Initial events in non-canonical Wnt signaling

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Perfer et obdura; dolor hic tibi proderit olim

Publi Ovidi Nasó, *Ovidi*

TABLE OF CONTENTS

ABBREVIATIONS

aa	Amino acid
AP	Anterior-posterior
AP-1	Activator protein 1
AP-2	Adaptor protein 2
ARM	Armadillo domain
ARVCF	Armadillo-repeat protein deleted in velo-cardio-facial syndrome
BDB	Brachydactyly type B
BDC	Brachydactyly type C
CA-LRP	Constitutively active low-density lipoprotein receptor-related protein
CAM-1	CAN abnormal migration 1
CAMK	Ca2+/calmodulin-dependent protein kinase
CK1	Casein kinase 1
CE	Convergent-extension
CM	Conditioned medium
CRD	Cysteine-rich domain
Daple	Dvl-associating protein with a high frequency of leucine residues
DEP	Dishevelled, egl-10, pleckstrin domain
DIX	Dishevelled, axin domain
DKK	Dickkopf
DRS	Dominant form of Robinow syndrome
Dvl	Dishevelled
ECM	Extracellular matrix
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1 or 2
Fz	Frizzled
GEF	Guanine exchange factors
GSK3	Glycogen synthase kinase 3
ICL	Intracellular loop
JNK	c-Jun N-terminal kinase
KD	Kinase-dead
LEF	Lymphoid enhancer factor
LRP5/6	Low-density lipoprotein receptor-related protein 5 or 6
MDC	Monodansylcadaverine

MMP	Matrix metalloproteinase
NLK	Nemo-like kinase
PAGE	Polyacrylamide gel electrophoresis
PDZ	Postsynaptic density 95, *Drosophila* disc large tumor suppressor, zonula occludens-1
PKC	Protein kinase C
P-R	Proline-rich
PCP	Planar cell polarity
PP2A	Protein phosphatase 2A
Ror1/2	Receptor tyrosine kinase-like orphan receptor 1 or 2
RRS	Recessive form of Robinow syndrome
RS	Robinow syndrome
RTK	Receptor tyrosine kinase
shRNA	short hairpin RNA
S/T-R	Serine/threonine-rich
TAK	TGF-β -activated kinase
TCF	T-cell factor
Vangl2	Vang-like 2
Wnts	Wnt-family proteins

Introduction

1. Wnt function at early stages

The developmental pathways are signaling cascades usually conserved among species that control complex processes such as embryogenesis or cell growth. Although the targets of these signaling cascades may differ among organisms, the pathways are still homologous[1]. Inside this classification fall several pathways, such as receptor tyrosine kinases/Ras/MAP kinases, TGF-β, Hedgehog, Wnt, and Notch[2]. These pathways tightly regulate cell signaling activation, inhibition, gene transcription, gene repression and many other functions. Deregulation of these pathways may promote several diseases, such as cancer.

During development, all embryonic tissues are developed along dorso-ventral, anterior-posterior and left-right body axes. Throughout this process, the Spemann's Organizer in *Xenopus*[3] or the node in mammals[4] is formed. This is a specialized region of the embryo that confers positional information to all embryo tissues and appears when the three embryo layers become apparent. At this moment, convergent and extension movements during gastrulation promotes the involution of mesoderm and endoderm and the dissemination of the ectoderm all along the outside of the embryo covering it. This step sets up the basic body plan of future embryonic tissues. One of the relevant pathways controlling this process is the Wnt pathway.

The Wnt pathway comprises two different branches that traditionally have been attributed a different function. The canonical Wnt/β-catenin pathway has been associated with stemness[5] and also cell fate specification through β-catenin mediated gene transcription[6], while the non-canonical Wnt pathway is more related to morphogenetic events[7]. In *Xenopus* embryos after fertilization and during blastula stage, nuclear β-catenin marks the region where the Spemann's Organizer will form. Whereas ventral cells only have cytoplasmic or membrane associated β-catenin[8, 9], nuclear expression in ectopic regions is sufficient to induce dorsalization and axis duplication[10, 11]. At late blastula stage, starts the gastrulation process that will pattern the basic body plan. In order for this to happen, the cells or group of cells suffer a process of polarization and single- or collective-migration towards the inside of the embryo. These cell intercalations generate pushing forces that extend the anterior-posterior (AP) body axis of the embryo in a process referred to convergent extension (CE). In this last process of cell polarization and

migration, non-canonical Wnt pathway has been proven essential. Defects in its signalization leads to alterations in gastrulation, as well as in AP extension[12] (see figure I-1 for summary).
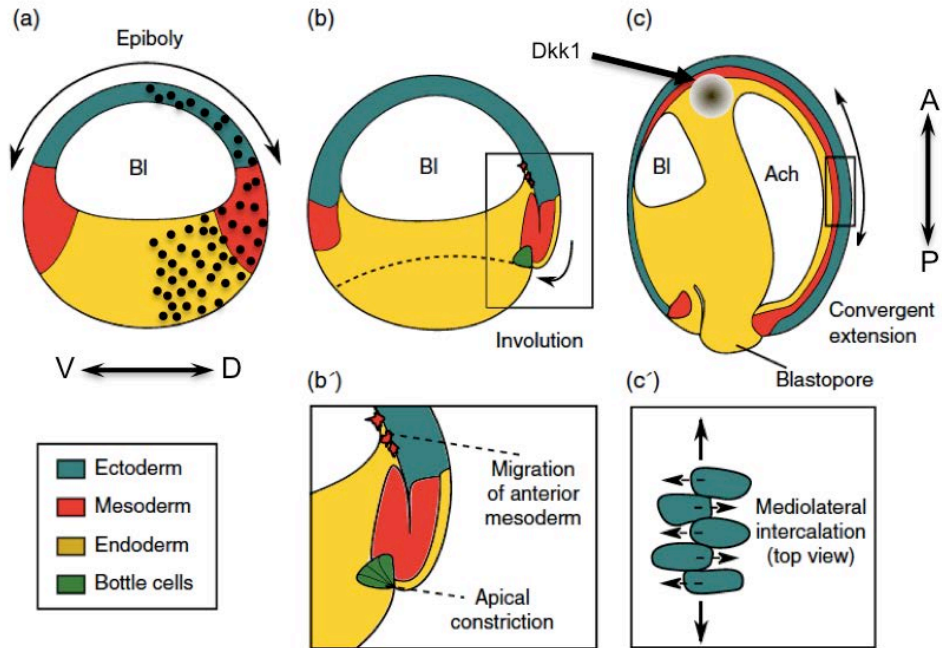
Figure I-1. Scheme of early *Xenopus* development. a) Black dots represent nuclear β-catenin in dorsal region of the embryo in late blastula. **b)** In early gastrula, apical cell constriction marks the region where the dorsal blastopore lip originates. This is followed by tissue invagination by individual or collective cell migration. **c)** Late gastrula stage. Wnt repression by Dkk1 expressed in this stage will mark the anterior region of the embryo. Mediolateral intercalation of cells in the ectoderm allows the extension of the anterior-posterior axis of the embryo in a process called convergent extension. To achieve proper gastrulation as well as CE, cells need to undergo processes of cell polarization and migration. Adapted from [13].

2. Components of the Wnt signaling pathway

2.1. Wnt ligands

Wnt factors are signaling molecules conserved among all metazoans. In mammals this family comprises 19 members. These factors are glycoproteins 350 to 400 amino acids-long enriched in cysteine residues and with an N-terminal signaling peptide that targets them for secretion and several putative N-glycosylation sites[14]. Although they are secreted factors, they are mostly insoluble due to cysteine palmitoylation (Figure I-2). Wnt ligands act as morphogens at short or long range depending on concentration gradients[6, 15]. It is still unclear how these concentration gradients are generated but one of the hypotheses is that Wnt proteins hide its lipophilic domains through different interactions, and thus are able to travel long distances[16].
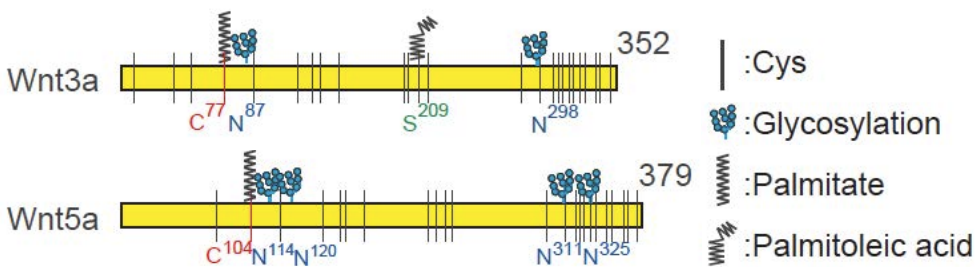
Figure I-2. Scheme of Wnt ligands. The image shows the main representative ligands of canonical (Wnt3a) and non-canonical Wnt pathway (Wnt5a). Main glycosylated and acylated residues are represented. Adapted from [37].

Wnt proteins are glycosylated and lipid-modified while they move from the endoplasmic reticulum (ER) through the exocytic pathway[17]. In *Drosophila* Porcupine, a transmembrane protein in the ER, is responsible for Wnt secretion[18] producing Wnt palmitoylation[19, 20]. Therefore, Porcupine deficiency causes Wnt accumulation in the ER and reduced palmitoylation[19]. Other three components have also been described necessary for Wnt secretion. One of them is p24, the second one is Wntless(Wls)/Evennes interrupted (Evi)/Sprinter (Srt) and the third one is the multiprotein complex retromer[21, 22, 23]. P24 family members of transmembrane proteins are necessary for the early secretory pathway of Wnt: they allow the exit of Wnt proteins from the ER and transit to the Golgi apparatus[24]. The second

component, Wls, is a seven-pass transmembrane protein located mainly in the Golgi apparatus but also in endosomal compartments and in the plasmatic membrane. In the absence of Wls, Wnt is also confined inside the cell and cannot be secreted. Wls function is thought to be Wnt transport and delivery from Golgi to the membrane where it is released for exocytosis[25, 26]. Once Wnt is secreted, Wls is recycled back via endosomes to the Golgi apparatus by the action of the retromer complex[27]. This last complex is crucial for secretion and gradient distribution of Wnts [28, 29, 30]. Retromer complex lost or mutation leads to lysosomal degradation of Wls, which leads to lower levels of Wls protein and, as a consequence, of Wnt secretion[31].

The molecular Wnt signaling structure is overwhelmingly complex. It is formed by myriad of components that are subjected to a high degree of regulation and crosstalk mechanisms. At cellular membrane level several Wnt receptors and co-receptors that participate in the activation of the pathway can be found: Frizzled[32], low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6)[33], receptor tyrosine kinase-like orphan receptor 1 or 2 (Ror1 or Ror2) and receptor tyrosine kinase (Ryk)[34], protein Tyrosine kinase 7 (PTK7)[35], muscle skeletal receptor tyrosine kinase (Musk)[36] and proteoglycan families[37].

2.2. Frizzled

In humans, the Frizzled (Fz) protein family is encoded by a group of 10 different genes. Structurally Fz is a seven-transmembrane protein with an extracellular cysteine-rich domain (CRD) resembling a G-protein-coupled receptor[38]. The CRD is formed by a cysteine-rich conserved sequence of 120 amino acids connected to the first transmembrane helix through a very variable region (70 to 120 amino acids). The CRD is necessary for Fz interaction with Wnt ligands, which bind to with a high affinity (K_d 1-10nM)[39]. The intracellular part of Fz, specifically the carboxyl tail with KTxxxW motif (motif III) and two regions in the intracellular loop 3 (ICL3) (motif I and motif II), are necessary for Dishevelled interaction and therefore, for signaling[33, 40] (Figure I-3). The utmost cytoplasmic binding partner of Frizzled is the scaffold protein Dishevelled (Dvl). Upon activation of the Wnt pathway, Dvl interacts with most Wnt co-receptors and transduces all major Wnt signals. It represents an almost ubiquitous scaffold protein downstream of Wnt receptors[37].
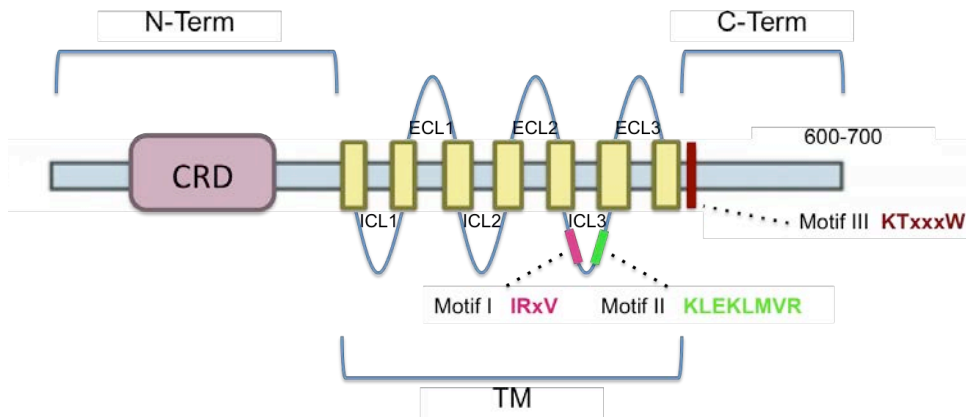
Figure I-3. Schematic diagram of Frizzled receptor. The representation shows the different domains of Frizzled with the extracellular cysteine-rich domain (CRD), the seven transmembrane domains with their extracellular loops (ECL) and intracellular loops (ICL) and the region where Dvl binds. This region is formed by the motif III in the carboxyl tail and motifs I and II in the ICL3. Figure adapted from [37].

2.3. Dishevelled

Dishevelled is a scaffold protein that is considered a hub in canonical as well as in non-canonical Wnt pathway. It was first described in *Drosophila* and in human being presents 3 isoforms: Dvl-1, Dvl-2 and Dvl-3. Dvls are 700 amino acid proteins composed by mainly 3 domains: DIX (Dishevelled, Axin) domain connected to PDZ (Postsynaptic density 95, *Drosophila* disc large tumor suppressor, Zonula occludens-1) through an unstructured region that contains a serine and threonine-rich (S/T-R) domain. Following PDZ there is a region enriched in proline residues (P-R region) that connects to the DEP (Dishevelled, Egl-10, Pleckstrin) domain. This last domain is finally followed by a disordered C-terminal tail (Figure I-4).

The interaction of Dvl with Fz involves the PDZ domain in Dvl and the KTxxxW (Motif III) in the carboxyl tail of Fz[41, 42]. DEP Dvl domain has been described to be also necessary for motif III interaction in Fz and C-terminus of Dvl also binds to Motif I and II of Fz ICL3[40]. The Dvl DEP domain is required for membrane localization. It presents a positively charged region that is crucial to the binding with the negatively charged phospholipids in the plasma membrane[43]. For canonical Wnt pathway, DIX and PDZ domains are necessary

not only for Fz interaction (PDZ), but also for homotypic and heterotypic interactions with Dvl and Axin that allows polymerization and pathway activation[44, 45, 46, 47].
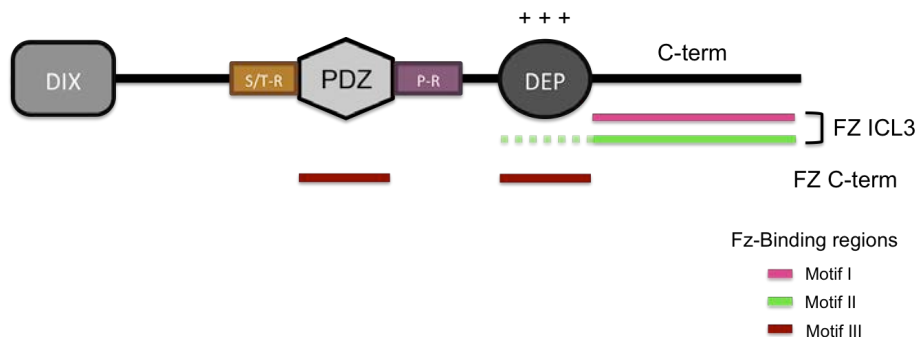
Figure I-4. Schematic representation of Dvl domains. Dishevelled domains DIX, PDZ and DEP are represented together with S/T-R and P-R region. Above DEP domain are also depicted sum signs to highlight that this domain is positively charged to enhance membrane interaction. Below the structure of Dvl is showed, using the same color scheme as in figure I-3, the regions required for the interaction with Fz. Adapted from [47].

Dvl is associated with kinases and is dynamically phosphorylated upon Wnt stimulus. This phosphorylation, independently of Wnt ligand type, triggers a mobility shift of Dvl on SDS-PAGE[48, 49]. This effect is used as an indicator of Wnt pathway activation.

2.4. Wnt co-receptors

2.4.1. LRP5/6

LRP5 and LRP6 form a unique group inside the low-density lipoprotein receptor family. Structurally they are characterized by a unique transmembrane segment, an extracellular domain with four tandem β-propellers and four epidermal growth factor repeats followed by three repetitions of LDLR type A domain[50]. The four YWTD domains are crucial for the interaction of LRP5/6 with canonical Wnt ligands or the pathway inhibitor Dickkopf (DKK1)[51]. The cytoplasmic region is formed by an amino acid cluster of serines and threonines (S/T cluster) followed by five PPPSPxS motifs (P = Proline, S = Serine and x = any aminoacid) (Figure I-5). The S/T cluster and the

two serines of the motif PPPSPxS are phosphorylated after Wnt stimulation and are necessary for β-catenin stabilization[52, 53].

Mutant forms of co-receptor LRP5/6 lacking the intracellular region (LRP5/6ΔC) act as dominant inhibitor of the Wnt pathway[54]. Alternatively, other versions of the co-receptor without the extracellular domain, but with transmembrane region (LRP5/6ΔN or CA-LRP5/6) to allow its proper membrane localization, work as a constitutively active form and display basally phosphorylated PPPSPxS motifs[55, 56].
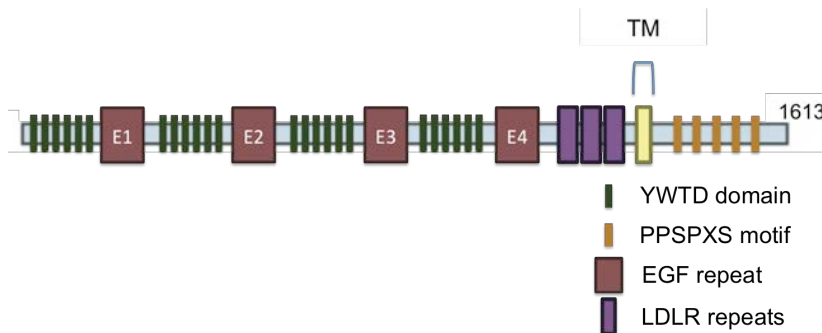
Figure I-5. Diagram of the LRP5/6 receptor domains. The scheme shows the four tandem YWTD domains and EGF repeats followed by the triplet of LDLR-A domain. Intracellularly are represented the five PPPSPxS phosphorylation motifs. Adapted from [37].

2.4.2. Ror2

2.4.2.1. Structure and function

The family or Ror tyrosine kinase receptors were first identified as orphan receptors in a PCR screening for novel receptor tyrosine kinases (RTKs)[57]. Hence its name: RTK-like Orphan Receptors. In vertebrates, the family consists of two closely related proteins: Ror1 and Ror2. The Ror family is quite conserved and structurally it is formed by different domains. The more conserved are Fz-like CRD, Kringle (KR) and tyrosine kinase domain. In some species, it can also be appreciated the presence of Ig-like, P-R and S/T-R domains (Figure I-6).

The CRD domain of the Ror family is related to the CRDs of Fz family receptors of the Wnt pathway. Ror and Wnt proteins can interact through the CRD domain[58, 59, 60]. One of the main Wnt interactors with Ror2 is Wnt5a. In this interaction Ror2 works as a co-receptor facilitating the role of Fz as a receptor. This association mediates Wnt5a signaling[61].

Although recent publications highlight the possible role of Ror1 also as Wnt5a co-receptor[62, 63], this interaction and its intracellular consequences are less known.

Wnt5a promotes the activation of β-catenin-independent Wnt pathways (non-canonical Wnt pathways). These pathways are grouped into two signaling cascades known as planar cell polarity (PCP) pathway, where Ror2 plays a relevant role, and Wnt-Ca^{2+} pathway[7]. The PCP has a major role in the regulation of CE movements, a process naturally occurring during embryo gastrulation[64].
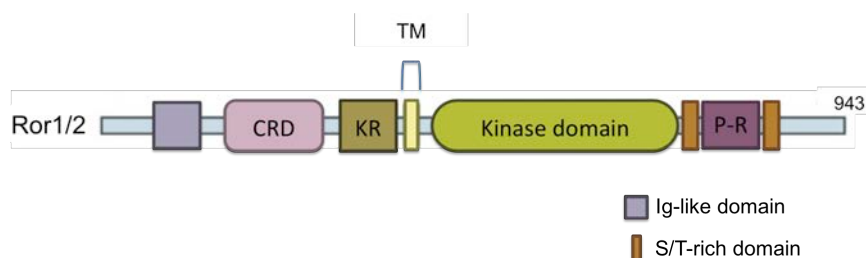
Figure I-6. Representation of the Ror-family RTKs. The depicted domains are Immunoglobulin-like (Ig-like) domain, Frizzled-like cysteine-rich (CRD) domain, Kringle domain (KR), Tyrosine kinase domain, proline-rich domain (P-R) and serine/threonine-rich domains (S/T-R 1 and 2). Figure adapted from [37].

2.4.2.2. Ror2 tyrosine kinase activity

Ror2 is a single-pass transmembrane tyrosine kinase receptor although it is still not clear if its tyrosine kinase activity is necessary for signaling. Ror2 has been described to interact with Casein Kinase 1ε[65]. The interaction with this kinase phosphorylates Ror2 on serine/threonine residues in its C-terminal S/T-R 2 domain and allows Ror2 tyrosine autophosphorylation on the cytoplasmic P-R domain[65]. According to these authors, impairment of Ror2 kinase activity affects the phosphorylation of the

Ror2-interacting substrate G protein-coupled receptor kinase 2 (GRK2). Induction of dimerization of Ror2 with Wnt5a ligand has been shown to enhance Ror2 tyrosine kinase activity and phosphorylation, which are markedly reduced in Ror2 kinase-dead (KD) mutant[66]. However, still some tyrosine phosphorylation has been detected in cells expressing Ror2-KD mutants, raising the possibility that another tyrosine kinase is responsible for Ror2 phosphorylation. Src tyrosine kinase has been shown to associate with Ror2 at the C-terminal P-R and/or S/T-R 1 domain and phosphorylates Ror2 in tyrosine residues after Wnt5a stimulation[67]. Although some publications[68, 69] support the relevance of Ror2 as a tyrosine kinase, other results argued that Ror2 barely has tyrosine kinase activity[70] and it is dispensable for its functions[71, 72]. Nevertheless, in Ror2 KD mutants there is a notably reduction of Src-Ror2 interaction[73].

2.4.2.3.   Ror2 function in development

In mice Ror2 and Wnt5a are expressed in a spatiotemporal similar manner during development in various organs and tissues[74, 75]. Any alteration may cause several abnormalities in the musculoskeletal development, in the formation of heart, lungs, urogenital and intestinal tract and nervous system[12, 76, 77]. Ror as well as Wnt5a mutants usually die neonatally[76, 77, 78]. Although Ror2 is critical during development, it becomes almost undetectable in normal adult tissues, including humans[79].

2.4.2.4.   Ror2 malfunction and human diseases

In humans Ror2 deletion causes pathological consequences. Robinow Syndrome is a rare disorder that can appear as recessive Robinow Syndrome (RRS) or autosomal dominant Robinow Syndrome (DRS). Robinow syndrom (RS) patients are characterized by short-limbed dwarfism with abnormal formation of the face, hemivertebrae and genital hypoplasia. Patients can also present cardiac malformations, nephrological and endocrinological abnormalities[80, 81]. RS is caused by different type of mutations in various domains of Ror2 protein that triggers deletion of tyrosine kinase domain or ER retention and degradation due to improper protein folding[80, 82, 83, 84]. Missense mutations in the Wnt5a ligand can also cause RS but in this case is the dominant form (DRS)[85].

Brachydactyly is a medical term used to describe a condition where fingers and toes are abnormally reduced. In the case of the autosomal dominant Brachydactyly Type B syndrome, there is finger and toe shortening with rudimentary or absent distal and middle phalanges. This underdevelopment normally is accompanied by nail aplasia. Other characteristics may include syndactyly and symphalangism[86, 87]. This features appear as a consequence of Ror2 nonsense or frameshift mutations located N-terminal or C-terminal of the tyrosine kinase domain[87, 88, 89].

2.4.2.5. Ror alterations in cancer progression

Ror implication in cancer is very controversial due to the fact that it has been described both, as a pro-tumorigenic and as an anti-tumoral agent depending on cancer type. In table I-1 the involvement of Ror proteins in cancer development is resumed.

Cancer	Ror expression	Tumor Role	References
Chronic Lymphocytic Leukemia	Ror1	Oncogenic	90, 91
Melanoma	Ror1, Ror2	Oncogenic	92, 93, 94
Breast cancer	Ror1, Ror2	Oncogenic	95, 96, 97
Lung cancer	Ror1	Oncogenic	98
Gastric cancer	Ror1	Oncogenic	99
Osteosarcoma	Ror2	Oncogenic	100, 101
Prostate carcinoma	Ror2	Oncogenic	102
Renal carcinoma	Ror2	Oncogenic	103, 104
Colon carcinoma	Ror2	Tumor Suppressor	105, 106
Hepatocellular carcinoma	Ror2	Tumor Suppressor	107
Medulloblastoma	Ror2	Tumor Suppressor	108

Table I-1. Ror co-receptor involvement in tumor progression.

These opposing results might be explained on the basis of Wnt-pathway dependence in the different tumors [107, 109] (see section non-canonical inhibition of the canonical Wnt pathway).

2.5. Other intracellular components involved in Wnt signaling

Additionally to receptor and co-receptors at the membrane and the ubiquitous protein Dvl, other intracellular components have also been shown to be necessary for Wnt pathway activation. Among them, two factors are required for initial signalization after ligand stimulation: p120-catenin and casein kinase family members.

2.5.1. Casein Kinase 1 protein family

The CK1 family of serine and threonine protein kinases has been involved in the regulation of several cellular processes such as cell division, DNA repair, membrane transport, nuclear localization and circadian rhythms[110]. This monomeric protein family is very conserved in eukaryotic organism from yeast to mammals. In mammals, there are seven distinct genes encoding for the isoforms α, β, γ1, γ2, γ3, δ and ε. All these isoforms present a highly conserved catalytic domain (51-98% homology) with the closest similarity between CK1ε and δ. However, in addition to the catalytic domain, this protein family presents a N- and C-terminal domains that are quite divergent (at aa sequence level and length of domains)[110, 111].

CK1 kinases are usually constitutively active with the exception of CK1ε/δ. These two proteins present a C-terminal domain of approximately 100 aa that when phosphorylated, inactivates the catalytic domain[112,113]. The phosphorylation, usually carried out by the same enzyme[114], induces a conformational change of the C-terminal domain that blocks the catalytic cleft thus impeding the access of substrates[115] (Figure I-7). This inhibition by the C-terminal domain can be released by dephosphorylation, leaving the catalytic domain accessible again[114, 116].

All family members were thought to have similar specificity for their substrates *in vitro*[117]. The consensus phosphorylation sequence is S/TpXX**S/T**. This means that to be able to phosphorylate a serine (S) or a threonine (T) in bold, the kinase requires a S or a T previously phosphorylated and followed by any two aminoacids (XX). Therefore, CK1 protein family requires a priming phosphorylation[118]. Other atypical phosphorylation sequences, however, have also been described[119].
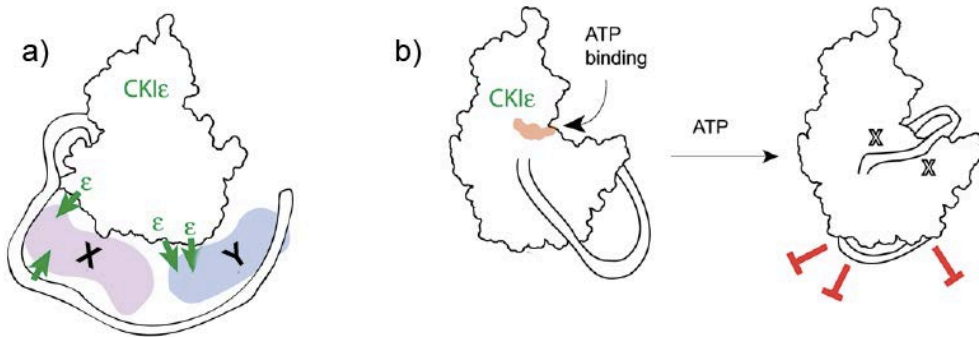
Figure I-7. Inhibition scheme of CK1ε. a) When the C-terminal is dephosphorylated, CK1ε can interact with its substrates with the aid of its C-terminal tail. **b)** When CK1ε is active and not bound to substrates, autophosphorylates its C-terminal tail and this makes it fold over its catalytic domain thus blocking substrate access. Adapted from [115].

Substrate specificity is determined by subcellular localization *in vivo*, determined by splicing variants in the case of CK1α[120] or acylation in the case of CK1γ that anchors it at the membrane (lipid rafts)[52, 121]. Generally, there is a localization overlap between CK1α, CK1δ and CK1ε[122].

2.5.2. P120-catenin

The relevance of p120-catenin has been well established in the canonical Wnt pathway by our group[123, 124, 125, 151]. Its function is important at different stages of the Wnt signalization. It directly interacts with CK1ε and it has been described to be necessary during initial signaling events to approach the constitutively bound CK1ε to the signaling complex[123]. It has been shown to be also relevant, once released from cadherins, to activate Rac1 and promote β-catenin translocation to the nucleus[124] and, finally, to release Kaiso repression from TCF/β-catenin promoters[125]. Since CK1ε kinase is important in canonical and non-canonical Wnt pathway for Dishevelled phosphorylation[48, 63, 126] and p120-catenin has also been involved in the release of Kaiso-mediated repression of the non-canonical Wnt11 ligand[127], we considered that p120-catenin might be relevant also in other signaling steps of the non-canonical Wnt pathway.

2.5.2.1.   P120-catenin structure

P120-catenin was first described in a screening of substrates of the tyrosine kinase Src[128]. It belongs to the family of proteins with an armadillo-repeat domain (ARM). Structurally, p120-catenin is formed by a central armadillo domain flanked by N-terminal and C-terminal regions (Figure I-8). The armadillo domain is the region through which p120-catenin interacts with the juxtamembrane region of cadherins and with the transcriptional repressor Kaiso. Depending on their similarities to p120-catenin, there are two subgroups inside this family of proteins. The first group shares 45% homology in the ARM domain and it comprises ARVCF, δ-catenin and p0071. The second group, with only 30% of homology, corresponds to proteins that are present in desmosomes, such as plakophilins.
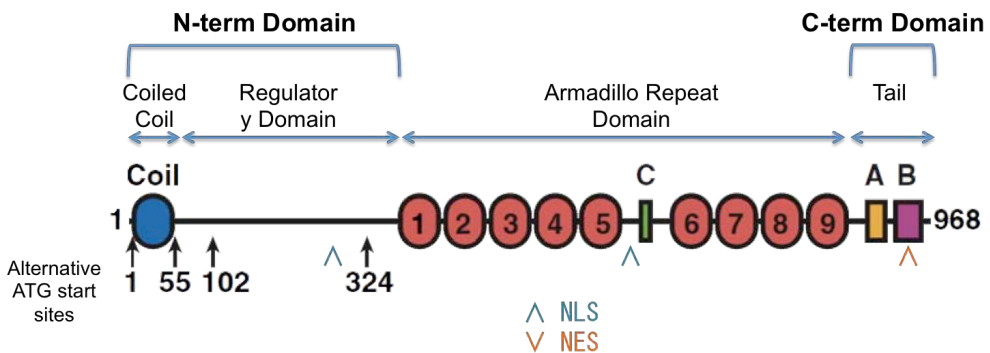
Figure I-8. Diagram of the p120-catenin domains. p120-catenin is formed by a tail corresponding to C-terminal domain, a central Armadillo Repeat domain (9 ARM domains) and the N-terminal domain. This last one is composed of coiled coil domain and the regulatory domain. In the figure the 4 different transcription start sites are shown and the 3 exons (A, B and C) whose presence is determined by alternative splicing. Localization of nuclear export signal (NES) and nuclear localization signal (NLS) are also depicted. Figure adapted from [129] and [130].

P120-catenin presents different isoforms originated by alternative splicing. The C-terminal tail contains exons depicted as A and B, the latter with a nuclear export signal (NES). The N-terminal domain is formed by the coiled coil domain and the regulatory domain. This N-terminal tail has four different transcriptional start sites that give name to four main p120-catenin isoforms:

Isoform I (starts at amino acid 1), Isoform II (starts at aa 55), Isoform III (starts at aa 102) and Isoform IV (starts at aa 324)[131] (see also figure I-8). The regulatory domain contains most of tyrosine, serine and threonine phosphorylable residues in p120-catenin[132].

The different transcriptional start sites and the splicing variants generate myriad forms of p120-catenin that are differentially expressed depending on the cell tissue. This divergent expression shows functional dissimilarities between the isoforms. Roughly, the shorter isoforms (Isoform III) are associated with E-cadherin and are representative of epithelial tissue, whereas longer isoforms (Isoform I) are found in association with N-cadherin in cells with more mesenchymal and mobile phenotype[133, 134].

2.5.2.2. P120-catenin function

2.5.2.2.1. *Adherent junctions regulation*

P120-catenin colocalizes with cadherins and is aberrantly localized in the cytoplasm in those cell lines that have lost E-cadherin. Cadherins are then necessary and sufficient to promote membrane recruitment of p120-catenin[135]. P120-catenin is required for the formation of strong adherent junctions and lateral oligomerization of cadherins[136]. P120-catenin is also important in cadherin transport to the membrane [137] and in adhesion complexes organization[138, 139]. However, the main role of p120-catenin in adherent junctions is to regulate cadherin stability at the plasmatic membrane[129]. P120-catenin dissociation of adherent junctions promotes a fall in cadherins protein levels[140] due to endocytic internalization[141, 142]. The juxtamembrane (JMD) region of E-cadherin, where p120-catenin binds, contains residues implicated in clathrin-mediated endocytosis and Hakai-dependent ubiquitination. Thus, p120-catenin release from cadherins exposes binding sites for the adaptor protein complex 2 (AP-2) and the E3 ligase Hakai. AP-2 promotes clathrin-dependent endocytosis of cadherins capable to be recycled back to the membrane. On the other hand, Hakai induces cadherins ubiquitination and degradation via proteasome.

P120-catenin association to E-cadherin is regulated by phosphorylation. P120-catenin was first described as a Src substrate, but can also be phosphorylated by growth factors receptors, EGF[143], HGF[144] and

VEGF[145]. Concerning Src kinase, eight different phosphorylation sites have been described in p120-catenin, all localized in the N-terminal domain comprising residues 250-350[146]. P120-catenin binds to different tyrosine kinases such as Fer and Fyn [147, 148], tyrosine phosphatases and to the serine/threonine kinase CK1ε[123]. Phosphorylation on p120-catenin tyrosine residues increases interaction with cadherins[147, 149, 150]. On the other hand, tyrosine phosphorylation of E-cadherin on residues Y755/Y756[129] or serine phosphorylation of p120-catenin S268/S269 by CK1α induces the dissociation of p120-catenin from cadherins[151].

2.5.2.2.2. *Control of Rho family GTPases*

The GTPases of the Rho family (RhoA, Rac1 and Cdc42) are involved in the regulation of cell migration by the remodeling of the actin cytoskeleton[152, 153, 154]. P120-catenin has been described to be relevant in the regulation of the GTPases. For this purpose, p120-catenin must be uncoupled from cadherins and accumulate in the cytosol where it activates Rac1 and Cdc42 and inhibits RhoA[155, 156]. The mechanism by which p120-catenin affects the activities of RhoGTPases is pleiotropic:

1. Direct association of p120-catenin with GTPases. P120-catenin acts as a guanine-nucleotide dissociation inhibitor suppressing RhoA activity[157, 158]. This association of p120-catenin is stabilized by Src tyrosine phosphorylation and destabilized by Fyn phosphorylation[158]. On the other hand, p120-catenin also interacts with Rac1. This association however does not impede intrinsic GTPase activity of Rac1 and promotes cell motility[159]
2. P120-catenin association with RhoGEFs or RhoGAPs. P120-catenin bound to cadherin in newly formed adherent junctions, locally interacts with Vav2 to activate Rac1 and Cdc42 and promotes the remodelation of the actin cytoskeleton[160]. This interaction has also been reported in the activation of the canonical Wnt pathway; cadherin-released p120-catenin that have undergone tyrosine dephosphorylation and Ser/Thr phosphorylation associates with Vav2 and activates Rac1[124]. Another function of p120-catenin consists in promoting the cortical localization of p190 RhoGAP and this mediates Rac-dependent suppression of RhoA activity[161].

3. P120-catenin interacts with Rho GTPases effector proteins. Rho-associated protein kinase 1 is recruited to cell-cell contacts by its association with p120-catenin[162]. In this context p120-catenin acts as a scaffold protein mediating the interaction of GTPases with its effectors. In agreement with this hypothesis, p120-catenin facilitates Rac1 association to PAK1 in newly formed cell-cell contacts[163].

Furthermore, p120-catenin isoforms act differently regulating Rho GTPases. Isoform I inhibits RhoA while Isoform IV activates it. Epithelial isoform III, however, has no effect upon RhoA activity[134]. Moreover, the p120-catenin stable transfection induces cell motility[164] but this effect on cell motion is affected by the cadherin context in the cell[165].

2.5.2.2.3. *Nuclear signaling*

Another function of p120-catenin is the transcriptional regulation of canonical Wnt-dependent genes. Cytosolic p120-catenin (released from adherent junctions) promotes, through Rac1 activation, β-catenin entry to the nucleus once it has been stabilized[166]. Another mechanism of action of p120-catenin regulating gene transcription is dependent on its binding to the transcriptional repressor Kaiso. Inside the nucleus, Kaiso is bound to TCF-4[167] preventing the interaction of the transcription factor with the DNA. Moreover, it is also associated to β-catenin thus inhibiting TCF-4 and β-catenin interaction[125]. Cadherin released p120-catenin triggered by Wnt enters the nucleus and associates with Kaiso [168]. This interaction releases the transcriptional inhibition of Kaiso over β-catenin/TCF-4 complex and allows gene transcription[125, 169, 170].

3. Canonical Wnt pathway

Canonical Wnt signaling or β-catenin-dependent pathway is defined by the capability of the signalosome to stabilize the cytoplasmic β-catenin levels and translocate it to the nucleus where it activates TCF/LEF dependent genes.

In the absence of Wnt ligand, the β-catenin degradation complex formed by Axin, APC and the kinases GSK-3 and CK1α, phosphorylates cytoplasmic β-catenin promoting its degradation via proteasome. Upon Wnt activation, the degradation complex is inhibited and thus, β-catenin degradation is blocked and allowed to accumulate. Stabilized β-catenin then translocates to the nucleus and activates its target genes[171].

Co-receptor LRP5/6 is constitutively bound to cadherins and, through this interaction, to proteins such as p120-catenin[123] (Figure I-9a). P120-catenin, apart from interacting with cadherin, also binds to CK1ε. This interaction includes the casein kinase in the complex and indirectly associates to cadherin and LRP5/6[123].

When Wnt ligand is present, Fz and LRP5/6 interact (Figure I-9b) and CK1ε kinase gets closer to Fz receptor constitutively-bound PR61ε (the regulatory subunit that confers specificity to the phosphatase PP2A). PR61ε then regulates the dephosphorylation and activation of CK1ε by PP2A[172] (Figure I-9b). CK1ε activation is followed by Dvl-2 recruitment to Fz[42] (Figure I-9c). It is not clear the exact role of CK1ε: one possibility is that it directly modifies Dvl-2 increasing its affinity for Fz or, alternatively, it phosphorylates Fz motifs required for Dvl-2 interaction.

A form of LRP5/6 lacking the extracellular domain is capable of activating Wnt pathway without the presence of the ligand, since it promotes signalosome clustering. Hence the name of constitutively active LRP5/6 (CA-LRP)[55]. CA-LRP promotes Dvl-2 hyperphosphorylation, Dvl-2 binding to LRP5/6 and the reactions downstream even in the absence of CK1ε[172]. This means that the point of action of the casein kinase is upstream of receptor clustering, and Dvl-2 hyperphosphorylation is probably a consequence and not the cause of the signalosome formation.
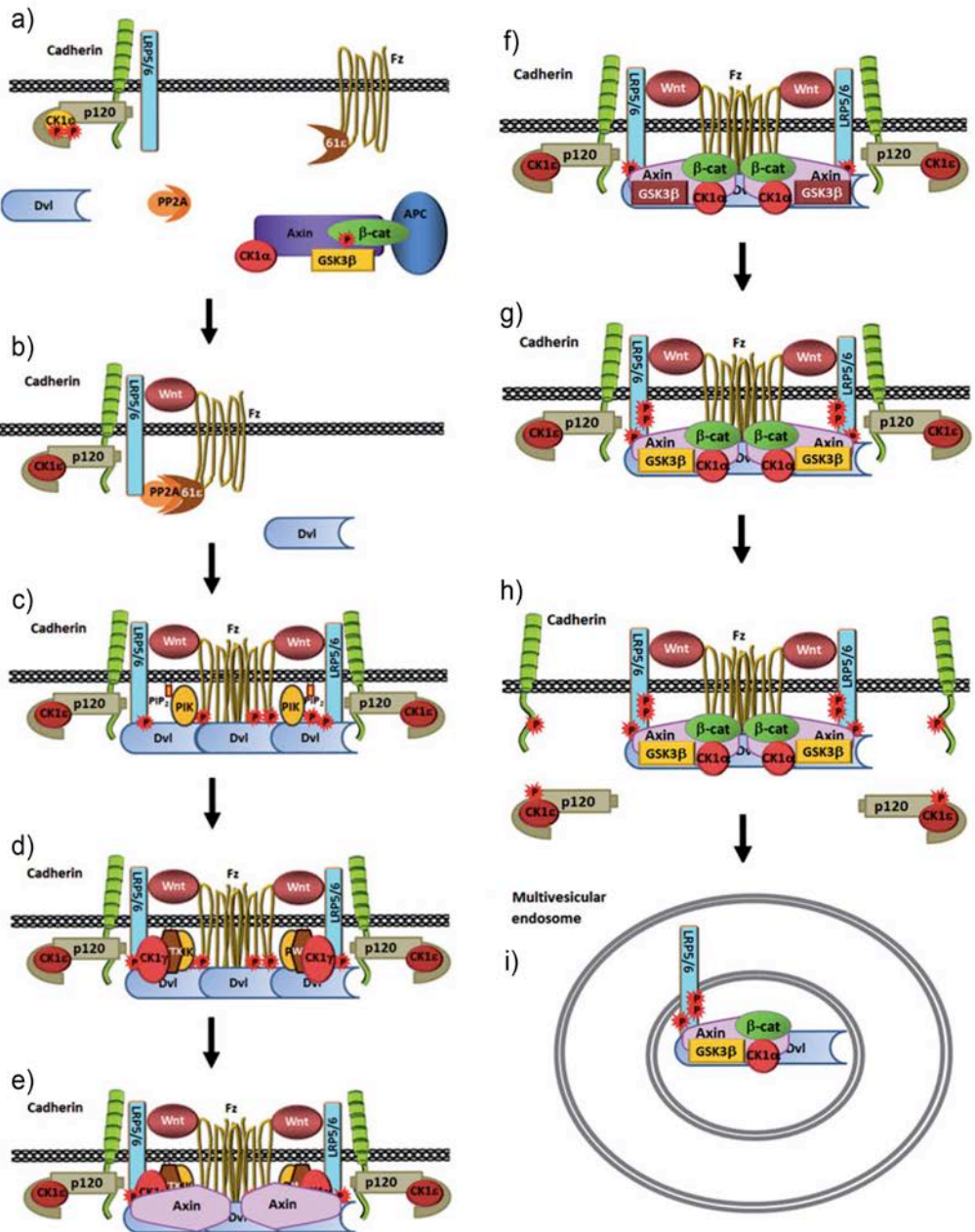
Figure I-9. Canonical Wnt Pathway. Representation of signaling steps from canonical Wnt ligand stimulation to MVB formation. Details are provided in the text. Adapted from [180]

Dvl-2 binding induces the recruitment and activation of phosphatidyilinositol 4-kinase IIα and phosphatydilinositol 4-phosphate 5-kinase 1β generating PtdIns(4, 5) P_2 [173] (Figure I-9c). This phospholipid promotes recruitment of Amer/WTX to the plasma membrane and Amer/WTX-mediated association of CK1γ to LRP5/6 [174] (Figure I-9d). CK1γ phosphorylates LRP5/6 on Thr1479 and probably other residues creating docking sites for Axin.

This process enhances the interaction of Axin, and associated protein kinases GSK3 and CK1α, with LRP5/6[52] (Figure I-9e).

Axin association to LRP5/6 promotes the rapid inhibition of GSK3 (Figure I-9f). A more recent model indicates that Axin binding to LRP5/6 promotes GSK3 phosphorylation of PPPSPxS LRP5/6 motifs (such Serine 1490) thus creating and inhibitory site for GSK3[175, 176](Figure I-9g).

Afterwards, p120-catenin is phosphorylated on Ser268/269 by CK1α disrupting cadherin and p120-catenin interaction and releasing this catenin from the complex[151] (Figure I-9h). At this point, and once p120-catenin has been dissociated, cadherin is also phosphorylated by CK1α promoting its dissociation from the complex[151] (Figure I-9h). This separation enables the internalization of LRP5/6 together with Dvl-2 in multivesicular bodies (MVB)[177, 178] (Figure I-9i), providing an additional level of GSK3 inhibition because the access of the kinase to substrates is prevented by physical separtion. The process has been named Wnt/STOP since Wnt increases half-life of many proteins whose stability is sensitive to GSK3 inhibition[179].

Cadherin-released p120-catenin phosphorylated on Ser268/269, now participates in the activation of Rac1. For this process to happen, p120-catenin interacts with the Guanine Exchange Factor (GEF) Vav2. Apart from Ser phosphorylations, Wnt induces tyrosine dephosphorylation of p120-catenin to favor p120-catenin interaction with Vav2 and Rac1[124, 164]. Vav2 function allows Rac1 exchange of GDP by GTP and PAK1 activation. In turn PAK1 phosphorylates JNK2 kinase and finally, JNK2 phosphorylates β-catenin which then shuttles from the cytoplasm to the nucleus[166]. β-catenin then, once p120-catenin releases repressor Kaiso, activates Wnt dependent genes (see p120-catenin *nuclear signaling* section) (See [180] for a review and figure I-9).

4. Non-canonical Wnt pathway

Non-canonical Wnt pathway has been normally divided into two major groups: the Wnt-Ca^{2+} pathway and the PCP/CE pathway (Figure I-10).

4.1. Wnt/Ca^{2+} pathway

The characteristic feature of this pathway is that Wnt signaling induces an increase in intracellular calcium. This pathway implicates also downstream factors such as phospholipase Cβ (PLCβ) that cleaves phospholipid phosphatidylinositol 4,5-bisphosphate (PIP$_2$) and form inositoltrisphosphate (IP$_3$) and diacylglycerol (DAG). PLCβ activation is regulated by heterotrimeric G-protein and probably involves $G_{i/o}$ or G_t family[181]. However, the mechanism is still not clear. The intracellular calcium release triggered by IP$_3$ activates calcium-sensitive enzymes: protein kinase C [182, 183] (PKC) and calcium/calmodulin-dependent kinase II[184, 185] (CamKII), the calcium-sensitive phosphatase calcineurin (CaCN) [186] and the calcium-sensitive protease calpain[187, 188]. The activation of these calcium-sensitive enzymes triggers different downstream effects. CamKII induces TGFβ-activated kinase (TAK) phosphorylation and activation which, in turn, activates Nemo-like kinase (NLK)[185]. At the same time, PKC phosphorylates retinoic acid-related orphan nuclear receptor α (RORα) enabling its nuclear functions[189].

4.2. Planar Cell Polarity or Convergent Extension pathway

PCP pathway is necessary for embryo morphogenesis. This prominent biological phenomenon arranges epithelial cells within the plane of a cell sheet. This process is important in cellular movements occurring during gastrulation and neural tube closure[190] and is regulated by Wnt ligands Wnt5a or Wnt11[191].

Given the phenotypical similitudes observed between Ror2- and Wnt5a-deficient mice, Ror2 has been considered as the main receptor for Wnt5a in non-canonical PCP Wnt pathway. Mice deficient for Ror2 or Wnt5a show a shortened AP axis due to improper CE movements[12]. Ror2 binds Wnt5a through its CRD. This interaction has been proven necessary to mediate Wnt5a signals intracellularly[61, 192]. Moreover, Ror2 binds to Fz also through its CRD[61,

[71]. This interplay of Ror2 with Wnt5a and with Fz, leads to the activation of several cellular functions that lead to cell polarization, migration and invasion.

4.2.1. Cell polarity, migration and invasion

In cell polarity Dvl plays a key role activating Rac1 and RhoA and its downstream effectors JNK and Rho-associated protein kinase, respectively. This leads to reorganization of the actin cytoskeleton to induce directional cell migration within tissues. For this pathway to be activated, Ror2 forms a trimeric complex with receptor Fz and the ligand Wnt5a[71, 193, 194]. Collagen triple helix repeat containing protein 1 (Cthrc1) selectively promotes PCP pathway forming a complex with Wnt5a-Fz-Ror2[195]. Ror2 has also been shown to interact with the four-pass transmembrane protein Vangl2. In this complex, Ror2 promotes Vangl2 activation through its serine and threonine phosphorylations (by CK1δ)[196].

Wnt5a together with Ror2 increases the migration of mouse embryonic fibroblast[72]. According to the Ror2-deficient mouse fibroblastic cell line L, Wnt5a is only capable of stimulating its migration after ectopic expression of full-length Ror2. The expressed Ror2 requires the CRD and PRD to bind to Wnt5a and Filamin A, respectively. This interaction induces filopodia formation and promotes cell migration[72].

Wnt5a has been shown to stimulate wound closure in a wound-healing assay using NIH-3T3 by inducing the formation of lamellipodia at the leading edge through JNK activation. In this context, wound closure is impaired when Ror2 expression is suppressed in NIH-3T3[193]. This emphasizes the role of Wnt5a-Ror2 in the induction of polarized cell migration. In wound closure, Dvl is phosphorylated after Wnt5a stimulation and, moreover, is required for Wnt-induced cell migration[72, 197]. For this reason, Dvl interacts with Dvl-associating protein with a high frequency of leucine residues (Daple), which promotes its association with aPKC and regulates Wnt5a-induced Rac1 activation[198].

To move from the plain migration to the invasion step, cells have to degrade the extracellular matrix (ECM). Wnt5a-Ror2-Fz axis regulate gene expression of activator protein 1 (AP-1) target genes[71]. Ror2 ectopically expressed in L cells induce, after Wnt5a stimulation, Dvl-2 polymerization that activates Rac1 and, in turn, leads to AP-1-dependent gene transcription. In

SaOS2 osteosarcoma cell line, Wnt5a-Ror2 axis activates c-Jun and ATF-2 through Dvl2–Rac1–JNK pathway[101]. This induces c-Jun/ATF-2 binding to the AP-1-binding site within the matrix metalloproteinase-13 promoter and the transcription of MMP-13[101, 199]. MMP-13 allows ECM breaking and cell escaping from the primary tumor site increasing invasiveness.
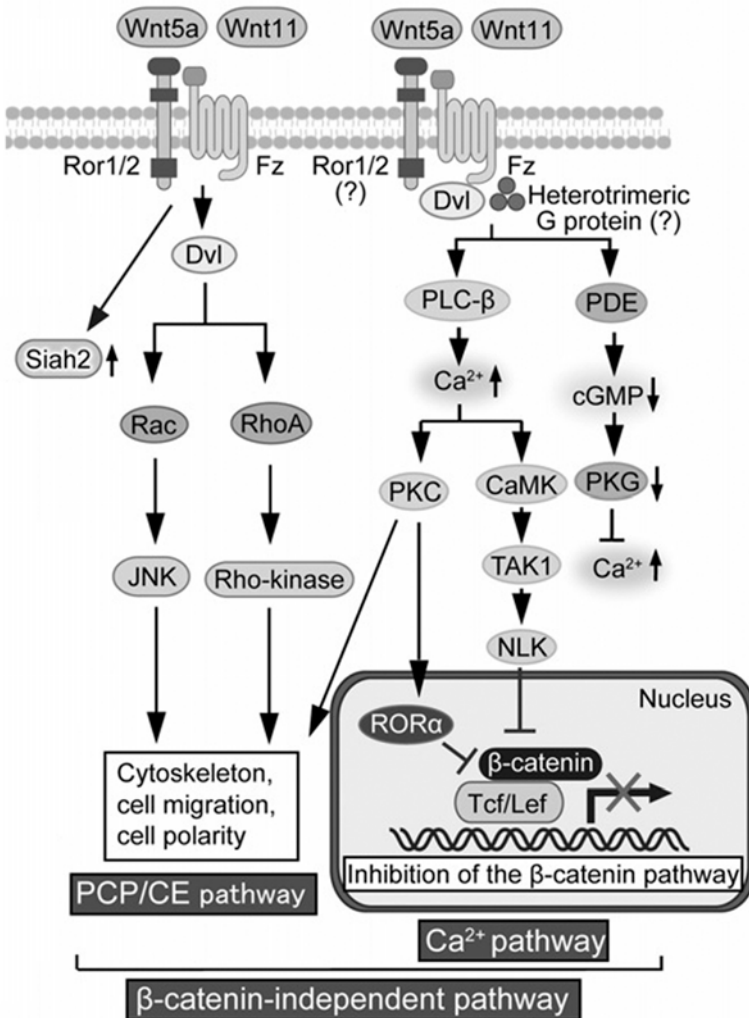
Figure I-10. Summary of general non-canonical Wnt pathway activation. For more details of specific signals upon activation, see text. Adapted from [37].

4.3. Inhibition of β-catenin-dependent pathway by Wnt5a

Initial evidence from *Xenopus* embryos indicates that Wnt5a suppresses axis duplication induction by XWnt8, which activates canonical Wnt pathway [200]. Different mechanisms have been proposed for this suppressive activity of Wnt5a over canonical signalization. One of them is that Wnt5a inhibits β-catenin dependent pathway because it limits Wnt3a access to Fz2 receptor by competing for the interaction[194]. The second mechanism is that Wnt5a induces the expression of Siah2, an ubiquitin E3 ligase that degrades β-catenin[201]. In this scenario, Wnt5a directly affects the stability of β-catenin inducing another degradation system, in this case, GSK3-independent. Moreover, Wnt5a signaling affects the transcription of β-catenin-dependent genes through a mechanism that has been shown to be dependent on Ca^{2+} pathway activation, since it involves the activity of NLK and RORα. Wnt5a triggers the kinase cascade CaMK-TAK-NLK which at the end phosphorylates and inhibits the transcriptional activity of β-catenin dependent TCF[185]. Besides, Wnt5a activation of PKCα phosphorylates RORα and this, in turn, inhibits the recruitment of coactivators (p300 and CBP) to the promoter region of β-catenin-dependent genes[189]. To sum up, Wnt5a affects canonical Wnt pathway activation at multiple levels (Figure I-10).

Objectives

OBJECTIVES

Canonical and non-canonical Wnt pathways have been described to require CK1ε to promote ligand-dependent pathway activation. Although they use a different co-receptor, LRP5/6 in canonical and Ror2 in non-canonical, both pathways promote Dvl-2 phosphorylation and recruitment to Fz.

The goal of this thesis is to study the initial events triggered by binding of the non-canonical Wnt ligand to its receptor and:

- Determine if p120-catenin is also relevant for non-canonical Wnt signaling.

- Characterize if CK1ε is activated and elucidate the mechanism.

- Demonstrate that p120-catenin and CK1ε are relevant for late responses to non-canonical Wnt.

Results

1. Canonical and non-canonical Wnt ligand share a common Fz receptor and a similar initial step but differ in the final response

The Wnt pathway has been long described to be a complex signaling pathway that is activated through the binding of Wnt ligands to the transmembrane Fz receptors and depending on the co-receptor used, Wnt-Fz complexes activate the canonical or the non-canonical Wnt pathway. In the case of the canonical Wnt pathway, Wnt interacts with the receptor Fz and the co-receptor LRP5/6 promoting β-catenin stabilization[202]. In the case of the non-canonical Wnt pathway, much less studied, although the receptor is also Fz, several co-receptors have been reported[202]. We centered this study in the transmembrane tyrosine kinase-like co-receptor Ror2. As main representative Wnt factors, we used the well-documented Wnt3a for the canonical signaling and Wnt5a for the non-canonical. These ligands are produced and secreted by the stably transfected L-M(TK-) cells with Wnt3a or Wnt5a plasmids (see Materials and Methods).

1.1. After ligand stimulation, both pathways phosphorylate JNK and Dishevelled

The project was started comparing initial responses, as Dvl-2 and JNK phosphorylation, since they have been described to be common to both signaling pathways[101, 124, 126]. We confirmed these results in a time course of Wnt3a and Wnt5a-treated HEK 293T cells (Figure R-1). Dvl-2 phosphorylation kinetics were similar between both pathways, but JNK phosphorylation occurred faster and more efficient with Wnt5a. Dvl-2 phosphorylation was detected by the shift produced in the electrophoretic mobility of this protein. As observed, each ligand showed specific phosphorylation patterns. Thus, LRP5/6 was only phosphorylated in Thr1490 by Wnt3a whereas ERK1/2 was strongly phosphorylated just by Wnt5a.

1.2. Canonical and non-canonical Wnts recruit Dvl-2 to Fz receptor, although they differ in the co-receptor.

Both Wnt factors promote Dvl-2 interaction with Fz, as shown by co-immunoprecipitation experiments in figure R-2. Moreover, each signaling

pathway specifically recruited its own co-receptor: LRP5/6 in the case of canonical Wnt3a and Ror2 when cells were stimulated with Wnt5a. As expected, Wnt3a also increased the association with Axin, but not Wnt5a. Other canonical Wnt responses, such as recruitment of CK1γ to LRP5/6 and Dvl-2 were only observed upon Wnt3a addition to the cells (Figure R-3). These results demonstrate that Wnt5a does not activate the canonical Wnt pathway.
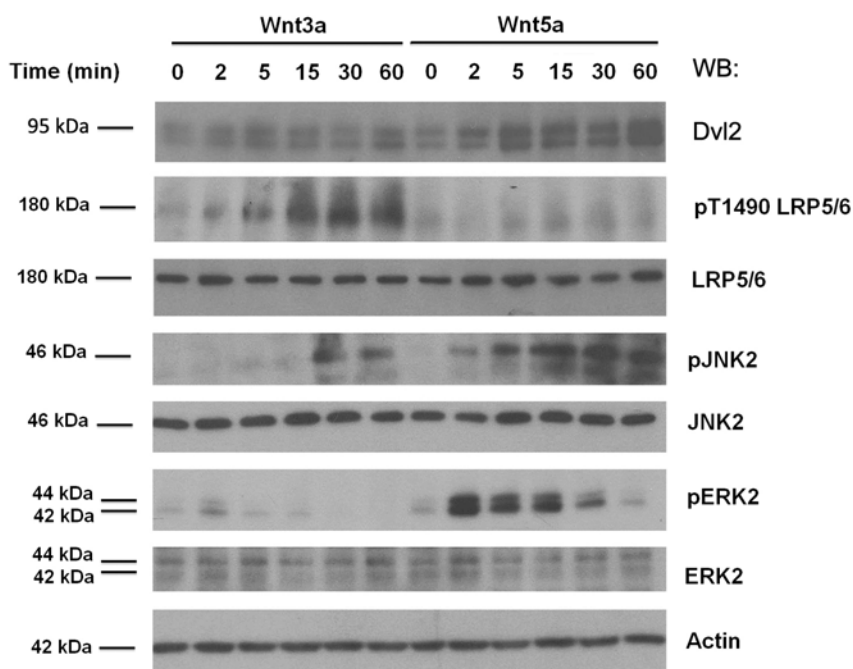
Figure R-1. Canonical and non-canonical Wnt ligands promote Dvl-2 and JNK phosphorylation. HEK293T cells were stimulated with Wnt3a or Wnt5a-conditioned medium for the indicated times. Cells were lysed and proteins were analyzed by WB with specific antibodies.

1.3. CK1ε and p120-catenin are also recruited to Fz upon non-canonical Wnt stimulation

Since CK1ε and p120-catenin have been previously described by our group to be required for Dvl-2 phosphorylation and recruitment to Fz by Wnt3a[123, 172] (see Introduction), we investigated whether these two proteins were also involved in non-canonical Wnt signaling. As shown in figure R-2, Wnt5a also increased p120-catenin and CK1ε binding to Fz receptor. It has been previously found in our group that the canonical co-receptor LRP5/6

constitutively interacts with cadherins and through these proteins with p120-catenin and CK1ε[123]. The result found in figure R-2 raised the possibility that an interaction between cadherin and Ror2 might also occur.
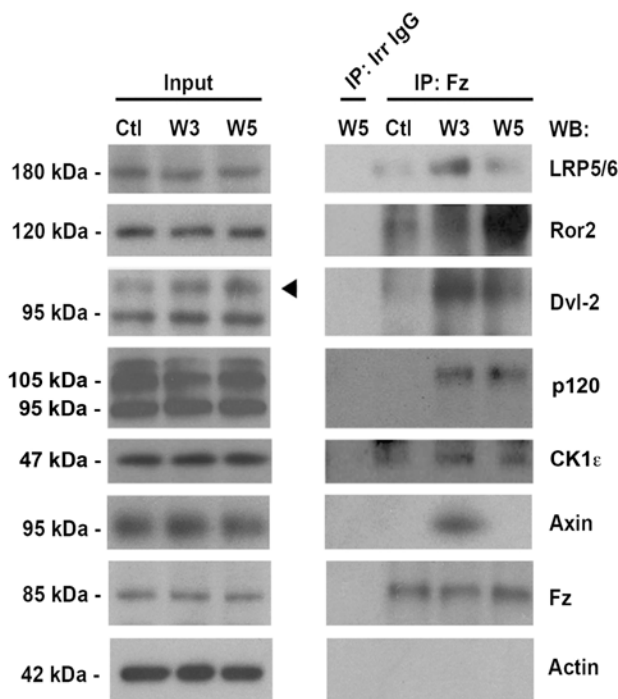
Figure R-2. Canonical and non-canonical Wnt factors promote Dvl-2, p120-catenin and CK1ε recruitment to Fz. HEK293T cells treated with control, Wnt3a or Wnt5a-conditioned medium for 15 min were lysed and Fz2 was immunoprecipitated with a specific antibody. Associated proteins were analyzed by WB. This experiment was done in collaboration with Aida Villarroel.

1.4. N-cadherin interacts only with the canonical Wnt co-receptor LRP5/6 and not with Ror2

In order to find out the mechanism leading to the binding of p120-catenin to Fz by Wnt5a, N-cadherin was immunoprecipitated. HEK293T cells were treated with control or Wnt5a-conditioned medium at a time point where Dvl-2 is already recruited to Fz. Whereas LRP5/6, p120-catenin and CK1ε were immunoprecipitated with cadherin, Ror2 was not present in the immunocomplex neither in control nor Wnt5a-stimulated cells (Figure R-4). Therefore these results indicate that p120-catenin and CK1ε form part of the Wnt5a signalosome through a different mechanism.
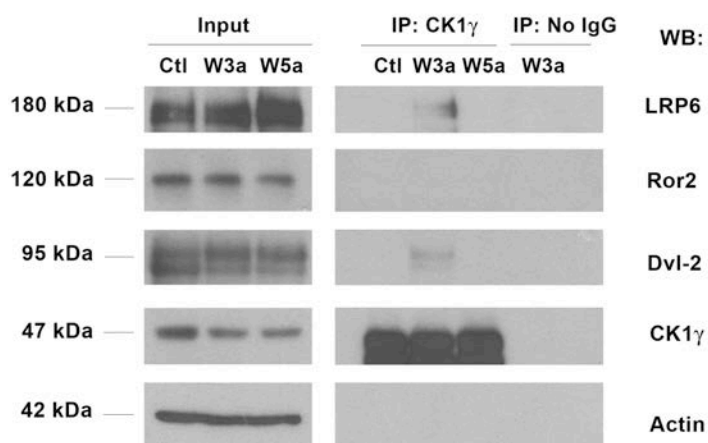
Figure R-3. Casein Kinase 1 gamma is recluted to LRP5/6 only after Wnt3a stimulation. HEK293T cells treated with control, Wnt3a or Wnt5a-conditioned medium for 15 min, were lysed and CK1γ was immunoprecipitated with a specific antibody. Associated proteins were analyzed by WB.
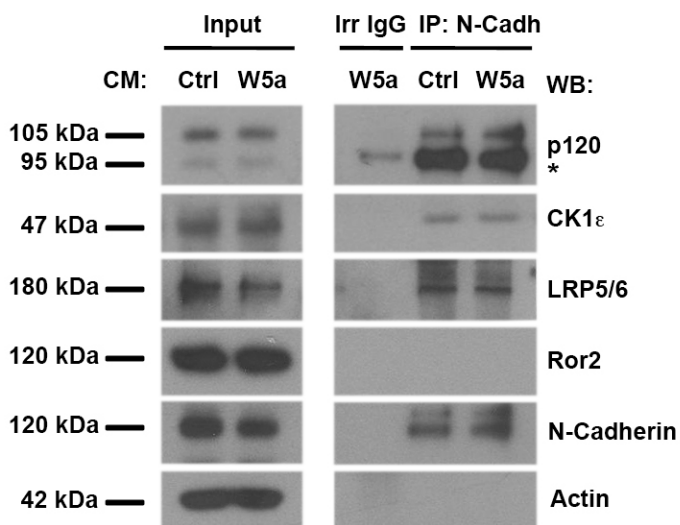
Figure R-4. N-cadherin interacts only with the canonical Wnt co-receptor LRP5/6 and not with Ror2. HEK293T cells treated with control or Wnt5a-conditioned medium for 5 min were lysed and N-cadherin was immunoprecipitated with specific antibody. Associated proteins were analyzed by WB.

2. The Non-canonical Wnt pathway depends on Ror2, CK1ε and p120-catenin complex

Since our group has previously observed that upon canonical Wnt activation, Dvl-2 phosphorylation and interaction with Fz is dependent on CK1ε activity and p120-catenin is required for this activation[123, 151], and since these two elements were also present in the non-canonical Wnt5a signalosome (Figure R-2), we further investigated the implication of p120-catenin and CK1ε in the non-canonical Wnt pathway.

2.1. Dishevelled recruitment is abolished in the absence of Ror2, CK1ε or p120-catenin

We determined the relevance of p120-catenin and CK1ε in the formation of the Wnt5a signalosome. We generated HEK mutants lacking p120-catenin or CK1ε using the CRISPR technology. After stimulation of these cells with Wnt5a, Fz was immunoprecipitated. As shown in figure R-5, when p120-catenin or CK1ε were absent, after stimulation with Wnt5a, Dvl-2 phosphorylation was decreased as well as its recruitment to Fz. Moreover, Ror2 interaction with Fz promoted by Wnt5a was also abolished in the absence of p120-catenin or CK1ε.

These results suggest that p120-catenin and CK1ε are needed for assembling the Wn5a signalosome. A similar requirement was found for the Wnt3a signalosome complex[151, 172, 178].

In canonical Wnt signaling CK1ε is activated by PP2A phosphatase; the action of this phosphatase on CK1ε is mediated by PR61ε regulatory subunit that interacts with Fz cytosolic C-tail[172]. As shown in figure R-6, depletion of PR61ε also prevented Wnt5a-induced Dvl-2 interaction with Fz. Therefore, these results indicate that similarly to canonical Wnt signaling, CK1ε is also activated by Wnt5a through the action of PR61ε-PP2A complexes and is required for Dvl-2 recruitment to Fz receptor.
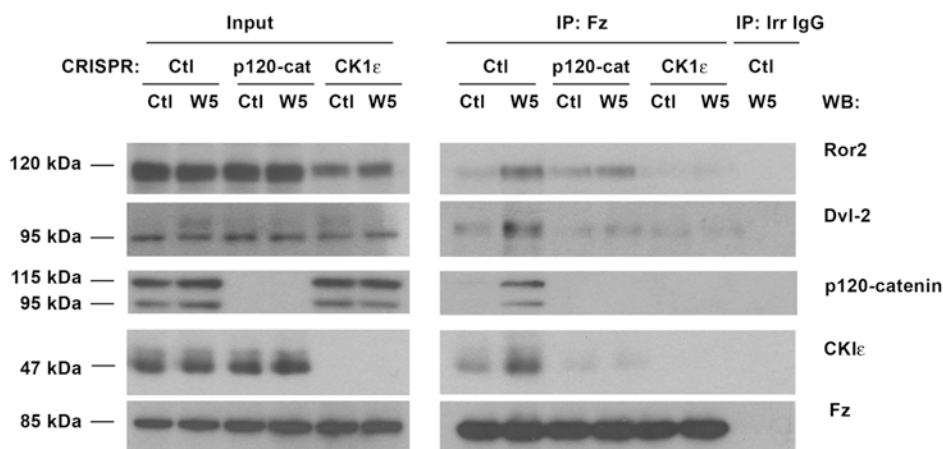
Figure R-5. . The Wnt5a-induced Dvl-2 and Ror2 recruitment to Fz is dependent on p120-catenin and CK1ε. Fz2 was immunoprecipitated from control, p120-catenin or CK1ε HEK 293T CRISPR whole-cell extracts treated with control or Wnt5a-conditioned medium for 5 min. Protein complexes were analyzed by WB with the indicated antibodies.
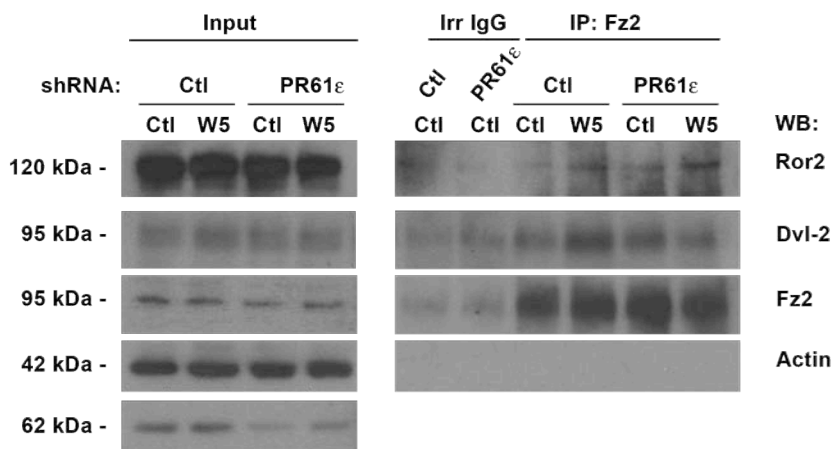
Figure R-6. The Wnt5a-induced Dvl-2 but not Ror2 recruitment to Fz is dependent on PR61ε. HEK293T cells depleted of PR61ε using specific shRNA or a scrambled shRNA as a control, were treated with control or Wnt5a-conditioned medium for 5 min. Fz2 was immunoprecipitated from total cell extracts and the protein complex analyzed by WB.

Despite this similarity, the mechanisms involving CK1ε in the non-canonical Wnt pathway seem to be distinct from those in Wnt3a. For instance, CK1ε depletion did not modify LRP5/6 total levels (see figure 3G from

Casagolda et al., 2010 ref. [123]) but curiously, total Ror2 levels were decreased (Figure R-5). This effect is analyzed below in Section 4. Moreover, p120-catenin depletion affected Ror2-Fz interaction upon ligand stimulation. One possibility is that Ror2, similar to cadherins, constitutively binds to p120-catenin and through this protein to CK1ε. P120-catenin was discovered as a substrate of Src-tyrosine kinase[128] and its interaction with several tyrosine kinases has also been described[147, 203]. For this reason we analyzed the effect of Ror2 downregulation in the Wnt5a initial events.

HEK 293T cells depleted of Ror2 by specific shRNA were stimulated with control or Wnt5a-conditioned medium. Ror2 downregulation not only blocked the Wnt5a-induced Dvl-2 binding to Fz, but also p120-catenin and CK1ε recruitment (Figure R-7).

All these results point out that p120-catenin and CK1ε are required for the initial events in the Wnt5a pathway and act upstream Dvl-2 recruitment to Fz. Next we examined whether they also affect other downstream responses to Wnt5a stimulation and how these proteins are coupled to the pathway.
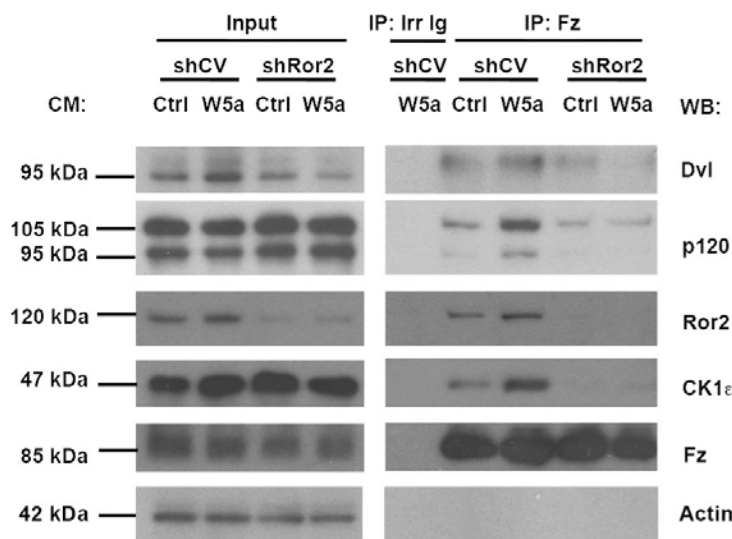
Figure R-7. The Wnt5a-dependent Dvl-2, p120-catenin and CK1ε recruitment to Fz depends on Ror2. HEK293T cells depleted of Ror2 using specific shRNA or a scrambled shRNA as a control, were treated with control or Wnt5a-conditioned medium for 5 min. Fz2 was immunoprecipitated from total cell extracts and the protein complex analyzed by WB.

2.2. Rac1 and JNK are not active when Ror2, CK1ε or p120-catenin are not present

Taking into account the effect of Ror2, p120-catenin and CK1ε in the recruitment of Dvl-2, downstream non-canonical Wnt responses were also analyzed. It has been described that JNK gets activated through Rac1 and that Rac1 is activated upon non-canonical Wnt stimulation[199, 204]. Therefore, HEK 293T cells were depleted of p120-catenin, CK1ε or Ror2 and assayed for Rac1 activation with the classic PAK pull-down assay. When the levels of these proteins were diminished, Rac1 was not activated in any of the cases (Figure R-8).
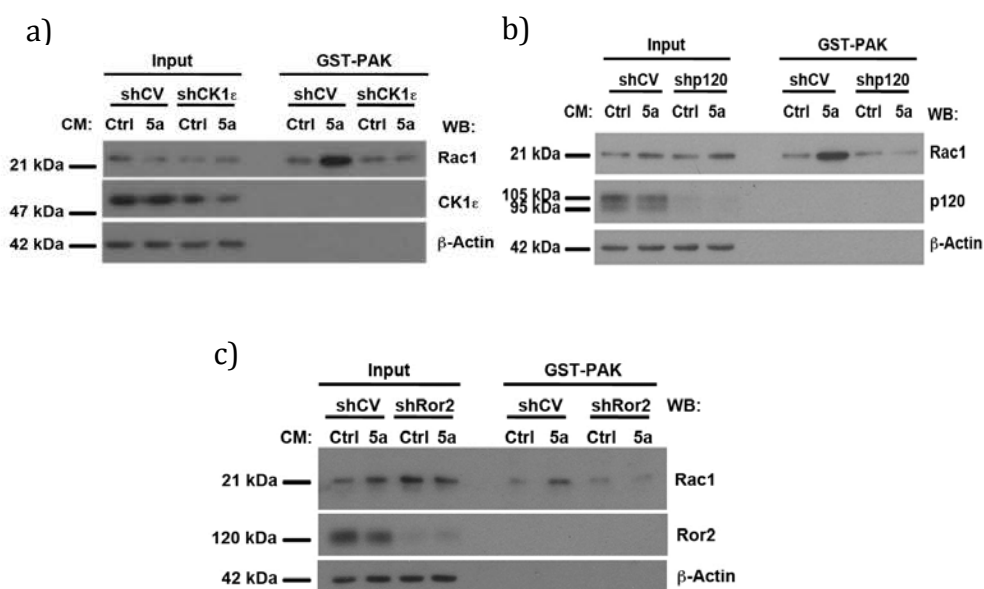
Figure R-8. Rac1 is not activated in the absence of Ror2, p120-catenin or CK1ε. a) HEK293T cells depleted of CK1ε were treated with control or Wnt5a-conditioned medium for 1 hour. GST–PAK pull-down assay was performed and active Rac1 was determined by WB. **b)** As in a) but p120-catenin was downregulated. **c)** Ror2 was downregulated.

In addition, the Rac1 downstream effector, JNK2, was not activated either when Ror2, p120-catenin or CK1ε were downregulated (Figure R-9). The Wnt5a-induced Dvl-2 phosphorylation was also abolished in all the cases. However, depletion of p120-catenin or CK1ε did not interfere with ERK2 activation; on the contrary this phosphorylation was enhanced, suggesting that

JNK2 and ERK2 belong to different branches of the non-canonical Wnt pathway.
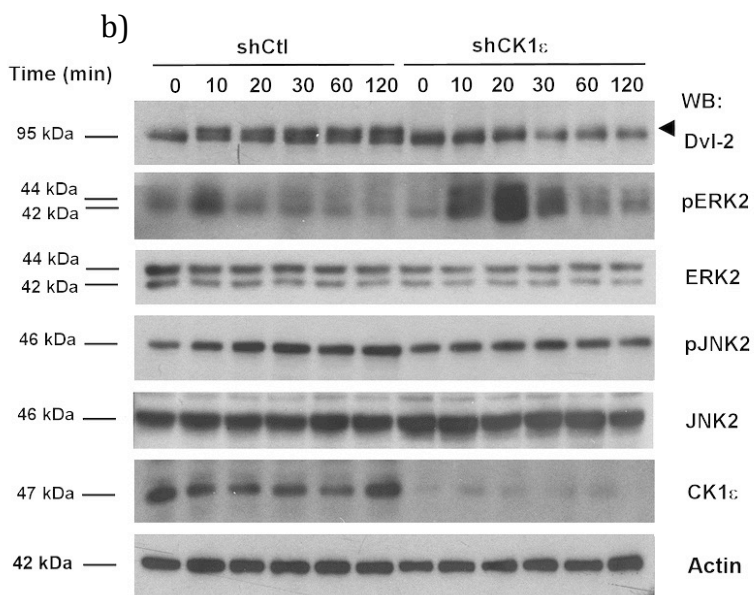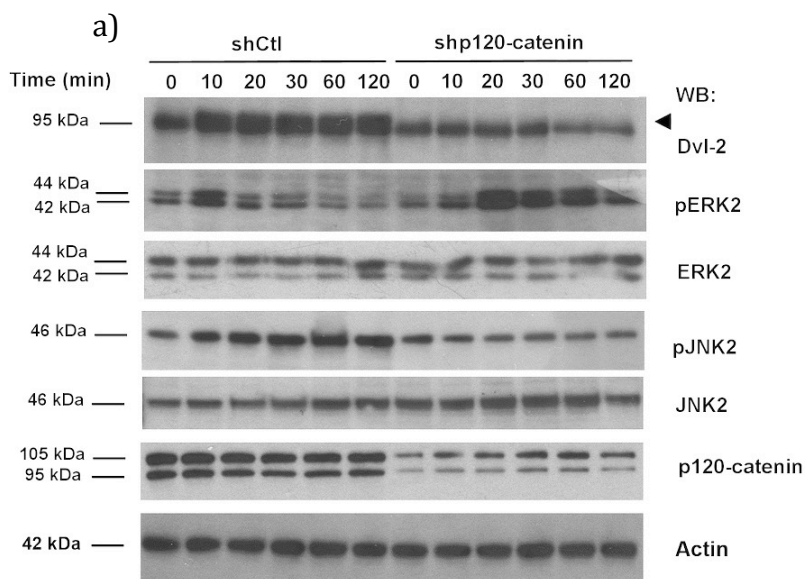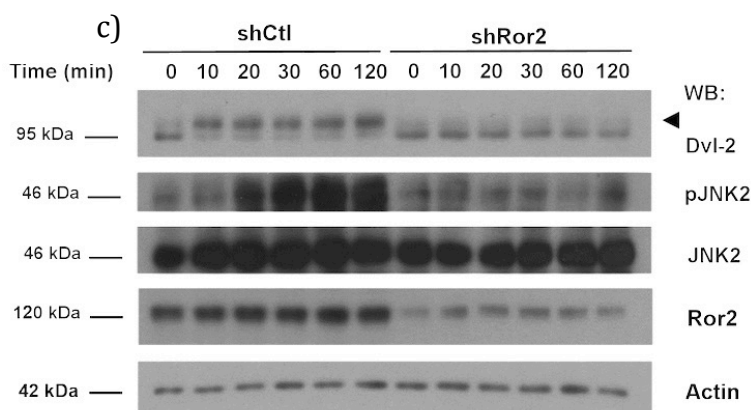
Figure R-9. JNK2 phopshorylation depends on Ror2, p120-catenin and CK1ε. a) HEK293T cells were depleted of p120-catenin using specific shRNA or a scrambled shRNA as a control. Cells were treated with control or Wnt5a-conditioned medium for the indicated times and total levels of phosphorylated proteins were determined by WB with specific antibodies. **b)** As in a) but CK1ε was downregulated. **c)** Ror2 was downregulated.

2.3. The Canonical Wnt stabilizes β-catenin while non-canonical Wnt downregulates β-catenin levels.

The characteristic event in the canonical Wnt pathway (or β-catenin dependent) is the ability to stabilize β-catenin protein levels. However, non-canonical Wnt activation has been described to produce the opposite effect, i. e. β-catenin downregulation[201]. As expected, Wnt3a stabilized β-catenin while Wnt5a promoted its down-modulation (Figure R-10).
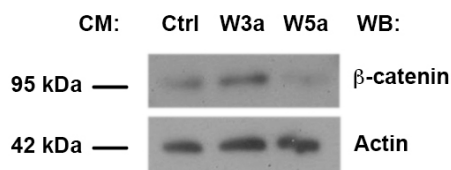
Figure R-10. Wnt3a stabilizes and Wnt5a downregulates β-catenin protein levels. HEK 293T cells were treated with Wnt3a or Wnt5a medium for 16 h and lysed. Protein levels were analyzed by western blot.

2.4. <u>P120-catenin and CK1ε are also required for the activation of down-stream Wnt5a signals, such as β-catenin downregulation</u>

We next determined whether p120-catenin and CK1ε were also necessary for other later responses to Wnt5a stimulation. Downregulation of Ror2, CK1ε or p120-catenin prevented the β-catenin down-modulation induced by non-canonical Wnt5a (Figures R-11a, R-11b, R-11d). P120-catenin depletion decreased β-catenin levels even in control conditions (compare lanes 1 and 5 in figure R-11b). The requirement of CK1ε was also demonstrated by the prevention of the Wnt5a-induced β-catenin down-modulation caused by depletion of PR61ε (Figure R-11c, R-11d), since this protein is required for CK1ε activation (see figure R-6).

β-catenin downregulation induced by non-canonical Wnt5a has been associated to the up-regulation of the β-catenin E3 ubiquitin ligase Siah2[201]. We observed that Siah2 shRNA prevented the β-catenin down-modulation by Wnt5a, since Siah2 depletion abolished the effect of the non-canonical Wnt ligand (Figure R-12a). To evaluate that the effect of the shRNA against Siah2 effectively downregulated the targeted mRNA, we performed a semiquantitative PCR of Siah2 in Wnt5a-treated cells (Figure R-12b). This result suggests the relevance of Siah2 upregulation for the Wnt5a-induced β-catenin decrease.

In accordance with results in figure R-11, where β-catenin was not downregulated in cells deficient of Ror2, PR61ε, p120-catenin or CK1ε, upon Wnt5a treatment they also failed to increase Siah2 levels (Figure R-13).
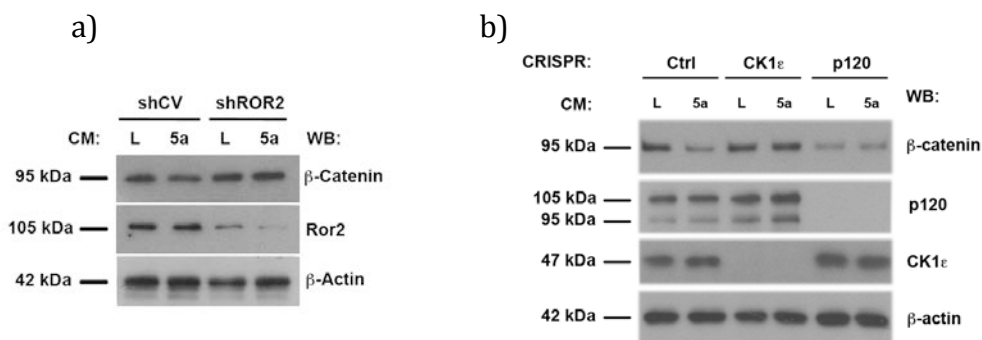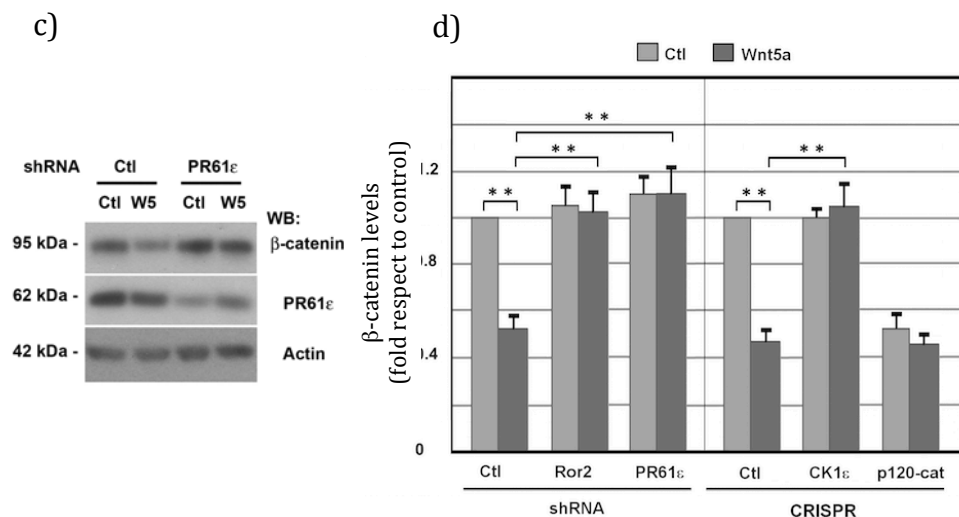
c)

d)

Figure R-11. β-catenin downregulation depends on Ror2, p120-catenin, CK1ε and PR61ε. HEK293T cells were depleted of Ror2 **a)** and PR61ε **c)** by using specific shRNAs or a scrambled. After 48 hours, cells were stimulated with control or Wnt5a-conditioned medium overnight, and β-catenin levels were analyzed by WB from total cell extracts. **b)** Control, p120-catenin and CK1ε HEK293T CRISPR cells were treated with control or Wnt5a-conditioned medium overnight. β-catenin levels were analyzed by WB. **d)** β-catenin levels were quantified by analyzing three independent experiments ± SD performed in a), b) and c) and p values < 0.01 are symbolized with two asterisks.

In order to better define the β-catenin downregulation in the non-canonical Wnt pathway, we also checked if this down-modulation was affected by inhibitors of kinases that are known to be activated by Wnt5a, such as PI3 kinase, ERK, JNK and Tyr kinases[205, 206, 207].

β-catenin downregulation was abolished only when JNK or tyrosine kinase activity (Herbimycin A) were inhibited (Figure R-14). Whereas ERK1/2 or PI3K inhibitors did not affect β-catenin down-modulation induced by Wnt5a.

All together these results suggested that β-catenin downregulation by Wnt5a occur under the axis we were trying to characterize, since JNK activity depends on Ror2, p120-catenin and CK1ε.
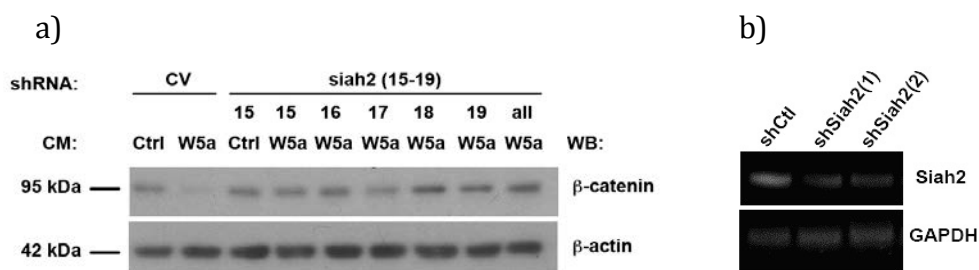
Figure R-12. Wnt5a-dependent β-catenin downregulation depends on Siah2. a) HEK293T cells were depleted of Siah2 using specific shRNAs or scrambled. After 48 hours, cells were stimulated with control or Wnt5a-conditioned medium overnight, and β-catenin protein levels were analyzed by WB from total cell extracts. **b)** as in a) but cells were only stimulated with Wnt5a-conditioned medium. Total RNA was extracted and a semi-quantitative PCR was performed.
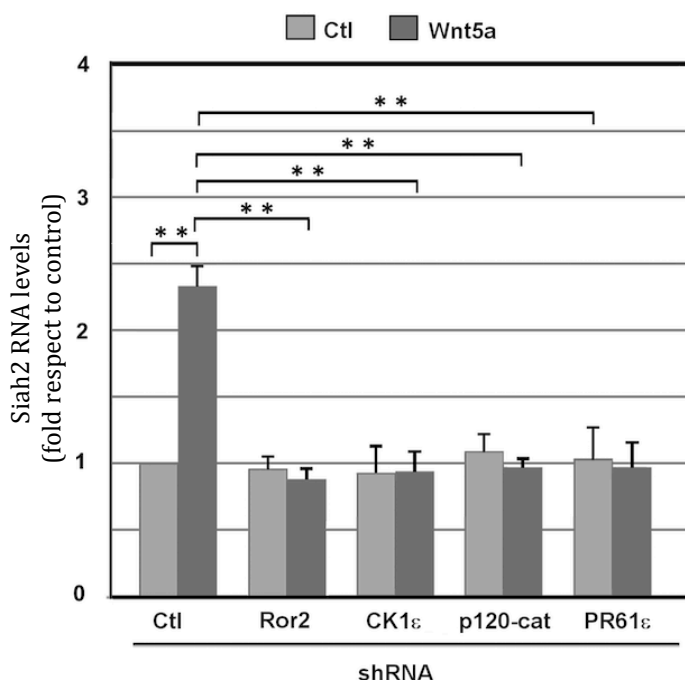
Figure R-13. Siah2 RNA upreglation depends on Ror2, p120-catenin, CK1ε and PR61ε. RNA was isolated from control, Ror2, CK1ε, p120-catenina and PR61ε-depleted HEK293T cells stimulated overnight with control or Wnt5a-conditioned medium. Expression of Siah2 was assessed by semiquantitative RT-PCR. Quantification of three independent experiments ± SD and p values < 0.01 are symbolized with two asterisks.

a)

b)
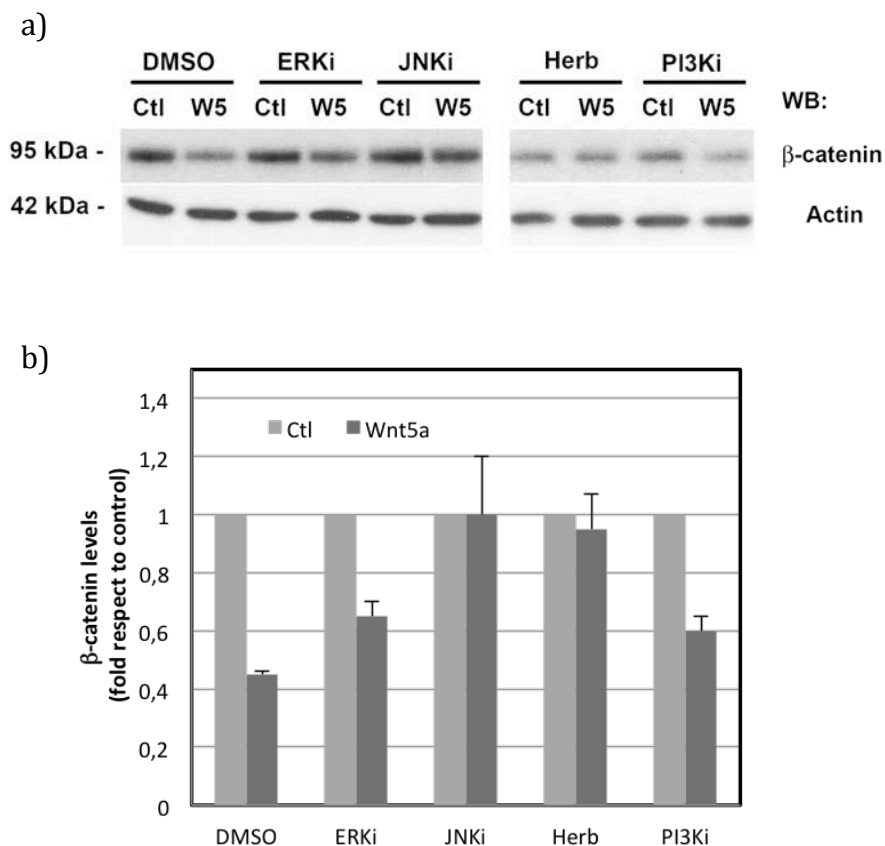
Figure R-14. β-catenin downregulation upon Wnt5a treatment depends on JNK and tyrosine phosphorylations. a) HEK 293T cells were treated overnight with ctrl or Wnt5a-CM plus different inhibitors: ERK1/2, JNK2, PI3K or a general tyrosine kinase inhibitor (Herbimycin A). Cell extracts were prepared and β-catenin levels were analyzed by Western Blot. **b)** Quantification of at least three independent experiments.

3. P120-catenin and CK1ε directly interact with Ror2

3.1. P120-catenin and CK1ε interact with Ror2

As shown in figure R-2, p120-catenin and CK1ε associated with Fz when cells were stimulated with Wnt5a, similarly to what occurs with Wnt3a. Binding of p120-catenin to Fz is not direct; in Wnt3a-stimulated cells it is dependent on N-cadherin and LRP5/6[123]. However, neither LRP5/6 (Figure-R3) was recruited to Fz nor N-cadherin interacted with Ror2 upon Wnt5a stimulation (Figure R-4). Therefore, we investigated the molecular basis for Fz-p120-catenin association.

P120-catenin and CK1ε interact with Ror2 as observed by co-immunoprecipitation experiments (Figure R-15). Formation of the complex was detected in non-treated cells and was stimulated by Wnt5a.
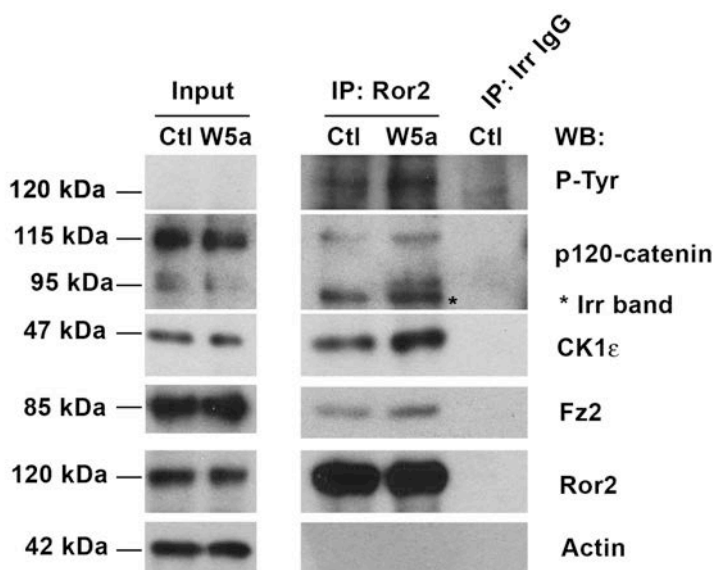
Figure R-15. P120-catenin and CK1ε interaction with Ror2 increases upon Wnt5a treatment. HEK293T cells were treated with control or Wnt5a-conditioned medium for 5 min. Cells were lysed and Ror2 was immunoprecipitated with a specific Ror2 antibody. Associated proteins were analyzed by WB.

Given that the increase in the interaction between endogenous Ror2 and p120-catenin was accompanied by an increase of CK1ε, and since we knew

that CK1ε and p120-catenin directly interact, we first thought that p120-catenin and CK1ε might come altogether to the complex.

3.2. The N-terminal domain of p120-catenin directly interacts with Ror2

P120-catenin directly interacts with several protein tyrosine kinases such as Fer and Fyn[147, 148]. Therefore, we examined if the p120-catenin-Ror2 complex happens through a direct association. Recombinant proteins corresponding to different domains of p120-catenin were used and the interaction with Ror2 was analyzed in a pull-down experiment. A GST-p120-catenin full-length fusion protein pulled-down Ror2 from cells extracts. Deletion of the first 102 amino acids did not affect this interaction; however, removal of the entire N-terminal regulatory domain (amino acids 1-350) prevented binding to Ror2 (Figure R-16). Therefore, these results suggest that binding to Ror2 takes place through amino acids 102-350 of p120-catenin.
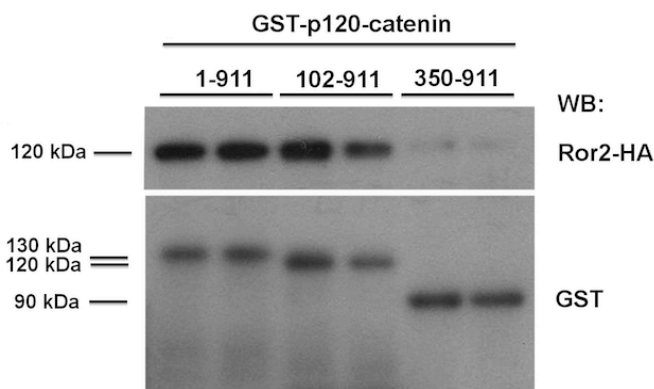
Figure R-16. The N-terminal regulatory domain of p120-catenin interacts with Ror2. GST–p120-catenin fusion proteins (7 pmol) were incubated with cells extracts overexpressing Ror2-HA and treated with Wnt5a-conditioned medium for 5 min. A pull-down assay was performed and bound Ror2-HA was analyzed by WB with anti-HA.

3.3. Tyrosine phosphorylation of p120-catenin increases its interaction with Ror2

Since the N-terminal regulatory domain of p120-catenin is phosphorylated by CK1 and Src[208], we determined the effect of p120-catenin phosphorylation in the interaction with Ror2. GST-p120-catenin was phosphorylated by recombinant Src or CK1 kinases and then a pull-down experiment was performed.

Src, but not CK1 phosphorylation increased p120-catenin/Ror2 interaction (Figure R-17).
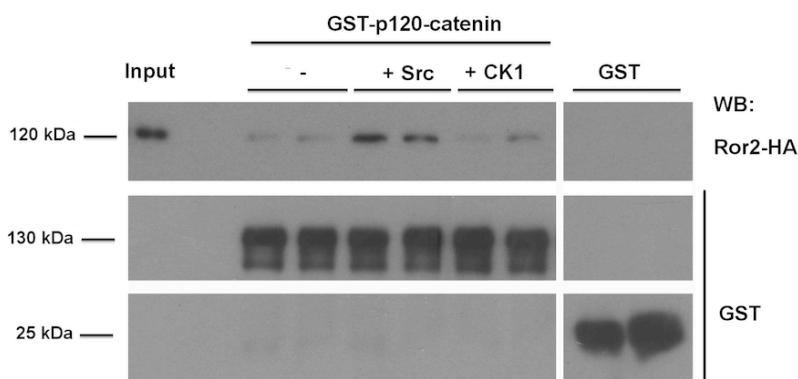
Figure R-17. p120-catenin tyrosine phosphorylation increases Ror2 affinity. Full-length GST–p120-catenin (7 pmol) was *in vitro* phosphorylated with recombinant Src or CK1 kinases for 2 hours. A pull-down assay was performed by incubating the fusion proteins with cell extracts overexpressing Ror2-HA. Protein complexes were affinity purified and analyzed by WB.

Next, a reversed binding assay was done with a phosphorylated GST-cytoRor2 fusion protein as bait. In this case, however, tyrosine phosphorylation of the recombinant cytosolic domain of Ror2 did not enhance its interaction with p120-catenin (Figure R-18).

These results suggest that the increased p120-catenin/Ror2 interaction observed upon Wnt5a treatment might be probably due to an enhanced p120-catenin tyrosine phosphorylation which increases its affinity for Ror2 co-receptor.

In this sense, both Ror2 and p120-catenin are phosphorylated in tyrosine residues after Wnt5a stimulation (Figure R-19).
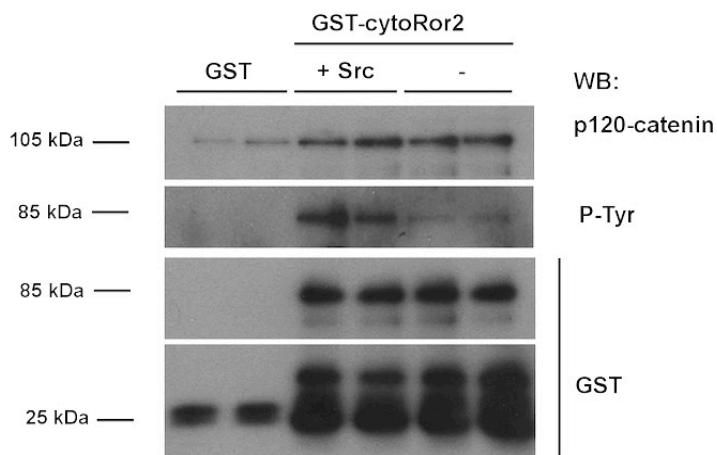
Figure R-18. Tyrosine phosphorylation of the cytosolic Ror2 domain does not increase interaction with p120-catenin. Recombinant GST–cytoRor2 (amino acids 426-944) was *in vitro* phosphorylated with recombinant Src kinase for 2 h. A binding assay was performed by incubating 2 pmol of GST-cytoRor2 with 1 pmol of recombinant p120-catenin.
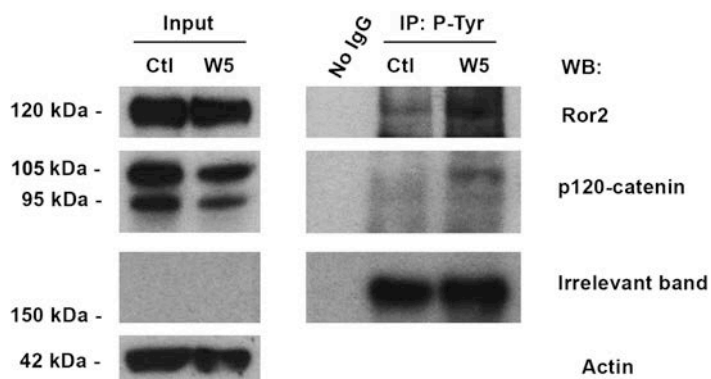
Figure R-19. P120-catenin and Ror2 are tyrosine phosphorylated after Wnt5a stimulation. HEK293T cells were treated with control or Wnt5a-conditioned medium for 5 min. Cells were lysed and tyrosine-phosphorylated proteins were immunoprecipitated with general anti-phosphotyrosine antibody. Associated proteins were analyzed by WB.

The requirement of p120-catenin tyrosine phosphorylation for Ror2 interaction was confirmed, since it was blocked with an inhibitor of these

protein kinases, such as Herbimycin A. As shown in figure R-20, p120-catenin tyrosine phosphorylation and interaction with Ror2 are increased upon Wnt5a treatment and are abolished by Herbimycin A.
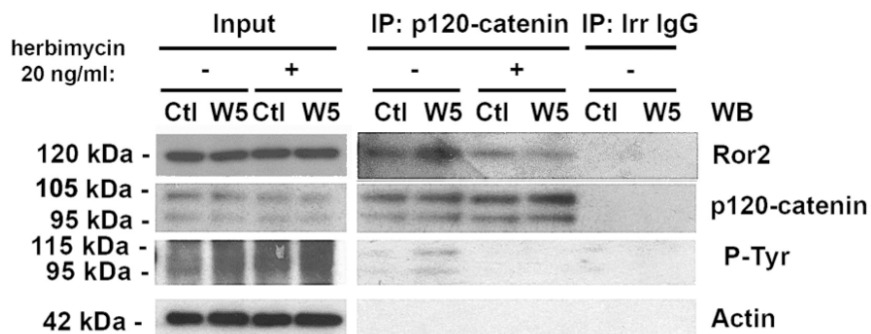
Figure R-20. p120-catenin interaction with Ror2 requires tyrosine phosphorylation. When indicated HEK293T cells were pretreated for 1 hour with 20 ng/ml Herbimycin A and then stimulated with control or Wnt5a-conditioned medium for additional 5 min. Cells were lysed and p120-catenin was immunoprecipitated. Tyrosine phosphorylated p120-catenin and associated Ror2 were analyzed by WB.

3.4. P120-catenin interacts with the juxtamembrane domain of Ror2 whereas CK1ε binds to Ror2 C-terminal sequence

We first considered that CK1ε was interacting with Ror2 through p120-catenin, since it was described that CK1ε and p120-catenin directly interact[123]. Moreover, the increased p120-catenin/Ror2 association observed with Wnt5a was also accompanied by an increase in CK1ε. To further analyze the interaction of CK1ε with Ror2, a pull-down assay was performed in cells CRISPR for CK1ε or p120-catenin using GST-cytoRor2 as bait to evaluate the interacting capability of each of these factors in the absence of the other. As shown in figure R-21, CK1ε as well as p120-catenin were able to interact with Ror2 even in the absence of the other protein. Therefore, p120-catenin was not required for CK1ε association with Ror2.
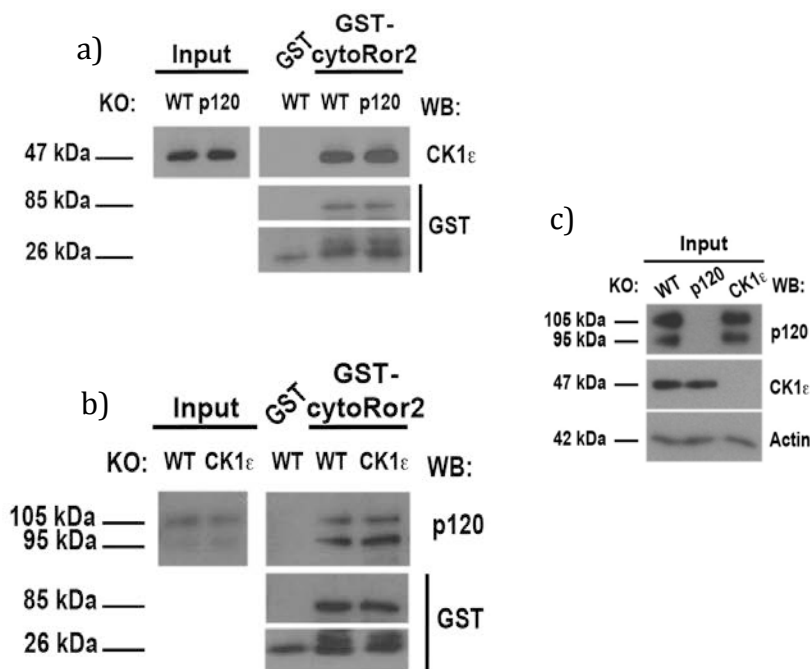
Figure R-21. p120-catenin and CK1ε independently interact with Ror2. a) Pull-down assays were performed incubating 700 μg total cell extracts from control and p120-catenin HEK 293T CRISPR cells with 10 pmol of GST-cytoRor2. Protein complexes were affinity purified and analyzed by WB. **b)** As in a) but CK1ε CRISPR cells with GST-cytoRor2 as bait. **c)** Total cell extracts of Ctrl, p120-catenin and CK1ε CRISPR cells.

We next examined in which domain of Ror2, p120-catenin and CK1ε were interacting and also if these complexes (p120-catenin/Ror2 and CK1ε/Ror2) are formed through a direct association. For this purpose, different recombinant GST-cytoRor2 fragments were generated (Figure R-22) and an *in vitro* binding assay was performed with recombinant full-length p120-catenin or CK1ε. A GST-cytoRor2 fusion protein bound recombinant p120-catenin (Figure R-23a). This association requires the juxtamembrane sequence of Ror2, since it was also observed with a GST fusion protein comprising amino acids 426-563 of Ror2; on the contrary the remaining Ror2 cytosolic domain (amino acids 563-944) did not significantly interact with p120-catenin. Differently to p120-catenin, the binding site for CK1ε was mapped in the C-terminal sequence of Ror2, aa 563-944 (Figure R-23b).
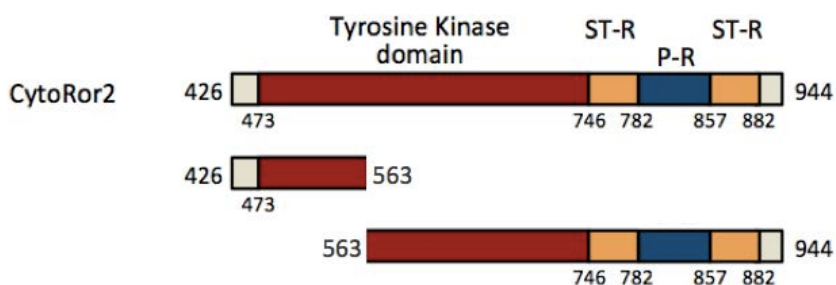
Figure R-22. Recombinant fragments of the cytosolic domain of Ror2.
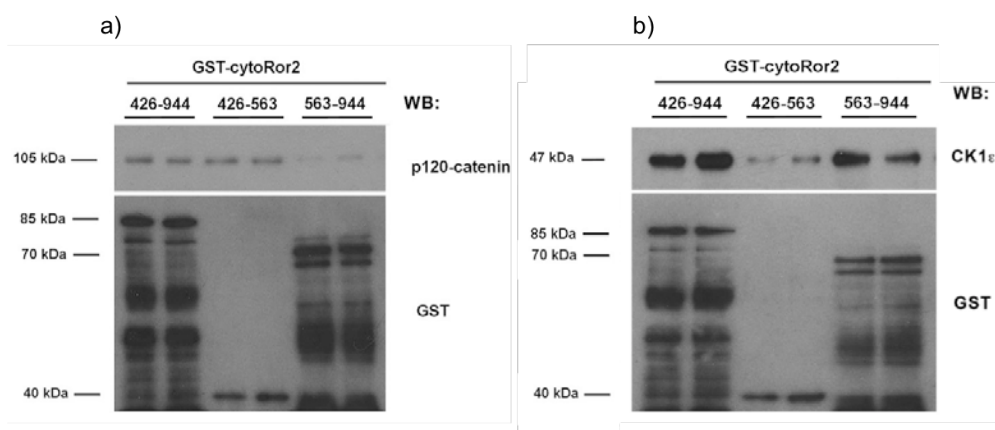
Figure R-23. P120-catenin binds to the juxtamembrane domain of Ror2, whereas CK1ε binds to the C-terminal part. a) Different GST–cytoRor2 fusion proteins (0.5 pmol) or GST as a control were incubated with recombinant p120-catenin (2 pmol). Protein complexes were affinity purified and analyzed by WB with anti-p120-catenin. **b)** As in a) but 2 pmol of CK1ε were assayed to bind with 0.5 pmol of GST-cytoRor2 fragments.

Thus, p120-catenin and CK1ε independently bind to Ror2 through different domains and both factors are required for the activation of the non-canonical Wnt pathway.

4. P120-catenin and CK1ε have different roles in the non-canonical Wnt pathway

4.1. CK1ε affects Ror2 stability

We further explored the previous observation that total Ror2 protein levels were decreased in CK1ε KO cells but not in p120-catenin KO cells (Figure R-5 and also figures R-24a and R-24d). This downregulation was also observed when Ror2-HA was overexpressed in CRISPR CK1ε KO cells compared to control (Figure R-24b).
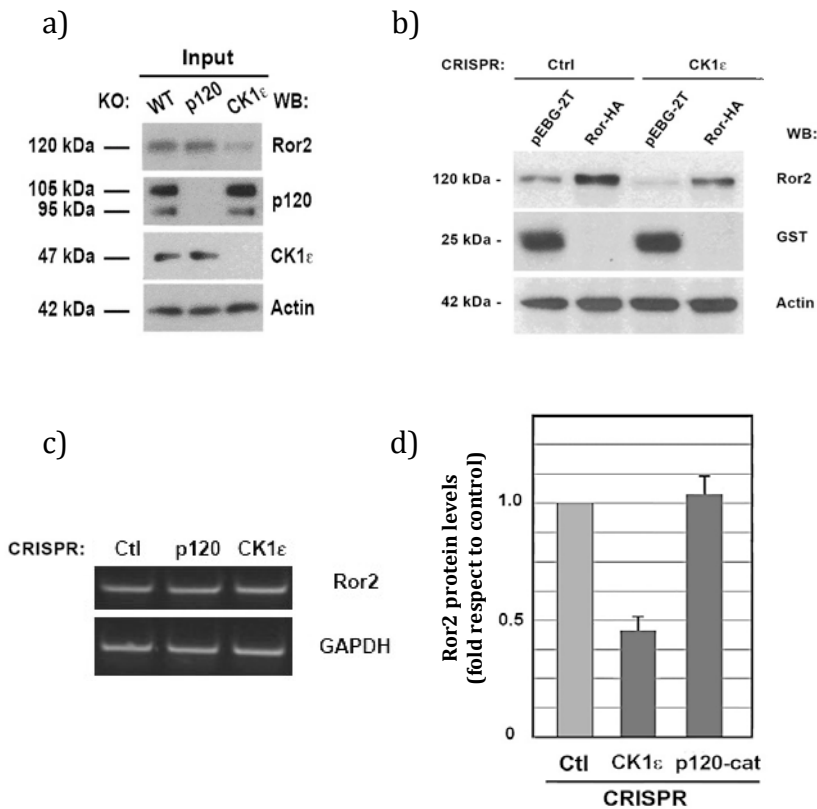
Figure R-24. CK1ε stabilizes total Ror2 protein levels. a) Control, p120-catenin and CK1ε HEK293T CRISPR cells were lysed and total Ror2 levels were analyzed by WB with a specific anti Ror2 antibody. **b)** Total Ror2 protein levels were determined by WB in control or CK1ε HEK293T CRISPR cells overexpressing Ror2-HA or GST as a control for 48 hours. **c)** RNA was isolated from control, p120-catenin and CK1ε HEK293T CRISPR cells. Ror2 RNA levels were measured by semi-quantitative RT-PCR. **d)** Autoradiograms from three different experiments in a) were quantified and total Ror2 protein levels are shown ± SD.

However this difference is not accompanied with altered levels of Ror2 mRNA (Figure R-24c), suggesting that it was due to a lower stability of Ror2 protein. Since Kani et al., 2004[65] described that CK1ε overexpression promoted Ror2 phosphorylation in Ser/Thr residues and this, in turn, allowed Ror2 tyrosine kinase activity, we tried to inhibit the tyrosine kinase or the CK1 activity. Neither IC261 (CK1ε/δ specific inhibitor) nor Herbimycin A affected Ror2 protein levels (Figure R-25).

a)

b)
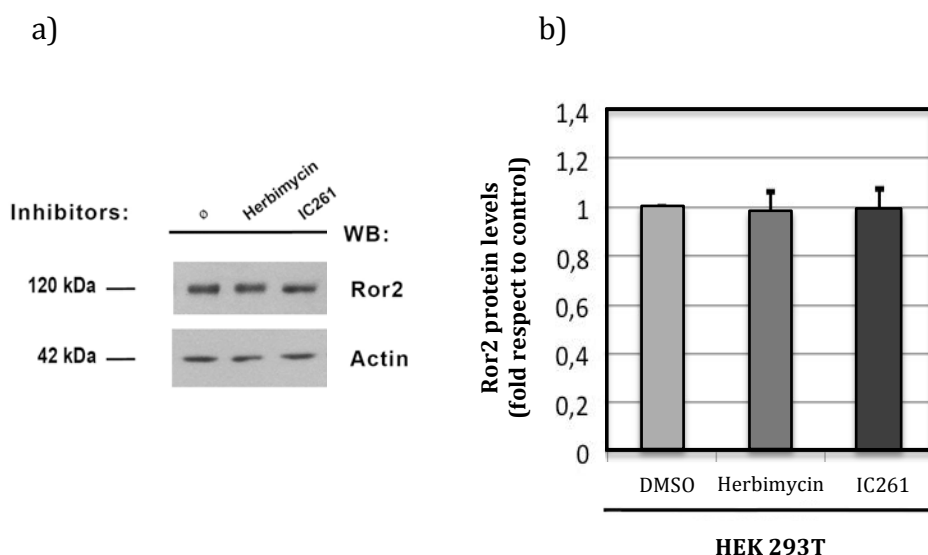
HEK 293T

Figure R-25. Ror2 protein levels are not affected by Ser/Thr or Tyr kinase inhibitors. a) HEK293T cells were treated with 20 ng/ml Herbimycin A or 15 µM IC261 for 1 hour. Total Ror2 protein levels were analyzed by WB with an anti-Ror2 antibody. **b)** Quantification of a) from three independent experiments ± SD is shown.

We next explored Ror2 protein stability with cycloheximide assays. Cells CRISPR Ctrl or CK1ε were treated with cycloheximide for different time points and total Ror2 levels were measured (Figure R-26a). A clear difference was detected and the results indicated that Ror2 protein half-life was decreased in CK1ε depleted-cells (Figure R-26c). However, this is a specific effect since CK1ε interaction with Ror2 controls the protein stability of this co-receptor but does not affect LRP5/6 total protein levels (Figure R-26b).

a)

b)

c)
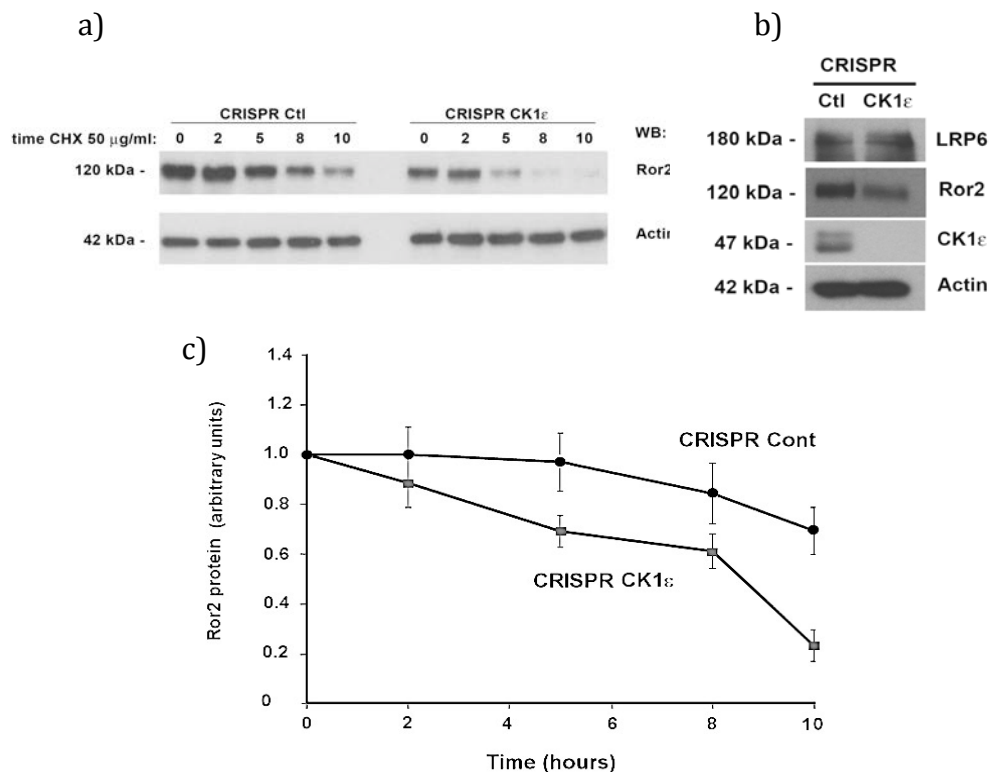
Figure R-26. CK1ε increases Ror2 protein half-life. a) Control or CK1ε HEK293T CRISPR were treated with 50 μg/ml cycloheximide for the indicated times. Cells were lysed and Ror2 protein levels were analyzed by WB. **b)** Control and CK1ε HEK293T CRISPR cells were lysed and Ror2 and LRP6 total protein levels were analyzed by WB. **c)** Autoradiograms from four different experiments performed were quantified using Quantity One software and represented for each time point ± SD.

Moreover, this new role of CK1ε in the non-canonical Wnt pathway is not the only one. Several papers suggest the involvement of CK1ε as a necessary step for the activation of the pathway through Dvl-2 phosphorylation, which would promote recruitment to Frizzled[48, 126]. We checked whether CK1ε activity was relevant for Dvl-2 phosphorylation and recruitment to Fz. Fz was immunoprecipitated from cells treated with de CK1 inhibitor IC261, Herbimycin A or vehicle (DMSO) before Wnt5a stimulation. Although the electrophoretic shift due to Dvl-2 phosphorylation was only abrogated with the CK1 inhibitor (Figure R-27, lanes 3 and 4), both inhibitors reduced Dvl-2 recruitment to Frizzled (Figure R-27, right panel). This result

suggests that not only phosphorylation in Ser/Thr residues is necessary for Dvl-2 association with Fz but also Tyr phosphorylation is relevant for this step.
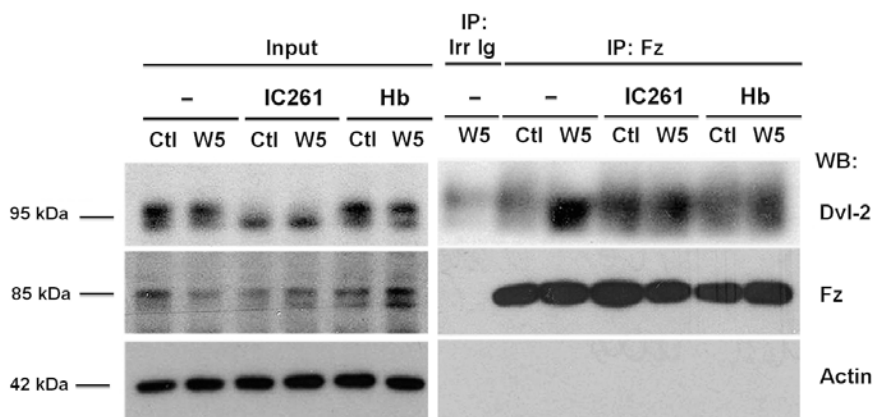
Figure R-27. IC261 and Herbimycin A abrogate Dvl-2 recruitment to Fz, while only IC261 inhibits the electrophoretic shift of Dvl-2. When indicated, HEK293T cells were pretreated for 1 hour with 15 μM IC261 or 20 ng/ml Herbimycin A and stimulated with control or Wnt5a-conditioned medium for additional 5 min. Fz2 was immunoprecipitated from cell extracts and associated Dvl-2 detected by WB.

4.2. P120-catenin controls the presence of Ror2 in the plasma membrane

We have previously shown that CK1ε and p120-catenin are required for Dvl-2 phosphorylation and recruitment to Fz by the non-canonical Wnt5a (see figure R-5) and this Wnt5a-induced Dvl-2 binding to Fz was sensitive to a general CK1 inhibitor (IC261) (Figure R-27). Also we observed that Wnt5a increased the association of both, p120-catenin and CK1ε with Fz (Figure R-2). With all this data we decided to analyze CK1ε activity upon Wnt5a stimulation. CK1ε was immunoprecipitated from control or Wnt5a-treated cells and its activity assayed *in vitro* using recombinant p120-catenin as substrate. As shown in figure R-28, phosphorylation of p120-catenin was increased when CK1ε was precipitated from Wnt5a-treated cells, although similar levels of CK1ε were present in the immunocomplex. No substrate phosphorylation was observed when ATP was omitted from the reaction. The increase was calculated to be approximately 3-fold as determined from three different

experiments (Figure R-28, right panel). Since p120-catenin is necessary for the activation of CK1ε by canonical Wnt ligands[123] we determined its relevance in the Wnt5a-induced stimulation of CK1ε activity. As shown in figure R-28, p120-catenin downregulation by shRNA prevented the Wnt5a-induced activation of CK1ε. This result is in agreement with figure R-9b where the Wnt5a-induced electrophoretic shift of Dvl-2 is not observed in p120-catenin downregulated cells (meaning, no CK1ε activity).
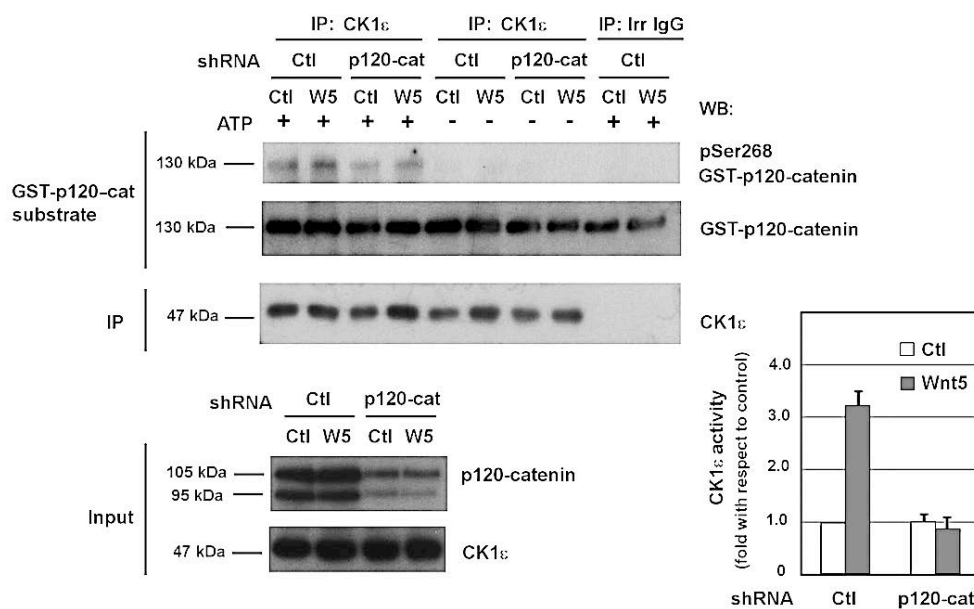
Figure R-28. CK1ε activation induced by Wnt5a is blocked in the absence of p120-catenin. Control or p120-catenin-depleted HEK293T cells were treated with control or Wnt5a-conditioned medium for 15 min. CK1ε was immunoprecipitated from total cell extracts and the immunocomplex was incubated with 2 pmol of recombinant GST-p120-catenin in CK1 phosphorylation conditions. Phosphorylation of Ser268 was analyzed by WB with a specific PSer268 p120-catenin antibody. The quantification of three different experiments is represented ± SD.

Since p120-catenin directly binds to the juxtamembrane domain of Ror2 and does not modulate CK1ε interaction with Ror2, we wonder what would be its role in the pathway, since in its absence CK1ε is not activated by Wnt5a. It is well established that p120-catenin stabilizes cadherins at the cell membrane by interacting through the juxtamembrane domain of cadherins[209]. When p120-catenin is downregulated or released from cadherins, the latter are endocytically internalized and can be degraded in the lysosomes or recycled back to the plasma membrane[141, 142, 210]. We thought that similarly to

cadherin, p120-catenin might be regulating Ror2 stability at the plasma membrane. It has been described that Wnt5a promotes clathrin-dependent Fz-receptor complex internalization[194, 211]. We therefore tried to reproduce these results by biotinylation of membrane proteins in intact cells to study Ror2 internalization. In a biotin pull-down experiment, Wnt5a caused a progressive decrease in biotinylated Ror2 (Figure R-29). An internal membrane protein used as control, EEA1, was not biotinylated. It has been reported[194] that monodansylcadaverine (MDC), an inhibitor of clathrin-dependent endocytosis, blocked non-canonical Wnt signaling. Another biotin pull-down assay was then performed in the presence of MDC. As shown in figure R-30, addition of MDC prevented Ror2 disappearance from the plasma membrane induced by Wnt5a. Moreover, Ror2 levels at cell surface were increased when the inhibitor was present (Figure R-30, compare lanes 1 and 2).
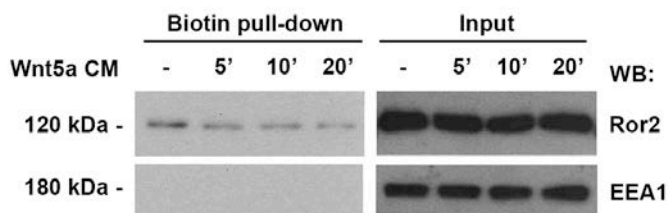
Figure R-29. Ror2 is internalized upon Wnt5a treatment. Surface proteins were biotinylated in HEK293T cells treated with control or Wnt5a-conditioned medium for the indicated times. A pull-down assay was performed with NeutrAvidin Agarose and biotinylated membrane proteins analyzed by WB. This experiment was done in collaboration with Guillem Fuertes.
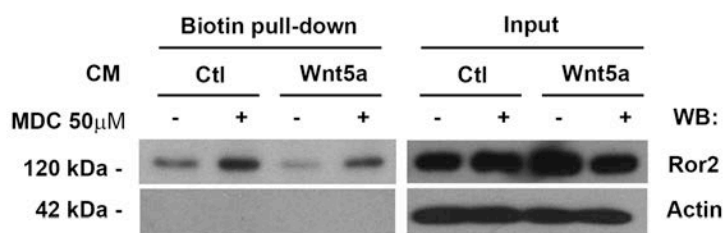
Figure R-30. Wnt5a-induced Ror2 internalization is inhibited by MDC. HEK293T cells pretreated with 50 μM MDC for 30 min. Then, cells were stimulated with control or Wnt5a-conditioned medium with the corresponding inhibitor for additional 20 min. Surface proteins were biotinylated and a pull-down assay was performed with NeutrAvidin Agarose. Biotinylated Ror2 was analyzed by WB with specific antibody. This experiment was performed in collaboration with Guillem Fuertes.

A similar experiment with MDC was performed by immunofluorescence to analyze endogenous Ror2 localization with a specific antibody. In control cells Ror2 is localized mainly into the plasma membrane and the pattern is more diffuse upon Wnt5a stimulation (Figure R-31, upper panels), whereas addition of MDC kept Ror2 into the membrane even in the presence of Wnt5a (lower panels).
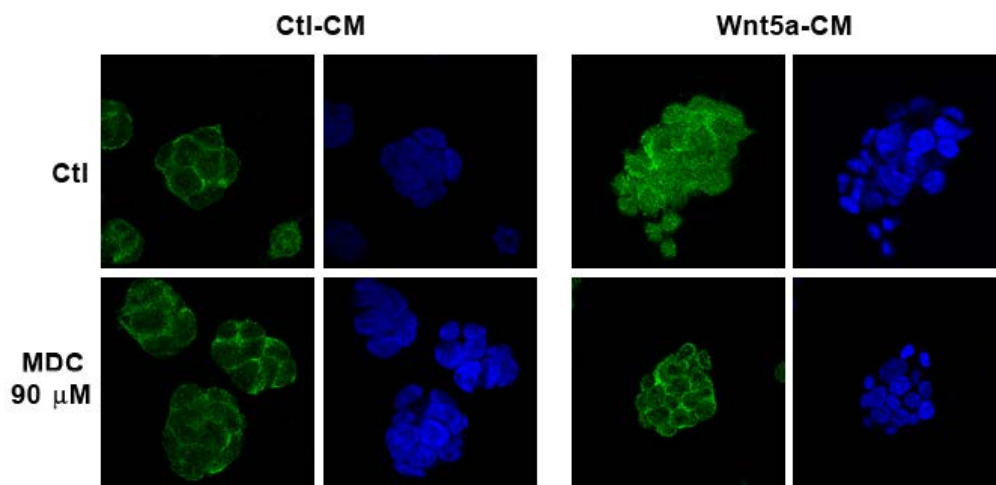
Figure R-31. MDC inhibits Ror2 internalization induced by Wnt5a. HEK 293T cells were pretreated with MDC for 30 minutes and stimulated with Ctrl or Wnt5a-CM for 15 minutes. Cells were fixed with paraformaldehyde (PFA) and used for immunofluorescence (IF) with Ror2 specific antibody. Nuclear staining was performed with DAPI. This experiment was done in collaboration with Guillem Fuertes.

These results suggest that Ror2 was internalized through a clathrin-dependent mechanism and, as shown in the biotin pull-down in figure R-30, these vesicles are also involved in the basal recycling of the co-receptor in unstimulated cells.

We next evaluated if in the absence of p120-catenin, the presence of Ror2 at the cell surface was affected. The biotin pull-down experiment in figure R-32 shows that in non-stimulated cells, in the absence of CK1ε or p120-catenin, Ror2 disappears from the plasma membrane. In the case of KO CK1ε cells the reason is because total Ror2 levels are diminished but, in KO p120-catenin cells, total Ror2 protein levels are not changed. This result points out the possibility that, through the interaction with Ror2, p120-catenin might be

controlling Ror2 internalization, similarly to its function on E-cadherin[208]. The presence in the plasma membrane of LRP5/6, the co-receptor of the canonical Wnt signaling pathway, was also diminished by elimination of p120-catenin (Figure R-33), although to a lower extent, whereas the presence of Fz was not modify in p120-catenin KO cells.
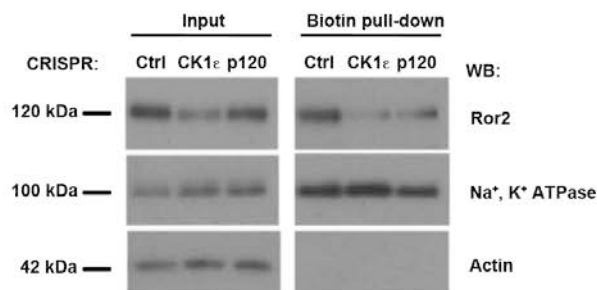
Figure R-32. P120-catenin controls levels Ror2 at the plasma membrane but does not affect total Ror2 protein levels. HEK 293T cells CRISPR Ctrl, CK1ε or p120-catenin surface proteins were labeled with biotin and total cell extracts were obtained with RIPA buffer. Biotin pull-down experiment was performed. Protein levels were analyzed by Western Blot.
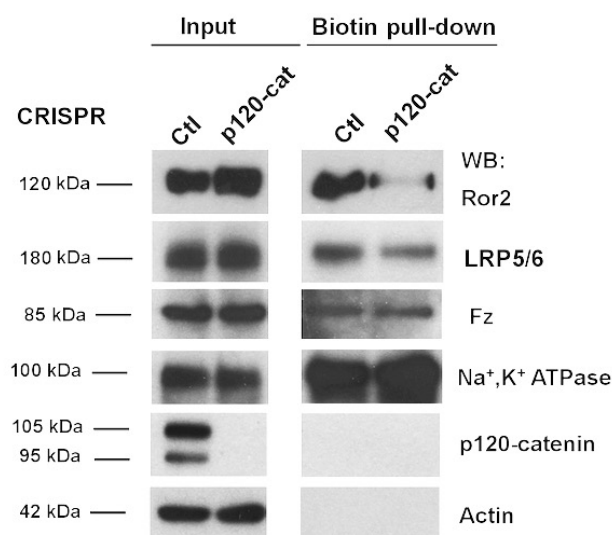
Figure R-33. p120-catenin only affects the presence of Ror2 in the plasma membrane. HEK 293T cells CRISPR Ctrl or p120-catenin were serum starved overnight. Next day, surface proteins were labeled with biotin and total cell extracts were obtained with RIPA buffer. Biotin pull-down experiment was then performed. Protein levels were analyzed by Western Blot. This experiment was performed in collaboration with Guillem Fuertes and Aida Villarroel.

This diminution of surface Ror2 in KO p120-catenin could be due to an increase of basal co-receptor recycling since, the absence of p120-catenin, makes Ror2 less stable at the cell membrane. As Ror2 may have a recycling process where clathrin coated-vesicles play a role, we tested if this increase in internalization upon p120-catenin depletion may be clathrin dependent. A proteinase K assay was performed, which works opposite to the biotin assay. A biotin assay measures Ror2 levels at the cell surface whereas a proteinase K experiment measures the amount of protease-resistant (intracellular) Ror2. The same conclusion was obtained in experiments where cells were treated with proteinase K; the levels of intracellular Ror2 were increased by Wnt5a in a MDC-sensitive fashion (Figure R-34). When clathrin internalization was abolished, the co-receptor was not internalized in the absence of p120-catenin and thus, was degraded by proteinase K. Intracellular Ror2 was also up-regulated in p120-catenin KO cells, further demonstrating the role of this protein in controlling clathrin-dependent internalization of Ror2.
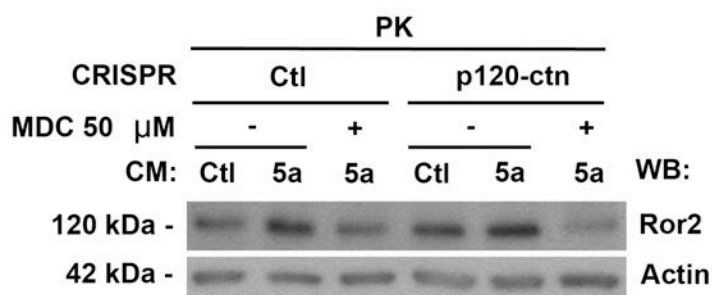
Figure R-34. Ror2 internalization in the absence of p120-catenin is abrogated upon MDC treatment. Control or p120-catenin HEK293T CRISPR cells were pretreated with 50 μM MDC for 30 min. Then, cells were stimulated with control or Wnt5a-conditioned medium for additional 20 min with MDC when indicated. Intact cells were treated with Proteinase K for 10 min. Total cell extracts were prepared and total Ror2 levels were analyzed by WB. This experiment was performed by Guillem Fuertes.

Therefore p120-catenin controls the presence of Ror2 at the membrane and regulates the internalization of Ror2 in a clathrin-dependent manner.

5. Ror2 tyrosine phosphorylation and oligomerization

5.1. Ror2 intrinsic tyrosine kinase activity is very low

Unlike the canonical Wnt co-receptor LRP5/6, Ror2 has a tyrosine kinase domain. Although it has a structure that resembles a single-pass transmembrane tyrosine kinase, whether it has intrinsic tyrosine kinase activity is still a matter of discussion. In order to decipher if Ror2 has kinase activity, an assay was performed in cells overexpressing Ror2-HA and treated with control or Wnt5a-conditioned medium. Ror2-HA was immunoprecipitated and assayed for kinase activity using a recombinant fragment of Ror2 cytosolic domain, GST-cytoRor2, as substrate. Immunoprecipitated Ror2-HA was able to phosphorylate GST-cytoRor2 and this kinase activity is just very slightly increased after Wnt5a stimulation (Figure R-35).
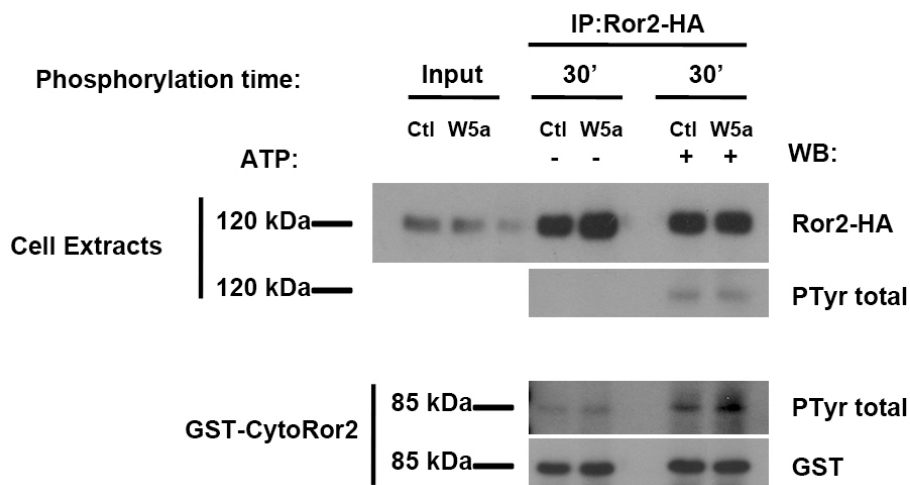
Figure R-35. Immunoprecipitated Ror2-HA phosphorylates Ror2 intracellular domain. HEK 293T cells were transfected with Ror2-HA. After 24h of overexpression cells were stimulated with Wnt5a or ctrl-CM for 10 minutes. HA-Ror2 was immunoprecipitated for 4 hours. Beads containing immunoprecipitated Ror2-HA were put in phosphorylating conditions with recombinant GST-tagged intracellular fragment of Ror2 and phosphorylation was performed during 30 minutes. Protein levels were analyzed by Western Blot.

Then, to ensure that this tyrosine kinase activity was really performed by Ror2 and was not due to a Ror2-associated kinase, the same experiment was done in NaCl restricting conditions and using recombinant p120-catenin as substrate. When washes were performed using a higher salt concentration prior to the kinase assay, Ror2 kinase activity was lost (Figure R-36), suggesting that a Ror2-associated tyrosine kinase is unbound in these restricting conditions.
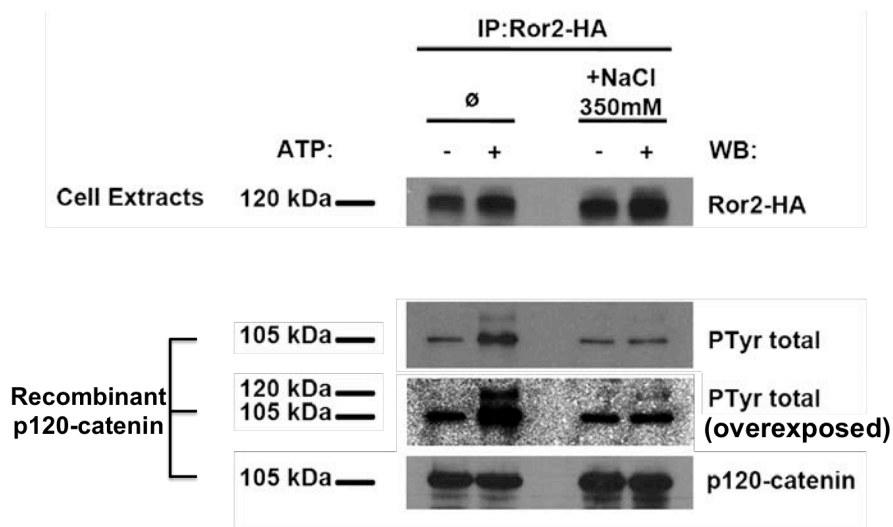
Figure R-36. Ror2 kinase activity is lost upon NaCl restricting washes. HEK 293T cells were transfected with Ror2-HA. After 24h of overexpression, cells were treated with Wnt5a-CM for 10 minutes and Ror2-HA was immunoprecipitated from cell extracts. Beads containing immunoprecipitated Ror2-HA were washed with PBS+NP-40 0,2% or PBS+NP-40 0.2% supplemented with 350 mM NaCl. Beads were added in phosphorylating conditions to recombinant p120-catenin and phosphorylation was performed during 30 minutes. Protein levels were analyzed by Western Blot.

This result reinforces the idea of Ror2 as a pseudokinase, where its inherent tyrosine kinase activity is negligible and probably performed by an associated kinase. However, what is clear is that a tyrosine kinase activity is necessary for Dvl-2 recruitment and hence for the activation of the pathway (see figure R-27).

The ability of receptor tyrosine kinases to dimerize has been widely described[212, 213] and for this polymerization, tyrosine kinase phosphorylation is necessary. Co-receptor Ror2 has not been an exception in terms of homodimerization[66] or heterodimerization[62] with Ror1.

The PTyr band at 120 kDa observed in figure R-36 in the overexposed film corresponds to tyrosine phosphorylated Ror2-HA. It has been published that Ror2 and Src interact[67, 73] and this interaction is diminished in kinase death Ror2[73]. These data suggest that Ror2 autophosphorylation maybe is necessary for Src recruitment and, further phosphorylation by Src in tyrosine residues, allows Ror2 oligomerization. This receptor aggregation could be necessary to form a more stable signaling complex allowing CK1ε activation and thus, Dvl-2 recruitment to Fz (Figure R-27, loss of Dvl-2 recruitment with Herbimycin A).

5.2. Tyrosine kinase activity is necessary for Ror2 polymerization

The ability of Ror2 to polymerize was assessed in HEK 293T cells overexpressing two forms of Ror2, alternatively labeled with HA or Flag tags. Ror2-HA was immunoprecipitated from control or Wnt5a-stimulated cells and Flag-labeled Ror2 interaction was analyzed. Ror2-HA and Ror2-Flag already interacted in untreated cells and Wnt5a barely favored this complex (Figure R-37).
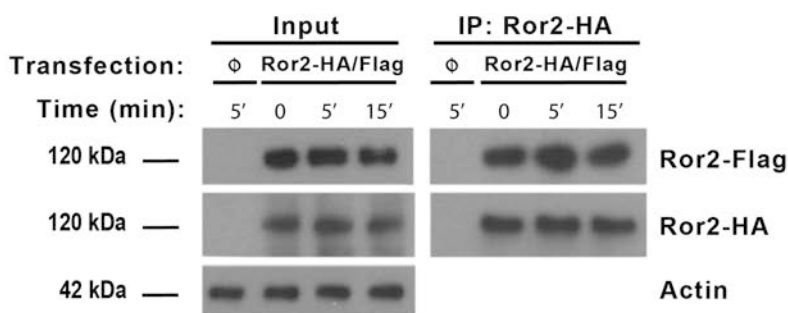
Figure R-37. Ror2-HA and Ror2-Flag polymerize. HEK 293T cells were overexpressed with Ror2-HA and Ror2-Flag. After 24h of overexpression cells were stimulated with ctrl or Wnt5a-CM for 5 and 15 minutes. Then, Ror2-HA was immunoprecipitated from cell extracts. Protein levels were analyzed by Western Blot with specific antibodies.
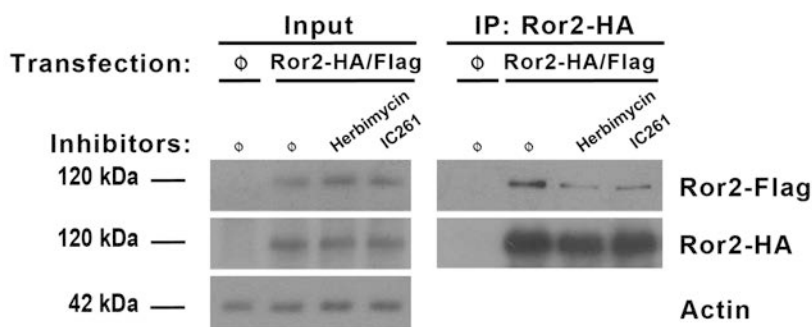
Figure R-38. Ror2 polymerization is inhibited with IC261 or Herbimycin A. Ror2-HA was immunoprecipitated from HEK293T cell extracts overexpressing both, Ror2-HA and Ror2-Flag, or the empty vector for 24 hours. Cells were treated with 20 ng/ml Herbimycin A or 15μM IC261 for 1 hour. Protein complexes were affinity purified and bound Ror2-Flag was analyzed by WB with anti-Flag.

To test whether Ror2-HA polymerization was affected by Ror2 phosphorylation, a similar experiment was performed with the CK1 inhibitor, IC261, and the tyrosine kinase inhibitor, Hermibycin A. The interaction was sensitive to both inhibitors that reduced Ror2 oligomerization (Figure R-38).

Ror2 oligomerization was further validated by a pull-down assay were overexpressed Ror2-HA was retained by a recombinant intracellular fragment of Ror2 (GST-cytoRor2) (Figure R-39).

This interaction was stimulated if GST-cytoRor2 was previously phosphorylated with recombinant Src (Figure R-40) but not with CK1, suggesting that tyrosine phosphorylation of the intracellular domain of Ror2 potentiates receptor oligomerization. It has been reported that the intracellular Ror2 domain needs CK1ε priming in Ser/Thr residues to then autophosphorylate in tyrosine residues[65]. This suggests that inhibition of CK1 activity might decrease the endogenous levels of Ror2 tyrosine phosphorylation and oligomerization because CK1ε stimulates Ror2 autocatalytic activity or its phosphorylation by another tyrosine kinase.
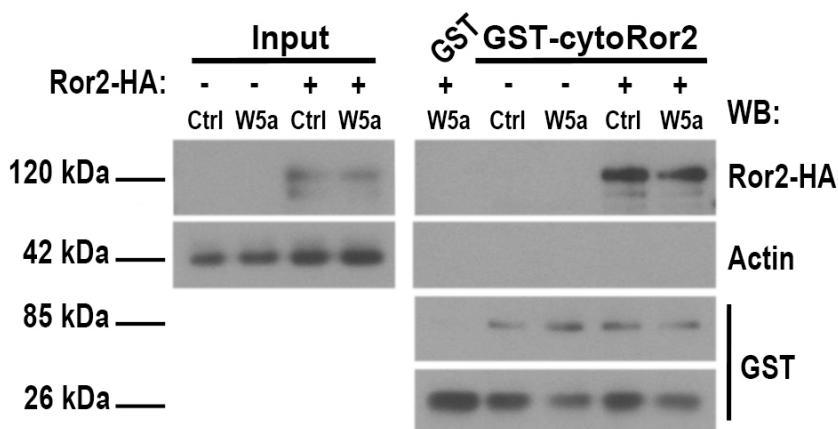
Figure R-39. GST-cytoRor2 polymerizes with overexpressed Ror2-HA. HEK 293T cells were overexpressed with Ror2-HA or empty vector. After 24h of overexpression, cells were treated with ctrl or Wnt5a-CM for 5 minutes. HA-Ror2 was pulled-down with GST-cytoRor2 (10 pmol) from total cell extracts at 4ºC during 2 hours. Protein levels were analyzed by Western Blot.
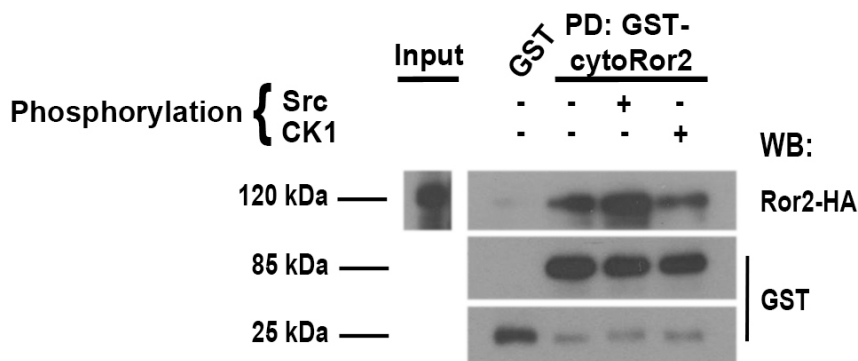
Figure R-40. Tyrosine phosphorylation of GST-cytoRor2 increases Ror2 polymerization. GST–cytoRor2 (10 pmol) was *in vitro* phosphorylated with recombinant Src or CK1 kinases for 2 hours. Pull-down assays were performed by incubating the fusion proteins with HEK293T cell extracts overexpressing Ror2-HA. Protein complexes were purified and analyzed by WB with an anti-HA antibody.

5.3. Ror2 overexpression activates the non-canonical Wnt pathway

We observed that Ror2 overexpression stimulated non-canonical Wnt-pathway signals even without any ligand treatment. Figure R-41 shows increased JNK and Dvl phosphorylation induced by overexpression of Ror2.
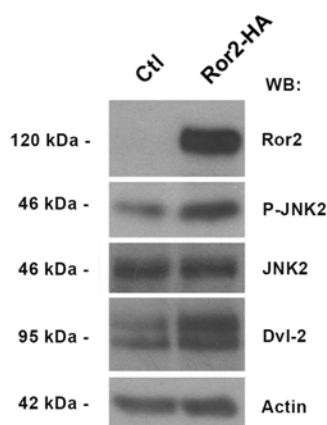
Figure R-41. Ror2 overexpression increases JNK and Dvl-2 phosphorylation. HEK 293T cells were transfected with Ror2-HA or GFP vector as a control. After 24h of overexpression, cells were lysed with RIPA buffer for total protein extraction. Protein levels were analyzed by Western Blot. This experiment was done in collaboration with Aida Villarroel.
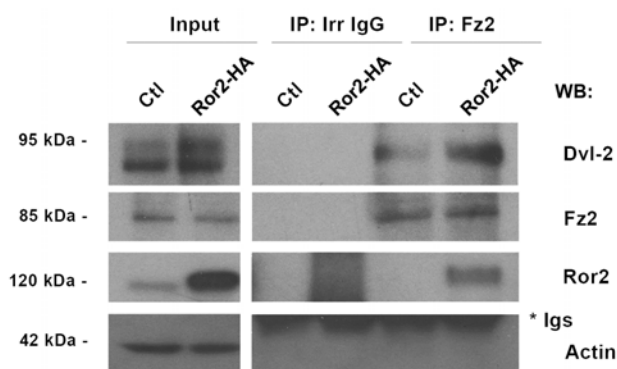
Figure R-42. Ror2 overexpression promotes the recruitment of Dvl2. HEK 293T cells were overexpressed with Ror2-HA or empty vector. After 24h of overexpression, Fz was immunoprecipitated overnight. Protein levels were analyzed by Western Blot with specific antibodies.

Overexpression of Ror2-HA also promoted Dvl2-recruitment to Frizzled in the absence of Wnt5a (Figure R-42). Moreover Ror2-HA was constitutively phosphorylated and the interaction with p120-catenin and CK1ε did not increase upon Wnt5a stimulation (Figure R-43).
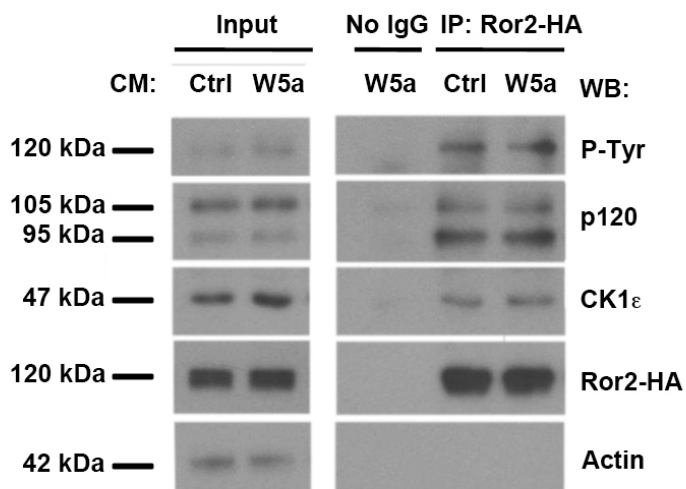
Figure R-43. P120-catenin and CK1ε interaction with overexpressed Ror2 does not increase upon Wnt5a treatment. Ror2-HA was overexpressed in HEK 293T. Cells were stimulated with ctrl or Wnt5a-CM for 5 minutes. Ror2-HA was immunoprecipitated with an anti-HA antibody and protein levels were analyzed by Western Blot.

To further analyze Dvl-2 recruitment to Fz receptor, immunofluorescence assays were performed. As expected, upon Wnt5a treatment Dvl-2 localization changed from the cytoplasm to the plasma membrane already after 5 minutes of ligand stimulation (Figure R-44). Since Dvl-2 recruitment to the membrane is a hallmark of the activation of the Wnt pathway, we tested if Ror2-HA overexpression was also able to induce Dvl-2 relocalization to the membrane by IF. Only in those cells were Ror2 was overexpressed, Dvl-2 co-localized with Ror2 at the plasma membrane. However, in the absence of Ror2 overexpression, Dvl-2 was stained as a diffuse pattern in the cytoplasm (Figure R-45).
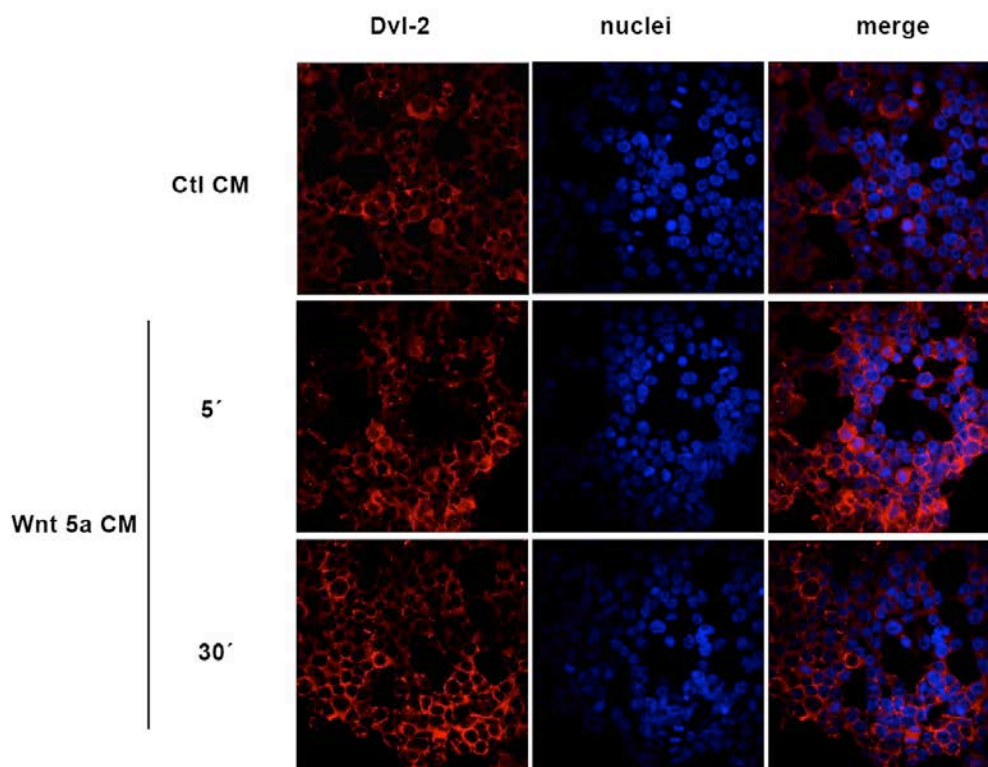
Figure R-44. Wnt5a stimulation promotes Dvl-2 recruitment to the membrane. HEK 293T cells were treated with Ctrl or Wnt5a-conditioned medium for 5 and 30 minutes. Cells were fixed with paraformaldehyde (PFA) and analyzed by immunofluorescence with the indicated antibodies. Nuclear staining was performed with DAPI.

Therefore these results indicate that overexpression of co-receptor Ror2 activates the non-canonical Wnt pathway in the absence of Wnt5a. An equivalent effect occurs in the canonical Wnt pathway that can be activated by overexpression of a cytosolic fragment of co-receptor LRP5/6 in the absence of Wnt ligand.
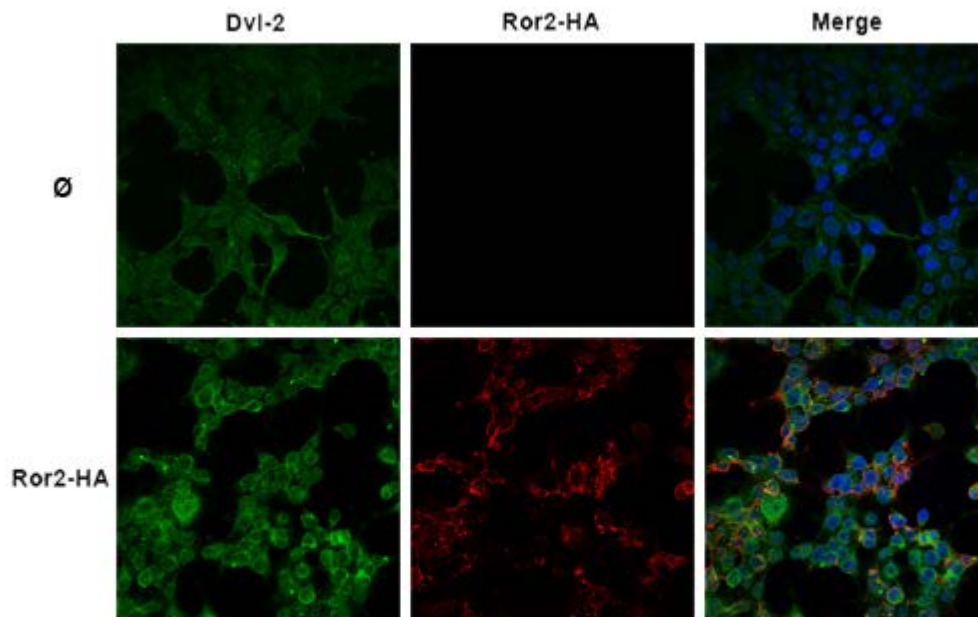
Figure R-45. Ror2-HA overexpression induces Dvl-2 relocation to the plasma membrane. HEK 293T cells transfected with Ror2-HA or empty vector. After 24h of overexpression cells were fixed with PFA and analyzed by immunofluorescence with the indicated antibodies. Nuclear staining was performed with DAPI.

6. P120-catenin and CK1ε are also required for later responses of the Wnt5a pathway

In previous experiments we have already analyzed that p120-catenin and CK1ε were necessary for the Wnt5a-upregulation of β-catenin E3 ligase Siah2 (see figure R-13). Downregulation of Ror2, CK1ε or PR61ε prevented the β-catenin down-modulation induced by Wnt5a (Figure R-11). P120-catenin depletion decreased β-catenin levels even in control conditions and also caused resistance to the Wnt5a effect on this parameter. Wnt5a also induces other cellular responses, such as cell invasion and actin polarization that will be analyzed below.

6.1. Mesenchymal cells deficient in Ror2, CK1ε or p120-catenin fail to invade upon Wnt5a stimulation

Wnt5a has been described to promote other cellular effects such as cell invasion and actin polarization in single cells[72, 193]. To test the relevance of p120-catenin and CK1ε in these processes, an invasion assay was first performed in a Boyden chamber coated with collagen I. Mouse mesenchymal stem cells (mMSC) control or deficient in CK1ε, p120-catenin or Ror2 were left overnight to invade towards the other side of the chamber using control or Wnt5a-conditioned medium as chemoattractant. Increased invasion was observed upon Wnt5a stimulation in cells transfected with control shRNA, but not when cells were depleted of Ror2, p120-catenin or CK1ε that failed to invade (Figure R-46). Therefore, elimination of any of these three genes totally prevented the Wnt5a stimulation of cellular invasion.

6.2. Mesenchymal cells deficient in Ror2, CK1ε or p120-catenin decreased Wnt5a-induced actin polarization

Cell polarity results from an asymmetric organization of intracellular organelles and cytoskeleton that ends up in extracellular differences at the level of morphology or membrane organization of the cell[214]. To induce polarization cells cannot be grown in plastic or glass cover and other materials such as collagen or matrigel are used. Few cells have the ability to induce Apical-Basal (AB) orientation when seeded as single cells (with no cell-to-cell contacts) and to grow in 3 dimensional matrices such as matrigel. In this

context, cells establish polarity with brush border formation (F-actin cap) away from the basal matrigel-interacting domain. This process has been described to be dependent on the non-canonical Wnt5a[215]. We tested the ability of mMSC to establish this AB polarity with the F-actin cap when seeded as single cells. The brush border at the apical domain was formed only when cells were stimulated with non-canonical Wnt5a and not with the canonical Wnt3a ligand (Figure R-47).
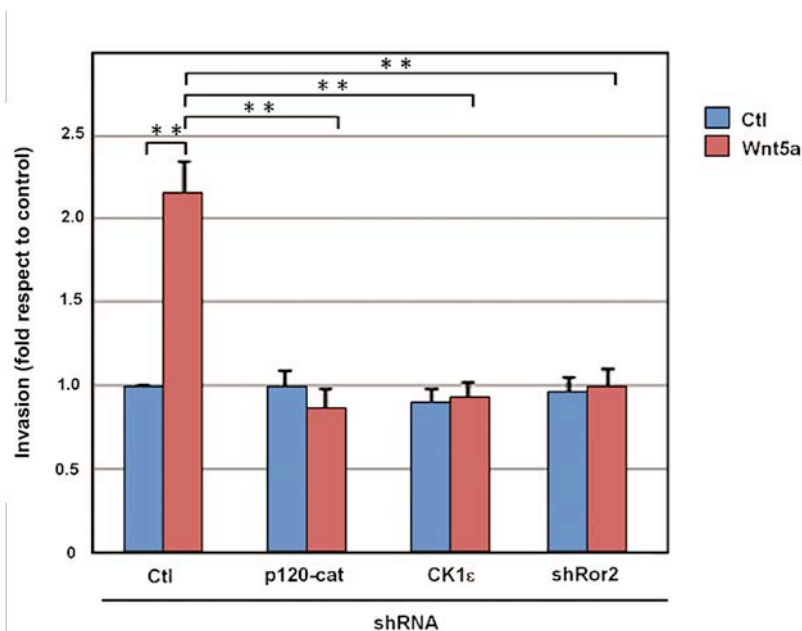
Figure R-46. Mesenchymal cells deficient in Ror2, CK1ε or p120-catenin fail to invade. Control, p120-catenin, CK1ε and Ror2-depleted mMSCs were seeded in transwell chambers containing 1 mg/ml collagen type I. Control or Wnt5a-CM was added to the lower chamber and after 16 hours of incubation, cells were fixed and stained with crystal violet and optical density was quantified at 590 nm. DAPI was used for nuclear staining. The graphic shows the quantification of at least three independent experiments ± SD and p values < 0.01 are shown with two asterisks.

Then, we determined whether Ror2, p120-catenin or CK1ε were also necessary for the Wnt5a-induced polarization response. Depletion of any of these genes very substantially downregulated the percentage of cells exhibiting a polarized distribution of cortical actin upon Wnt5a addition (Figure R-48).
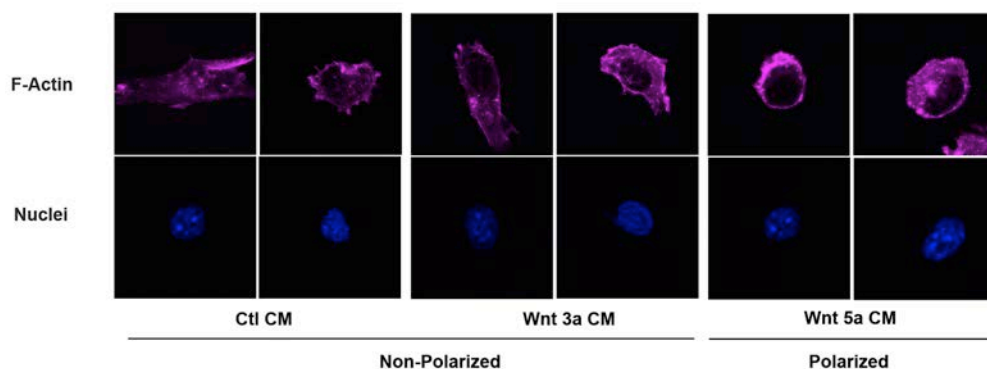
Figure R-47. mMSC polarize upon Wnt5a stimulation in matrigel single-cell assay. Control mMSCs show a polarized cell shape at the single-cell level in a Wnt5a-dependent. Cells were plated on Matrigel for 2h with control, Wnt3a or Wnt5a-conditioned medium, fixed and stained for F-Actin. DAPI was used for nuclear staining.

a)

b)
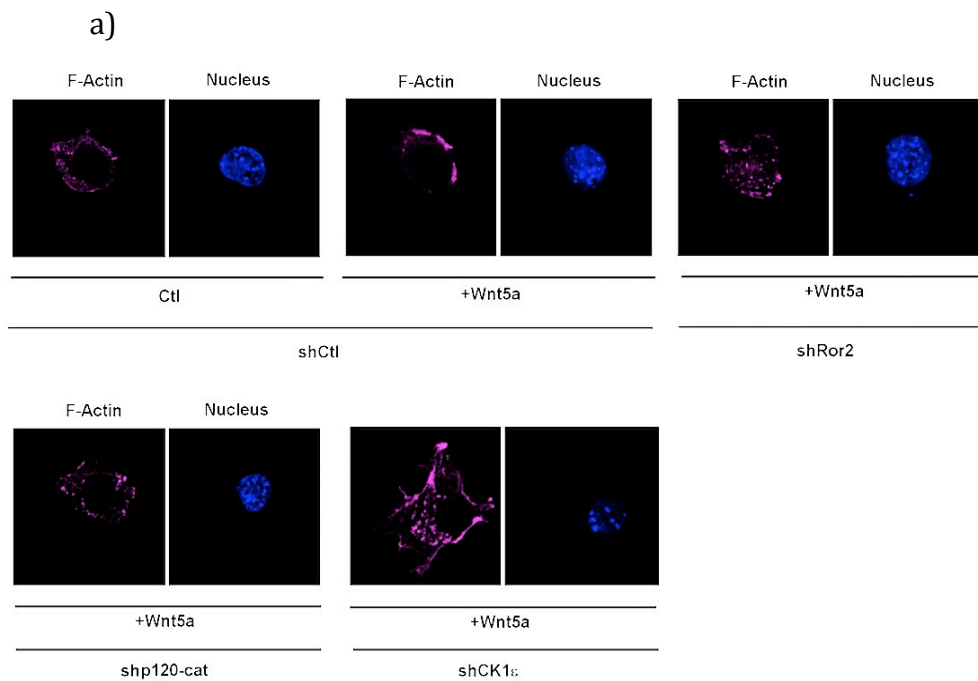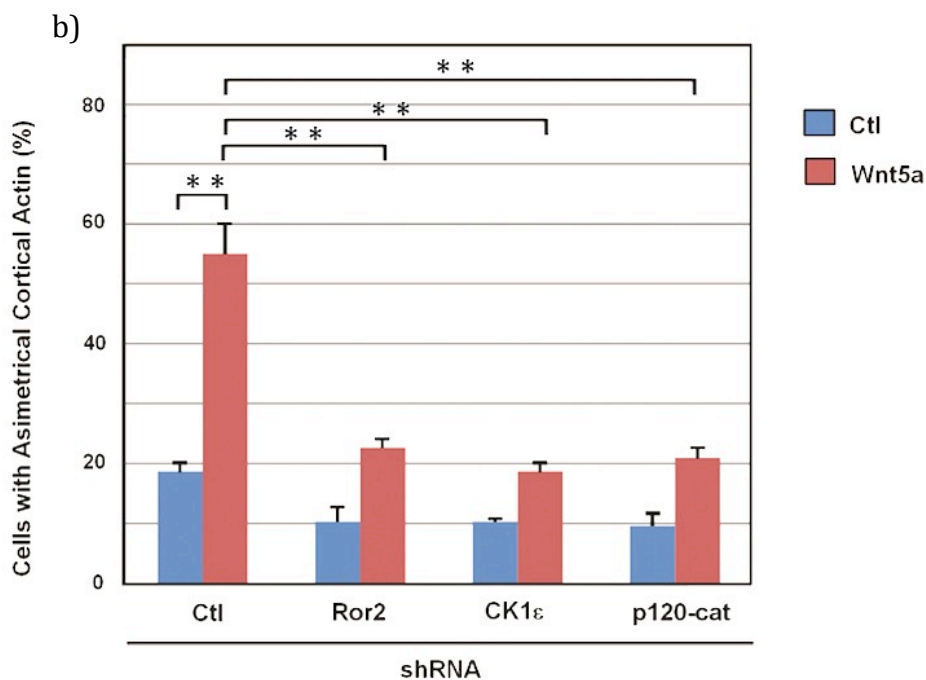
Figure R-48. Mesenchymal cells deficient in Ror2, CK1ε or p120-catenin decreased Wnt5a-induced actin polarization. a) Control, Ror2, CK1ε or p120-catenin depleted mMSCs were plated on Matrigel for 2h with control or Wnt5a-conditioned medium, fixed and stained for F-Actin. **b)** Polarized cells among at least 100 cells were counted for each condition and expressed as the percentage of polarized cells. Results are means ± SD from three independent experiments and p values < 0.01 are symbolized with two asterisks.

Discussion

1. CK1ε is necessary in initial signaling events in canonical and non-canonical Wnt pathway

Wnt signaling pathway was initially described when Mouse Mammary Tumor Virus (MMTV) was discovered inserted in integrated-1 gene that was responsible of mammary tumors[216]. This gene was later named Wnt1, one of the main representative of canonical Wnt pathway. It was not until several years later that members of non-canonical Wnt pathway started to be characterized[217]. The distinction between canonical and non-canonical Wnt pathway was originally defined according to the ability of some Wnt ligands to promote β-catenin stabilization: those whom up-regulate β-catenin were called canonical Wnt ligands whereas the others, non-canonical Wnts.

Although members of non-canonical Wnt pathway do not have the ability to stabilize β-catenin, they share a similar initial signaling structure and some effectors with canonical Wnts. Both receptor complexes involve Fz as a receptor and transduce the signal intracellulary using the adaptor protein Dvl[63, 71, 178]. In canonical Wnt pathway, Wnt ligand stimulation induces phosphorylation and recruitment of Dvl to the receptor Fz[218]. This step promotes oligomerization of Dvl causing the formation of a signaling complex named signalosome[218, 219, 220]. This process requires the rapid activation of CK1ε after Wnt stimulation, as it is required for Dvl phosphorylation and interaction with Fz. Although non-canonical Wnt pathway uses a different co-receptor, it induces Dvl phosphorylation and interaction with Fz[71].

These processes previously defined, like in canonical pathway, are dependent on the action of casein kinase 1ε; this kinase is activated upon Wnt5a stimulation and its downregulation prevents Dvl2 interaction with Fz. In both pathways, CK1ε activation requires the regulatory subunit of PP2A, PR61ε. In fact, this subunit constitutively interacts with Fz receptor and its downregulation abolishes Dvl phosphorylation and interaction with Fz in canonical and non-canonical Wnt pathway. Therefore, aside from co-receptor differences, the first actions triggered upon Wnt ligand stimulation are similar in canonical and non-canonical pathway requiring CK1ε activation.

The presence of CK1ε in each Wnt signaling pathway is mediated by the different Wnt co-receptors. In canonical Wnt pathway, p120-catenin

connects the constitutively bound CK1ε with LRP5/6[123] co-receptor. This interaction is mediated by LRP5/6 binding to cadherins, which in turn, interact with p120-catenin/CK1ε. On the other hand, in non-canonical Wnt pathway, CK1ε interacts directly with Ror2 co-receptor (Figure D-1b, see also [65]). The interaction formed upon ligand stimulation in both pathways results in a complex between receptor Fz and each of the co-receptors. This approximation allows Fz-constitutively bound PR61ε to specifically interact with CK1ε and that permits the activity of PP2A over CK1ε (Figure D-1c).

Once activated, CK1ε induces Dvl-2 binding to Fz and oligomerization (Figure D-1c). The mechanisms by which CK1ε positively regulate non-canonical Wnt pathway are still under discussion. One possibility is that CK1ε modifies Fz to increase its affinity for Dvl-2 since it is a rather weak interaction[40, 42]. This increased affinity for Fz translocates Dvl-2 to the plasma membrane, a limiting step in non-canonical Wnt signaling[46, 221, 222]. Another hypothesis, perfectly compatible with the previous one, is that CK1ε phosphorylates several residues of Dvl allowing Dvl polymerization and interaction with Fz.

Recently has been published that Dvl-2 exists constitutively in a "closed" conformation where the C-terminus is bound to the PDZ domain thus impeding the interaction of PDZ and DEP domains with their interactors and at the same time, constricting the membrane recruitment of Dvl by Fz[223]. Any modification which may disrupt this closed conformation, such as phosphorylation on PDZ domain, would promote Dvl function. It has been published that upon activation by Wnt simulation, CK1ε phosphorylates Dvl on the PDZ domain[224]. This may promote the open conformation of Dishevelled allowing Fz recruitment and all downstream signaling events. Afterwards, CK1ε extensively phosphorylates Dvl C-terminus, which then forms the PS-Dvl and finishes the signaling cascade[126, 224].

Last but not excluding hypothesis is that Dvl polymerization may be dependent on an intermediate substrate. Since Ror2 is required for Dvl-2 polymerization, CK1ε may be promoting Ror2 oligomerization by increased tyrosine phosphorylation through the priming effect on Ser/Thr phosphorylations. Ror oligomerization would favor Dvl recruitment to the membrane.

2. CK1ε regulates Ror2 total levels independently on its serine/threonine kinase activity

Apart from functions stated above requiring kinase activity, CK1ε also acts by direct binding to Ror2. This interaction has been mapped to the C-terminal of Ror2; more specifically, to the proline-rich region[65]. Binding to CK1ε increases half-life of Ror2 and, therefore, its total levels. Ror2 is degraded in a kinase-independent manner since IC261, a general inhibitor of CK1, does not modify Ror2 stability. Ror2 is destabilized only by the absence of CK1ε. The independence of CK1ε-kinase function for this regulation was reinforced by the fact that downregulation of the regulatory subunit PR61ε of PP2A did not affect Ror2 total levels, although it is necessary for CK1ε activity.

CK1ε bound to Ror2 PR-region is likely hiding a degradation mark revealed only in the absence of CK1ε. The fact that this downregulation affects endogenous HEK 293T Ror2 levels (human origin) as well as overexpressed Ror2 (cDNA from mouse Ror2), means that it is conserved among species. It has been described that some receptor tyrosine kinases, such as EGFR are regulated by ubiquitination and subsequent lysosomal degradation[225, 226]. EGFR, through E3 enzymes function, is ubiquitinated and marked for clathrin-mediated endocytosis and subsequent lysosome degradation[227]. The E3 enzymes family responsible for this function belongs to the same family as Hakai, the E3 ubiquitin-ligase that regulates cadherin levels[228]. The EGFR ubiquitination seems to be a multi-ubiquitination (several single monoubiquitin modifications) instead of a polyubiquitination (several ubiquitin molecules attached one after another forming a chain), classically targeting to proteasomal degradation[227, 229]. Moreover, Bache et al., 2004[227] have described that just one ubiquitin molecule is enough to trigger the lysosomal degradation process. Recent data from our laboratory indicates that Ror2 might suffer the same degradation process. HEK 293T CRISPR for CK1ε cells treated with the lysosomal inhibitor chloroquine, recover levels of Ror2 protein similar to control cells. Besides, analysis of the cytoplasmic Ror2 region with an ubiquitination prediction software (UbPred[230]), reveal a lysine residue (K, 858) with high confidence of ubiquitination at the region where CK1ε binds (P-R domain). Altogether, *in silico* data and results from our laboratory suggest that CK1ε regulates Ror2 protein levels through a mechanism independent on its activity but dependent on its interaction. This

interaction probably avoids Ror2 lysosomal internalization and degradation (Figure D-1a).
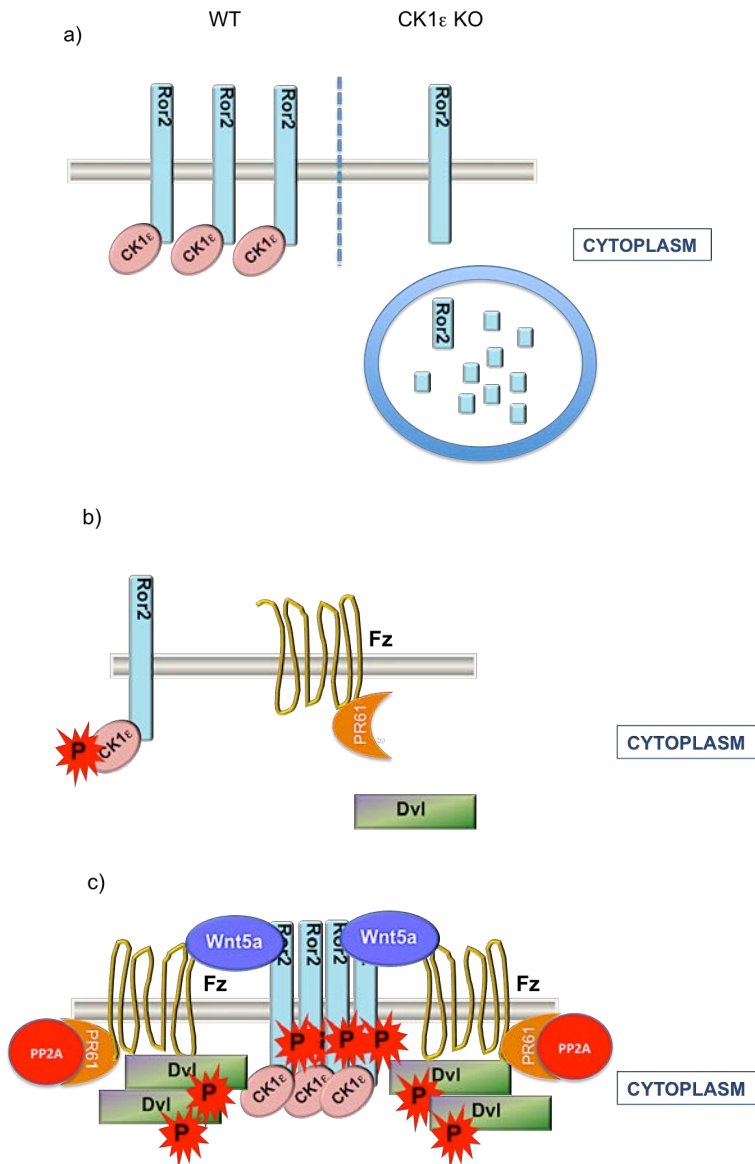
Figure D-1. Functions of CK1ε in non-canonical Wnt signaling. a) CK1ε through direct interaction with Ror2 avoids Ror2 internalization and lysosomal degradation. **b)** In non-stimulated pathway, Fz is bound to PR61ε and CK1ε is inactive and bound to Ror2. **c)** Upon Wnt5a binding, PR61ε is able to interact with CK1ε. CK1ε is then, through PP2A, dephosphorylated and activated. This step allows phosphorylation and recruitment of Dvl to Fz and Ror2 and Dvl oligomerization permitting downstream signalization.

3. P120-catenin stabilizes Ror2 at the plasma membrane

Since p120-catenin and CK1ε were necessary for Dvl binding to Fz in canonical Wnt signaling, the initial hypothesis considered that the complex was also required in the non-canonical Wnt pathway. Moreover, this idea was reinforced by the fact that p120-catenin depletion affected Wnt5a induced Dvl-2 phosphorylation and Dvl-2 binding to Fz. However, several differences were rapidly evidenced: for instance, CK1ε associates with the co-receptor Ror2 independently on p120-catenin. Accordingly, the difference in non-canonical Wnt pathway is that p120-catenin does not mediate CK1ε binding to Ror2 but maintains this protein at the plasma membrane. The latter function, preventing co-receptor internalization, is shared in both Wnt pathways although to a lesser extent in canonical Wnt pathway. Recent results from our laboratory show that p120-catenin also regulates the levels of LRP5/6 at the plasma membrane. This indirect function is performed through the well-described p120-catenin dependent regulation of cadherin stability at the plasma membrane preventing its internalization[208, 210]. Lack of p120-catenin promotes a downregulation of cadherin levels that, in turn, induces LRP5/6 internalization without affecting the total amount of protein[178]. On the other hand, the effect of p120-catenin depletion is direct over Ror2. Consequently, the absence of p120-catenin induces a more accused effect since it promotes internalization through a clathrin-dependent mechanism.

This p120-catenin function is similar to the one performed over cadherins; in both cases p120-catenin bind at the juxtamembrane region. This binding requires the N-terminal part of p120-catenin (1-350 amino acids), a domain also involved in the interaction with other tyrosine kinases, such as Fer and Fyn[147, 148]. Recently it has been described that δ-catenin, another member of p120-catenin family, has also a role regulating the stability of EGFR tyrosine kinase[231]. In this case, δ-catenin not only affects membrane stability of EGFR but also its protein levels. Upon EGF signaling, δ-catenin is phosphorylated and this promotes its release from EGFR receptor. This release, however, does not seem necessary for EGFR internalization. Oppositely, phosphorylated N-terminal p120-catenin, a modification that is upregulated upon Wnt5a pathway activation, increases its affinity and interaction for Ror2. This is likely a mechanism to increase the signaling complex stability at the membrane enhancing p120-catenin interaction. It is

still unclear if p120-catenin binding will act like in cadherins, blocking the access of AP-2 and the endocytic machinery to an internalization sequence in the juxtamembrane domain of Ror2[209, 232]. When controlling cadherins, p120-catenin binding protects the dileucine LL motif that mediates clathrin-dependent internalization of cadherins[129]. The affinity of p120-catenin for the JMD is the same as AP-2 affinity for the LL motif[233]. Therefore, the final output is a steady-state determined by an equilibrium between the two possible states, p120-catenin- or AP-2-bound E-cadherin. Any change in this balanced state (e.g. p120-catenin downregulation) will promote an imbalance of membrane versus internalized cadherin. This steady-state is also present in the regulation of membrane Ror2 since monodansylcadaverine treatment in unstimulated cells increases the presence of Ror2 in the membrane. This reflects that there is also an equilibrium between p120-catenin- and AP-2-bound Ror2.

The precise p120-catenin-binding sequence of Ror2 is still unknown although clearly differs from the one present in cadherins. Ror2, unlike cadherins, has a classical [D/E]xxx[L/I] internalization motif at position 453-458 based on a software to predict internalization motifs by protein sequence[234]. This sequence fulfills the requirements since it is placed at the region where p120-catenin binds and is in the juxtamembrane region of Ror2. Juxtamembrane position of this type of clathrin-internalization sequence has been shown in several other proteins[235]. This raises the possibility of this mechanism being shared among different members of the first group of p120-catenin family. Two members of this group, δ-catenin and ARVCF, have been described to also have the ability to associate with cadherins and this binding is mutually exclusive with p120-catenin[236, 237]. Moreover, δ-catenin and p120-catenin are crossregulated[236]: they regulate each other protein levels since when p120-catenin is overexpressed δ-catenin is downregulated and vice versa.

Interestingly, in prostate cancer, where non-canonical Wnt pathway is activated, p120-catenin is downregulated. However, δ-catenin level (CTNND2) is otherwise upregulated, suggesting that it might compensate for p120-catenin downregulation (Table D-1).

ARVCF role in cancer is not as widespread as other catenin members but its function is better described in development. This protein was first

described in a screening to determine the molecular causes of patients with Velo-cardio-facial or DiGeorge syndrome[238]. The outcome of this pathology is characterized by a wide spectrum of phenotypes that includes conotruncal heart defects, cleft palate, and facial dysmorphology. This phenotype is reminiscent of some of the pathological consequences of Ror2-deletion in Robinow syndrome. As in RS, some of DiGeorge syndrome malformations have been published to be due to impaired Wnt5a signalization[239]. These similarities strengthen the hypothesis that p120-catenin function in non-canonical Wnt pathway, as it happens with cadherin regulation, might be done by other closely related p120-catenin family members.

Fold change	Gene	Comparison	Gene	Fold change
Upregulation	CTNND1	Lung carcinoma aggressiveness	CTNND2	Downregulation
Upregulation	CTNND1	Medulloblastoma vs. normal	CTNND2	Downregulation
Downregulation	CTNND1	Prostate cancer vs. normal	CTNND2	Upregulation
Upregulation	CTNND1	Osteosarcoma vs. normal	CTNND2	Downregulation

Table D-1. Comparison of p120-catenin and δ-catenin expression in different cancers. Gene expression data of p120-catenin (CTNND1) and δ-catenin (CTNND2) in different cancers was extracted from EMBL-EBI expression atlas[240].

On the other hand, clathrin-mediated internalization of non-canonical Wnt signaling complex is required for downstream signaling events activation, such as Rac1 and JNK[194, 241]. It has to be determined if this same region occupied and hidden by p120-catenin binding is the one involved in Ror2 internalization upon long-term non-canonical Wnt treatment[211, 241]. If this were the case, p120-catenin should be released to allow Ror2 internalization. This mechanism might be dependent on Ser/Thr phosphorylation of p120-catenin since CK1ε phosphorylation decreases catenin association with tyrosine kinases Fyn and Fer[124]. There is a possibility that also alters the interaction with Ror2. Besides, if p120-catenin were released from Ror2, it would be important to determine if it has other functions downstream of the initial signaling complex. In canonical Wnt pathway, after being released from the signalosome, p120-catenin is required for Rac1 activation by mediating the interaction of Vav2 GEF with Rac1[124]. Since Wnt5a signalization also elicits the

activation of the small GTPase Rac1, if p120-catenin is released it may mediate Rac1 activation too. However, whether p120-catenin is released from Ror2 or not, it would probably not affect Ror2 internalization upon Wnt5a stimulation. It has been published that this is a function developed by Dvl itself. Two regions of Dvl-2 are required for the strong interaction with the clathrin adaptor AP-2: one involves the DEP domain and the other a conventional tyrosine-based tetrapeptide YHEL motif placed C-terminal to the DEP domain[242]. Impairment of any of these two regions specifically affects receptor complex internalization, JNK activation and PCP in frog embryos[242]. β-arrestin2 internalization component may also be required for clathrin-dependent endocytosis[243]. Then, internalizing Dvl-2 interacts with the GEF Tiam1 and, together with Daple and PKCλ, trigger Rac1 activation[198, 215, 244]. Active Rac1, through stimulation of PAK1, activates JNK[245].

We cannot discard other functions in which, this hypothetical p120-catenin released from Ror2, might be involved. For instance it might be involved in derepressing Kaiso-inhibited genes. In canonical Wnt pathway this function has been well-described[125, 168, 170]. Moreover, it has been reported the requirement of p120-catenin to release Kaiso-dependent repression of Wnt11[127], a known non-canonical Wnt ligand. Given that Kaiso has also been described regulating the expression of some matrix metalloproteinases, such as MMP-7[246], it is possible that is also repressing the expression of other Wnt5a/AP-1 dependent genes[71]. Among them it could be Siah2, MMP-9 and MMP-1 since all of them have Kaiso-binding sites in within their respective gene enhancer[247] (data extracted from GeneCards® - Human Gene Database).

In any case, the results shown here demonstrate that p120-catenin regulates the membrane levels of other proteins different from cadherins (see figure D-2).
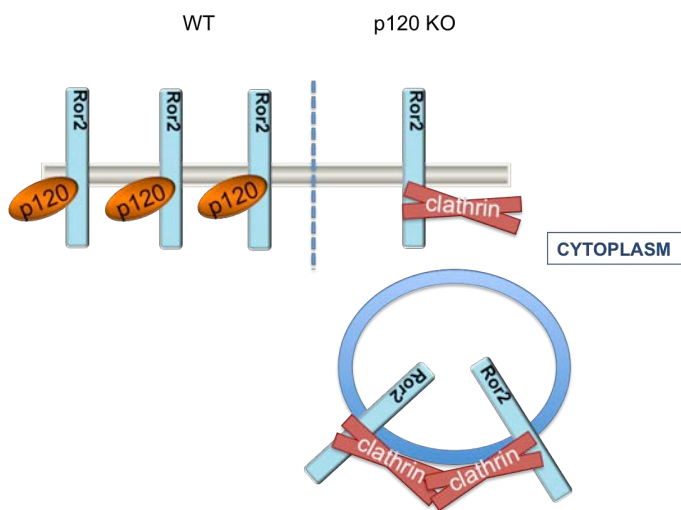
Figure D-2. P120-catenin avoids Ror2 internalization. P120-catenin interacts in the JMD of Ror2 with it N-terminal domain. This interactions maintains Ror2 at the plasma membrane. As a similar mechanism to what happens with cadherins, p120-catenin lost induces Ror2 internalization. However, in this case, internalized Ror2 is not targeted for degradation.

4. Ror2 tyrosine phosphorylation is required for non-canonical Wnt pathway activation

It is well established that many transmembrane tyrosine kinases undergo oligomerization upon ligand activation that normally leads to trans-phosphorylation and activation of kinase domain[212, 213]. In the case of Ror2, although presents a putative tyrosine kinase domain, is considered a pseudo-kinase and its tyrosine phosphorylation activity has not been well demonstrated. Compared to well-characterized transmembrane tyrosine kinases, it does not contain consensus cysteine, aspartic or glutamic acid residues in the glycine rich P-loop required for ATP binding and, as a consequence, for enzymatic activity[70, 248]. *In vitro* autophosphorylation assays show that Ror2 only have a weak tyrosine kinase activity. This is in agreement with the little autophosphorylation signal detected after restrictive NaCl washes of the immunoprecipitated Ror2-HA and also agrees with data from other authors[67, 73]. In these publications, a KD overexpressed Ror2 shows very similar autophosphorylation to the wild-type form. It is possible that this modification is consequence of the activity of an associated kinase. Our results suggest that this associated kinase is Src. Accordingly, a kinase dead overexpressed Src almosty completely abolished Ror2 phosphorylation[67]. If any, the low Ror2 intrinsic kinase activity might be required for facilitating Src binding[73].

Tyrosine kinase activity is necessary for non-canonical Wnt pathway to be active since the general inhibitor Herbimycin A avoids Dvl-2 recruitment to Fz upon non-canonical Wnt pathway stimulation. Dvl-2 tyrosine phosphorylation has been reported in canonical Wnt3a pathway to be required for Dvl-2-mediated β-catenin phosphorylation[249]. This tyrosine phosphorylation of β-catenin occurs at late times upon activation. At this time Dvl-2 is already phosphorylated in Ser/Thr residues and recruited to Fz[172]. So, Dvl-2 tyrosine phosphorylation does not seem to be necessary for Dvl-2/Fz interaction. However, it has been reported that Dishevelled tyrosine phosphorylation by Abelson tyrosine kinases in residue 473 in *Drosophila* is required for PCP[250].

On the other hand, Ror2 tyrosine phosphorylation is also sensitive to the CK1 inhibitor IC261. The requirement of CK1ε activity for Ror2 tyrosine

autophosphorylation has been previously described[65]. This reduction in Ror2 tyrosine phosphorylation is accompanied by less Ror2 oligomerization. So, IC261 might diminish Ror2 tyrosine phosphorylation and oligomerization because Ser/Thr phosphorylation must precede tyrosine modification either by Ror2 itself or by Src. This Ser/Thr phosphorylation induced by CK1ε may induce conformational changes on the intracellular part of Ror2 or create docking sites that allow the following tyrosine phophorylations. Ror2 tyrosine phosphorylation would promote Ror2 oligomerization[66]. This dimerization of non-canonical Wnt pathway co-receptors is likely required to mediate its function. Ror1 and Ror2 heterooligomerizate in CLL patients to allow RhoA and Rac1 activation[251]. In the case of *Drosophila*, the co-receptor homolog of vertebrate Ryk, Derailed, homo- or heterooligomerize with Derailed family members upon Wnt5a stimulation promoting recruitment of a Src family kinase[252]. According to this model, Ror2 oligomerization would facilitate Dvl-2 binding to Fz since Dvl-2 would interact better with multimeric than single binding sites, a possibility that might be also extended to its role in canonical Wnt signalling[173]. This model explain why Ror2 overexpression activates non-canonical Wnt pathway since it potentiates co-receptor function, in a similar way as CA-LRP overexpression in canonical Wnt pathway[55]. Overexpression of both co-receptors would bypass the requirement of CK1ε activity (and therefore PR61ε) for the intial steps of both pathways although in the case of non-canonical Wnt, the specific requirement of CK1ε activity still has to be determined.

Altogether, based on our results and literature in the field we propose the following model for non-canonical Wnt pathway activation (Figure D-3).
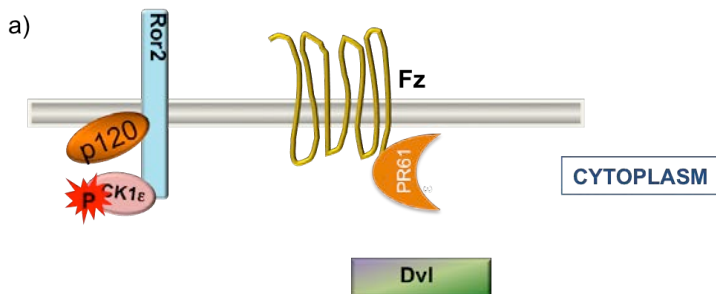
a) When the Wnt5a ligand is not present, non-canonical Wnt pathway is inactive.

b)
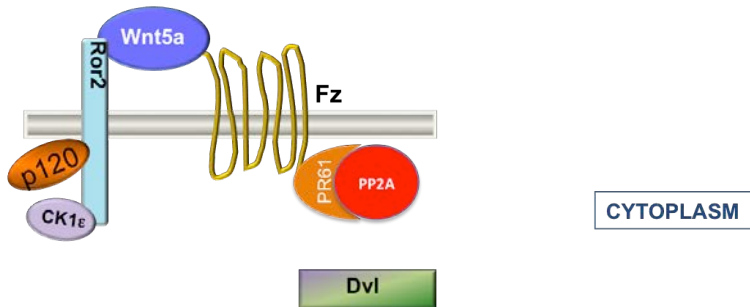
b) Upon ligand stimulation, there is a rapid complex formation between Fz, Ror2 and Wnt5a. CK1ε now, in close proximity with Fz-constitutively bound PR61ε, can be dephosphorylated and activated thanks to the action of the PP2A.

c)
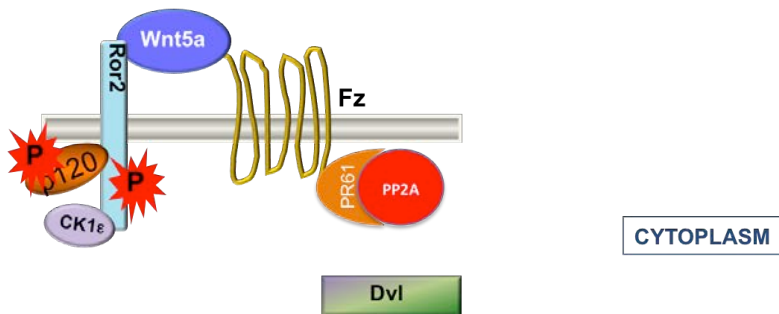
c) Active CK1ε can now phosphorylate multiple residues such as Fz, Dvl or Ror2. This will lead to Ror2 and p120-catenin tyrosine phosphorylation. P120-catenin tyrosine phosphorylated increases Ror2 stability at the membrane.
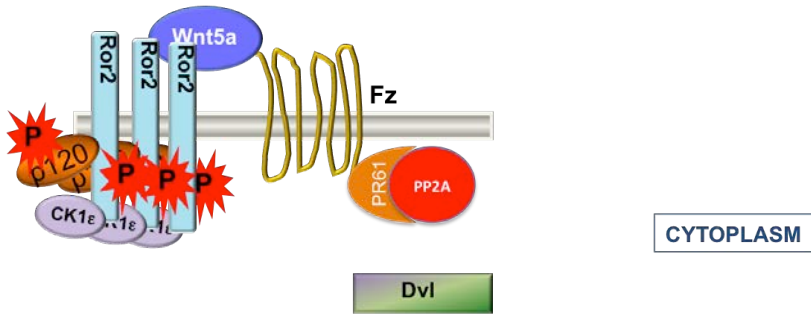
d) Ror2 tyrosine phosphorylated oligomerize with more Ror2.
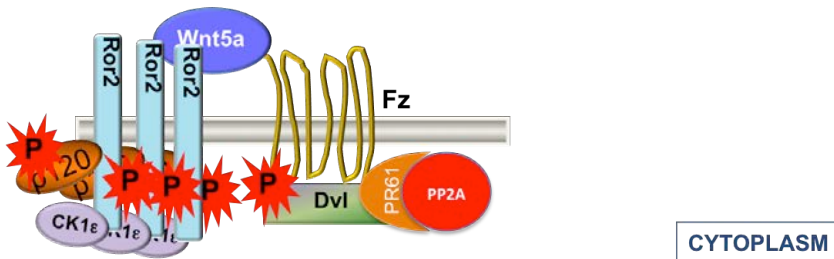
e) This will probably create a more suitable environment to permit Dvl recruitment.
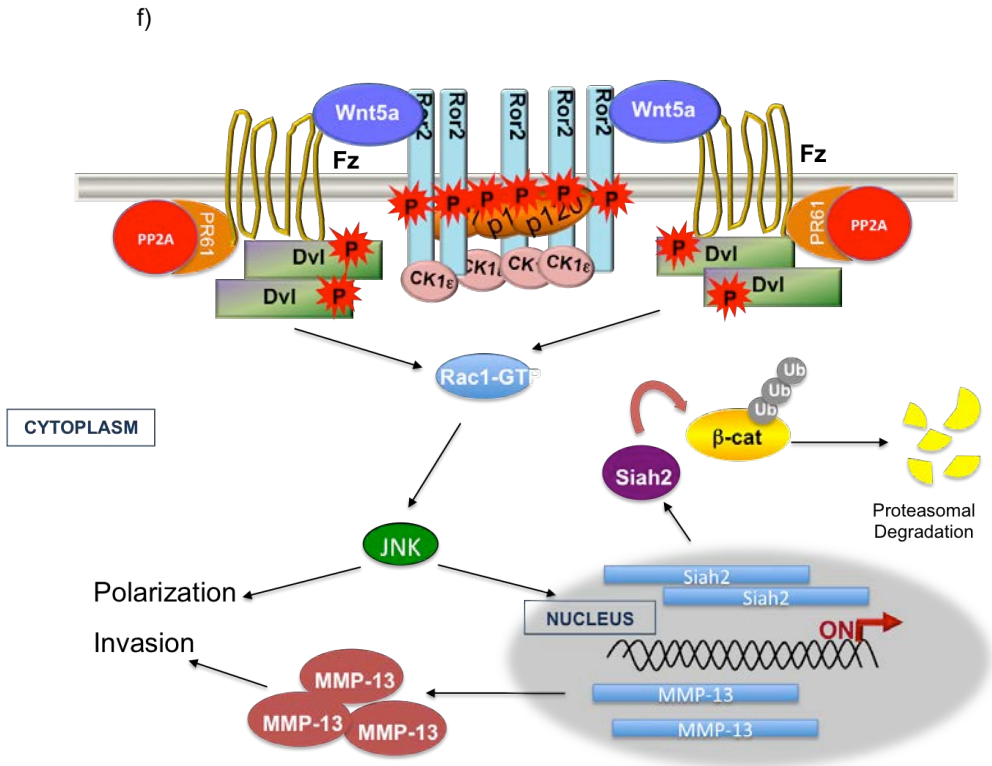
f) Dvl recruitment to Fz has the ability to polymerize with more Dvl proteins and thus, form a signaling platform that permits downstream events. Rac1 directly interacts with Dvl and gets activated. Rac1 activates JNK, which induces Siah2 upregulation and β-catenin downregulation together with increased production of matrix metalloproteases. Rac1 activation also promotes cytoskeleton reorganizations to induce polarized cell migration.

Figure D-3. Non-canonical Wnt signaling Model.

Conclusions

CONCLUSIONS

- CK1ε is activated by non-canonical Wnt through the action of PR61ε-PP2A complex in a Ror2 dependent manner.

- CK1ε activity and p120-catenin are required for assembling the non-canonical Wnt signalosome.

- CK1ε binds to the C-terminal domain of Ror2 and is also needed to stabilize Ror2 protein levels.

- P120-catenin directly interacts with the juxtamembrane domain of Ror2 and maintains Ror2 at the plasma membrane avoiding its internalization.

Final conclusion:

- **P120-catenin and CK1ε are essential for the initial and late responses in the non-canonical Wnt signaling.**

Materials and methods

1. Cell culture

1.1. Eukaryotic cell lines

1.1.1. Employed cell lines

The following cell lines were used in the development of this project:

Cell Line	Origin	Characteristics
HEK 293T	Human Embryonic Kidney	• Epithelial Phenotype • N-cadherin expression • Transformed with Adenovirus 5
HEK 293T CRISPR Ctrl	Human Embryonic Kidney	• Comes from parental HEK-293T • CRISPR process with non-targeting shRNA
HEK 293T CRISPR CK1ε	Human Embryonic Kidney	• Comes from parental HEK-293T • KO for CK1ε
HEK 293T CRISPR p120	Human Embryonic Kidney	• Comes from parental HEK-293T • KO for p120-catenin
L cells	Subcutaneos Mouse Connective tissue	• Fibroblastic Phenotype • Cell line L-M(TK)
L-Wnt3a Cells	Subcutaneos Mouse Connective tissue	• L selected clones with plasmid Wnt3a plasmid integrated • G418 Selection
L-Wnt5a Cells	Subcutaneos Mouse Connective tissue	• L selected clones with plasmid Wnt5a plasmid integrated • G418 Selection
mMSC (primary cell culture)	Mesenchymals stem cells coming from mouse bone marrow	• Mesenchymal Phenotype • Differentiation: adipocytes, chondrocytes and osteoblasts

Table M-1: Enumeration and Characteristics of the cell lines used in the current thesis.

1.1.2. Maintenance of cell lines

The different cell lines were maintained with Dulbecco's Modifyed Eagle's Medium (D-MEM, High glucose, Biological Industries) supplemented with 10 % Fetal Calf Serum (FCS, Biological Industries), 4.5 g/L of Sodium Pyruvate (Life Technologies), 2 mM of Glutamine, 56 U/mL of Penicillin, 56 µg/L Streptomycin and non essential amino acids (Life Technologies). Cells were kept at a temperature of 37 ºC and 5 % of CO_2.

Stock cell lines were grown in treated plastic flasks of 75 cm^2. Prior to reach confluence, medium was removed, cells were gently washed two times with PBS and then, allowed to detach with 0.5 % trypsin (Life Technology) (exposure time to trypsin before detachment depends on every cell line. Ranging from 2 to 5 minutes at 37 ºC). Then, cells were seeded again in 75 cm^2 flasks (stock cell line) or in treated plastic plates of different sizes depending on the experiments that were going to be done.

The number of cells present in a 75 cm^2 flask was determined with an optical microscope and a Neubauer chamber. The addition of trypan Blue to the mix of re-suspended cells, allowed cell integrity and viability determination.

The long-term preservation of each cell line was performed with D-MEM plus 10 % FCS and 10 % DMSO, as a cryoprotective, in liquid nitrogen. The freezing process was done at -80 ºC overnight and with a freezing container that contained isopropanol to slow down the cryogenic process. Next day frozen cells were moved to liquid nitrogen for long-term storage.

1.1.3. Wnt3a and Wnt5a conditioned medium

Control as well as Wnt5a or Wnt3a conditioned media (CM) were obtained from parental L, Wnt5a-L or Wnt3a-L respectively. Cell line maintenance was performed with D-MEM 10 % FCS for L cells and supplemented with G418 at 0.4 mg/mL for L-Wnt3a and L-Wnt5a (to avoid losing plasmid expression).

To obtain CM, all L cell lines were seeded in 75 cm^2 flasks with D-MEM plus 1 % FCS but without G418. After 4 days, first batch was recovered and

freshly D-MEM plus 1 % FCS was added to the cells. Second batch was recovered after 3 more days and mixed with first batch. First and second batch were centrifuged to get rid of the cells that could be present. Supernatant was stored at 4 ºC up to one month. Control-CM came from parental and untransfected L cells while Wnt3a or Wnt5a came from L-Wnt3a or L-Wnt5a, respectively.

1.1.4. Cellular treatments

Depending on the experiment performed, different cellular treatments were done:

- Treatment with conditioned media Wnt3a or Wnt5a: Cells were treated with the corresponding CM depending on which pathway activation was looked for: Wnt3a for canonical pathway activation and Wnt5a for non-canonical. Prior to CM treatment, cells were serum depleted (D-MEM plus 1 % FCS) overnight or over a whole day. Then, CM treatment varies from 5 minutes to up to 16 hours and is specified in each experiment.
- Herbimycin A treatment: Herbimycin A (Sigma, H6649) was used as a general tyrosine kinase inhibitor at a concentration of 20 ng/mL. The duration of the treatment depends on the experiment. When combined with short-time CM treatment, cells were first pre-treated with the inhibitor alone for 1 hour. When the treatment with inhibitor and conditioned media was overnight, cells were not pretreated with the inhibitor alone.
- IC261 treatment: IC261 (CalbioChem, 400090) is an inhibitor that targets specifically casein kinase 1 family with a preference for casein kinase 1δ and casein kinase 1ε. Also inhibits CK1α but at higher concentration. IC261 was used at a concentration of 15 μM and the duration of the treatment varied according to the experiment. When used in combination with CM, cells were first pretreated with IC261 alone for 1 hour.
- Monodansylcadaverine (MDC) treatment: (Sigma, 30432) is an inhibitor that targets the clathrin-mediated internalization of proteins. MDC was used at a concentration of 50 μM 30 minutes before conditioned-medium treatment.

- <u>Cycloheximide treatment:</u> (Sigma, C4859) Cycloheximide is a eukaryote protein synthesis inhibitor. It exerts its function blocking translational elongation step. Cycloheximide was used at a final concentration of 50 µg/mL during different time points specified in the results part.
- <u>JNK inhibitor treatment:</u> (CalbioChem, 420119) is a selective and reversible inhibitor that targets c-Jun N-terminal kinase (JNK) by impeding ATP binding. JNK inhibitor was used at a concentration of 50 µM overnight together with CM treatment.
- <u>PI3K inhibitor (LY294002) Treatment:</u> (Biomol International, Cay70920-10) LY294002 is a strong and reversible inhibitor of PI3 kinase. It was used at 50 µM altogether with CM in overnight treatments.
- <u>ERK1/2 inhibitor (U0126) Treatment:</u> (Cell Signaling, 9903S) It is a highly selective inhibitor of MEK 1 and MEK 2. It was used at a final concentration of 10 µM together with CM treatment overnight.

1.2. <u>Prokaryotic cell lines</u>

1.2.1. <u>Employed cell lines</u>

Based on the application, two different *Escherichia coli* strains were used. DH5α strain was employed for transformation processes of different plasmids whose amplification and ulterior purification was looked for. Otherwise, when high recombinant protein production was the objective, BL21 strain was used.

1.2.2. <u>Prokaryotic culture medium</u>

All the different prokaryotic strains were grown in the rich medium *Luria Broth* (LB) in liquid or solid state (LB and LB-Agar respectively). The medium is sterilized in an autoclave machine for 20 minutes at 120 ºC. To select the appropriate strain based on the transformed plasmid (that contain the antibiotic resistance *cassette*), different antibiotics were used: Ampicillin at 150 mg/L, Kanamycin at 30 mg/L.

The prokaryotic cell culture was grown in an incubator at 37 ºC with LB-Agar (and appropriate antibiotics) in a petri dish when we wanted to isolate single colonies. These petri dishes can be stored at 4 ºC for weeks.

For plasmid or protein purification, cells were grown in liquid LB at 37 ºC and agitation. Cellular colonies grown in liquid LB can be stored at -80 ºC for years, when supplemented with glycerol at a final concentration of 15 %.

2. Methods of DNA purification, manipulation and analysis

2.1. Plasmid DNA transformation and purification

To transform DNA into *E. Coli*, we used the Ultracompetent cells derived from the Inoue Method. Aliquoted ultracompetent cells are stored in -80 ºC and, when needed, thawed on ice. 100 μL of cells were used for up to 10 μL of DNA (5-50 ng). The mix was kept on ice for 60 seconds and then, thermal shock of 90 seconds at 42 ºC. After thermal shock, cells were put back to ice for 5 more minutes, 900 μL of LB were added without antibiotic and the cells were let to recover 1 hour at 37 ºC with agitation. Finally, cells were plated in a LB-agar plate with the antibiotic of the transformed plasmid.

For plasmid DNA isolation from a prokaryotic saturated cell culture grown in liquid LB, the commercial Wizard Plus SV Minipreps (Promega) or Maxipreps (Qiagen) kits were used.

2.2. DNA cloning

2.2.1. Subcloning

To be able to subclon a DNA insert of interest between different plasmid vectors, the next scheme was followed:

1. Vector and Insert linearization
2. DNA bands purification in an agarose gel
3. Vector Dephosphorylation
4. Ligation

2.2.1.1.   Vector and insert linearization

To obtain a determined DNA insert and vector for subcloning, the plasmids DNA were digested with restriction enzymes to be able to release the insert and linearize the vector of choice. The enzymes and reagents used were from New England Biolabs and the total amount of DNA was of 1 µg in a reaction final volume of 50 µL.

In some clonings, the DNA insert did not come from a former plasmid but from eukaryotic cellular mRNA. In those cases, total mRNA was extracted from eukaryotic cells using total RNeasy Mini Kit (Qiagen) followed by a step of reverse transcription (RNA → DNA;) with Transcriptor First Strand cDNA Synthesis Kit (Roche).

In those cases when the insert came from eukaryotic cellular RNA or from a DNA vector to which it was necessary to add new restriction sites for subcloning, a Polymerase Chain Reaction (PCR) was done. This PCR was performed in a final reaction volume of 50 µL and using up to 500 ng of DNA. It was also used 1 unit of Taq polymerase (NE Biolabs), DNA primers at 0.2 µM and the four deoxyribonucleotide triphosphates (dNTPs; Invitrogen) at a concentration of 0.3 µM. The PCR conditions were as follows:

1.   2 minutes at 95 ºC
2.   15 seconds at 95 ºC
3.   30 seconds at 50-55 ºC (depending on primers melting temperature)
4.   1 minute per kilobase pair (Kbp) at 68 ºC

Steps from 2 to 4 were repeated 30 times to get an exponential amplification of the fragment.

5.   2 minutes at 68 ºC

2.2.1.2.   DNA bands purification from agarose gel

The DNA fragments coming from a digestion reaction with restriction enzymes or PCR were electrophoretically separated in an agarose gel. To isolate the fragment of interest, DNA bands were visualized under an UV lamp.

Then using a sharp scalpel, the band was excised by cutting the gel surrounding it. It is important to try to minimize the size of gel slice to just contain the DNA band. Finally, the DNA band was purified using the protocol for DNA extraction from agarose gel of the GFX-PCR DNA and Gel Band Purification kit (Amersham).

2.2.1.3. Vector dephosphorylation

After DNA purification from agarose gel, the vector was dephosphorylated to avoid self-ligation. For this, 0.5 units of calf intestinal phosphatase (CIP, NE Biolabs) per µg of DNA was used with the supplied buffer. The reaction was incubated at 37 ºC for 1 hour. Once the reaction was finished, another purification step with GFX-PCR DNA and Gel Band Purification kit (Amersham) was done but following the DNA purification protocol from a reaction solution.

2.2.1.4. Ligation

For this step, insert and vector must have been digested with compatible restriction enzymes. Once the vector was dephosphorylated, it was mixed with the insert in a molar ratio of 3:1 (insert:vector) using the ligation buffer (Roche) and 1 unit of T4-DNA Ligase (Roche) all in a reaction final volume of 10 µL. The ligation mix was incubated during 16 h at 16 ºC. The ligation product was transformed as explained in the section 2.1.

2.3. DNA analysis

2.3.1. Agarose gel analysis

To analyse and identify the different DNA fragments, electrophoresis in agarose gels was performed. These gels were prepared at different agarose concentrations (0,5-1,5 %) depending on the size of the DNA fragments to be analysed. TAE buffer was used for the electrophoresis (and also to prepare the agarose gel) and Ethidium Bromide (BrEt, Sigma) in the agarose gel (at 5 µg/mL) to be able to visualize the DNA bands under the UV lamp. The DNA 1 Kbp Ladder (NE Biolabs) was used as a molecular weight marker.

2.3.2. <u>DNA sequencing</u>

To verify that the DNA has the correct sequence it was sent to "Servei the Genòmica de la UAB" who performed an automated DNA sequencing reaction developed by Applied Biosystems. This method is based on Sanger Sequencing reaction.

2.4. <u>Description of the vectors and DNA constructs</u>

2.4.1. <u>Vectors</u>

Vector	Characteristics
pGEX-6P (Amersham)	• Prokaryotic expression • Recombinant protein expression system in three reading frames • The product is expressed as a fusion protein with GST (Glutathione S-transferase) • Tac-promoter inducible by IPTG (Isopropyl β-D-1-thiogalactopyranoside) • Ampicillin resistance • Contains specific cleavage site for PreScission protease (PSP) to remove the GST from the recombinant protein
pcDNA3	• Expressed in eukaryotic cells • Multiple cloning sites in the forward (+) and reverse (-) orientations • Ampicillin and Neomycin resistance
pLKO	• Expressed in eukaryotic cells • Encoding short hairpin RNAs (shRNAs) to diminish the targeted endogenous protein levels • Ampicillin and Puromycin resistance
pEBG-2T	• Expressed in eukaryotic cells • GST tag expression • Ampicillin resistance

Table M-2. Backbone of the used vectors.

2.4.2. <u>DNA constructs</u>

Vector	Origin
pGEX-6P	
pGEX-6P1 p120-catenin (full length)	Derived from previous work in our laboratory.
pGEX-6P1 p120-catenin (102-end)	Derived from previous work in our laboratory.
pGEX-6P1 p120-catenin (350-end)	Derived from previous work in our laboratory.
pGEX-6P3-cytoRor2 (426-end)	Cloned from pcDNA3.1 Ror2-HA vector into pGEX-6P3 using the following primers: FW: 5'-AATAAGGGATCCTGCATGTGCCGCAAC-3' (Flanked by BamH1) and RV: 5'-TTAATAGAATTCCATGAGCCGCCTCGG-3' (Flanked by EcoRI).
pGEX-6P3-cytoRor2 (426-563)	pGEX-6P3-cytoRor2 (426-end) was cut whit EcoR1 giving two fragments. We kept the fragment of 5870 bp and religate itself after isolating it. The vector in which is placed is still pGex 6P3.
pGEX-6P3-cytoRor2 (563-end)	pGEX-6P3-cytoRor2 (426-end) was cut with EcoR1 and the fragment of 1230 bp was kept. Then, pGEX-6P1 was cut with EcoR1 and dephosphorylated with CIP to avoid self-ligation. Finally, the cytoRor2 fragment was ligated in the pGEX-6P1.
pGEX-6P3-CK1ε (full length)	cDNA of CK1ε was obtained by PCR from HEK293T cells with the following primers: FW: 5'-TAACCCGGGATGGAGCTACGTGTGGGGA-3' (flanked by XmaI) and RV: 5'-TTAGCGGCCGCTCACTTCCCGAGATGGTCA-3' (flanked by NotI). Then, after enzymatic digestion, cDNA was placed in pGEX-6P3.
pEBG-2T	
pEGB-2T	Empty vector. We had it in our laboratory.
pcDNA3.1	
pcDNA3.1 Ror2-HA	Given by Dr. Y. Minami
pcDNA3.1 Ror2-Flag	Given by Dr. Y. Minami

pLKO	
pLKO shRNA-non targeting	SIGMA (#SHC001, #SHC002)
pLKO shRNA-p120 catenin	SIGMA (#TRCN122988, #TRCN122987)
pLKO shRNA-CK1ε	SIGMA (#TRCN1837, #TRCN1836)
pLKO shRNA-Ror2	SIGMA (#TRCN1492, #TRCN10625)
pLKO shRNA-PR61ε	SIGMA (#TRCN2558, #TRCN2560)

Table M-3. DNA constructs employed during the project

3. Methods of protein expression and purification

3.1. Recombinant protein expression and purification in prokaryotic cells

Recombinant GST fusion proteins were expressed in *E. Coli* and were purified by affinity chromatography using glutathione-Sepharose beads.

Initially a starter culture from the glycerol stock with the desired protein-coding vector was grown overnight at 37 ºC in LB-Ampicillin and agitation. Next day, the starter culture was diluted 40 times in LB-Ampicillin and left growing at 37 ºC until the exponential phase of growth (Absorbance of 0.6 at a wavelength of 600 nm). At that moment, protein expression was induced with the addition of 0.1 mM IPTG and the cell culture was left for 2 more hours at 37 ºC and agitation. Finally, cells were centrifuged at 3134 g for 10 minutes in a temperature of 4 ºC.

Depending on the solubility of the protein expressed, there are two different ways of lysing the cells:

- Soluble proteins: Cellular pellet was resuspended in PBS with phosphatases (10 mM NaF, 0.1 mM sodium orthovanadate and 2.5 mM β-glycerol phosphate) and proteases inhibitors (0.3 μM, aprotinin, 1 μM leupeptin, 1 μM pepstatin, 1 mM AEBSF), sonicated 5 times with 30 seconds resting between them and finally, Triton X-100 (Sigma) was added at a final concentration of 1 %. The cell extract was left with

gentle agitation at 4 ºC for 30 minutes after which was centrifuged at 4 ºC, 10 minutes at 17000 g.

- <u>Low-Soluble proteins</u>: Pellet was resuspended in buffer STE + 0.1 µg/mL lysozyme + protease and phosphatase inhibitors and left 15 minutes on ice. Sarcozyl was then added at 1 % concentration and the mix is vortexed for 1 minute. Next, the extract was sonicated 5 times alternating with rest periods of 30 seconds. At the end, Triton X-100 (Sigma) was added at 1 % final concentration and the mix was centrifuged at 17000 g for 20 minutes at 4 ºC. Pellet was discarded, 2 % more of Triton X-100 was added to the supernatant and the mixture was vortexed for 2 minutes.

At this moment, 1:50 volume of glutathione-Sepharose-4B (Pharmacia) was added to the supernatant and then left in the cold room (4 ºC) for 1 hour with gentle agitation. After 1 hour, the beads were washed 3 times with PBS (resin is centrifuged for 1 minute at 500 g and 4 ºC between washes). Now, whether we wanted the protein of interest bound to GST or not, there are two ways of proceeding:

- <u>GST-bound:</u> Resin was incubated with glutathione elution buffer for 1 hour at 4 ºC in a tube stirrer. In this step, GST gets unbound from glutathione-Sepharose beads and, then, to eliminate the glutathione from the supernatant the protein was dialyzed overnight.
- <u>GST-unbound</u>: Resin was incubated with PreScission Protease (PSP, Amersham-Pharmacia) buffer, 1 unit of PSP for every 50 µg of fusion protein and left overnight at 4 ºC in a tube stirrer.

Whether the final recombinant protein was bound or not to GST, it was aliquoted and stored at -20 ºC in 10 % glycerol. The amount of protein and its purity were assessed by Polyacrylamide gel electrophoresis (PAGE) and Comassie Blue (Sigma) staining.

3.2. <u>Protein expression in eukaryotic cell lines</u>

Cell line of interest was seeded in different plate sizes depending on the experiment that was going to be done. After seeding, cells were left 8-24

hours to properly adhere and grown until the confluence was about 50 %. That was the moment when the cells could be transfected.

The main cell line used was HEK 293T because they have not known mutations in the proteins associated with Wnt pathway and can be transfected routinely with Polyethilamine (PEI, Polysciences Inc.). The amount of DNA transfected depended on the size of the plates were the cells had been seeded.

Plate Diameter	Plate Surface	DNA
10 cm	60 cm^2	12 µg
60 mm	20 cm^2	3-4 µg
35 mm (6 well)	10 cm^2	2 µg
15 mm (24 well)	2 cm^2	0.4 µg

Table M-4. Amount of plasmid DNA transfected depending on the size of the plate

DNA was firstly mix with D-MEM (without antibiotics and serum). Then, 10 µL of PEI was added per each µg of DNA and the whole solution was gently vortexed for 15 seconds. The mixture was incubated for 20 minutes at room temperature and gently dispersed over the plate dropwise. After 6-12 hours of transfection cell medium was replaced by fresh D-MEM with 10 % serum to avoid PEI toxicity. Transfected cells were used to perform experiments past 24-72 hours of transfection based on the plasmid DNA used and the experiment to be done (high or low expressing plasmid and overexpression vs protein downregulation).

4. Techniques for protein study

4.1. Electrophoresis and Western Blot

To analyse the results of the performed experiments, since we mainly work at protein level, the foremost method was Western Blot. PAGE was performed in denaturing conditions with Sodium dodecyl sulphate (SDS). Used gels were 1.5 mm thick and had 8-12 % of polyacrylamide. The electrophoresis was performed with the Mini-Protean III/Tetra system (Bio-Rad). Prior to loading the protein samples into the gel, loading buffer was added and the mixture was boiled 5 minutes at 95 ºC. The gels were run with 1x Tris-Glycine-SDS buffer (TGS 10x, Bio-Rad) at different voltage:

- 100 V: While protein sample run through the stacking gel.
- 160 V: Once protein sample had already entered resolving part.

The molecular weight marker used was the Precision Plus Protein Dual Color Standard (Bio Rad).

To visualize the protein samples directly after SDS-PAGE, we performed a staining of the gel with Comassie Brilliant Blue during 30 minutes at room temperature with slow agitation. Later the gel was washed several times (until protein bands could be clearly seen) with destaining solution and finally the gel was left in water.

Otherwise in Western Blot the SDS-PAGE is transferred to a membrane by means of electrotransference to specifically immunodetect the proteins of interest. For this purpose, after gel electrophoresis the proteins were transferred to a Polyvinylidene difluoride (PVDF) membrane (Immobilion-P Transfer Membrane 0.45 μm; Millipore) in a wet system (Bio-Rad) at 100 V for 60 minutes with transference buffer.

Before immunoblotting, the PVDF membrane was blocked with a solution of 1 % BSA in TTBS (Tris-Buffered Saline plus Tween 20) during 45 minutes at room temperature in a shaker. Next, the membrane was incubated 2 hours at room temperature or overnight at 4 ºC with the desired primary

antibody diluted in 0.1 % BSA-TTBS at a concentration determined experimentally.

The following antibodies were used in this project:

Primary Antibody	Host	Epitope	Dilution	Reference	Producer
CK1ε	Mouse	248-414	1/2000	610445	BD Transduction Labs
CK1γ	Rabbit	170-393	1/1000	64829	Abcam
Dvl-2	Rabbit	Around Ser638	1/1000	3216	Cell Signaling
Dvl-2	Rabbit	623-697	1/500	sc-13974	Santa Cruz
Axin	Goat	210-410	1/500	AF3287	R&D Systems
Fz-2	Rabbit	N-terminal	1/1000	52565	Abcam
N-cadherin	Mouse	735-883	1/1000	610921	BD Transduction Labs
GST	Goat	-	1/10000	27-4577-01	GE Healthcare
LRP5/6	Rabbit	Residues around M1409	1/1000	2560S	Cell Signaling
pLRP5/6 Ser1490	Rabbit	PSer-1490	1/500	2568	Cell Signaling
p120-catenin	Mouse	326-632	1/1000	610134	BD Transduction Labs
p120-catenin-pS268	Mouse	pS268	1/500	sc-293000	Santa Cruz
β-actin	Mouse	N-terminal	1/10000	A5441	Sigma
β-catenin	Mouse	696-750	1/1000	610153	BD Transduction Labs
GSK-3β		1-160		610201	BD Transduction Labs
EEA1	Mouse	3-281	1/1000	610457	BD Transduction Labs
PP2A Regulatory Subunit PR61ε	Rabbit	-	1/500		Jin et al, 2009

Ror2	Mouse	868-943	1/500	sc-374174	Santa Cruz
Rac1	Mouse	-	1/1000	610650	BD Transduction Labs
P-Tyrosine general	Mouse	P-Tyrosine	1/1000	610000	BD Transduction Labs
P-ERK1/2	Rabbit	pT202/Y204	1/1000	4370	Cell Signaling
ERK1/2	Mouse	-	1/1000	9107	Cell Signaling
P-JNK	Rabbit	JNK peptide P-Thr183/P-Tyr185/P-Thr221/P-Tyr223	1/500	07-175	Millipore
JNK2	Rabbit	C-term JNK2	1/1000	178953	Abcam
HA tag	Rat	-	1/5000	11867423001	Roche
Flag tag	Mouse	-	1/5000	F3165	Sigma

Table M-5. Used Antibodies

After primary antibody incubation, membrane was washed three times of 10 minutes each with TTBS. Once done, membrane was incubated with secondary antibody for 1 hour at room temperature. Finally, membrane was again washed 3 times of 10 minutes each of them with TTBS. Secondary antibody signal was developed with Immobilon™ Western Chemiluminiscent HRP Substrate (Millipore) and photographic films (Kodak).

When the same membrane had to be blotted with different primary antibodies the first antibody was removed incubating the membrane for 20 minutes at 55 ºC with stripping buffer, generously washed with TTBS, blocked with TTBS + 1 % BSA and then, the membrane ccould be blot once more.

4.2. Eukaryotic cell lysis

In order to obtain the protein extracts, plated cells were washed two times with cold PBS (4 ºC) and scrapped with the elected lysis buffer (amount of buffer depends on the size of the plate and cellular confluency to get an approximate protein concentration of 1 to 2 μg/μL). Lysis buffer must contain the following proteases and phosphatases inhibitors to avoid losing protein or its modifications: 0.3 μM Aprotinin, 1 μM Leupeptin, 1 μM Pepstatin, 1 mM AEBSF as inhibitors of proteases and 10 mM NAF, 2.5 mM β-glycerol-phosphate and 0.1 mM of sodium orthovanadate as phosphatases inhibitors. For this cellular lysis different buffers can be used and the election of one in front of another will depend on the experiment and what is needed to solubilize. Having this in mind, different buffers were used:

- NP-40 0.5 % Buffer: Used to mainly solubilize cytosqueleton or membrane proteins. After scrapping cell extracts were centrifuged at 16000 g 10 minutes at 4 ºC and supernatant was kept.
- RIPA buffer: Used to solubilize almost all cellular compartments and keeping only the strongest interactions. Once cell layer had been scraped, cell extracts were passed 10 times through a 25-gauge syringe. Later, 16000 g centrifugation 10 minutes at 4 ºC and kept the supernatant.
- PAK buffer: it is a little variation of NP-40 0.5 % having, as principal differences, NP-40 at a final concentration of 1 % and addition of glycerol 10 % to increase protein solubility and stability as active GTP-bound Rac1 is a very labile protein. Once solubilized, cell extracts were centrifuged at 16000 g 10 minutes at 4 ºC and supernatant was kept.

4.3. Protein-protein interaction assays

4.3.1. Binding assay

This assay is based on the capability of two or more recombinant proteins to interact with each other. To analyse this interaction, on of the proteins is GST-tagged (the one that will be bound to the glutathione-Sepharose beads) and the other lacks the GST tag (it has been removed with PSP).

The indicated amount of each protein was added to a final volume of 300 μL of binding buffer and the mix was incubated at 4 ºC for 1 hour in a rotatory wheel. 20 μL of effective glutathione-Sepharose beads were added and was left 1 hour more at 4 ºC in the same rotatory wheel. Finally, the resin was washed 3 times with 500 μL of PBS+NP-40 0.1 % to reduce the unspecific binding of recombinant proteins. Solubilisation of resin-bound complexes was done with loading buffer at 1x final concentration and 5 min boiling at 95 ºC. Protein levels were analysed by western blot.

As a control of the binding experiments, the non-GST tagged recombinant proteins were assayed to interact with GST alone to eliminate the possibility of GST acting as the interacting partner.

This experiment has also been done with previously phosphorylated recombinant proteins. The phosphorylation protocol is explained below.

4.3.2. Pull-down assay

This assay studies the ability of recombinant GST-tagged proteins to interact with proteins present in a cellular extract.

The indicated amount of recombinant protein and cellular extracts (600 μg of extracts lysed with NP-40 0.5 % buffer) are added to a final volume of 600 μL with PBS and incubated 1 hour at 4 ºC in a rotatory wheel. 20 μL of effective glutathione-Sepharose beads were added and left 1 hour at 4 ºC. To finish, beads were washed 3 times with 500 μL of PBS+NP-40 0.1 % and complexed proteins were released with loading buffer at 1x final concentration and 5 minutes boiling Protein products were analysed by Western Blot.

In this experiment the recombinant protein can also be phosphorylated prior to the binding step.

4.3.2.1.   PAK pull-down assay

This specific form of pull-down assay uses the Cdc42/Rac interactive binding region (named also p21 Binding Domain, PBD) of the Cdc42/Rac effector protein, p21 activated kinase 1 (PAK). PAK specifically binds the active

GTP-bound form of Rac and/or Cdc42 proteins. The fusion protein GST–PAK was purified from bacteria and bound to glutathione-Sepharose beads to precipitate GTP–Rac1. Cells were, after the specified treatments, washed two times with PBS, lysed in PAK buffer and centrifuged for 5 minutes at 16,000 g. The supernatant was incubated at 4°C with recombinant GST–PAK for 60 minutes. Samples were washed three times with PBS+NP-40 0.1 % and bound proteins were analyzed by western blot.

4.3.3. Coimmunoprecipitation assay

This assay determines the interaction of a protein contained in a cellular extract with another protein or proteins present in the same cellular extract. The analysed protein is bound to an antibody that specifically recognizes it and then the antibody (plus the protein bound and its interactors) is pulled-down with a special type of beads that bind to its heavy chains. This assay can be performed with endogenous proteins or overexpressing the protein of which we want to study its interactors.

The cellular extracts were obtained with 0.5 % NP-40 lysis buffer supplemented with protease and phosphatase inhibitors. When indicated, prior to cell lysis, the inhibitors of CK1 IC261 (CalbioChem, 400090) and Src tyrosine kinases Herbimycin A (Sigma, H6649) were added. Extracts were left on ice for 10 min and centrifuged at 16000 g for 10 minutes at 4 ºC. Supernatants constituted the cell extracts. Proteins were immunoprecipitated from cell extracts (400 to 1000 µg) using 1 µg/mL of the appropriate antibody or an irrelevant IgG as control for 16 h at 4 ºC. The higher amount of cell extracts was used to immunoprecipitate phosphorylated proteins with the general anti-phospho-tyrosine antibody. Lowest amount was used when overexpressing proteins. Range between 500-900 µg was used for intracellular proteins immunoprecipitation. The final protein concentration in the coimmunoprecipitation assay was of 1 µg/µL. When this assay was performed to evaluate the kinase activity of the immunoprecipitated protein, cell extracts were incubated with the specific antibody for just 4 hours at 4 ºC. After antibody binding step, samples were incubated for 2 h with 20 µL of γ-bind G-Sepharose (GE-Healthcare). Immunoprecipitates were washed three times with PBS+0.1 % NP-40 and bound proteins were eluted with electrophoresis sample buffer and analyzed by WB.

4.4. Cellular protein localization assay

4.4.1. Immunofluorescence

This assay is used to study the presence and the distribution of target molecules in a sample using specific antibodies. This technique can be primary (or direct) or secondary (indirect) depending on which is the antibody conjugated to fluorescent dyes. In our laboratory we use the indirect method since we use two antibodies. The first (primary) recognizes specifically the target molecule, and the secondary antibody, which is the one chemically linked to the fluorophore, recognizes the primary antibody heavy chains.

Cells were seeded in 24 well-plates over sterile round coverslips (15 mm diameter) previously treated with poly-D-lysine. After appropriate cellular treatment with or without conditioned media and or overexpression of proteins of interest, cells were fixed with 4 % paraformaldehyde for 15 minutes and washed 3 times with PBS. Then, cells were permeabilized to allow antibody entrance to the intracellular compartment with a solution of 0.2 % triton x-100 in PBS for 5 minutes. Cells were washed 3 times more with PBS after permeabilization. After, cells were rinsed with 3 % BSA solution in PBS for 30 minutes at room temperature (RT) to block non-specific protein interactions. After 30 minutes, blocking solution was removed and the desired specific primary antibody applied at 1/50 dilution in PBS+3 % BSA for 1 hour at RT. Next, coverslips were washed again 3 times more with PBS and cells were then incubated with secondary antibody at a concentration of 2 µg/µL in PBS+3 % BSA for another 1 hour at RT. Since secondary antibodies were labelled with fluorophores, from this step on, the coverslips were protected from light to avoid the photobleaching of the fluorophores. The secondary antibodies used were Alexa Fluor 555 goat anti-rabbit IgG, Alexa Fluor 647 rabbit-anti Rat IgG i Alexa Fluor 547 goat anti-mouse IgG. Coverslips were washed 3 times more with PBS to remove the excess of unbound secondary antibody and incubated with DAPI at 500 ng/mL in PBS for 10 minutes at RT to stain nuclei. To finish, 3 more washes with PBS, 1 with distilled water to remove the excess of salts and coverslips were fixed over a slide with Mowiol (Calbiochem).

Once the mowiol had dried, cells were observed under a confocal Leica microscope (Leica spectral confocal TCS-SL), exciting the fluorochromes with the laser at the required wavelength.

4.4.1.1. Polarization assay

A little modification of immunofluorescence protocol was used just in the case of the <u>Single-cell cortical actin polarization analysis:</u> 40 µl of Matrigel (BD Bioscience, 354230) were deposited on a round coverslip (15 mm diameter) and incubated at 37 °C for 30 min to let the gel solidify. mMSC cells were trypsinized to a single-cell suspension and 5×10^4 cells in Control or Wnt5a conditioned media and 2 % Matrigel (V/V) were seeded on top of the solidified gel. Cells were incubated for 2 h with the indicated media and analyzed by immunofluorescence. Cells were then fixed with 4 % paraformaldehyde for 15 min, and incubated with PBS+0.2 % Triton X-100 for 5 min. After blocking with 3 % BSA in PBS for 30 min at room temperature, CytoPainter Phalloidin-iFluor 647 Reagent (Abcam, 176759) was added for 1 h at room temperature. Slides were washed three times with PBS and incubated for 10 min with DAPI for nucleus identification. Coverslips were mounted on glass slides with Fluoprep (BioMérieux, 75521), and immunofluorescence was analyzed with a Leica confocal microscope (LEICAspectral confocal TCS-SL).

4.4.2. <u>Cell surface biotinylation assay</u>

This assay is used to analyse which proteins are placed at the plasmatic membrane exposed to the extracellular side of the cell. After the corresponding cellular treatments, medium was removed, HEK 293T cells were washed 3 times with PBS and then incubated with 0.5 mg/mL sulfo-NHS-LC-biotin (Pierce, Rockford, IL) at 4 °C for 30 minutes. Excess of biotin was washed away with PBS and finally quenched with 50 mM NH_4Cl. This step was followed by 3 more washes with PBS and, after, cells were lysed in 0.5 % NP-40 lysis buffer. Lysates were incubated with Streptavidin-Agarose (Sigma) beads for 1 hour at 4 ºC. Beads were collected with low-speed centrifugation at 500 g, washed three times with PBS+0.1 % NP40 buffer and analyzed by Western Blot with specific antibodies.

4.4.3. Cell surface protein digestion assay with Proteinase K

This assay is used for the same purpose as the previous one, i.e., analyse membrane proteins exposed to the extracellular side but with a different approach. In this case, all the accessible proteins to which proteinase K is in contact will be digested. Therefore, the proteins at the cellular membrane will be broken down due to the action of the proteinase K but, the proteins that are inside the cell or membrane proteins that have been internalised, will be physically protected. For this assay, cells were seeded in plates of 10 cm in diameter. After the desired treatments, cells were washed 3 times in PBS and treated with Proteinase K (Invitrogen) at 1 μg/mL in resuspension buffer for 10 min at room temperature. Digestion was stopped with AEBSF 20 mM in PBS. Then, this solution is removed and cells were lysed with RIPA lysis buffer and heated at 60 ºC for 5 min.

4.5. Phosphorylation assays

4.5.1. With recombinant protein

In this assay a purified recombinant protein is phosphorylated with a commercial recombinant kinase. The recombinant phosphorylated protein is later used in pull-down or binding assays.

Phosphorylation assays were done in a final volume of 50 μL, with a specific buffer that depended on the kinase used and with an ATP concentration of 100 μM. The solution contained the specific phosphorylation buffer with the recombinant protein, the commercial kinase and the same protease and phosphatase inhibitors used in cell lysis. The two commercial kinases used in this project to phosphorylate are the catalytic domain of CK1 kinase (CK1δ C-terminal truncated, New England Biolabs P6030) and Src kinase (Full length, Millipore 14-326). Phosphorylating condicions were as follows:

	Kinase (CK1/Src)
Temperature	30 ºC
Time	30 minutes
Kinase concentration (mU/pmol substrate)	300 mU
Substrate	2-10 pmol
Buffer	Phospho-Buffer
Final Volume	50 µL

Table M-6. Recombinant kinases phosphorylation conditions.

4.5.2. <u>With purified cellular kinases</u>

<u>CK1ε activity assay:</u> Protein was immunoprecipitated from NP-40 0.5 % buffer lysed HEK293T with CK1ε Ab for 4 hours at 4 ºC. Immunoprecipitates were washed three times with PBS+0.1 % NP-40 and once with phosphorylation buffer. Then, the immunocomplexes were incubated with 10 pmol of recombinant GST-p120-catenin or GST-cytoRor2 and phosphorylation buffer supplemented with 100 µM ATP in a final volume of 50 µL, for 30 min at 30 ºC. Specific phosphorylation on GST-p120-catenin Ser268 was analyzed by WB with a phospho-specific Ser268-p120-catenin Ab.

<u>Ror2-HA activity assay:</u> 4 µg Ror-HA were overexpressed in HEK293T. After 24 hours, cells were lysed with NP-40 0.5 % buffer and protein was immunoprecipitated with HA Ab for 4 hours at 4 ºC. Immunoprecipitates were washed three times with PBS+0.1% NP-40 and once with phosphorylation buffer (60 mM Tris-HCl pH 7.3, 12 mM $MgCl_2$). Then, the immunocomplexes were incubated with 10 pmol of recombinant GST-p120-catenin or GST-cytoRor2 and phosphorylation buffer supplemented with 100 µM ATP in a final volume of 50 µl, for 30 min at 30 ºC.

5. RNA isolation and PCR semiquantitative

After HEK293T cells were treated with the required conditions RNA was isolated using the RNeasy Mini Kit (Qiagen). Purified RNA was quantified using and spectrophotometer. Next, RNA was retrotrasncrived and amplified with One-Step RT-PCR kit (Qiagen), using manufacturer instructions and starting with 2 µg of total RNA amount with specific primers:

Primer	Sequence
Siah2	**Fw:** 5'-GCTAATAAACCCTGCAGCAA-3'
	Rv: 5'-ACTTCTGGCGGCATTGGTTA-3'
Ror2	**Fw:** 5'-ATCGCCCGCTCCAACCCTCTCATC-3'
	Rv: 5'-ATCCCCATCTTGCTGCTGTCTCG-3'
GAPDH	**Fw:** 5'-ACCACAGTCCATGCCATCAC-3'
	Rv: 5'-TCCACCACCCTGTTGCTGTA-3'

Table M-7. Sequences of the primers used for semiquantitative-PCR.

6. Invasion assay

This assay is used to evaluate the capability of the cells to migrate towards a chemoattractant using a Boyden chamber. When the transwell is coated with extracellular components before cell seeding, it can also be used to test the invading capabilities of the cell line through the deposited ECM. Several components can be used to artificially resemble an ECM. In our assay collagen type I was used.

Collagen type I invasion assay: For this experiment forty thousand mMSC cells were resuspended in 150 µL DMEM 0.1% BSA. Cells were seeded on a Transwell filter chamber (Costar 3422, Thermo Fisher Scientific, Waltham, MA, USA) coated with 1 mg/mL Collagen Type I (Corning, 354249) and incubated for 16 h. Control or Wnt5a conditioned media were used as chemoattractant. Non-invading cells were removed from the upper surface of the membrane, while cells that adhered to the lower surface were fixed with MetOH 100 % for 20 min and stained with Crystal Violet. Cells were eluted with 30 % Acetic Acid and absorbance was measured at 590 nm with a spectrophotometer.

7. Statistical analyses.

All the results shown were representative from at least three independent experiments. Data are presented as mean ± SD. When appropriate, statistical analyses were conducted using GraphPad Prism software (GraphPad, La Jolla, CA, USA) and data were analyzed for significance using Unpaired T-Test. P values < 0.01 are symbolized with two asterisks.

8. Reagents and solutions

If not specified, buffers or reagents are diluted in distilled water.

Binding Buffer: 50 mM Tris-HCl pH 7.8, 150 mM NaCl, 1 mM EDTA, 1mM DTT, 3 mM $MgCl_2$, 0.1 % w/v triton X-100.

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

Destaining Buffer: 40 % methanol, 10 % glacial acetic acid.

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

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

LB: 0.5 % w/v yeast extract, 1 % w/v bacto-tryptone, 170 mM NaCl, pH 7.0.

LB Agar: 0.5 % w/v yeast extract, 1 % w/v bacto-tryptone, 170 mM NaCl, 1.5 % Agar w/v, pH 7.0.

Loading Buffer 5x: 200 mM Tris-HCl pH 6.8, 15 % glycerol, 8 % SDS, 0.3 % β-mercaptoethanol, 0.5 % bromophenol blue.

NP-40 0.5 % Lysis Buffer: 0.5 % NP-40, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$.

PAK Buffer: 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, 1 % NP-40, 10 mM $MgCl_2$, 10 % Glycerol.

PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4/1.8 mM KH_2PO_4 (pH 7.4).

PEI: 1 mg/mL PEI pH 7.0.

Phospho-Buffer (for recombinant proteins): 50 mM Tris–HCl pH 7.5, 10 mM MgCl2, 0.1 mM EDTA, 2 mM DTT, with 2.5 mM β-Glycerophophsate (just for CK1 kinase) or 0.25 mM sodium orthovanadate (just for Src kinase), pH 7.0 and supplemented with the remaining protease and phosphatase inhibitors.

PreScission Protease (PSP) Buffer: 50 mM Tris-HCl pH 7.8, 300 mM NaCl, 1 mM DTT, 1 mM EDTA.

Resuspension Buffer: 100 mM K_2HPO_4/KH_2PO_4 pH 6.7, 5 mM $MgCl_2$, 250 mM sucrose

RIPA lysis Buffer: 1 % NP-40, 0.5 % Sodium deoxycholate, 0.1 % SDS up to final volume with PBS.

STE: 10 mM Tris-HCl pH 8.0, NaCl 150 mM, EDTA 1 mM.

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

TAE Buffer: 40 mM Tris-Acetate pH 8.3, 0.1 mM EDTA.

Transference Buffer: 192 mM glycine, 25 mM Tris-HCl, methanol 10 %.

Trypan Blue Solution: 0.4 % w/v Trypan Blue in PBS.
TTBS: 25 mM Tris-HCl pH 7.5, 0.1 % Tween-20, 135 mM NaCl.

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Als *cateninos* (see figure A-1) no sé per on ni per qui començar… Willy, *nuestro pequeño niño rata*: amb tu he après com fer una bona gestió conservadora de la economia, que cinc cèntims de canvi són cinc cèntims, que a un karaoke tu, jo i *Avalancha* ho petaríem; i que es poden trobar ofertes de cafè als llocs més inesperats. Sap greu no haver-te conegut abans però ereh un grande tete.

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Figure A-1. Scheme of Catenin team. From left to right, oneself, Queen of the Westernos, Neusi, Mireia, Aidasita and the little rat boy. For details, see text.

Aida, lo siento si esperabas algo más por aquí pero lo que te quiero decir te llegará de otra forma. Es lo mínimo.

Suades...Suades...Suades...qui t'ho hauria dit que acabaries parlant amb el tio aquell que borratxo feia tests de duresa i capacitat de deformació màxima als retrovisors dels cotxes i posava a prova la resistència al pH de les rajoles de casa de la Txell. Crec que ets la persona amb la que més he gaudit de discutir intel·ligentment qualsevol cosa per absurda que fos perquè tens discurs per qualsevol tema. Evidentment que no oblidaré les teves preguntes magistrals que posen a prova els principis més profunds del ser humà ni

l'humor negre que compartim i espero seguim compartint durant anys. Al final segur te n'hauràs adonat que tenim més similituds que diferències tot i que hi hagi temes que mai ens posarem d'acord. I si, sé que t'ho estàs preguntant. A Filipines pagaré els meus deutes.

Maribelilla, vam començar tard a començar a parlar però es que realment no ho posaves fàcil. Al principi tenies tanta conversa com un Erasmus Amish de Lancaster, Pennsylvania. Per sort després et vas començar a relaxar i vaig descobrir una persona meravellosa. Tens un humor molt bo tot i que encara el comparteixes poc i, personalment, ets de les persones que més admiro. No has tingut una vida fàcil però en cap moment has renunciat ni renunciaràs als teus principis ni a aquelles coses que vols fer. Ets una persona molt vital i sempre tens un somriure a la cara per compartir amb la gent. Tot i les dificultats sé que aconseguiràs qualsevol cosa que et proposis perquè, ho has més que demostrat, a lluitadora i perseverant ningú et guanya.

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