five different experiments performed in triplicate were quantified and the mean \pm s.d. was obtained for each condition. Each value is presented relative to that obtained in non-depleted cells treated with control medium.

6.3 THE ASSOCIATION BETWEEN P120CTN, RAC1 AND VAV2 IS UP-REGULATED UPON WNT TREATMENT

Although Vav2 seemed to be a good candidate for being the GEF associated to p120ctn involved in the Wnt-mediated Rac1 activation, we also checked for another GEF of Rac1, Tiam1, that had recently been shown to cooperate in the activation of Wnt-responsive promoters (139). Tiam1 was immunoprecipitated from SW-480 cell extracts treated with control or Wnt-conditioned medium for 2 hours and associated proteins were analyzed by Western Blot (Figure 35). As already described by other groups (139), Tiam1 increased the association with β -catenin upon Wnt stimulation. We also observed p120ctn/Tiam1 association but no significant change in this interaction was detected upon Wnt stimuli.

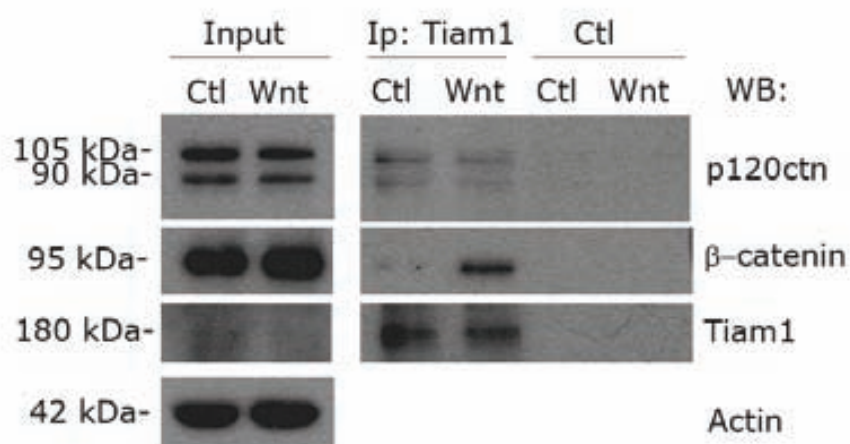


Figure 35. Tiam1 association with p120ctn does not change upon Wnt stimulation. Tiam1 was immunoprecipitated from HEK-293 whole-cell extracts treated with control or Wnt3a-conditioned medium for 2 hours. Protein complexes were analyzed by Western Blot.

In contrast, when Vav2 was immunoprecipitated from control and Wnt-stimulated cell extracts, an up-regulation of Vav2 association with p120ctn as well as with Rac1 was observed (Figure 36).

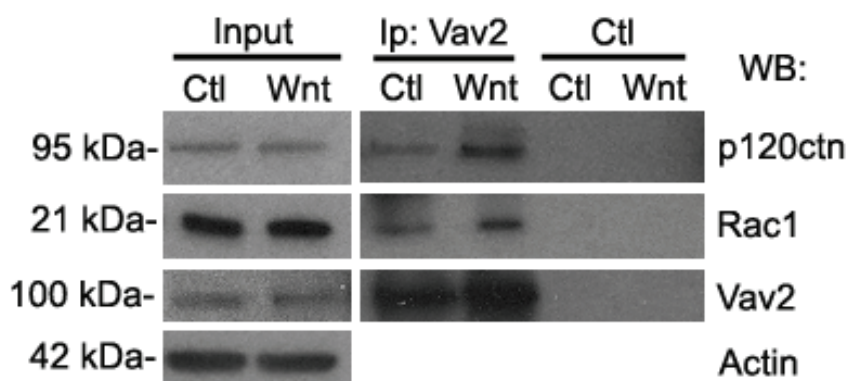


Figure 36. Vav2 association with p120ctn is up-regulated upon Wnt stimulation. Vav2 was immunoprecipitated from SW-480 whole-cell extracts treated with control or Wnt3a-conditioned medium for 2 hours. Protein complexes were analyzed by Western Blot.

Results

To further confirm these results, the interaction of p120ctn with Vav2 and Rac1 was also verified by an inverse coIP. p120ctn was immunoprecipitated from SW-480 cell extracts at different times of Wnt activation. The presence of Rac1 and Vav2 in p120ctn immunocomplexes was up-regulated after 2 hours of incubation with Wnt3a concomitantly with Rac1 activation, a time when p120ctn has been released from its interaction with E-cadherin (Figure 37). This Wnt-stimulated p120ctn release from E-cadherin is promoted by CK1 phosphorylation of p120ctn as shown before (Figure 18) and has been described by others members of the group (151).

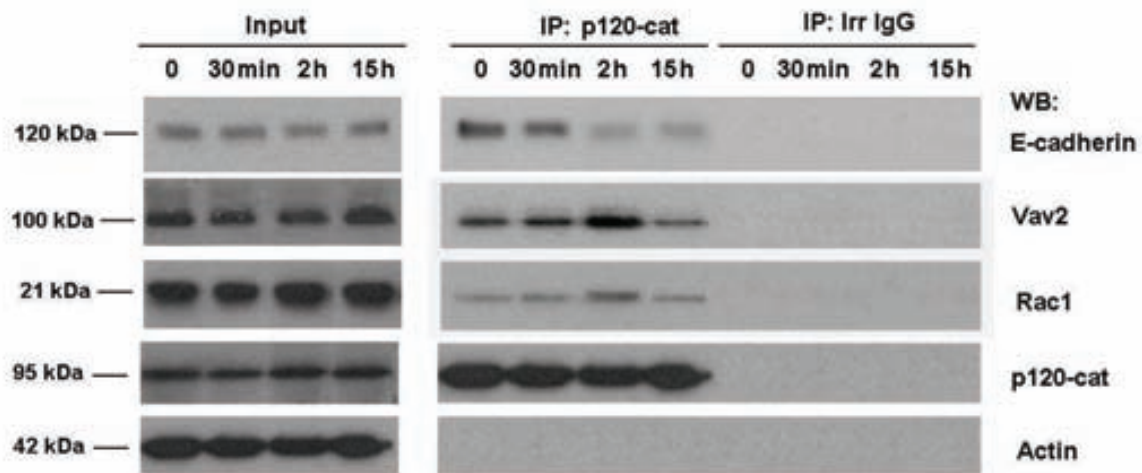


Figure 37. p120ctn/Vav2/Rac1 complex is up-regulated after 2 hours of Wnt activation. p120ctn was immunoprecipitated from SW-480 whole-cell extracts treated with control or Wnt3a-conditioned medium for the indicated times. Protein complexes were analyzed by Western Blot.

The presence of Rac1 and p120ctn in Vav2 immunocomplexes was also increased after 2 hours of Wnt3a incubation (Figure 38).

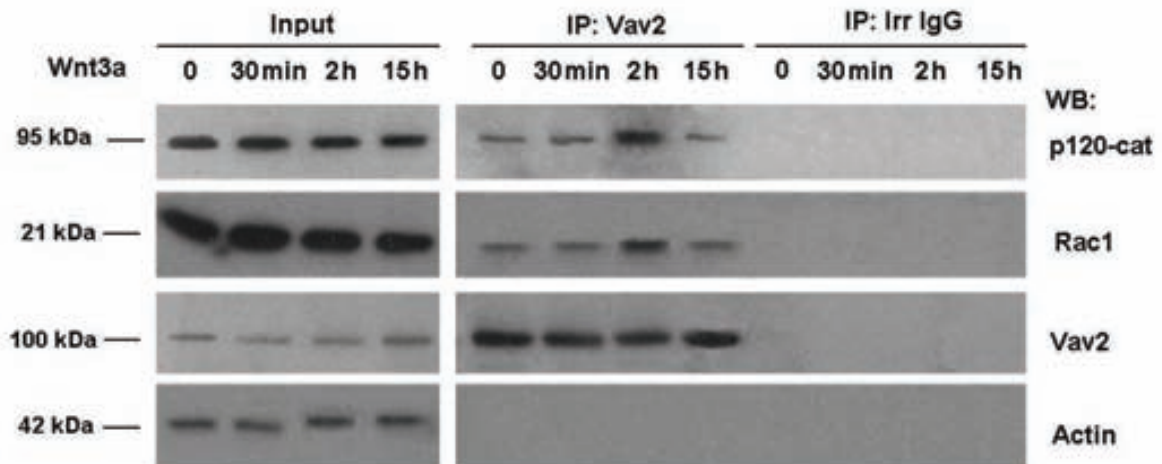


Figure 38. Vav2/p120ctn/Rac1 complex is up-regulated after 2 hours of Wnt activation. Vav2 was immunoprecipitated from SW-480 whole-cell extracts treated with control or Wnt3a-conditioned medium for the indicated times. Protein complexes were analyzed by Western Blot.

7. p120CTN IS DEPHOSPHORYLATED IN TYROSINES AND PHOSPHORYLATED IN SERINES AFTER WNT3A STIMULATION

We also wanted to verify if the phosphorylation-dependent regulation of p120ctn/Vav2/Rac1 interaction was observed *in vivo* upon Wnt3a activation.

As shown before and in the following Figure 39, Wnt3a enhances p120ctn interaction with Rac1 and Vav2. This increase was accompanied by p120ctn phosphorylation by CK1, determined analyzing Ser268 modification (Figure 39, 4th panel). Conversely, tyrosine phosphorylation on p120ctn was decreased by Wnt3a (Figure 39, 3rd panel). This Wnt-dependent down-regulation was also accompanied with a lower association of p120ctn to Fyn, since the amount of this tyrosine kinase co-immunoprecipitated with p120ctn was decreased upon 2 hours of Wnt3a addition (Figure 40). This is likely the consequence of p120ctn phosphorylation by CK1 since an *in vitro* phosphorylation of recombinant p120ctn phosphorylated by CK1 decreased its interaction with Fyn (Figure 41).

Results

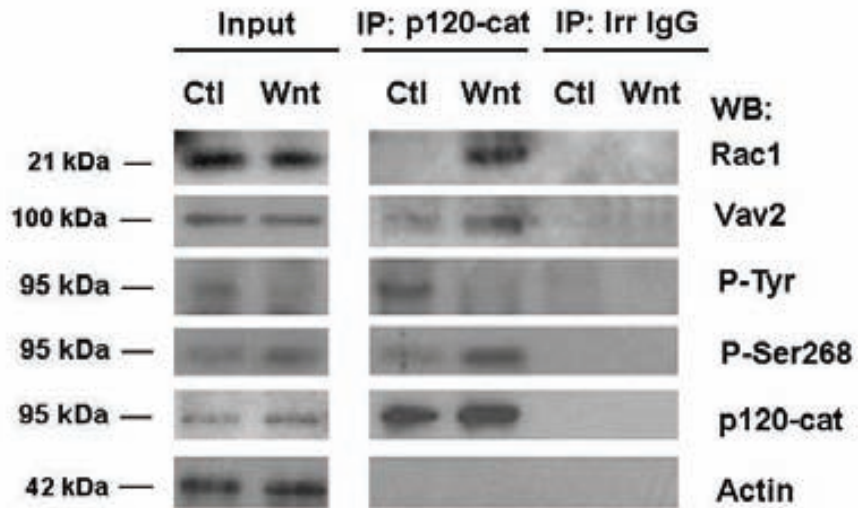


Figure 39. p120ctn is phosphorylated in serines and dephosphorylated in tyrosines upon Wnt-stimulation. p120ctn was immunoprecipitated from SW-480 whole-cell extracts treated with control or Wnt3a-conditioned medium for 2 hours. Protein complexes were analyzed by Western Blot.

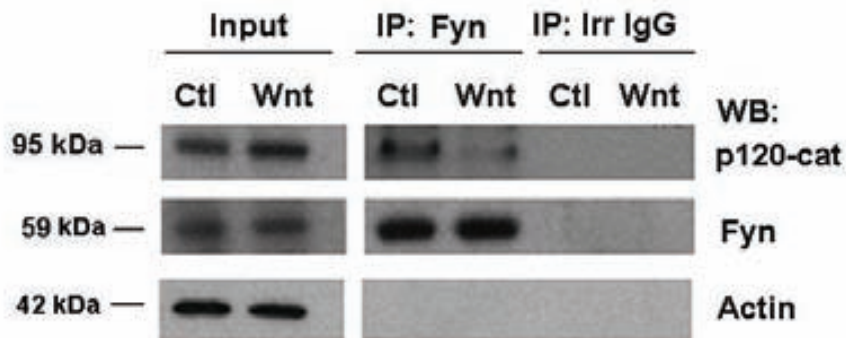


Figure 40. Fyn association with p120ctn decreases upon Wnt stimulation. Fyn was immunoprecipitated from SW-480 whole-cell extracts treated with control or Wnt3a-conditioned medium for 2 hours. Protein complexes were analyzed by Western Blot.

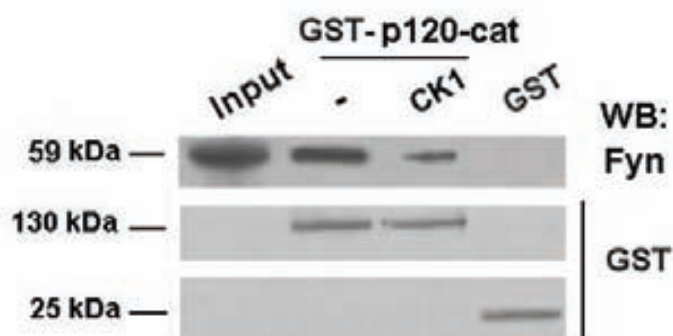


Figure 41. p120ctn association with Fyn decreases *in vitro* after CK1 phosphorylation. GST-p120ctn was phosphorylated by CK1 and pull-down assay was performed incubating 5 pmols of GST-p120ctn or GST with cell extracts from SW-480 cells. Protein complexes were affinity purified and analyzed by Western Blot. 5 μ g of SW-480 lysate were included as an internal reference (Input).

8. OTHER WNT3A RESPONSES DOWNSTREAM OF RAC1 ARE ALSO AFFECTED BY P120 OR VAV2 DEPLETION

8.1 JNK2 PHOSPHORYLATION

As mentioned before, JNK2 phosphorylation (catalyzed by Rac1-dependent PAK kinase) has been described to be essential for β -catenin nuclear translocation after canonical Wnt stimulation (140). Total cell extracts from control, p120ctn or Vav2-depleted cells were prepared after 2 hours stimulation by Wnt3a. An increase in phospho-JNK2 was observed in Wnt-stimulated cells whereas it was not detected in p120ctn or Vav2-depleted cells (Figure 42A and 42B, respectively).

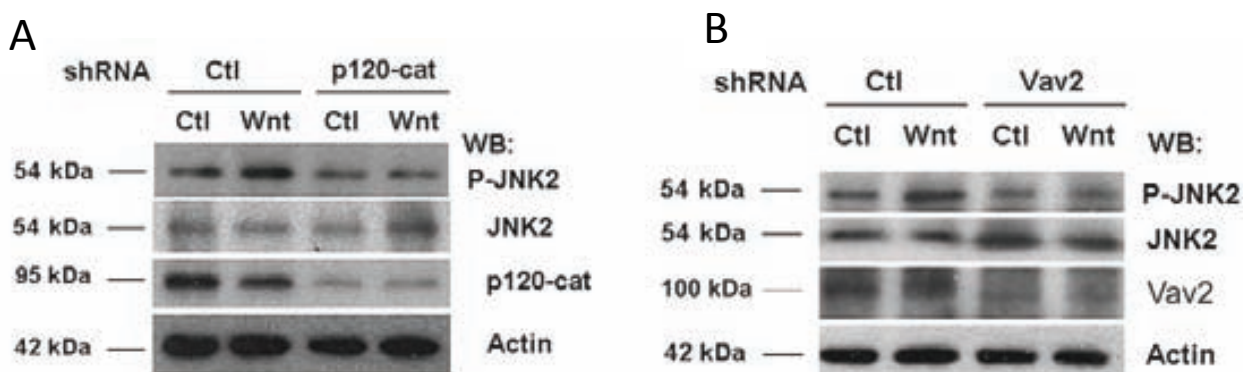


Figure 42. JNK2 phosphorylation upon Wnt stimulation is inhibited in p120ctn and Vav2-depleted cells. SW-480 stably expressing control or shRNA specific for p120ctn (A) or Vav2 (B) were treated with Wnt3a-conditioned media for 2 hours and lysed. Phospho-JNK2 and total JNK2 were detected from extracts by Western Blot.

8.2 β -CATENIN NUCLEAR TRANSLOCATION

In accordance with previous observations indicating a role for Rac1 in β -catenin translocation to the nucleus (140), transfection of an inactive mutant of Rac1 that works as a dominant negative (Rac-N17) decreased the nuclear β -catenin levels and blocked the up-regulation in nuclear β -catenin caused by Wnt3a (Figure 43).

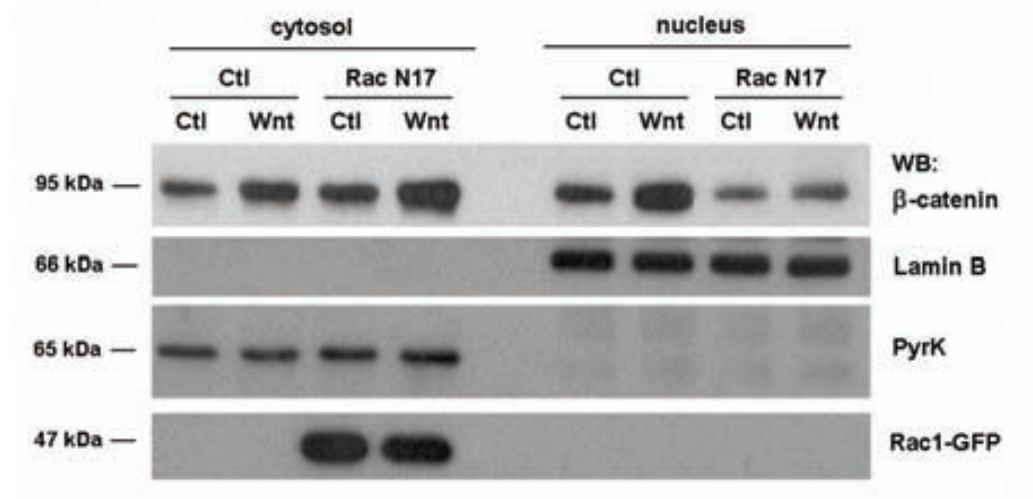


Figure 43. β -catenin nuclear translocation after Wnt stimulation is inhibited when over-expressing a dominant negative construct of Rac1. Dominant negative Rac1 (Rac1-N17) or the empty vector were over-expressed in HEK-293 cells and treated with control or Wnt3a-conditioned medium for 15h. Nuclear fraction was separated from the cytosolic and membrane-associated fraction as detailed in Experimental Procedures and β -catenin levels in each cell compartment were analyzed by Western Blot. Lamin- β 1 was used as a nuclear marker; pyruvate kinase was used as a marker for the cytosolic-plus-membrane fraction.

The increase in nuclear β -catenin promoted by Wnt3a stimulation was also prevented in p120ctn-depleted cells. Similar effects were observed in SW-480 cells (Figure 44) and HEK-293 cells (Figure 45).

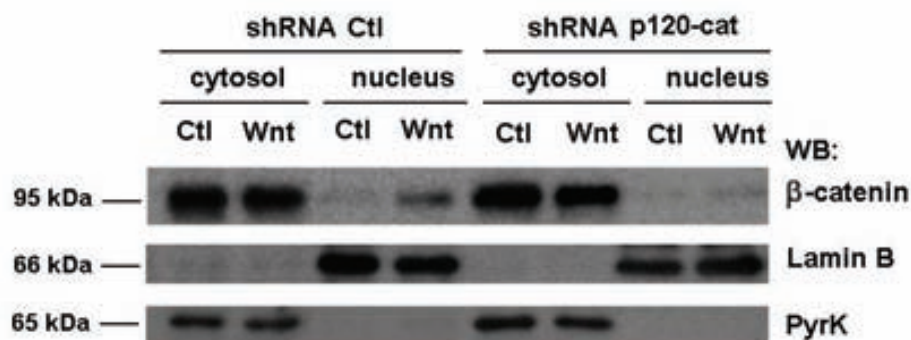


Figure 44. β -catenin nuclear translocation after Wnt stimulation is inhibited in p120ctn-depleted SW-480 cells. Cytosolic and nuclear lysates were obtained from control and p120ctn-depleted SW-480 cells treated with control or Wnt3a-conditioned medium for 15h. β -catenin distribution between the two cell compartments was analyzed by Western Blot.

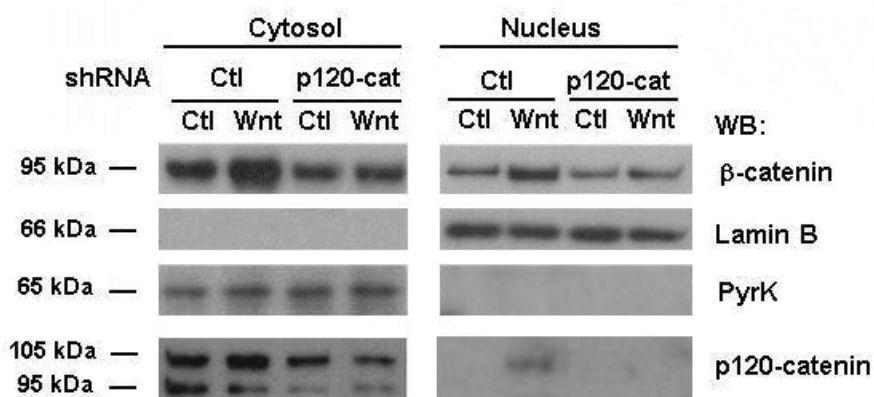


Figure 45. β -catenin nuclear translocation after Wnt stimulation is inhibited in p120ctn-depleted HEK-293 cells. Cytosolic and nuclear lysates were obtained from control and p120ctn-depleted HEK-293 cells treated with control or Wnt3a-conditioned medium for 15h. β -catenin distribution between the two cell compartments was analyzed by Western Blot.

Moreover, Vav2-depleted SW-480 cells also blocked the nuclear translocation of β -catenin promoted by Wnt3a (Figure 46).

Results

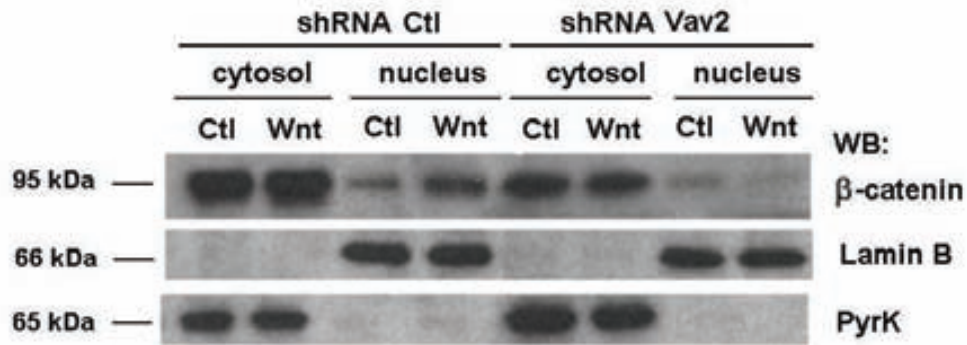


Figure 46. β -catenin nuclear translocation after Wnt stimulation is inhibited in Vav2-depleted cells. Cytosolic and nuclear lysates were obtained from control and Vav2-depleted SW-480 cells treated with control or Wnt3a-conditioned medium for 15h. β -catenin distribution between the two cell compartments was analyzed by Western Blot.

On the contrary, transfection of a constitutively active Rac1 mutant (Rac1-V12) increased the amount of nuclear β -catenin in SW-480 cells (Figure 47). This effect was not sensitive to p120ctn down-regulation (Figure 47), indicating that Rac1 acts on β -catenin nuclear translocation down-stream p120ctn.

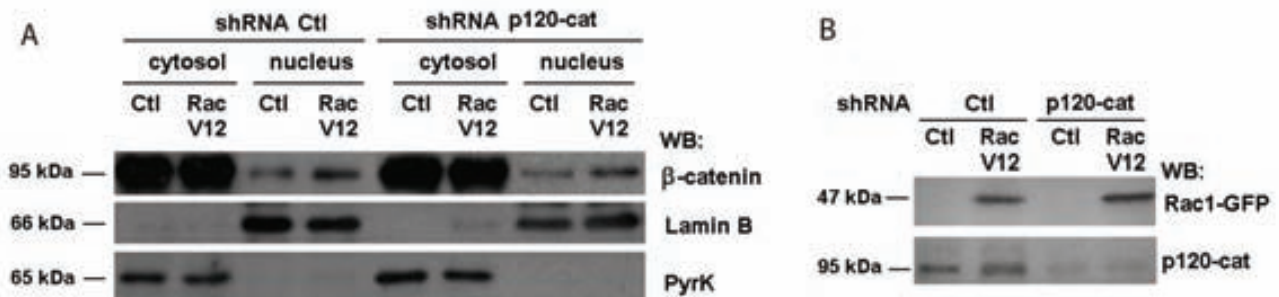


Figure 47. Over-expression of a constitutive active Rac1 mutant increases the amount of nuclear β -catenin in p120ctn-depleted cells. Active Rac1 (Rac1-V12) or the empty vector were over-expressed in SW-480 cells and treated with control or Wnt3a-conditioned medium for 15h. Nuclear fraction was separated from the cytosolic and membrane-associated fraction as detailed in Experimental Procedures and β -catenin levels in each cell compartment were analyzed by Western Blot.

Rac1 has also been described to be activated in canonical Wnt signaling through a signaling cascade involving LRP5/6, Dvl, $G_{\alpha_{q/11}}\beta\gamma$, and PI3K (140). However, using a potent inhibitor of PI3Ks (LY-294002) we did not observe any significant changes in β -catenin nuclear translocation after Wnt3a stimulation comparing with non-treated cells (Figure 48). Thus, in our system, PI3K seems not to be involved in the Wnt-mediated Rac1 activation effects.

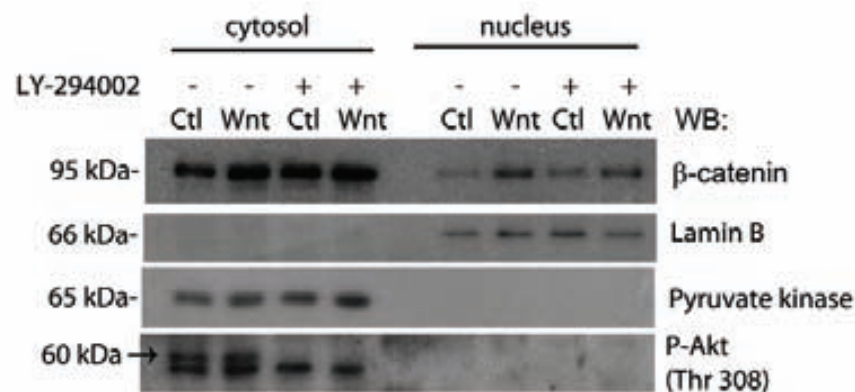


Figure 48. LY-294002 treatment is not able to inhibit β -catenin nuclear translocation after Wnt stimulation. HEK-293 cells were treated with control or Wnt3a-conditioned medium with and without LY-294002 (50 μ M) for 4 hours. Nuclear fraction was separated from the cytosolic and membrane-associated fraction as detailed in Experimental Procedures and β -catenin levels in each cell compartment were analyzed by Western Blot.

9. WNT SIGNALING IN *XENOPUS LAEVIS* REQUIRES RAC1 AND VAV2 BINDING TO P120CTN.

Recently, it has been addressed the requirement of *Xenopus* ARVCF (xARVCF) and *Xenopus* p120ctn (xp120ctn) in vertebrate embryogenesis. The work was conducted in *X.laevis* embryos and using a depletion-rescue strategy in conjunction with biochemical and cell biological analyses. Depletion of xp120ctn resulted in disrupted gastrulation and axial elongation and these effects were rescued upon co-injection of a xp120ctn construct, therefore demonstrating that xp120ctn is developmentally required (116).

We used an established morpholino (MO) to deplete *X. laevis* p120ctn. As shown in Figure 49, this MO efficiently decreased p120ctn protein levels when injected into both

Results

blastomeres at the two-cell stage. Although introduced at early stages (cleavage) of embryogenesis, when maternal mRNAs predominate, morpholinos continue to function after the mid-blastula transition (stage 8) when zygotic transcription/translation is initiated in *X.laevis*.

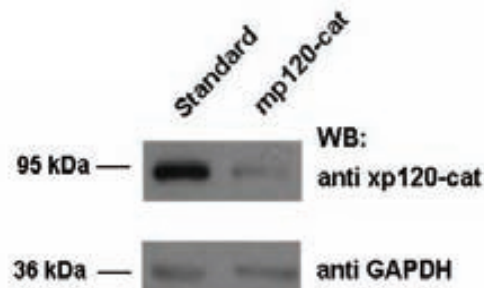


Figure 49. Xp120ctn depletion upon M.O injection. xp120ctn protein from total embryo extracts isolated at 11-12 stage was detected via SDS-PAGE/Western blotting using anti-Xp120ctn polyclonal antibody. Xp120ctn displayed a SDS-PAGE mobility of ~ 90 kD. Protein loads were assessed by Western Blotting samples for GAPDH.

At the morpholino dose employed, approximately 40% of the injected embryos displayed aberrant gastrulation compared with controls injected with a standard control (SC) morpholino (Figures 50 and 51). This phenotype was rescued upon ectopic expression of myc-tagged murine p120ctn constructs (a transcript that lacks the morpholino binding sequence).

All the experiments were performed separately in groups of mutants and repeated several times to find the adequate injection conditions. Some pictures from different experiments and different batch of embryos are shown in Figures 53, 54, 55 and 56. A compilation of different experiments performed is shown in diagrams from Figures 50 and 51.

Wild-type or Y112F forms of murine p120ctn were equally effective in rescuing the gastrulation defects (Figure 50). On the contrary, Y112E or Y217E failed to rescue in these assays (Figures 50 and 51, respectively), although they were expressed at similar levels to the other p120ctn forms (Figure 52). These results suggest that p120ctn binding to Vav2 and Rac1 is required for p120ctn to fulfill its role in *X.laevis* early development.

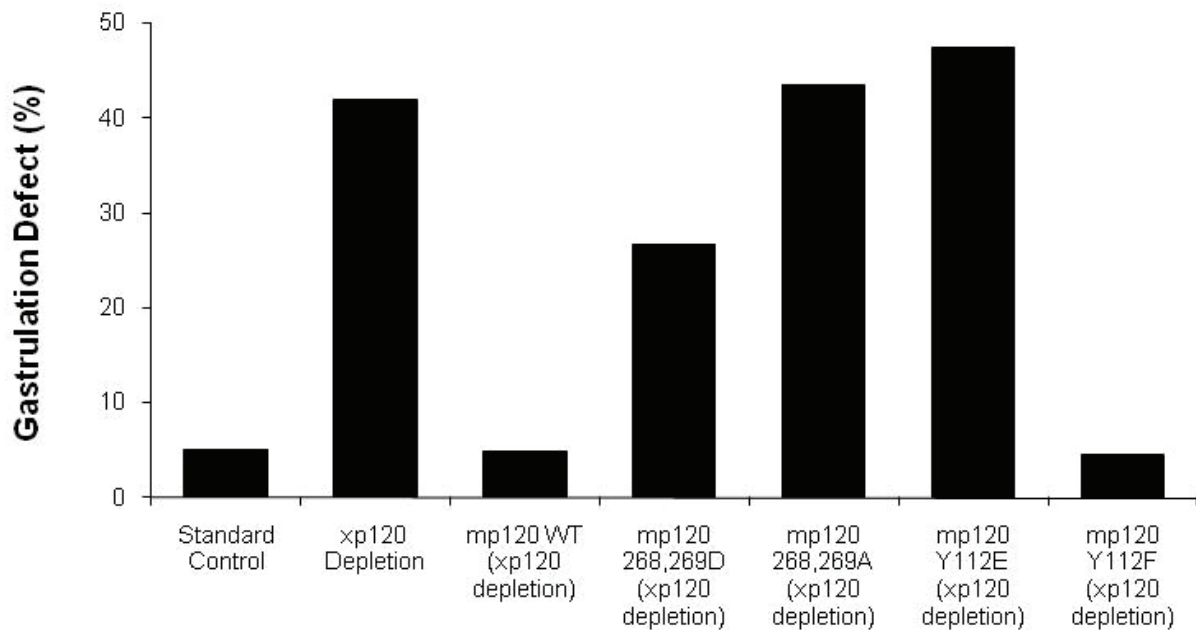


Figure 50. Only mp120ctn WT and mp120ctn 112F constructs are capable to rescue gastrulation defects. Embryos at the 2-cell stage were injected with a control morpholino or a combination of xp120ctn morpholinos (20 ng each of xp120ctn MO I and MO II per blastomere). Rescues were effected by co-injection of xp120ctn morpholinos with mp120ctn WT, mp120ctn Y112E, mp120ctn Y112F, mp120ctn S268-269DD and mp120ctn S268-269AA *in vitro* transcribed mRNAs. Embryos were scored at stage 12 (gastrula) for defects including partial/improper closure of the blastopore. Resulting gastrulation phenotypes are shown in Figures 53, 54 and 55 with percentages presented in this figure. To avoid over-expression phenotypes, the injection doses of all rescuing constructs were carefully titrated to produce minimal effects when injected.

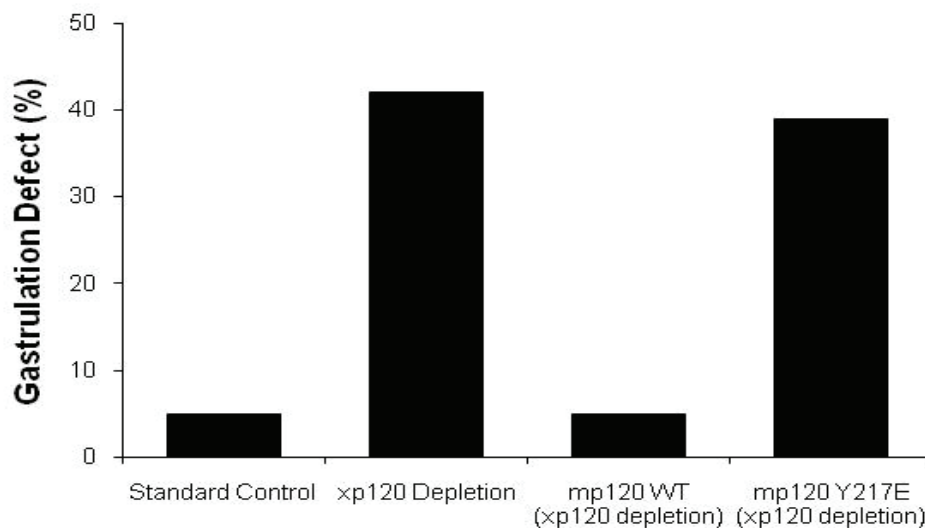


Figure 51. A mp120ctn construct unable to bind Rac1 (Y217E) is not capable to rescue gastrulation defects. Embryos at the 2-cell stage were injected with a control morpholino or a combination of xp120ctn morpholinos (20 ng each of xp120ctn MO I and MO II per blastomere). Rescues were effected by co-injection of xp120ctn morpholinos with mp120ctn WT and mp120ctn Y217E *in vitro* transcribed mRNAs. Embryos were scored at stage 12 (gastrula) for defects including partial/improper closure of the blastopore. Resulting gastrulation phenotypes are shown in Figure 56 with percentages presented in this figure. To avoid over-expression phenotypes, the injection doses of all rescuing constructs were carefully titrated to produce minimal effects when injected.

Results

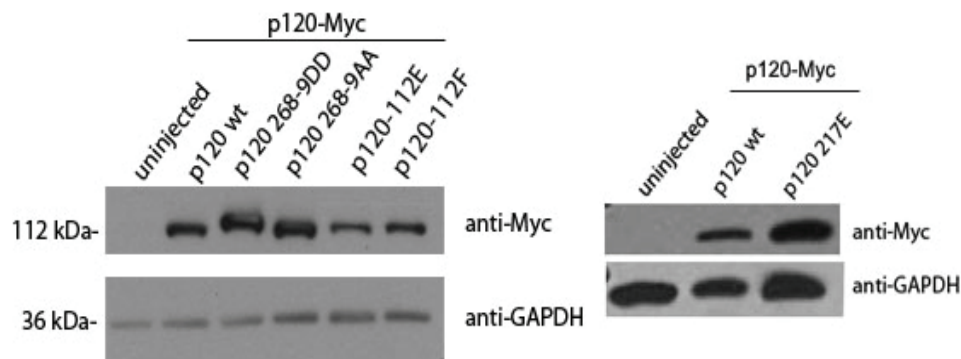


Figure 52. The different p120ctn mutants were expressed at similar levels. The expression of mp120ctn WT and Y112E, Y112F, Y217E, S268-269DD and S268-269AA mutants was detected via SDS-PAGE/Western blotting using anti-myc antibody. Protein loads were assessed by Western Blotting samples for GAPDH.

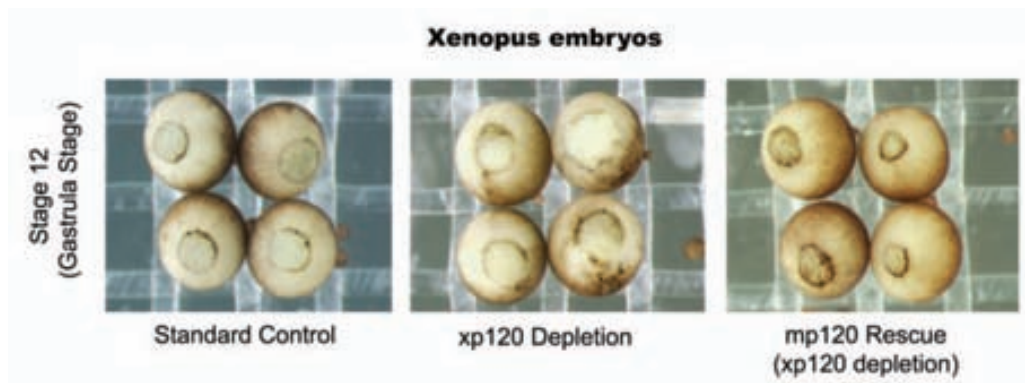


Figure 53. mp120ctn WT construct rescued gastrulation defects induced by p120ctn M.O. Resulting gastrulation phenotypes after co-injection of xp120ctn morpholinos with mp120ctn- WT *in vitro* transcribed mRNA.

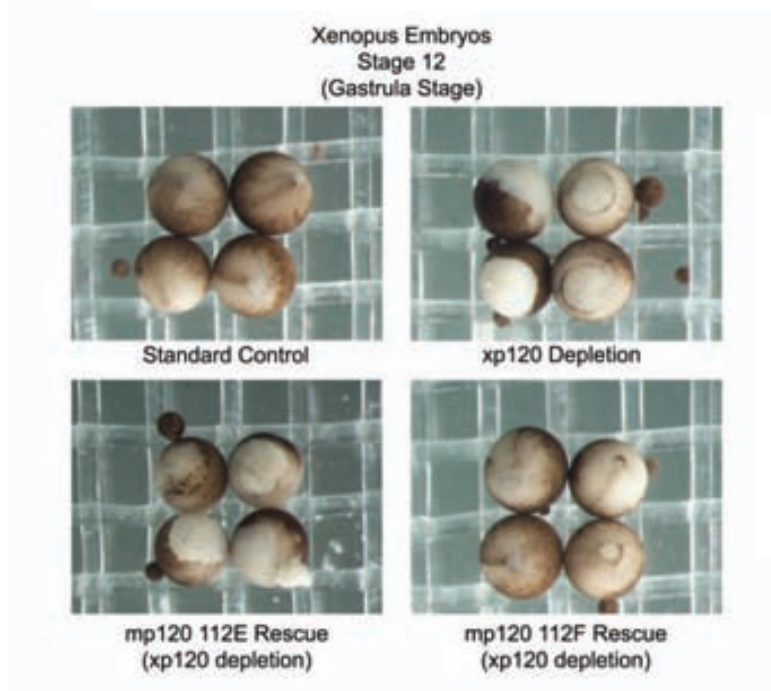


Figure 54. mp120ctn 112F construct rescued gastrulation defects induced by p120ctn M.O. Resulting gastrulation phenotypes after co-injection of xp120ctn morpholinos with mp120ctn Y112E and mp120ctn Y112F *in vitro* transcribed mRNAs.

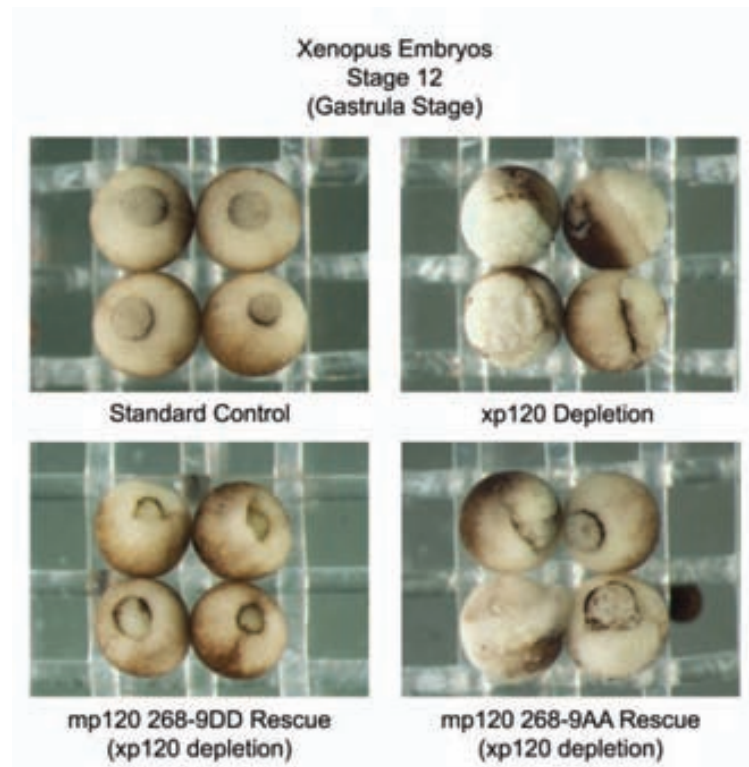


Figure 55. mp120ctn 268-9DD construct partially rescued gastrulation defects induced by p120ctn M.O. Resulting gastrulation phenotypes after co-injection of xp120ctn morpholinos with mp120ctn S268-269DD and mp120ctn S268-269AA *in vitro* transcribed mRNAs.

On the other hand, the S268-269DD/AA forms of murine p120ctn were also injected in the embryos to rescue the p120ctn depletion effects. We first looked at localization of both constructs by Animal Caps Immunofluorescence. As observed *in vitro*, mutant S268-269AA localized at the membrane while the mutant S268-269DD had a more disperse localization (Figure 57). Surprisingly, none of both mutants were effective in rescuing the gastrulation defects, although the mutant S268-269DD displayed a partial rescue phenotype (Figures 50 and 55). This result suggests that p120ctn localization in the cell is very important for its function but also shows that a low E-cadherin affinity (as it is the case of S268-269DD construct, totally effective in activating Rac1 *in vitro*, Figure 28) is not capable enough to fulfill the role in early development.

Results

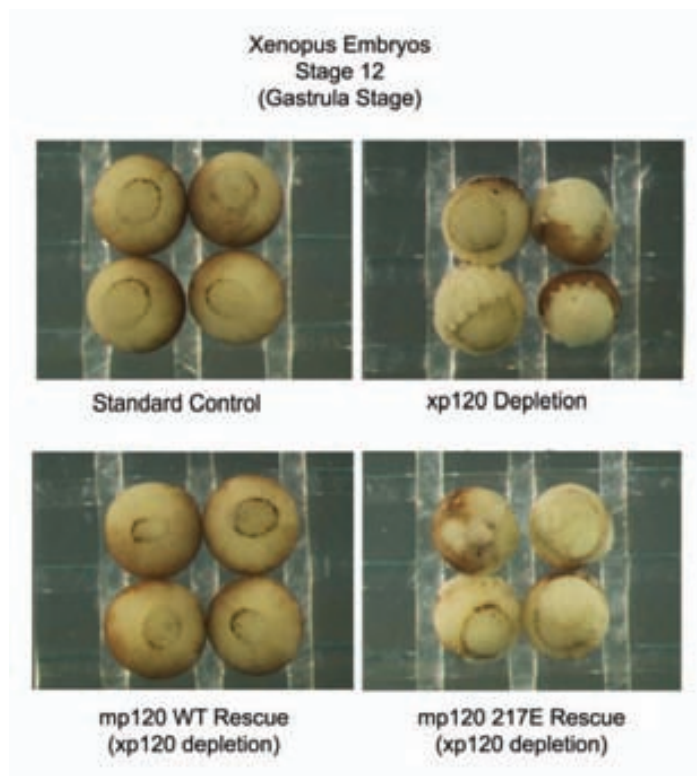


Figure 56. Only mp120ctn WT construct rescued gastrulation defects induced by p120ctn M.O Resulting gastrulation phenotypes after co-injection of xp120ctn morpholinos with mp120ctn WT and mp120ctn Y217E *in vitro* transcribed mRNAs.

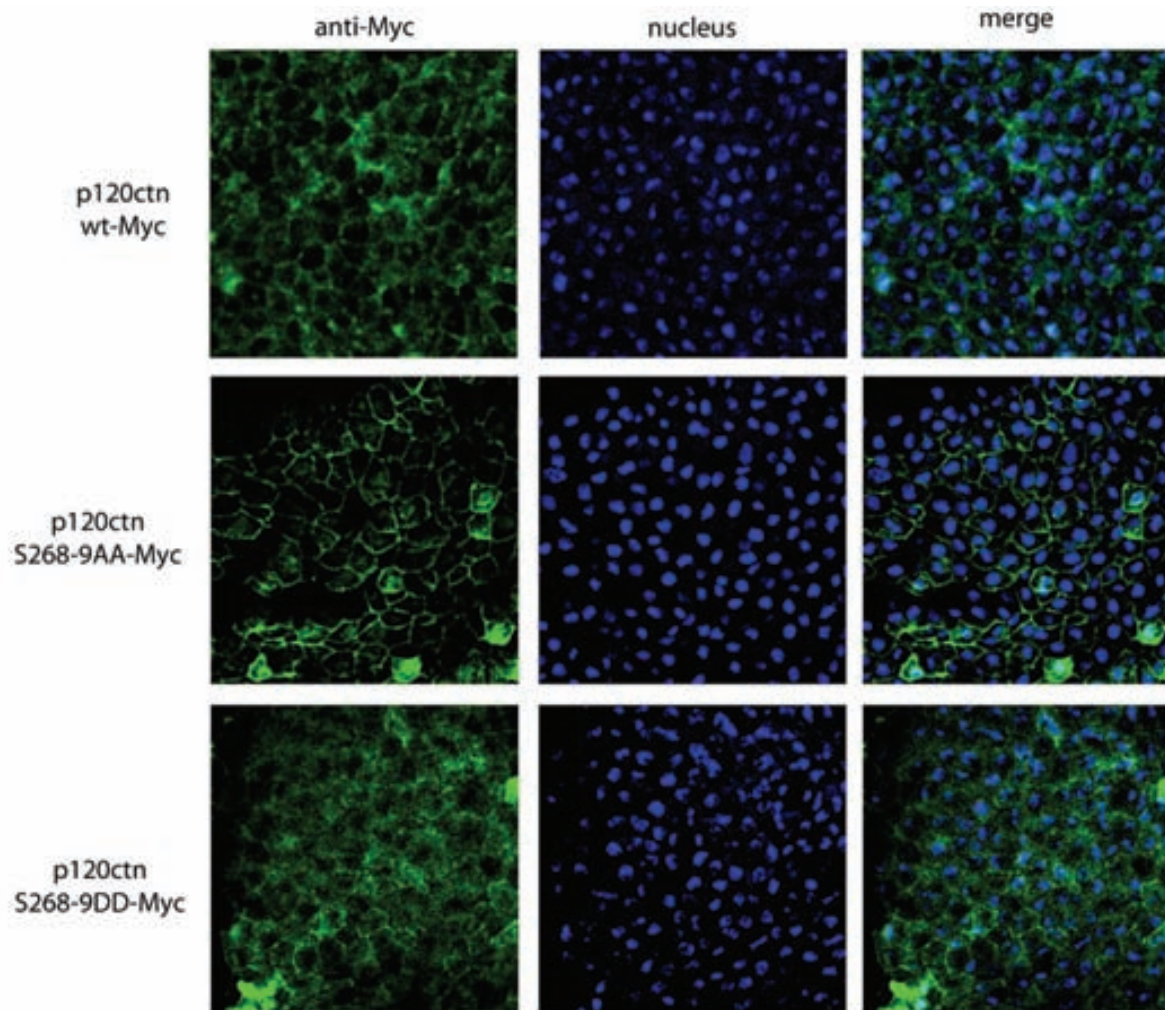


Figure 57. Different subcellular localization of p120ctn S268-269DD/AA constructs in *X.laevis* embryos. Animal Caps Immunofluorescence after 1 ng injections of mp120ctn S268-269DD/AA constructs at 1 cell stage embryos. Animal caps were obtained from embryos at gastrula stage.

As well as happened with p120ctn phosphorylated by Src and Fyn (see Figure 18), we knew from previous results from our lab that the two tyrosine point mutants (mp120ctn Y112E and Y217E) had a higher affinity for E-cadherin (58). For this reason, and since these two mutants were not able to rescue the gastrulation defects, we verified *in vitro* that CK1 phosphorylation had a dominant effect on the p120ctn tyrosine point mutants. That is to prove that the stronger affinity for E-cadherin was not producing this failure in gastrulation rescues and that the Wnt-induced CK1 phosphorylation would promote the release of p120ctn from E-cadherin independently of the tyrosine phosphorylation status of p120ctn. In this case we may attribute the gastrulation failure of the mutants Y112E and Y217E to not being able to bind Vav2 or Rac1, respectively (and not for showing a higher affinity for E-cadherin).

Results

As shown in Figure 58, CK1 phosphorylation had a dominant effect on the release of Y112E and Y217E mutants from E-cadherin (lanes 5, 7 and 9), although they had a higher affinity for E-cadherin compared to wild-type p120ctn (compare lane 4 with 6 and 8). Thus, the defects in rescuing the gastrulation phenotype of Y112E and Y217E mutants can be attributed to not being able to bind Rac1 and/or Vav2, as these mutants would be acting normally regarding its release from E-cadherin after Wnt stimulation.

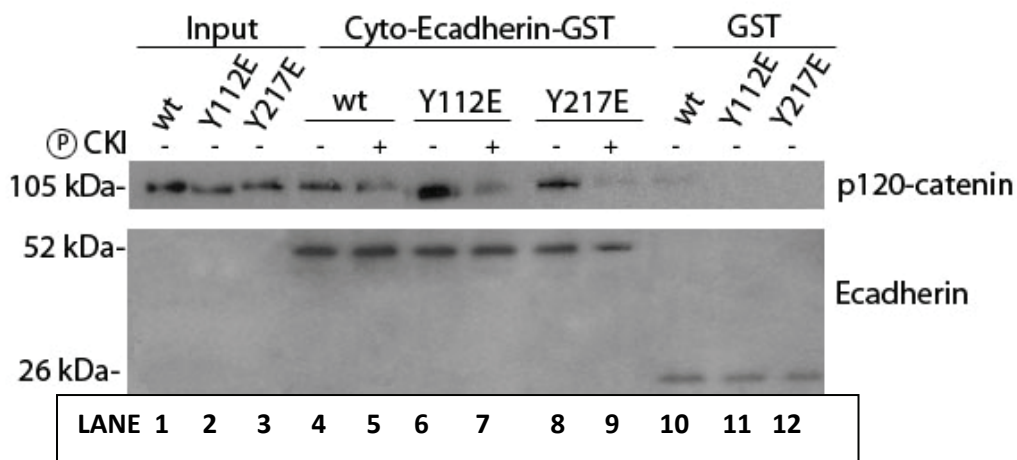


Figure 58. CK1 phosphorylation of tyrosine mutants has a dominant effect on its interaction with E-cadherin. 1.5 pmols of cyto-Ecadherin-GST were incubated with 1.5 pmols of p120ctn WT, Y112E and Y217E *in vitro* phosphorylated by CK1. Protein complexes were affinity purified and analyzed by Western Blot. 0.1 pmols of p120ctn ctn WT, Y112E and Y217E were included as internal reference (Input).

DISCUSSION

IV. DISCUSSION

p120ctn directly binds Rac1 and Vav2 and the interaction is regulated by phosphorylations in the N-terminal domain of p120ctn

p120ctn is a pleiotropic protein that was initially isolated in a screen for Src tyrosine kinase substrates (34). From then and on, phosphorylations in this protein have been analyzed in depth. It is often assumed that tyrosine phosphorylations of armadillo proteins, like β -catenin and p120ctn, represent the main mechanism of adherens junctions regulation, and this mechanism is used to the cell to rapidly respond to environmental cues and to contribute to the plasticity of adhesive contacts. For instance, tyrosine phosphorylations of catenins have been reported in nascent adhesive contacts (47, 48), whereas relatively low levels of tyrosine phosphorylated junctional proteins is been observed in confluent cells (48). These results suggest that tyrosine phosphorylation of adherens junction's components is a short-lived and tightly controlled regulatory event.

Src family kinases (SFKs) co-localize with cadherins at cell-cell contacts and at least one member, Fyn, is found physically associated to p120ctn (43). A scaffold role for p120ctn to regulate tyrosine kinase activity at cadherin complexes is suggested by its ability to recruit another Src family member, activated Yes, which in turn at least *in vitro* activates Fyn and Fer kinases. As reported, Fer or Fyn *in vitro* phosphorylation of p120ctn increase its binding to E-cadherin (43, 57)

Conversely, disassembly of the adherens junctions complexes is associated with the disruption of the interaction of β -catenin with E-cadherin (and with α -catenin) and is accompanied by increased tyrosine phosphorylation of β -catenin. Tyrosine 654 has been described to be important for the modulation of E-cadherin/ β -catenin interaction *in vitro* and *in vivo* (57). Tyrosine phosphorylation is requires for β -catenin to be transported to the nucleus and act as a transcriptional coactivator (164-166).

Discussion

Another example of p120ctn regulation by phosphorylations is the regulation of p120ctn and RhoA interaction by tyrosine kinases. As mentioned, it has been described by other members of our lab the direct interaction between p120ctn and RhoA and its regulation by Src and Fyn tyrosine phosphorylations (58). Specifically, Src phosphorylation of p120ctn in tyrosines 217 and 228 increases p120ctn/RhoA binding whereas Fyn phosphorylation in tyrosine 112 disrupts it.

Our results show a similar mechanism for p120ctn in regulating Rac1 binding/activity. We show that upon Src and Fyn phosphorylation of p120ctn there is a down-regulation of the p120ctn association with Rac1 and Vav2. Moreover, we have indentified two specific residues in p120ctn involved in this regulation: tyrosine 112 phosphorylated by Fyn totally disrupts the interaction between p120ctn and Vav2 while slightly decreases p120ctn/Rac1 binding. On the other hand, tyrosine 217 phosphorylated by Src disrupts p120ctn interaction with Rac1 while does not affect p120ctn/Vav2 binding at all. The different ability of these two point phosphorylated-mutants (Y112E and Y217E) to bind Rac1 or Vav2 has been relevant in this work for the study of the requirement of both interactors to fulfill p120ctn capability to activate Rac1.

In addition, we present innovative data showing that p120ctn directly interacts with Rac1 and Vav2 and we map the region of interaction of both co-factors within p120ctn. Not surprisingly, the relevant fragments for the interactions were shown to be located within the N-terminal regulatory domain of p120ctn, which is a highly tyrosine phosphorylated domain and where another GTPase like RhoA has been described to interact (58). More in detail, Vav2 has been mapped to interact within the amino acids 234-350 of p120ctn (Figures 14 and 15). Conversely, all p120ctn domains have been shown to be necessary to fulfill its interaction with Rac1, except for the very N-terminal part of p120ctn (amino acids 1-97) that does not interact with Rac1 (Figures 2 and 3). However, p120ctn isoform 1 (amino acids 1-911) showed a higher affinity for Rac1 than isoform 3 (amino acids 102-911). These results suggest that a specific folding of p120ctn tails might be necessary to fully interact with Rac1. Similarly, RhoA has been described to interact with the N-terminal domain of p120ctn (amino acids 102-234) but the Armadillo domain of p120ctn is also needed to fulfill its action as a GDI inhibiting RhoA intrinsic GDP/GTP exchange activity (58, 118). In the same way, other

armadillo-family proteins like β -catenin have been described to fold in a way that N- and C-terminal tails interact with the central armadillo domain and they regulate the different co-factors association with β -catenin (158, 167).

Unlike to what has been described for p120ctn and RhoA (58, 111), we did not see any preference of binding of p120ctn for Rac1 when bound to GDP (nor bound to GTP) (Figure 4) and p120ctn has been shown to be unable to inhibit GDP/GTP exchange activity of Rac1 (111).

Apart from showing that tyrosine phosphorylation of p120ctn decreases its binding to Vav2 and Rac1 (Figure 18) we have also analyzed the effects of p120ctn phosphorylated by a serine/threonine kinase, CK1, which has recently seen to highly phosphorylate the N-terminal domain of p120ctn (151). Contrary to what we observed with Src and Fyn kinases, CK1 phosphorylation enhanced p120ctn interaction with Rac1 and Vav2 (Figures 18, 23A and 24). This regulation by phosphorylations of p120ctn can occur in a more physiological context when we stimulate cells with Wnt3a factor (as I will discuss further on).

It is also noteworthy, that phosphorylations performed by Src/Fyn and CK1 inversely affect the p120ctn/E-cadherin binding. Src and Fyn phosphorylations increase p120ctn/E-cadherin binding (57, 58) whereas CK1 phosphorylations disrupt this binding (151). These results give us insights about the context where p120ctn/Vav2/Rac1 complex up-regulation is taking place. We have seen that p120ctn is able to interact with E-cadherin and Vav2/Rac1 simultaneously (Figures 5 and 16). However, it is likely that p120ctn/Vav2/Rac1 association is favored at the same time that p120ctn/E-cadherin binding is being lost (tyrosine-unphosphorylated serine-phosphorylated p120ctn). On the other hand, it is likely that p120ctn association with Rac1 and Vav2 is decreased when p120ctn/E-cadherin binding is enhanced (tyrosine-phosphorylated serine-unphosphorylated p120ctn). As a result, these interactions may occur when p120ctn is released from E-cadherin in the cell and correlates with the well-established idea of being E-cadherin-unbound p120ctn the protein involved in the regulation of Rho GTPases (113, 114, 121).

E-cadherin-unbound p120ctn activates Rac1

There are some evidences indicating that p120ctn over-expression leads to Rac1 activation (113, 114) and this regulation could be mediated by association of p120ctn with Vav2 (113). In this work we show that p120ctn directly binds to Vav2 between amino acids 234-350 of p120ctn (Figures 14 and 15). Moreover, we show that the association between Rac1 and Vav2 is decreased when p120ctn is depleted from the cells (Figure 13), suggesting that p120ctn could be acting as a scaffold providing the platform necessary for Rac1 and Vav2 binding.

Some evidences reinforce the idea that Rac1 and Vav2 binding to p120ctn is necessary for Rac1 activation. Point mutants unable to bind Rac1 or Vav2 (Y217E and Y112E, respectively) failed to activate Rac1 whereas wild-type p120ctn efficiently activated it. In the same way, p120ctn isoform 1 was significantly more active than isoform 3 was; we attribute this result to the fact that p120ctn isoform 1 showed higher affinity for Vav2 and Rac1 than isoform 3 (Figures 2, 14 and 25). Thus, p120ctn ability to activate Rac1 is enhanced as p120ctn capability to bind both co-factors increases and it is restricted when p120ctn is not able to interact with one of the 2 co-factors.

p120ctn isoform 1 is predominantly expressed in motile cells such as fibroblasts and in epithelial tumors, while isoform 3 is the predominant isoform in sessile epithelial cells. p120ctn expression has become an independent prognostic factor of breast cancer survival, and isoform 1 expression predicts metastatic disease (168). In addition, isoform 1 expression and metastasis are also significantly correlated in both lung (169) and renal (118) carcinoma. These results also agree with molecular data proposing that only p120ctn isoform 1, and not shorter isoforms, inhibits RhoA activity and promotes invasiveness. All these data would perfectly accommodate with our data suggesting that isoform 1 more efficiently binds Rac1 (and Vav2, Figures 2 and 14) and activates Rac1, therefore expecting a more motile and invasive phenotype in cells that predominantly express isoform 1.

Another important aspect discussed in this work is the localization of the p120ctn pool involved in activating Rac1 through Vav2. As mentioned before, binding of p120ctn to E-cadherin and to Rac1/Vav2 can occur simultaneously. The same has been shown to

occur with RhoA (58), although the inhibition of RhoA activity by p120ctn is blocked by E-cadherin over-expression suggesting that a conformational change in p120ctn when associated with E-cadherin allows RhoA activation (111, 113, 114). This work presents data supporting the idea that the p120ctn pool that activates Rac1 through Vav2 is an E-cadherin-unbound pool of p120ctn. First, there are evidences that suggest that Vav2 localization is restricted to the soluble, cytoplasmic fraction of the cell and that endogenous E-cadherin is not detectable in Vav2 immunoprecipitates (113). We have addressed this question using a p120ctn mutant that mimetizes a CK1 phosphorylation in a residue which has been shown to be responsible for p120ctn release from E-cadherin after Wnt stimulation (mutant p120ctn S268-269DD). This mutant shows low affinity for E-cadherin, while the corresponding control (mutant p120ctn S268-269AA) displays stronger association with E-cadherin. These 2 mutants co-expressed in cells with an E-cadherin construct display different abilities to activate Rac1 (Figure 28). We observed higher Rac1 stimulation in cells overexpressing the S268-269DD mutant (low affinity for E-cadherin). In contrast, the S268-269AA mutant slightly activated Rac1 and this effect was nearly insignificant when E-cadherin was co-expressed (Figure 28). This is in accordance with other works showing that effects of ectopically-expressed p120ctn are blocked by expression of various cadherin constructs that sequester the excess of p120ctn by providing new juxtamembrane available domains to interact with and therefore redirecting p120ctn to the membrane (111, 113, 114).

However, it is likely that only these CK1-phosphorylated specific residues are not sufficient to favor the p120ctn/Rac1/Vav2 complex, as the phosphorylated mutant does not show stronger interaction with Rac1 and Vav2 when we look at direct interaction with recombinant proteins (Figures 19, 20 and 21). This made us reinforce the idea that other p120ctn modifications, like tyrosine modifications mentioned before, may be needed *in vivo* to favor this complex formation.

So far we propose that in normal situations, the E-cadherin-unbound pool of p120ctn is small (32), but the level of this pool will be affected by events that influence the binding of p120ctn to cadherins (such as Wnt stimuli) and by the amount of cadherins that are present. This p120ctn change in localization (due to serine 268-269 modifications) may go accompanied by tyrosine modifications, among them

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dephosphorylation of tyrosines 112 and 217, and altogether these modifications may promote the increase in association of p120ctn with Rac1 and Vav2 and the subsequent Rac1 activation.

Rac1 activation through p120ctn and Vav2 is stimulated upon Wnt signaling and it is necessary for the translocation of β -catenin to the nucleus

In addition, we demonstrate in this work that a physiological signal that promotes these mentioned molecular events is the cell stimulation by canonical Wnt3a factor. Moreover, we propose that Wnt-mediated Rac1 activation through p120ctn and Vav2 is required for the cell to culminate the very last events of Wnt signaling pathway, which are the β -catenin translocation to the nucleus and the activation of Wnt-target genes.

Rac1 has also been implicated in the nuclear transport of other proteins. It was shown to regulate nuclear accumulation of an armadillo protein SmgGDS through a mechanism dependent upon both the C-terminal polybasic region (PBR) and the activation of Rac1 (170). More recently, Rac1 was reported to control nuclear translocation of STAT transcription factors through interactions with a GTPase-activating protein MgcRacGAP and tyrosine-phosphorylated STAT3 or STAT5A (171). The present study has identified a p120ctn-dependent molecular mechanism by which Rac1 activation controls β -catenin nuclear localization through JNK2-dependent phosphorylation (that I will explain further on).

Recent results from our lab suggest that E-cadherin-dependent adherens junctional complexes are closely associated with Wnt receptors. Besides β -catenin, p120ctn has emerged to be also indispensable for canonical Wnt signaling (151-153). Through its interaction with E-cadherin (or N-cadherin), p120ctn associates with the LRP5/6 co-receptor and enables the binding of this complex to CK1 ϵ , a protein kinase constitutively associated with p120ctn. This catenin is required for CK1 ϵ activation and for the early responses to Wnt3a, such as Dvl-2 or LRP5/6 phosphorylation (153). A current model depicting our vision of this process is presented in Figure 59. Panel **A**

shows the protein complexes in non-stimulated cells whereas panel **B** represents the formation of the Wnt receptor complex upon addition of Wnt3a ligand.

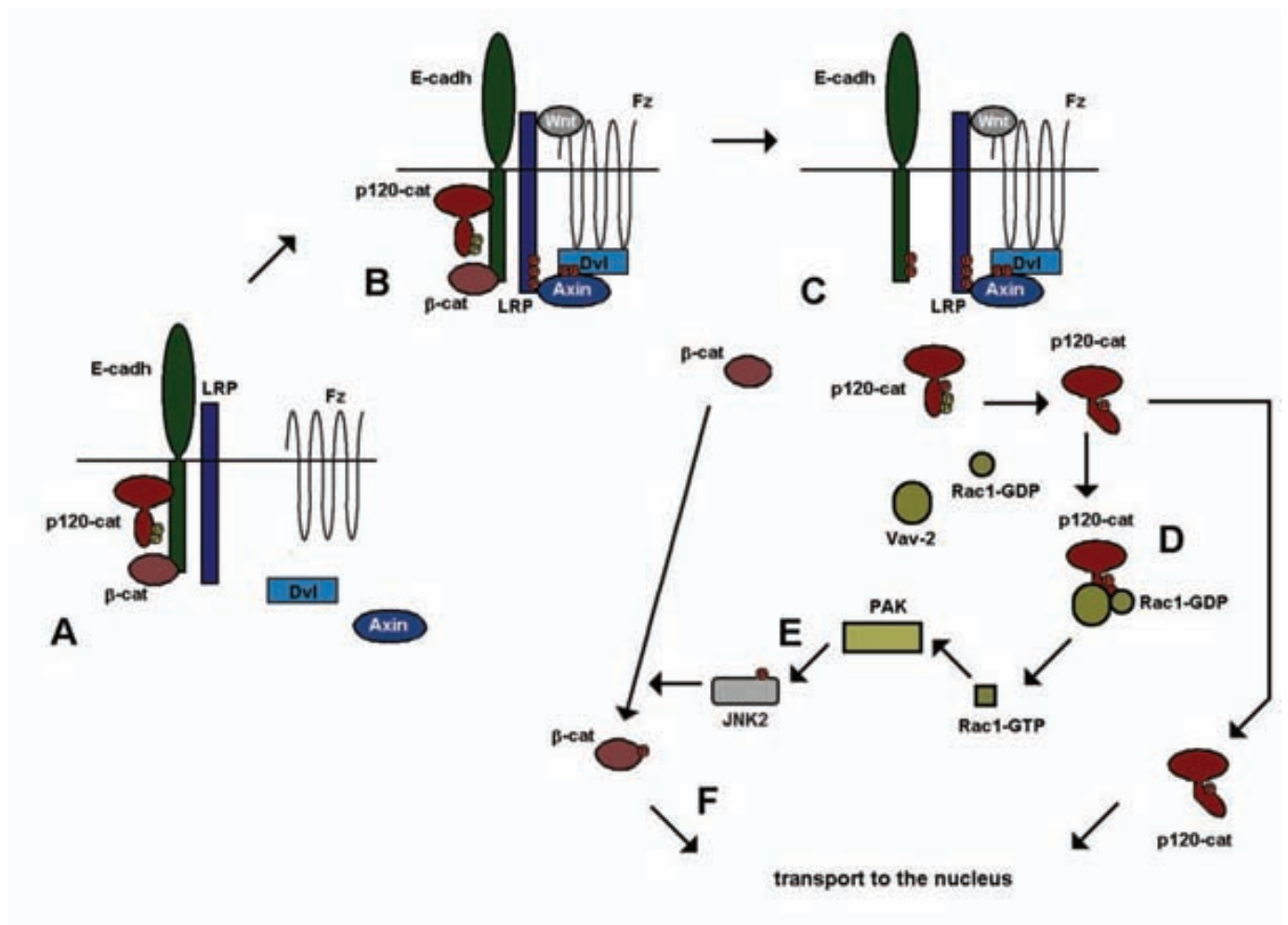


Figure 59. Model for Wnt-induced Rac1 activation through p120ctn and Vav2. In unstimulated cells (A), p120ctn is bound to E-cadherin through the Armadillo domain. Upon binding of Wnt factor (B), a receptor complex is formed including p120ctn, E-cadherin, LRP5/6, Axin, Dvl and Frizzled (Fz). At longer times, p120ctn is released from E-cadherin upon its phosphorylation in Ser268 and Ser269 by CK1 α (red spheres) (C). This phosphorylation decreases Src/Fyn interaction with p120ctn and consequently tyrosine phosphorylation (yellow spheres). Ser-phosphorylated Tyr-unphosphorylated p120ctn presents a higher affinity for Vav2 and Rac1 (D). Association with p120ctn enhances Vav2 activity towards Rac1 and activates this GTPase. GTP-bound Rac1 stimulates PAK kinase that phosphorylates and activates JNK2 (E). This protein kinase phosphorylates β -catenin in two serines, necessary for the translocation of this protein to the nucleus (F).

Approximately 30 minutes after Wnt addition, p120ctn is released from the E-cadherin/receptor complex upon its phosphorylation at Ser268 and 269 by CK1 α (C). Consequently, the levels of cytosolic p120ctn increase (151). The modification of p120ctn by CK1 is probably the cause of the p120ctn tyrosine dephosphorylation that we detect after Wnt addition (Figure 39), since it decreases the interaction with

Discussion

tyrosine kinases active on this catenin, such as Fyn, that binds to the N-terminus of p120ctn (Figure 40). Moreover, it also removes p120ctn from the vicinity of other tyrosine kinases active on this protein, such as Src, which remain associated to the membrane. These tyrosine modifications, in addition to the serines modifications, are likely to be the signal cues necessary to promote the above mentioned complex formation between p120ctn/Vav2/Rac1.

Ser-phosphorylated, Tyr-unphosphorylated p120ctn interacts with higher affinity with Vav2 and Rac1 (Figure 39). As mentioned before, these two proteins associate preferentially to the N-terminal domain, although the armadillo repeats are also relevant for full Rac1 binding to p120ctn (**D**). Association with p120ctn enhances Vav2 activity towards Rac1. There are several possibilities to explain this fact. First, it is possible that the p120ctn/Vav2 association can potentiate the tyrosine phosphorylation of Vav2 by a tyrosine kinase, still associated to p120ctn. However, we think that this hypothesis is unlikely since we have not detected an increase in Vav2 tyrosine phosphorylation upon Wnt addition (not shown). Alternatively, it is also possible that the interaction could free the Vav2 catalytic Dbl homology domain from the coordinated auto-inhibition by the Acidic and Calponin homology domains (109). Thus, p120ctn interaction could be mimicking the effects of Vav2 phosphorylation in the Acidic domain. Moreover, it is also plausible that p120ctn binding to E-cadherin might prevent its functional interaction to Vav2 and the release from E-cadherin would allow p120ctn to interact with and activate Vav2.

We did not observe any change in RhoA activity upon Wnt3a stimulation. Although these data is in agreement with data presented from other authors (140), we expected to see a decrease in active RhoA, as p120ctn is released from adherens junctions and cytosolic p120ctn has been shown to act as a GDI of RhoA (58, 111). However, and further investigation will be needed, this molecular mechanism of RhoA inactivation through p120ctn may not be valid in a canonical Wnt signaling pathway context. One molecular explanation for this could be in relation with the p120ctn recruitment of Vav2 upon Wnt stimulation. As mentioned before, Vav2 is a GEF not only for Rac1 but also for Cdc42 and RhoA (172), therefore it is plausible that Vav2 could also affect RhoA activity producing an equilibrium with the GDI activity supplied from p120ctn

itself. As a result, it seems that p120ctn-regulated RhoA activity modulation may involve different molecular mechanisms. Another explanation for this could be that upon Wnt stimulation p120ctn is being modified in some specific residues still unidentified which would prevent p120ctn/RhoA association, therefore preventing p120 ability to act as a GDI for RhoA. Further investigation will be needed in this regard.

Furthermore, we propose that Wnt-mediated active Rac1 stimulates PAK protein kinase that in turn phosphorylates and activates JNK2 (E). This protein kinase modifies two residues in β -catenin, Ser191 and 605, necessary for the translocation of this protein to the nucleus (F) (140). This β -catenin might proceed from the pool associated to E-cadherin and be released upon phosphorylation of this protein by CK1 α (173) or from phosphorylation of β -catenin by tyrosine kinases (43). Alternatively, or additionally, it might come from other signaling pools of β -catenin, normally degraded through the action of the β -catenin destruction complex either in the cytosol or in the membrane (174). In any case, Wnt blocks β -catenin degradation by these complexes and stimulates further phosphorylation increasing Rac1-dependent JNK2 activity. As presented in this work by using protein depletion approaches, both p120ctn and Vav2 seem to be required for the cell to stimulate Rac1-dependent JNK2 phosphorylation upon Wnt stimulation.

Besides this role in Rac1 activation, E-cadherin-unbound p120ctn plays an additional function in Wnt signaling since it translocates to the nucleus and participates in releasing the inhibition caused by Kaiso on the expression of several target genes (152, 175-177). It has been recently proposed that inhibition of the Wnt pathway by Kaiso is dependent on its association with Tcf-3/4, which precludes the binding of this factor to the DNA (178). Moreover, Kaiso is also capable to repress the expression of other genes not modulated by β -catenin, through direct interaction with methylated CpG sequences in their promoters (179). Recent results from our lab describe a novel interaction involving β -catenin and Kaiso and describe that CK1-phosphorylation of p120ctn after Wnt stimulation enhances its binding to Kaiso precluding the association of this factor to Tcf-4 and β -catenin and therefore the inhibition of Tcf-4 transcriptional

Discussion

activity. However, binding to p120ctn does not alter Kaiso interaction with methylated sequences (152).

Finally, this model reinforces the idea that proteins associated with adherens junctions, in addition to but distinct from β -catenin, contribute to Wnt signaling; particularly p120ctn plays a multiple role in activating this signaling pathway.

p120ctn binding to Rac1 and Vav2 is required for early development in *Xenopus laevis*

Finally, we have also demonstrated the *in vivo* relevance of the molecular mechanism that we propose. We used an established morpholino (MO) approach to deplete endogenous p120ctn in *Xenopus laevis* (xp120ctn). Xp120ctn depletion produces gastrulation failure in the embryos and the effects can be rescued upon expression of exogenous xp120ctn or of exogenous constitutively active Rac1 (116). We worked on these rescue assays using different p120ctn constructs and we demonstrated that p120ctn binding to both Rac1 and Vav2 is required for the embryo to fulfill gastrulation events.

Regarding the mutants that differently bind E-cadherin (S268-269AA/DD), we show that they were not capable to fully rescue the defects in gastrulation. We attribute this to the fact that both p120ctn localizations (bound or unbound to E-cadherin) may be needed for the embryo to complete gastrulation events in development. A prerequisite for gastrulation in all vertebrates is the specification of dorsal mesoderm capable to undergo morphogenetic movements and therefore adherens junctions may be needed to be dynamic and assembly or disassembly during the process. However, we got a greater rescue with the p120ctn S268-269DD construct (E-cadherin unbound p120ctn) meaning that p120ctn involvement in Rac1 activation may probably be of substantial importance. This would perfectly fit with other data showing that only a constitutively active Rac1 mutant is able to rescue the xp120ctn depletion defects (116). In addition, we should also consider that predominant early embryonic cadherin in *X.laevis* is the C-cadherin and that other p120ctn subfamily members like ARVCF and δ -catenin have shared roles. Of the Armadillo proteins, ARVCF is the most closely related to p120ctn, sharing significant homology within the central Armadillo domain

(55% identity), and even within the generally more divergent N and C-terminal domains (respectively, 42% and 31% identity). Moreover, we also show in this work that ARVCF directly binds to Rac1 and Vav2 and therefore this protein could also be taking part in Wnt-mediated Rac1 activation.

CONCLUSIONS

V. CONCLUSIONS

1. Rac1 and Vav2 directly bind p120ctn and their interaction is required for p120ctn-mediated Rac1 activation.
2. p120ctn interaction with Rac1 and Vav2 is regulated by phosphorylations in the N-terminal regulatory domain of p120ctn. Specifically, CK1 phosphorylation increases the interaction with Vav2 and Rac1 whereas Src and Fyn phosphorylations disrupt it.
3. E-cadherin unbound p120ctn pool is the one involved in Rac1 activation through Vav2.
4. Serine-phosphorylated p120ctn is released from E-cadherin after Wnt stimulation and it is then unphosphorylated in tyrosines. Ser-phosphorylated tyr-unphosphorylated p120ctn has higher affinity for Rac1 and Vav2 and activates Rac1.
5. Activation of Rac1 mediated by p120ctn and Vav2 after Wnt stimulation is necessary for JNK2 phosphorylation and for subsequent β -catenin nuclear translocation.
6. p120ctn and Vav2-mediated activation of Rac1 is required to accomplish gastrulation events in *Xenopus* early development.

EXPERIMENTAL

PROCEDURES

VI. EXPERIMENTAL PROCEDURES

1. CELL CULTURE

1.1 EUKARYOTIC CELL LINES

Cell lines were obtained from our Institute Cell Bank. Control L fibroblasts or fibroblasts stably transfected with a plasmid encoding Wnt3a (L-Wnt3a) were obtained from the *American Type Cell Collection, ATCC*.

Cell lines were grown in *Dulbecco's modified Eagle's medium* (DMEM, Invitrogene). Medium was supplied with 4.5 g/l Glucose, Sodium Pyruvate (Life Technologies), 2 mM Glutamine, 56 U/ml Penicillin, 56 µg/l Streptomycin and 10% Fetal Bovine Serum (FBS; GIBCO). Cell lines were grown at 37°C in a humid atmosphere containing 5% CO₂ and 95% air.

When indicated, cells were treated with conditioned medium from L or L-Wnt3A cells during different periods of time.

Cell Line	Origin	Characteristics
SW-480	Human Colon Adenocarcinoma	Epithelial-like in morphology Mutated APC High β-cat/TCF transcriptional activity Oncogenic K-ras
HEK-293T	Human Embryonic Kidney	Fibroblast morphology Expression of N-cadherin
L	Mouse fibroblasts	Subcutaneous connective tissue
L-Wnt3A	Mouse fibroblasts	L-cells were transfected with a Wnt3a expression vector and stable clones were selected in medium containing G418 (0.4 mg/ml)

Table 1: Cell lines used in the project.

Experimental procedures

Cell lines were grown in plastic treated flasks and weekly sub-cultured into new flasks. Cells in monolayer were treated with 0.5% Trypsin (Life Technologies) and seeded either to plates for performing the experiments or to flasks for maintenance.

Different cell numbers were seeded to plates depending on the experiment to perform. Cell count was determined by adding *Trypan Blue* (Sigma) to one cell sample and using an optical microscopy. Cell integrity and cellular survival was controlled and number of cells per ml was determined.

Stocks from different cell lines are kept in liquid nitrogen adding 10% DMSO as a cryoprotector.

1.2. BACTERIAL CULTURES

Two bacterial strains were used depending on the application. *Escherichia coli* DH5 α strain was used for purifying DNA and *Escherichia coli* BL21 DE3 RIL for efficiently purify recombinant proteins. BL21 cells must be maintained in Chloramphenicol in order to maintain the plasmids.

Bacterial cells were grown in LB (*Luria Broth*) rich media either in liquid or solid states (LB and LB Agar, respectively). Media was sterilized in an autoclave for 20 minutes at 120°C. Antibiotics were added to media for the correct selection of transformed bacteria (Table 2).

ANTIBIOTIC	LB
Ampicillin	150 mg/L
Kanamycin	30 mg/L
Chloramphenicol	50 mg/L

Table 2: Different antibiotics added to bacterial cultures.

Cultures were grown at 37°C, shaking at 110-220 rpm in the case of liquid media. Colonies were kept at 4°C during 4-6 weeks for solid media cultures and frozen at -80°C for years in the case of liquid media, 15% glycerol final concentration.

2. OBTAINING AND MANIPULATING DNA

2.1 TRANSFORMATION AND DNA EXTRACTION

“Ultra-Competent” *E.Coli* (Inve Methods) strain was used for transformation procedures. An aliquot of competent cells was thawed on ice and a maximum of 10 µl of DNA (5-50 ng) to 100 µl of competent cells were added. The mixture was incubated for 60 seconds on ice and cells were then heat-shocked for 90 seconds at 42°C. Cells were allowed to cool down on ice for 5 minutes, 900 µl of LB were added to the culture and incubated for 1h at 37°C shaking. Cells were then plated on suitable agar plates and incubated overnight at 37°C.

Each colony obtained belonged to one single cell that had incorporated the vector, thus, gaining the antibiotic resistance. Miniprep for colonies was carried out (*WizardR Plus SV Minipreps* (Promega), followed by digestion of purified plasmid DNA with specific restriction enzymes. An agarose gel was then runned to check the insert length.

Plasmidic DNA can be purified from a dense bacterial culture. The commercial kits used for the project were *WizardR Plus SV Minipreps* (Promega) or *Maxipreps* (Quiagene).

2.2 VECTORS AND DNA CONSTRUCTIONS USED

Vector	Description
pGEX-6P (Amersham-Pharmacia)	<ul style="list-style-type: none">- For prokaryotic expression- It is used for expression of recombinant proteins in 3 reading frames (6P-1, 6P-2 and 6P-3)- It allows expressing the product as a GST-fusion protein (<i>Glutathione-S-Transferase</i>)- It contains the promoter Tac that allows high levels of expression of the product and it is chemically inducible by IPTG (<i>isopropyl β-thiogalactoside</i>)- It confers Ampicillin resistance- It contains a <i>PreScission Protease</i> (PSP) recognition site (Amersham-

Experimental procedures

	Pharmacia) to cut between the GST and the protein once the fusion protein has been purified
pGFP.N1 (Stratagene)	<ul style="list-style-type: none"> - For eukaryotic expression - It allows to produce proteins fused to hrGFP (Green Fluorescent Protein) - It confers Ampicillin resistance - Constitutive expression - Protein has been humanized to minimize expression/degradation problems
pCDNA3 (Invitrogene)	<ul style="list-style-type: none"> - For eukaryotic expression - It allows expression of proteins with or without tag (Myc/His/HA) depending on the variety used - Constitutive expression - It confers Ampicillin and Neomycin resistance
pBEG-2T	<ul style="list-style-type: none"> - Kindly provided by Dr. Lizcano (UAB, Barcelona) - It allows producing proteins fused to GST and express them in eukaryotic cell lines, to detect or purify them - Constitutive expression - Ampicillin resistance
pSUPER (Oligoengine)	<ul style="list-style-type: none"> - It allows the expression of shRNAs in eukaryotic cell lines for decreasing the levels of a target endogenous protein - It confers Puromycin resistance for selecting cells that express the construct

Table 3: Mostly used vectors in the project

Construction	Obtaining
pGEX	
pGEX-6P3-Rac1	Kindly provided by Dr. Servitja (IDIBAPS-Hospital Clínic, Barcelona, Spain). Obtained from pGEX-4T3-Rac1 by standard cloning procedures using the flanking EcoRI and BamHI sites present in the polylinker of pGEX-6P3 plasmid.
pGEX-6P2-Vav2	Kindly provided by Dr. X. Bustelo (CIC, Salamanca, Spain). Generated from pECFL-HA-Vav2 by digestion with EcoRI and cloned into the digested plasmid with the same enzyme.
pGEX-6P3-p120ctn (1-911)	Generated from pcDNA3 digesting with EcoRI/NotI and cloned into a pGEX-6P3 digested with same enzymes.
pGEX-6P3-p120ctn (102-911)	Obtained by PCR using oligonucleotides including restriction sites for EcoRI and SacII at their 5' ends. The PCR product was digested with SacII, and overhang ends were filled with Klenow polymerase. The product was then digested with EcoRI and ligated in pGEX-6P1 that was digested with EcoRI and SmaI.

Experimental procedures

pGEX-6P3-p120ctn (350-911)	Generated from pcDNA3-p120ctn (1-911) by PCR introducing EcoRI/NotI sites and cloned into pGEX-6P1 digested with same enzymes.
pGEX-6P3-p120ctn (1-390)	Generated from pGEX-p120ctn (1-911) by using oligonucleotides corresponding to the CDS nucleotide sequences 1 to 18 and 1156 to 1170 that contained EcoRI and XhoI sites. Amplification product was cloned into pGEX-6P3 digested with EcoRI and XhoI enzymes.
pGEX-6P3-p120ctn (234-911)	Generated from digesting pcDNA-p120ctn (1-911) with SmaI/NotI and cloned into pGEX-6P1 digested with same enzymes.
pGEX-6P3-p120ctn (102-350)	Generated from pGEX-p120ctn (1-911) by using specific primers. Amplification product was cloned into pGEX-6P3.
pGEX-6P3-p120ctn (347-911)	Generated from pGEX-p120ctn (1-911) by using specific primers. Amplification product was cloned into pGEX-6P3.
pGEX-6P3-p120ctn (1-96)	pGEX-p120ctn (1-911) plasmid was digested with NotI and BsrGI, filled in, and religated.
pGEX-6P2-p120ctn (1-234)	A DNA fragment encoding amino acids 1 to 234 of p120ctn was amplified by PCR from pcDNA3.1-p120ctn-catenin by using appropriate primers containing either EcoRI or BamHI sites at their 5' ends. The 0.7-kbp amplification fragment was then digested with EcoRI and BamHI and cloned into the same sites of pGEX-6P2.
pGEX-6P3-p120ctn (Y112E)	Obtained using site-directed mutagenesis kit and using GFP-p120ctn-catenin wild type as a template. Sense primers used are detailed in Castaño et al, Mol Cell Biol 2007.
pGEX-6P3-p120ctn (Y217E)	Obtained using site-directed mutagenesis kit and using GFP-p120ctn-catenin wild type as a template. Sense primers used are detailed in Castaño et al, Mol Cell Biol 2007.
pGEX-6P3-p120ctn (268-9DD)	Obtained by site-directed mutagenesis using pGEX p120ctn (102-911) as template. Mutagenic sense primers used are described in Casagolda et al, J Cell Sci 2010.
pGEX-6P3-p120ctn (268-9AA)	Obtained by site-directed mutagenesis using pGEX p120ctn (102-911) as template. Mutagenic sense primers used are described in Casagolda et al, J Cell Sci 2010.
pGEX-6P3 cyto E-cadherin (aa 732-883)	Obtained from pBATEM2 (complete E-cadherin) by PCR adding new restriction sites BamHI/EcoRI and cloned into pGEX-6P3 digested with BamHI/EcoRI.
ARVCF-pGEX-6P1	Total RNA extracts from HCT116 cells were obtained and cDNA was generated using <i>RT-Roche Single Strand kit</i> . A DNA fragment that corresponds to human ARVCF was amplified by PCR using the following sense and antisense primers, respectively: 5'-CCAGAATTCATGGAGGACTGCAA-3' and 5'-CGAGTCGACCTAGACCCAGGAATC-3' that contained EcoRI and Sall sites at

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	their 5'-ends, respectively. The 3-kilobase amplification fragment was digested with EcoRI and Sall and cloned in the same sites of pGEX-6P1 plasmid. The absence of mutations in this fragment was verified by sequencing.
shortARVCF-pGEX-6P1	Prepared by PCR amplification from the pGEX-6P-ARVCF and digesting the insert and vector with BamHI/EcoRI. The 1.2-kilobase fragment obtained was religated into the vector.
phrGFP-N1	
phrGFP-p120ctn (8-911)	A DNA fragment encoding amino acid 8 to the C terminus of p120ctn-catenin was obtained from pGEX-p120ctn (1-911) by using oligonucleotides corresponding to nucleotide sequences 22 to 36 and 2717 to 2733 that contained EcoRI and SacII sites at the respective 5' ends. The 2.7-kbp amplification fragment was digested with EcoRI and SacII and cloned in the same sites of phrGFP-N1 (Stratagene).
phrGFP-p120ctn (102-911)	Extracted from pGEX-p120ctn (102-911) by PCR adding EcoRI/SacII sites and cloned into phrGFP digested with same enzymes
phrGFP-p120ctn (350-911)	Obtained from pGEX-p120ctn (350-911) with oligonucleotides that contained EcoRI and SacII sites and cloned into phrGFP-N1 digested with the same enzymes.
phrGFP-p120ctn (1-234)	Generated from pGEX-p120ctn (1-911) cutting with BamHI/EcoRI and cloned into phrGFP cut with same enzymes.
phrGFP-p120ctn (Y112E)	Obtained using site-directed mutagenesis kit and using GFP-p120ctn wild type as a template. Sense primers used are detailed in Castaño et al, Mol Cell Biol 2007.
phrGFP-p120ctn (Y217E)	Obtained using site-directed mutagenesis kit and using GFP-p120ctn wild type as a template. Sense primers used are detailed in Castaño et al, Mol Cell Biol 2007.
phrGFP-p120ctn (268-9DD)	PCR from pGEX-p120ctn (1-911) 268-9DD using primers for EcoRI and SacII sites. phrGFP digested with same enzymes and re-ligated.
phrGFP-p120ctn (268-9AA)	PCR from pGEX-p120ctn (1-911) 268-9AA using primers for EcoRI and SacII sites. phrGFP digested with same enzymes and re-ligated.
pEGFP	
pEGFP-C1-RacWT	Kindly provided by Dr F. Sánchez Madrid (Hospital de la Princesa, Madrid, Spain).
pEGFP-C1-RacV12	Kindly provided by Dr F. Sánchez Madrid (Hospital de la Princesa, Madrid, Spain).
pEGFP-C1-RacN17	Kindly provided by Dr F. Sánchez Madrid (Hospital de la Princesa, Madrid, Spain).

pCS2-MT	
pCS2-MT constructs	Generated from phrGFP-p120ctn constructs described above. The constructs were digested with EcoRI and SacII and cloned into the pCS2 plasmid digested with same enzymes.
pCMV-HA	
pCMV-Vav2-HA	5' EcoRI, 3' EcoRI Kindly provided by Dr. Xosé R Bustelo (Centro de Investigación del Cancer, Salamanca)
pECFP-N1	
pECFP-Ecadherin	Generated from pBATEM2 digesting with BglII and HindIII. pECFP cut with same enzymes and re-ligated.
pSUPER	
Scrb.pSUPER	Sigma
shRNA-p120ctn pSUPER	Kindly provided by Dr.Eduard Batlle (Institut de Recerca de Barcelona (IRB), Barcelona, Spain)
shRNA-Vav2pSUPER	Kindly provided by Dr.Eduard Batlle (Institut de Recerca de Barcelona (IRB), Barcelona, Spain)

Table 4: Constructs used in the project.

3. EXPRESSION AND PURIFICATION OF PROTEINS

3.1 RECOMBINANT PROTEINS EXPRESSION AND PURIFICATION IN PROKARYOTES

GST-fusion proteins were expressed in *E. coli* and purified by affinity chromatography in Glutathione-Sepharose beads.

An overnight pre-culture was first grown from the desired stock of bacterial cells. The following day, the culture was diluted 100 times (adding the corresponding bacterial resistance) and was allowed to rest for 3 hours. Protein synthesis was induced after cells reached the exponential phase ($Abs=0.6$ at $\lambda=600nm$) with IPTG (*Isopropyl β -D-1-thiogalactopyranoside*) at a final concentration of 0.1 mM. After 2 more hours of

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growth, cells were centrifuged for 10 minutes at 8.000 rpm (*Beckman Coulter Avanti J-25*).

Depending on the solubility of the protein, the experiment was proceeded in either of two different ways:

1. Soluble proteins: bacterial pellet was resuspended in PBS (*Phosphate-Buffered-Saline*), cells were 5 times sonicated in periods of 15 seconds each and 1/20 parts of 20% TX-100 (1% final concentration) was added. The culture was incubated shaking at 4°C during 30 minutes and lysed bacterial cells were centrifuged for 10 minutes at 10.000 rpm and 4°C (*Beckman Coulter Avanti J-25*).
2. Less soluble proteins: bacterial pellet was resuspended in 10 ml *STE Buffer* + 100 µg/ml STE of lysozyme (Sigma) and was allowed to rest for 15 minutes on ice. Sarcozyl to 1% final concentration was added and vortexed at full speed for 1 minute. The mixture was sonicated in periods of 30 seconds and Triton X-100 (Sigma) to 1% was added. Lysed cells were centrifuged at 10.000 rpm/4°C for 20 minutes (*Beckman Coulter Avanti J-25*), the supernatant saved and Triton to a 2% was added and vortexed at full speed for 2 minutes.

1/50 parts of effective Glutathion-Sepharose beads were added to the supernatant. The culture was kept for shaking for at least 1 hour with the beads and followed by 3 washes in PBS (beads were centrifuged for 1 minute at 1.000 rpm/4°C, *HETTICH Mikro 22R*). GST could be either cleaved of not from the fusion protein:

1. To obtain full GST-fusion protein: beads were incubated with the *Glutathione Elution Buffer* (GEB) for 1 hour at 4°C. GST detaches from the glutathione bound to the beads. To eliminate glutathione from media the supernatant was transferred to a dialysis bag and dialyzed for 20 hours at 4°C.
2. To remove GST from the protein: beads were incubated with the *PreScission Protease* (PSP, Amersham-Pharmacia) buffer. 1 unit of PSP was added for each 50 µg of fusion protein and incubated with beads shaking for 16h at 4°C.

The supernatant was finally collected, aliquoted and kept at -20°C.

Electrophoresis in polyacrylamide gels and *Comassie Blue* (Sigma) staining were carried out to quantify the protein obtained and the purity of it.

3.2 PROTEIN EXPRESSION IN EUKARYOTIC CELL LINES

TRANSITORY TRANSFECTIONS

Cells were seeded in the appropriate plate size, depending on the experiment to perform. It was preferable to let cells adhere and grow for at least 16-24h before transfecting them. Optimal conditions for a good transfection were a 60-80% of cell confluency. Two types of transfection methods were used depending on the cell type:

PEI (*Polyethylamine*, Polysciences, Inc.): used for easily transfectable cells (e.g. 293T cells). The amount of DNA (μg) to transfect depended on the size of the plate in which the cells had been seeded, and at some point, in the cell type that was going to be transfected. The following conditions were used:

Culture vessel	Surface area per well	DNA
10 cm	60 cm ²	10-12 μg
60 mm	20 cm ²	3-4 μg
6-well	10 cm ²	2 μg

Table 5: Amounts of DNA transfected for different plate sizes.

The DNA and 100 μl of DMEM (without antibiotics and serum) for each μg of DNA transfected were mixed in a tub. The mixture was vortexed and 10 μl of PEI per μg of DNA transfected were added. The mixture was vortexed again and allowed to rest for 20 minutes before adding it to the plated cells. In order to avoid the toxicity of the product in some cell lines, media was changed 6-12h after the transfection. The peak of highest expression of the protein is 48 hours post-transfection. However, in some cell lines (293T cells for instance) was just necessary to wait 24 hours to see good expression protein levels.

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Lipofectamine 2000 (Invitrogene™): following the manufacturer's instructions, 0.4 µg of DNA with 25 µl of Opti-MEM^R (Life Technologies) per each cm² of the plate were added in a tub. 1 µl of Lipofectamine 2000 and 25 µl of Opti-MEM^R (GIBCO) for each cm² of the plate were added in another tub and after 5 minutes of incubation, both solutions were mixed and allowed to rest for 20 minutes at room temperature (RT). DMEM from the plates was replaced by Opti-MEM^R + 10% FBS, meanwhile, and the mix was finally added to the plates. Media could be replaced again for DMEM + 10% FBS after 3-5 hours.

STABLE TRANSFECTIONS (LENTIVIRAL INFECTIONS)

293T cells were first seeded at 80-90% confluency in DMEM 10% FBS. Cells were allowed to attach to the plate during 8-10 hours (the more recently the cells are seeded the more efficient is the transfection). The transfection mix solution was then prepared containing, for a T75-cm² flask:

- 1.5 µg Envelope Vector (VSVG)
- 1.5 µg Rev-expressing Vector (RTR2)
- Packaging vector (PKGPIR)
- Transfer vector (vector of interest)

Cells were transfected with PEI (described above) and the plates were immediately moved to a virus incubator (as cells start to express virus after the transfection). The following day, the media was replaced with 7 ml of fresh media (in a T75 flask). We then seeded in appropriate plates (T75 for instance) the cell line that we wanted to infect (it was important to consider having a 60% confluency for the following day to infect, since viruses are more efficient infecting dividing cells). Media from the 293T cells was collected after 24 hours and filtered to remove the cells from the viruses (0.45 µm pore diameter). Polybrene (Sigma) was added to the infection media (1:1000 dilution) before infecting the cells to help the entrance of the virus to the cell. Media from the cells that we wanted to infect was replaced with 7 ml of infection media (with

virus). The same process was repeated the following day and the 293T cells were then discarded. The following day, media from infected cells was replaced with fresh media with 2 µg/ml puromycin to select infected cells. Once control cells died from the antibiotic effects, resistant cells were kept and a western blot was performed to assure that infected cells expressed the infected construct (depletion or over-expression of the protein of interest).

4. TECHNIQUES FOR THE STUDY OF PROTEINS

4.1 ELECTROPHORESIS AND WESTERN BLOT

In order to analyze the results from the experiments, tridimensional electrophoresis in SDS polyacrylamide gels were used. 1.5 mm thickness glasses with *Mini-Protean III/Tetra* system (Bio-Rad) with an 8-12% acrylamide gels were used. Sample Buffer is added to the samples and boiled for 5 minutes before loading the gel. Gels were runned at constant voltage in Tris-Glycin-SDS (TGS 10X, Bio-Rad). The protein marker used was *Precision Plus Protein Dual Color Strand* (Bio-Rad).

To directly visualize the samples in the gel, *Comassie Brilliant Blue* staining, Distainer Buffer and water to hydrate the gel were used. Proteins SDS-PAGE resolved were eletrotransferred to a PVDF membrane (*Immobilon-P Transfer Membrane* 0.45 µm; Millipore) in a humid transfer camera (Bio-Rad) at 100 mV for 60-70 minutes in Transfer Buffer. Membranes were blocked in TTBS 1% BSA for 1 hour at RT and incubated with the desired primary antibody. Depending on the quality of the antibody, different dilutions of the antibodies in TTBS 0.1% BSA were made and incubated with the membrane for 1-3 hours at RT. Using certain antibodies it was advisable to extend the primary incubation up to 4-6 hours or even incubate it overnight at 4°C. The antibodies and dilutions used during the project are the following:

Primary antibody	Organism	Epitope	Dilution	Company
E-cadherin	Mouse	735-883	1/2500	BD Transduction Labs
Lamin B1	Rabbit	400-500	1/1000	Abcam
p120ctn-catenin (N-ter)	Rabbit	N-term.	1/10000	Sigma

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p120ctn-catenin (C-ter)	Mouse	790-911	1/1000	BD Transduction Labs
Piruvate kinase	Goat	-	1/2000	Sigma
β-actin	Mouse	N-term.	1/5000	Sigma
β-catenin	Mouse	696-750	1/1000	BD Transduction Labs
Rac1	Mouse	-	1/500	Cytoskeleton Inc.
RhoA	Mouse	1-155	1/200	BD Transduction Labs
Vav2	Rabbit	131-330	1/200	Santa Cruz
Tiam1	Rabbit	C-term.	1/200	Santa-Cruz
Phosphor-Akt	Rabbit	Thr308	1/1000	Cell Signaling
Phospho-Tyr	Mouse	Tyr-P	1/1000	BD Transduction Labs
p120ctn-catenin-pS268	Mouse	P-Ser268	1/1000	Kindly provided by Dr. Albert Reynolds
GFP	Mouse	-	1/1000	Molecular Probes
GST	Goat	-	1/2000	GE Healthcare
Fyn	Mouse	1-132	1/250	BD Transduction Labs

Table 6: Antibodies and dilutions used in the project

After the primary incubation, membranes were washed three times for 10 minutes each with TTBS to eliminate the excess of antibody and then incubated with the appropriate secondary antibody (Horse-Radish Peroxidase conjugated anti-IgG) for at least 1 hour at RT. Three times 10 minutes washes with TTBS were again done and the membranes were developed using the kit *ImmobilonTM Western Chemiluminiscent HRP Substrate* (Millipore) and films (Kodak). Developing system *SNAP* (Millipore) was used, alternatively.

For developing the same membrane several times using different antibodies, it was necessary to remove the antibody stuck to the membrane: the membrane was incubated with Stripping Buffer for 20 minutes at 55°C and washed with TTBS before blocking the membrane again (1% BSA TTBS) and incubating with antibodies.

4.2 CELL EXTRACTS PREPARATION

Depending on the requirements of each experiment, different buffers for extracting proteins from the cell were used. Plates seeded with cells were washed with PBS and the lysis buffer was added. The plate was scrapped and lysed cells were collected in an eppendorf. Then, on of these protocols were followed:

Kemler extracts: used to achieve a soft lysis, to extract the soluble proteins of the cell and to maintain the majority of the interactions between proteins. Kemler Buffer was used to lysate the cells. A syringe was used to homogenize the extracts and break the nucleus. The extracts were then rotated for 10-30 minutes at 4°C, centrifuged 15 minutes at 14.000 rpm (*Eppendorf Coulter Avanti J-25*) and the supernatant was saved.

Kemler Buffer: 25 mM Tris pH 7.6, 210 mM NaCl, 1 mM EGTA, 0.1% Nonidet-40, 5 mM MgCl₂, 1 mM DTT, 3 mM aprotinin, 10 mM leupeptin, 10 mM pepstatin, 1 mM AEBSF, 1 mM NaF, 2.5 mM β-glycerolphosphate, 0.5 mM Va³⁺.

NP-40 extracts: to obtain a stronger lysis 0.25-1% NP-40 was used. Different amounts of proteins were solubilised from the membrane and from the cytoskeleton depending on the percentage of NP-40 added to the lysis buffer. Extracts were homogenized using syringes and rotated for 10 minutes at 4°C. Extracts were centrifuged for 10 minutes at 14.000 rpm and the supernatant was saved.

NP-40 Buffer: 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ /1.8 mM KH₂PO₄ (pH 7.4), 0.25-1% Nonidet-40, 3 mM aprotinin, 10 mM leupeptin, 10 mM pepstatin, 1 mM AEBSF, 1 mM NaF, 2.5 mM β-glycerolphosphate, 0.5 mM Va³⁺.

RIPA extracts: used to achieve a stronger lysis and almost a total solubilisation of the proteins, preserving the strongest interactions. Cells were lysed with RIPA buffer, homogenized with syringes and centrifuged 10 minutes at 14.000 rpm.

RIPA Buffer: 25 mM Tris pH 7.6, 210 mM NaCl, 1 mM EGTA, 1% Nonidet-40, 0.5% Sodium Deoxicolate, 0.1% SDS, 3 mM aprotinin, 10 mM leupeptin, 10 mM pepstatin, 1 mM AEBSF, 1 mM NaF, 2.5 mM β-glycerolphosphate, 0.5 mM Va³⁺.

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Total extracts 1% SDS: used to solubilise all the cellular components. Extracts acquired high viscosity once lysed due to DNA extraction. For this reason, extracts were boiled 5 minutes at 95°C and homogenized with a syringe. Once the viscosity had disappeared, samples were centrifuged 10 minutes at 14.000 rpm.

1% SDS Buffer: 25 mM Tris pH 7.6, 210 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% SDS, 3 mM aprotinin, 10 mM leupeptin, 10 mM pepstatin, 1 mM AEBSF, 1 mM NaF, 2.5 mM β -glycerolphosphate, 0.5 mM Va^{3+} .

Digitonin extracts: used to maintain the integrity from the membrane proteins, so it is used when it is important to maintain the integrity of the membrane complexes and interactions. Extracts were homogenized with syringe, incubated on ice for 10 minutes and centrifuged for 10 minutes at 14.000 rpm.

Digitonin Buffer: 25 mM Tris pH 7.6, 150 mM NaCl, 1mM EDTA, 1% Digitonin, 3 mM aprotinin, 10 mM leupeptin, 10 mM pepstatin, 1 mM AEBSF, 1 mM NaF, 2.5 mM β -glycerolphosphate, 0.5 mM Va^{3+} .

4.3 BINDING

This assay consists on the addition of recombinant proteins to a tube and the analysis of the interactions between them after some time of incubation.

Indicated amounts of proteins were added in a final volume of 200 μ l of Binding Buffer and incubated at RT for 25-30 minutes.

Complexes formed were isolated adding 20 μ l of effective Glutathion-Sepharose beads (that specifically retains GST) and incubating them for 20 minutes. Beads were immediately washed three times with 500 μ l of PBS adding detergent to eliminate the remaining free protein unbound to the GST-fusion protein. Complexes bound to the beads were solubilised by the addition of Sample Buffer and boiled for 3-5 minutes.

Solubilised products were analyzed by polyacrylamide electrophoresis (SDS-PAGE) and gel was transferred to a PVDF membrane to be analyzed by Western Blot.

In all cases, GST alone control was performed to avoid unspecific interaction with GST protein.

Recombinant proteins were phosphorylated before in some cases. Phosphorylation protocol is described further down.

4.4 PULL-DOWN

Pull-down assays were performed using purified recombinant proteins fused to GST and cell extracts. Indicated amounts of proteins were incubated with a constant amount of cell extracts, in a final volume of 500 μ l of the corresponding Lysis Buffer. Incubation was performed during 45 minutes at RT. Complexes formed were isolated adding Glutathione-Sepharose beads and incubating it for 20 minutes at RT. Beads were immediately washed for 3 times with 500 μ l of PBS and detergent (0.1-0.5% NP-40) to remove free and not-specifically-bound proteins. Protein complexes were eluted in Sample Buffer and analyzed by Western blotting with specific antibodies.

In some cases, recombinant proteins were first phosphorylated and then used for pull-down assays.

- ❖ **PAK pull-down assay:** also called PBD-GST pull-down assay. It was used for the detection of endogenous active Rac1 and Cdc42 in cell extracts. PAK protein (p21-activated kinase) is one of the main substrates for Rac1 and Cdc42 GTPases and PBD (p21 Binding Domain) is the domain from PAK through which Rac1 and Cdc42 interact once they are activated. This PBD domain fused to GST is used to isolate active molecules of Rac1 and Cdc42 in the cell at a certain moment. PBD-GST protein was first purified like a normal recombinant protein, as described above, but the final purified protein was left bound to the Glutathione-Sepharose beads instead (we did not remove the GST tag nor elute the protein from the beads). 20 μ l (approximately 60 μ g of PBD-GST protein) of these beads were then added to cell extracts. Cells were lysed with RIPA Buffer (MgCl₂ modified) and 800 μ g of cell extracts were added for each condition in a final volume of 400 μ l (2 μ g/ μ l final extracts concentration). Pull-down incubation was performed at 4^oC for 30 minutes and beads were washed two

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times with 500 μ l of Wash Buffer. Active Rac1/Cdc42 was isolated adding Sample Buffer and detected by SDS-PAGE and Western Blot.

- ❖ **Rhotekin pull-down assay:** active RhoA was detected using *Rhotekin-RBD protein Agarose beads* (Cytoskeleton, Inc). Same process as PAK-GST pull-down was followed but beads were incubated for 1h at 4°C instead.

4.5 CO-IMMUNOPRECIPITATION (COIP)

Endogenous or over-expressed proteins were purified from cellular extracts and interacting proteins were analyzed. 500-1000 μ g of cellular extracts were incubated with 2 μ g/ml of the appropriate antibody in a final volume of 300-600 μ l of lysis buffer during 2-16 hours rotating at 4°C. Lysis buffer would change depending on the proteins that we wanted to extract and on the interactions that we wanted to look at. After incubation with the antibody, precipitated material was removed by centrifugation at 14.000 rpm (*Eppendorf Centrifuge 5418*) and the resulting supernatant was incubated for 120 minutes with 20 μ l of γ -bind G-Sepharose (GE-Healthcare). Immunoprecipitate was washed three times with PBS (adding 0.1% NP-40 if necessary) and bound proteins were eluted with electrophoresis Sample Buffer. Immunoprecipitated proteins were analyzed by Western Blot using specific mAbs.

4.6 PHOSPHORYLATION ASSAYS

In these assays a purified recombinant protein was phosphorylated with a commercial recombinant kinase. This phosphorylated recombinant protein obtained was used for different types of experiments like bindings, pull-downs, etc.

Phosphorylation assays were performed in a final volume of 50 μ l in a special phosphorylation buffer (depending on the kinase used) and 0.1 mM ATP. The recombinant protein, the kinase and appropriate inhibitors were added to the mix and incubated at the temperature and time required for the kinase.

The optimal conditions for the kinases used in the project were the following:

	CKIδ C-terminal Truncated	SRC	Fyn
Company	SIGMA	Upstate	Upstate
Temperature	30°C	22°C	30°C
Time	45 minutes	2 hours	45 minutes
Reaction concentration (mU/pmol substrate)	20-100 mU	4500 mU	20-100 mU
Substrate	2-10 pmols	2-10 pmols	2-10 pmols
Volume	25-50 μ l	50 μ l	50 μ l
Origin	Recombinant, <i>E.Coli</i>	Recombinant, Sf21 insect cells	Recombinant, Sf21 insect cells
Buffer	TEST	Tyr-kinase	Tyr-Kinase

Table 7: Conditions used for the different kinases

4.7 CELLULAR SUBFRACTIONATION

The nuclear fraction was isolated from the cytosol, membrane and cytoskeleton fraction. Cells were lysed in Buffer 1. Different volumes were added depending on the size of the plate: 700 μ l of Buffer 1 for 10 cm plates and 300 μ l for 60 mm plates. Extracts were allowed to rest on ice for 15 minutes and centrifuged for 15 minutes at 500xg and 4°C (*HETTICH* Mikro 22R). The supernatant contained the cytosol, membrane and cytoskeleton fraction. The pellet was lysed in half of the initial volume of the buffer adding SDS to a 0.5% final concentration (350 μ l for 10 cm plates and 150 μ l for 60 mm plates). Extracts were sonicated for 5 seconds and centrifuged for 15 minutes at 14.000 rpm and 4°C (*Eppendorf Centrifuge* 5418). The supernatant contained the nuclear fraction. Proteins were analyzed by Western Blot. It was important to load double of the volume for the nucleus part for each volume of cytosol loaded in the gel (as the nucleus had been resuspended in half of the volume of the cytosol).

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Buffer 1 (subcellular fractionation): 50 mM Tris pH 7.5, 1% TX-100, 137 mM NaCl, 10% Glicerol, 3 mM aprotinin, 10 mM leupeptin, 10 mM pepstatin, 1 mM AEBSF, 1 mM NaF, 2.5 mM β -glicerolphosphate, 0.5 mM Va^{3+} .

5. *IN VIVO* ASSAYS, MIGRATION AND PROLIFERATION ASSAYS

5.1 WOUND HEALING

Different conditions of cells were seeded in a 6 multi-well plate. The following day (or when confluence reaches 80-90%), a wound was performed in each small plate using a micro-pipette (a mark with a cross). Immediately pictures were taken from each condition at time zero and after 24, 48 hours... And further on until the wound from the control condition cells was totally healed.

5.2 GASTRULATION ASSAYS IN *XENOPUS LAEVIS* EMBRYOS

Xenopus laevis females were primed 5 days before the induction of ovulation. Priming consisted on injecting 50 Units of HCG (Human Chorionic Gonadotropin) to the females. The night before obtaining the oocytes, frogs were induced to lay eggs injecting 800 U of HCG per frog. The following day frogs were squeezed to obtain the oocytes and they were *in vitro* fertilized in 1x MMR (Marc's Modified Ringer's Solution). After 10 minutes of fertilization, 0.1x MMR was added to the plate and eggs were allowed to rest for 30 minutes. De-jelly Solution (1x MMR containing 2% (w/v) cysteine (pH 8.0)) was then added for 10 more minutes and eggs were washed three times with 0.1 MMR. Embryos were then placed to the incubators. The optimal temperature for the embryos is 17-18°C. However, in order to slow down the embryo development rate and to gain time for injecting the constructs of interest in a particular developmental stage, embryos were kept at cooler temperatures before the injections. Morpholinos and RNA constructs were microinjected into the animal hemispheres of one- or two-cell cleavage stage embryos. Injected embryos were cultured in 0.1x MMR containing 50 μ g/ml gentamycin until desired stages. The injection volume for morpholinos or RNAs was 20 nl at the one-cell stage, 10 nl per blastomere at the two-cell stage and beyond with the doses as follows: 40 ng for p120ctn-catenin morpholinos (20 ng each morpholino I and II) per blastomere; 1ng for

p120ctn WT, p120ctn 112E, p120ctn 112F, p120ctn 268-9DD and p120ctn 268-9 AA over-expression; 40 pg/embryo for p120ctn wt, p120ctn 112E and 112F rescues, 80 pg/embryo for p120ctn 268-9DD and AA and 10 pg/embryo for p120ctn 217E rescue. Gastrulation phenotypes, principally failure of blastopore closure, were visually scored at embryonic stages 11-12 using a standard binocular dissecting microscope (*Zeiss Stemi DV4*).

Embryos were microinjected with capped mRNA synthesized in vitro (*mMessage mMachine*, Ambion). All pCS2-based constructs were linearized by using NotI prior to *in vitro* transcription. *X. laevis* p120ctn-catenin morpholinos were obtained from *Gene Tools*, LLC and were the following:

MORPHOLINO	Nucleotide sequence
Xp120ctn-MO I	5'-ACTCTGGCTCATC- CATATAGAAAGG-3'
Xp120ctn-MO II	5'-AGAGACCCAAGTTCACACTC- CAGGC-3'
Standard MO (negative control)	5'-CCTCTTACCTCAGTTACAATTATA-3'

Table 8: Morpholino's sequences used

Morpholinos were targeted against the following mRNA sequences relative to the translational start site (ATG): Xp120ctn-MO I (-9 to +13), Xp120ctn-MO II (-34 to +10). GenBank searches failed to detect significant complementarity of the Xp120ctn morpholinos to xARVCF mRNA or of the xARVCF morpholinos to Xp120ctn mRNA. The morpholinos were resuspended in Nuclease-Free Water (Ambion) to the experimental concentrations required for microinjection.

6. BUFFERS:

LB: 0.5% w/v Yeast extracts, 1% w/v bacto-trypton, 170 mM NaCl pH 7.0.

LB Agar: 0.5% w/v Yeast extracts, 1% w/v bacto-trypton, 170 mM NaCl pH 7.0, 1.5% Agar pH 7.0.

PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ /1.8 mM KH₂PO₄ (pH 7.4).

Glutathione Elution Buffer, GEB: 20 mM reduced glutathione (Sigma), 50 mM Tris-HCl pH 8.5, 100 mM NaCl.

Dialysis buffer: 25 mM Tris pH 8.3, 120 mM NaCl, 1mM EDTA, 1mM DTT.

PSP Buffer: 50 mM Tris pH 7.8, 150-250 mM NaCl, 1 mM DTT, 1mM EDTA, pH 7.5

PEI: 1 mg/ml PEI in H₂O miliQ, adjust pH to 7.0 with HCl and sterilize by filtration.

Sample Buffer: 100 mM Tris pH 6.8, 4% Glycerol, 2% SDS, 0.06% 2-β-Mercaptoethanol.

Comassie Brilliant Blue: 0.1% Comassie Brilliant Blue, 40% methanol, 10% acetic acid.

Distainer Buffer: acetic acid 10%, methanol 40%

Binding Buffer: 50 mM Tris pH 7.8, 150 mM NaCl, 1mM EDTA, 1mM DTT, 3 mM MgCl₂, 0.1% (w/v) Triton X-100, proteases and phosphatases inhibitors.

TTBS: 25 mM Tris/HCl pH 7.5, 0.1% Tween-20 and 135 mM NaCl

Stripping Buffer: 2 % SDS, 62.5 Tris pH 6.8, 100 mM β mercaptoethanol

STE Buffer: 10 mM Tris/HCl pH 8.0, 150 mM NaCl, 1mM EDTA

Wash Buffer: 25 mM Tris pH 7.5, 30 mM MgCl₂, 40 mM NaCl

Buffer 1 (subcellular fractionation): 50 mM Tris pH 7.5, 1% TX-100, 137 mM NaCl, 10% Glycerol, 3 mM aprotinin, 10 mM leupeptin, 10 mM pepstatin, 1 mM AEBSF, 1 mM NaF, 2.5 mM β-glycerolphosphate, 0.5 mM Va³⁺.

Tyr-kinase Buffer 5x: 25 mM Tris pH 6.8, 25 mM MgCl₂, 5 mM MnCl₂, 0.5 mM EGTA, 1mM DTT, 3 mM aprotinin, 10 mM leupeptin, 10 mM pepstatin, 1 mM AEBSF, 1 mM NaF, 27.8 mM β-glycerolphosphate, 0.5 mM Va³⁺.

TEST Buffer 5x: 25 mM Tris pH 6.8, 9mM MgCl₂, 0.55 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 3 mM aprotinin, 10 mM leupeptin, 10 mM pepstatin, 1 mM AEBSF, 1 mM NaF, 27.8 mM β-glycerolphosphate, 0.5 mM Va³⁺.

Digitonin Buffer: 25 mM Tris pH 7.6, 150 mM NaCl, 1mM EDTA, 1% Digitonin, 3 mM aprotinin, 10 mM leupeptin, 10 mM pepstatin, 1 mM AEBSF, 1 mM NaF, 2.5 mM β-glycerolphosphate, 0.5 mM Va³⁺.

RIPA Buffer: 25 mM Tris pH 7.6, 210 mM NaCl, 1 mM EGTA, 1% Nonidet-40, 0.5% Sodium Deoxicolate, 0.1% SDS, 3 mM aprotinin, 10 mM leupeptin, 10 mM pepstatin, 1 mM AEBSF, 1 mM NaF, 2.5 mM β-glycerolphosphate, 0.5 mM Va³⁺.(Supplemented with 10 mM MgCl₂ when used for PAK pull-down assays).

NP-40 Buffer: 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ /1.8 mM KH₂PO₄ (pH 7.4), 0.25-1% Nonidet-40, 3 mM aprotinin, 10 mM leupeptin, 10 mM pepstatin, 1 mM AEBSF, 1 mM NaF, 2.5 mM β-glycerolphosphate, 0.5 mM Va³⁺.

Kemler Buffer: 25 mM Tris pH 7.6, 210 mM NaCl, 1 mM EGTA, 0.1% Nonidet-40, 5 mM MgCl₂, 1 mM DTT, 3 mM aprotinin, 10 mM leupeptin, 10 mM pepstatin, 1 mM AEBSF, 1 mM NaF, 2.5 mM β-glycerolphosphate, 0.5 mM Va³⁺.

MMR (*Marc's Modified Ringer's Solution*): 100 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 5 mM HEPES 5 mM pH 7.4.

De-jelly solution: 1x MMR containing 2% (w/v) cysteine (pH 8.0)

Binding Buffer: 3 mM MgCl₂, 50 mM Tris, 1 mM EDTA, 1 mM DTT, 150 mM NaCl, 0.1 % TX-100, pH 7.5

Trypan Blue: 0.4% Trypan Blue in PBS (w/v)

Transfer Buffer: 192 mM glycine, 25 mM Tris, 10% methanol.

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