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ABSTRACT

Snail1 transcriptional factor is a key driver of EMT and controls invasion and chemo-resistance of cancer cells. Snail1 expression in tumor cells has been associated to a poor prognosis and increased resistance to chemotherapy. The exactly regulation of Snail1 in tumor cells has not been identified although several extracellular factors can induce Snail1 expression in different tumor cells and fibroblasts, such as TGF-β or Wnt factors. Canonical and non-canonical Wnt pathways are activated by Wnt factors, that can be differentiated between canonical and non-canonical Wnts. Both use a common receptor, Fz, but differ in the co-receptor, LRP5/6 for the canonical and Ror2 in the non-canonical. Both pathways also us common downstream effectors, such as Dvl2 or Fyn, although the final response is totally different. While canonical pathway promotes the stabilization of β-catenin, non-canonical Wnts promote its degradation. However, previous results of our lab have demonstrated that both, canonical and non-canonical Wnt pathways regulate a common subset of genes associated to EMT, including Snail1. In this work we have found that non-canonical Wnt pathway, and not TGF- β, is regulating Snail1 expression in colon tumor cells. Accordingly, Ror2 knock-down decreases Snail1 expression, reducing transcription and protein stability. Ror2 and Snail1 participate in a positive feedback loop, since Ror2 depletion decreases Wnt5a production. By itself, Wnt5a induces the expression of Wnt5a, Ror2, Snail1and Fz2. This Ror2/Snail1 autoactivation loop controls invasion, tumor growth and chemoresistance to cisplatin. Inhibition of Wnt5a signaling with Porcupine inhibitors decreases Snail1, Ror2 and Wnt5a expression and reduces the resistance to cisplatin in Snail1-expressing cells. Together, these results described that Snail1 expression in colon tumor cells is controlled by an autoactivated non-canonical Wnt signaling that also controls invasion and chemoresistance. Thus, inhibitors of this pathway may be useful to prevent colon tumor progression, metastasis and chemoresistance.

RESUM

El factor de transcripció Snail1 és un dels reguladors més importants de la EMT i controla la invasió i la quimioresistència de cèl·lules tumorals. L'expressió d'Snail1 en cèl·lules tumorals ha estat associada a una mala prognosi i un augment de la resistència a quimioteràpia. La exacta regulació d'Snail1 en cèl·lules tumorals no ha estat encara identificada, tot i que l'expressió d'Snail1 pot induir-se en cèl·lules tumorals i fibroblasts per diferents factors extracel·lulars, com ara TGF-β o factors Wnt. Les vies Wnt canònica i no canònica s'activen pels factors Wnt, que poden diferenciar-se en canònics o no canònics. Ambdues vies usen un receptor comú, Fz, però difereixen en el co-receptor, LRP5/6 per la canònica i Ror2 per la no canònica. Ambdues vies utilitzen també efectors *downstream* comuns, com ara Dvl2 o Fyn, tot i que la resposta final és diferent. Mentre la via canònica promou l'estabilització de β-catenina, la no canònica promou la seva degradació. Tot i això, resultats previs del nostre laboratori han demostrat que ambdues vies, canònica i no canònica, regulen un grup comú de gens associats a EMT, incloent Snail1. En aquest estudi, hem trobat que la via de Wnt no canònica, i no TGF-β, regula l'expressió d'Snail1 en cèl·lules tumorals de colon. Com a conseqüència, el *knock-down* de Ror2 redueix l'expressió d'Snail1, reduint la transcripció i l'estabilitat de la proteïna. Ror2 i Snail1 participen en un *loop* positiu d'activació, ja que la depleció de Ror2 també redueix la producció de Wnt5a. A més, l'estimulació amb Wnt5a indueix l'expressió de Wnt5a, Ror2, Snail1 i Fz2. Aquest *loop* d'autoactivació format per Ror2 i Snail1 controla la invasió, el creixement tumoral i la quimioresistència a cisplatí. La inhibició de la senyalització per Wnt5a amb inhibidors de la Porcupina redueixen els nivells d'Snail1, Ror2 i Wnt5a i bloqueja la resistència a cisplatí en cèl·lules que expressen Snail1. Aquests resultats descriuen que l'expressió d'Snail1 en cèl·lules tumorals de colon està controlada per un *loop* d'autoactivació de la senyalització no canònica de Wnt que també controla la invasió tumoral i la quimioresistència. Per tant, inhibidors d'aquesta via podrien ser útils per prevenir la progressió del càncer de colon, la metàstasis i la quimioresistència.

ABBREVIATIONS

AP-1: Activator protein 1

APC: Adenomatous polyposis coli

ATF-2: Activating transcription factor 2

BSA: Bovine serum albumin

CAFs: Cancer associated fibroblasts

CA-LRP5/6: Constitutively active Low density lipoprotein receptor-related protein

CCND1: Cyclin D1

cDNA: Complementary deoxyribonucleic acid

CK1: Casein kinase 1 family

CM: Conditioned medium

CRD: Cysteine-rich domain

Ctl: Control

DKK1: Dickkopf1

DMEM: Dulbecco-s modified Eagle's medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DUB: Deubiquitinating enzyme

Dvl2: Disheveled 2

ECM: Extracellular matrix

EGF: Epidermal growth factor

EMT: Epithelial to mesenchymal transition

EndMT: Endothelial to mesenchymal transition

ER: Endoplasmic reticulum

ERK: Extracellular signal-regulated kinase

FBS: Fetal bovine serum

FBXL5/14: F-box leucine-rich-repeat protein 5/14

FGF: Fibroblast growth factor

FN1: Fibronectin 1

Fz: Frizzled

GFP: Green fluorescent protein

GSK3β: Glycogen synthase kinase 3β

GTPasa: Guanosine triphosphatase

IL: Interleukin

Jnk2: c-jun N-terminal kinase 2

KD: Kinase dead

kDA: Kilo Dalton

KN: Kringle

KO: Knock-out

LB: Luria broth

LEF1: Lymphoid enhancer factor 1

LRP5/6: Low density lipoprotein receptor-related protein 5/6

MEFs: Mouse embryonic fibroblasts

MET: Mesenchymal to epithelial transition

Mmp: Matrix metalloprotease

mMSCs: Mouse mesenchymal stem cells

mRNA: Messenger ribonucleic acid

MVB: Multivesicular bodies

NFκB: Nuclear factor κB

PAGE: Polyacrylamide gel electrophoresis

PARP1: Poly8ADP-ribose) polymerase 1

PBS: Phosphate buffered saline

PCP: Planar cell polarity

PDZ: Post synaptic density protein, Drosophila disc large tumor suppressor and zonula occludens-1 protein domain

PEI: Polyethylenimine

PP2A: Protein phosphatase 2A

P-R: Proline-rich domain

PR61ε: PP2A regulatory subunit 61ε

PTEN: Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase

PVDF: Polyvinylidene difluoride

Rac1: Ras-related C3 botulinum toxin substrate 1

RhoA: Ras homolog family member A

RIPA: Radioimmunoprecipitation assay buffer

Ror1/2: Receptor tyrosine kinase-like orphan receptor 1/2

RTK: Receptor tyrosine kinase

RT-qPCR: Real time quantitative polymerase chain reaction

Ryk: Receptor-like tyrosine kinase

S/T-R: Serine/threonine-rich domain

SDS: Sodium dodecyl sulfate

shRNA: short hairpin RNA

STAT3: Signal transducer and activator of transcription 3

TCF4: Transcription factor 4

TF: Transcription factor

TGF-β: Transforming growth factor β

TGS: Tris glycine SDS

Thbs1: Thrombospondin 1

TM: Transmembrane domain

Treg: Regulatory T lymphocyte

TTBS: Tris-buffered saline-Tween 20

TWIST1: Twist family BHLH transcription factor 1

USP: Ubiquitin specific peptidase

WB: Western blot

WIF: Wnt inhibitor factor

YWTD: β-sheet domain

Zeb: Zinc finger E-box-binding homeobox

ZnF: Zinc finger domain

α-SMA: α-smooth muscle actin

β-TrCP1: β-transducin-repeat containing protein

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INTRODUCTION

1. Wnt signaling pathway in colorectal cancer

Colorectal cancer is the second and third most common cancer in women and men, respectively, ant it is a major cause of cancer mortality[1,2]. Importantly, in the majority of the cases, patients are diagnosed at advanced ages, which has a high impact in mortality, making this cancer the fourth most common cause of cancer death worldwide[2]. In general, the majority of colorectal cancer cases are sporadic. However, there are hereditary colorectal cancer cases caused, principally, by mutations in the adenomatous polyposis coli (*APC*) gene, which controls activation of the Wnt signaling[2].

Wnt signaling pathway plays a key role during embryonic development and tissue homeostasis by controlling different cellular functions such as proliferation, differentiation, migration or invasion[3,4]. Therefore, as mentioned above, deregulation of the Wnt signaling pathway drives to problems in development and more importantly to tumorigenesis and metastasis.

Wnt signaling is divided in two different pathways, the canonical and the non-canonical Wnt pathway, depending on the capacity to stabilize β-catenin, the central protein in the Wnt signaling pathway[5,6]. Indeed, the canonical Wnt pathway is also known as β-catenin-dependent and the non-canonical as β-catenin -independent pathway.

In the intestine, canonical Wnt pathway is essential for the maintenance and self-renewal of epithelial stem cells, which are located at the base of the intestinal crypts[7,8]. In fact, the higher expression of canonical Wnt target genes is in the bottom of the crypt and decrease over the top, generating a Wnt gradient along the crypt axis[8] (Figure 1). Due to its important role in controlling the intestinal homeostasis, mutations in the canonical Wnt pathway lead to the appearance of colorectal cancer[9]. The majority of colorectal cancer cases present mutations in the APC gene, although mutations in other members of canonical Wnt machinery have also been described[8,9].

Despite the abnormal activation of the canonical Wnt signaling is the cause of most colorectal cancer cases, in recent years the contribution of the non-canonical pathway to the progression of colorectal cancer has also been considered. Specifically, increased expression of Wnt5a has been observed in colorectal cancer samples[10]. Moreover, this augmented Wnt5a expression correlates with poor prognosis of colorectal cancer patients[11].

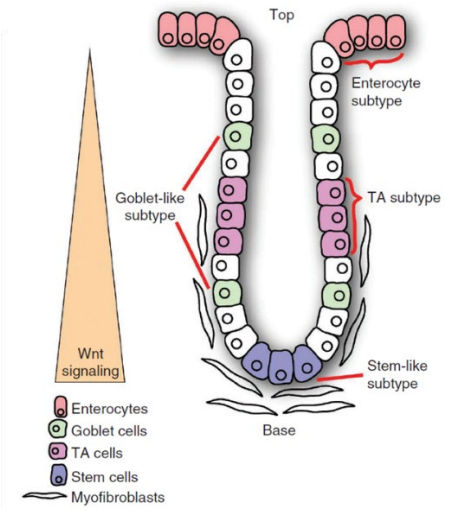

Figure 1: Intestinal crypt representation. The diagram shows the spatial distribution of the different cell types found in the intestinal crypts and the Wnt gradient. On the top, there are the enterocytes, in the middle the rapidly proliferating transit-amplifying (TA) cells and the goblet cells. In the bottom, where more Wnt signaling occurs, the intestinal stem cells are located. Adapted from [1].

In fact, while canonical Wnt pathway is mostly regulating the initiation of colorectal cancer, the non-canonical pathway could be more involved in the migratory and invasive capacities of the colorectal cancer cells[11]. Thus, non-canonical Wnt pathway also plays an important role in colorectal cancer malignancy.

2. General components of the Wnt signaling

2.1. The Wnt ligands

Activation of the canonical or the non-canonical pathway depends on the Wnt ligands that bind to the receptors. Wnt ligands are a family of secreted glycoproteins that in mammals is comprised by 19 members[12]. Wnt factors are proteins of about 350 to 400 amino acids enriched in cysteine residues and with a N-terminal signaling peptide that targets for secretion[12].

Wnt factors suffer posttranslational modifications in the endoplasmic reticulum (ER), such as glycosylation and palmitolaytion, are required for a correct secretion and activity[13]. Porcupine protein, a transmembrane protein in the ER, is the responsible of Wnt palmitoylation and is a key protein for Wnt secretion[14]. For this reason, in the last years, Porcupine inhibitors have come out as potent inhibitors of the Wnt pathway for different type of cancers[15,16] (Figure 2).

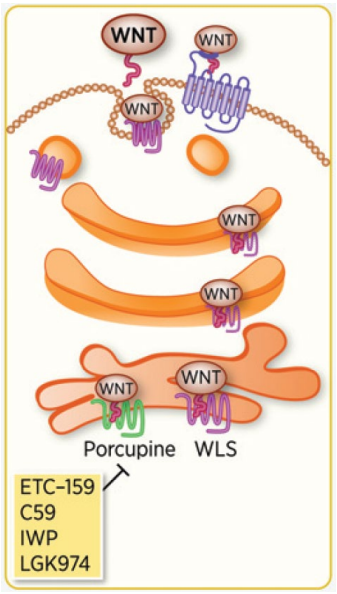

Figure 2: Wnt secretion route. Wnt factors suffer posttranslational palmitoylation by Porcupine in the ER. This modification is necessary for its transport to the plasma membrane by Wintless protein. Inhibitors of Porcupine, such as WntC59 or LGK974 prevent Wnt palmitoylation and secretion. Adapted from [17].

Although it is difficult to classify the Wnt factors among canonical and non-canonical, classically, Wnt1, Wnt3a, Wnt8 and Wnt8b have been considered canonical ligands, whereas Wnt4, Wnt5a and Wnt11 as non-canonical[18].

2.2. The receptor Frizzled

The Wnt ligands bind to Frizzled receptor (Fz)[19], which is a common element in both canonical and non-canonical pathways[20]. In humans, the Frizzled protein family is formed by 10 different genes. Fz receptors are seven-pass transmembrane proteins with an N-terminal extracellular cysteine-rich domain (CRD)[21]. This CRD is a conserved sequence of approximately 120 amino acids necessary for

the interaction of the Wnt ligands with the receptor[22] (Figure 3). In the intracellular C-terminal region, Fz receptors present a KTxxxW motif needed for Dishevelled interaction and signaling[23] (Figure 3).

Fz is considered the specific receptor for Wnt ligands, but for the activation of the signaling, other proteins act as co-receptors, that are specific for either the canonical or the non-canonical pathway. LRP5/6 is the co-receptor of the canonical pathway, while Ror1, Ror2 and Ryk are the co-receptors of the non-canonical[20,24].

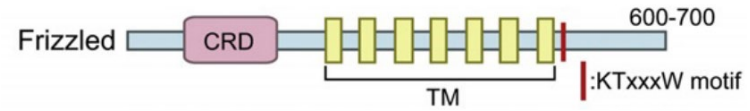

Figure 3: Frizzled receptor diagram. The representation shows the different domains of Frizzled receptor. The cysteine-rich domain (CRD), the seven transmembrane domains and the Dvl binding motif are represented. Figure from [18].

3. The canonical Wnt pathway

3.1. Canonical co-receptor: LRP5/6

The receptors LRP5 and LRP6 are homologs proteins that structurally are constituted by an extracellular part with 4 epidermal growth factor (EGF) domains and 3 low density lipoproteins (LDL) type A domains[25] (Figure 4). Moreover, LRP5/6 also present 4 β-sheet domains (YWTD) in the extracellular region that are required for the interaction with the canonical Wnt ligands and the inhibitor Dickkopf (DKK1)[26].

A cluster of serines and threonines followed by five PPPSPxP motifs constitutes the intracellular part of LRP5/6 (Figure 4). When the signaling is activated, some of these residues are phosphorylated[27,28]. The C-terminal region contains a tyrosine-rich YxxYxYxx motif that can be phosphorylated by the Src family of kinases, mostly by Src and Fer[29].

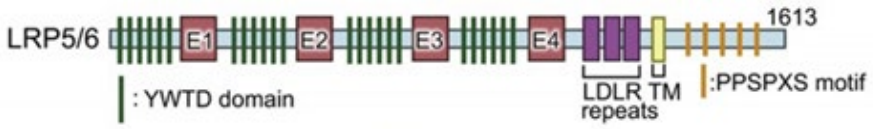

Figure 4: Representation of the LRP5/6 receptor. The diagram shows the four tandem YWTD and RGF domains followed by the LDL-A repeats. In the intracellular part, the five PPPSPxP motifs are also represented. Adapted from [18].

Several mutant forms of the LRP5/6 co-receptors have been described. For instance, a mutant lacking the intracellular part acts as dominant negative of the pathway, while a mutant without the extracellular domain, but maintaining the transmembrane domain, works as a constitutively active co-receptor (CA-LRP5/6)[30,31,32].

3.2. Canonical Wnt pathway activation

The canonical Wnt signaling or β-catenin -dependent is determined by the capacity to stabilize the cytoplasmic β-catenin that is translocated then to the nucleus promoting its transcriptional activity[5].

In the absence of canonical Wnt signaling, the levels of β-catenin are maintained low, by the action of its degradation complex. This β-catenin degradation complex if formed by Axin, APC and the kinases GSK-3 and CK1α. These two kinases phosphorylate the cytoplasmic β-catenin promoting its degradation by the proteasome[33] (Figure 5).

At the membrane, the co-receptor LRP5/6 is constitutively bound to E-Cadherin, which is also bound to p120-catenin. Additionally, p120-catenin binds to CK1ε as well, being these two factors

essential for the activation of the pathway[34] (Figure 5). Moreover, p120-catenin is also necessary to maintain cadherins at the cellular membrane[35]. In the absence of this interaction, cadherins are internalized by clathrin mediated endocytosis and degraded in the proteasome[36]. p120-catenin/cadherin interaction is regulated by the phosphorylation of specific residues by Src[37,38] and CK1 family kinases[39,34].

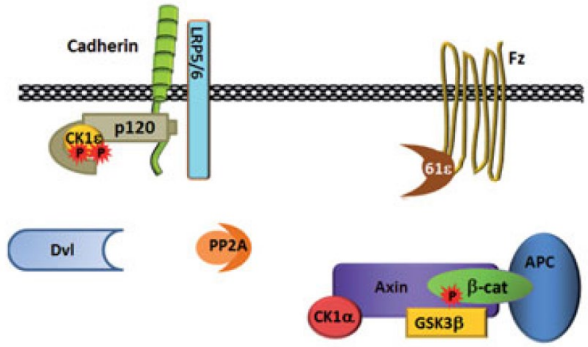

Figure 5: Canonical Wnt pathway and β-catenin degradation complex. Representative diagram of the canonical Wnt pathway elements and the β-catenin degradation complex in the absence of a Wnt ligand. In the absence of Wnt, β-catenin is phosphorylated by GSK3 and CK1α that target it for proteasomal degradation. Adapted from [40].

Upon activation of the pathway, the canonical Wnt ligand binds to the receptor Fz and the co-receptor LRP5/6, generating a signalosome that includes p120-catenin and CK1ε. Once formed, the phosphatase PP2A regulatory subunit PR61ε, constitutively bound to Fz, interacts with CK1ε causing its dephosphorylation and activation (Figure 6 A). CK1ε activation causes the recruitment of Dishevelled2 (Dvl2) to Fz receptor[41,42]. Dvl2 is a key element in the activation of the canonical Wnt pathway, but also participates in the non-canonical pathway. It is unclear whereas CK1ε activation promotes Dvl2 phosphorylation increasing its affinity to Fz or, alternatively, it phosphorylates Fz inducing Dvl2 recruitment (Figure 6 B).

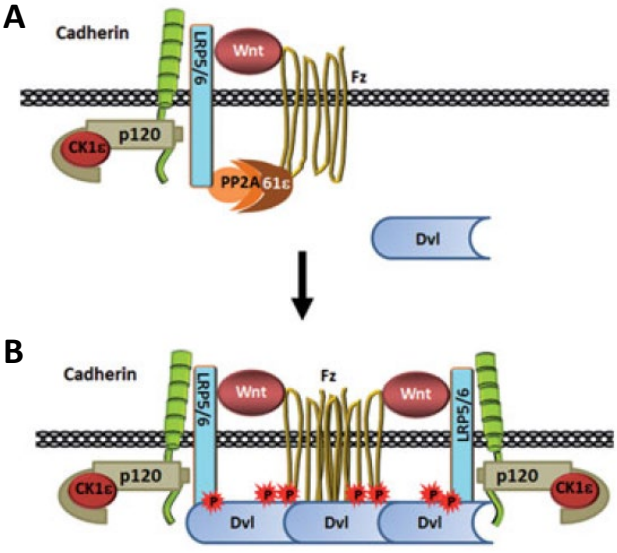

Figure 6: Canonical Wnt pathway activation. Representation of the first steps in the activation of the canonical Wnt pathway until Dvl recruitment to Fz. Upon binding of Wnt to Fz and LRP5/6, CK1ε is activated (A) causing Dvl recruitment to Fz and signalosome formation (B). Adapted from [40].

Dvl2 recruitment to Fz promotes then the phosphorylation of LRP5/6 in the residue Thr1479 by CK1γ (Figure 7 A) inducing the recruitment of Axin to LRP5/6 together with GSK-3 and CK1α, which

inhibits GSK-3[27] (Figure 7 B and C). Moreover, CK1α phosphorylates p120-catenin and cadherins disrupting this interaction and promoting the release of both proteins from the signalosome[39] (Figure 7 D and E). This dissociation induces the internalization of the signalosome in multivesicular bodies (MVB), which inhibits the β-catenin degradation complex promoting its stabilization in the cytosol[32] (Figure 7 F).

Once released from the cadherins, p120-catenin interacts and activates Rac1, a GTPase protein that controls cell adhesion and migration. Rac1 activation induces the activation of PAK1, which phosphorylates and activates JNK2 kinase[40]. Finally, JNK2 phoshporylates β-catenin and induces its nuclear translocation. In the nucleus, p120-catenin removes Kaiso from the TCF-4 transcriptional factor and allows TCF-4/β-catenin interaction[43], activating canonical Wnt target genes[44].

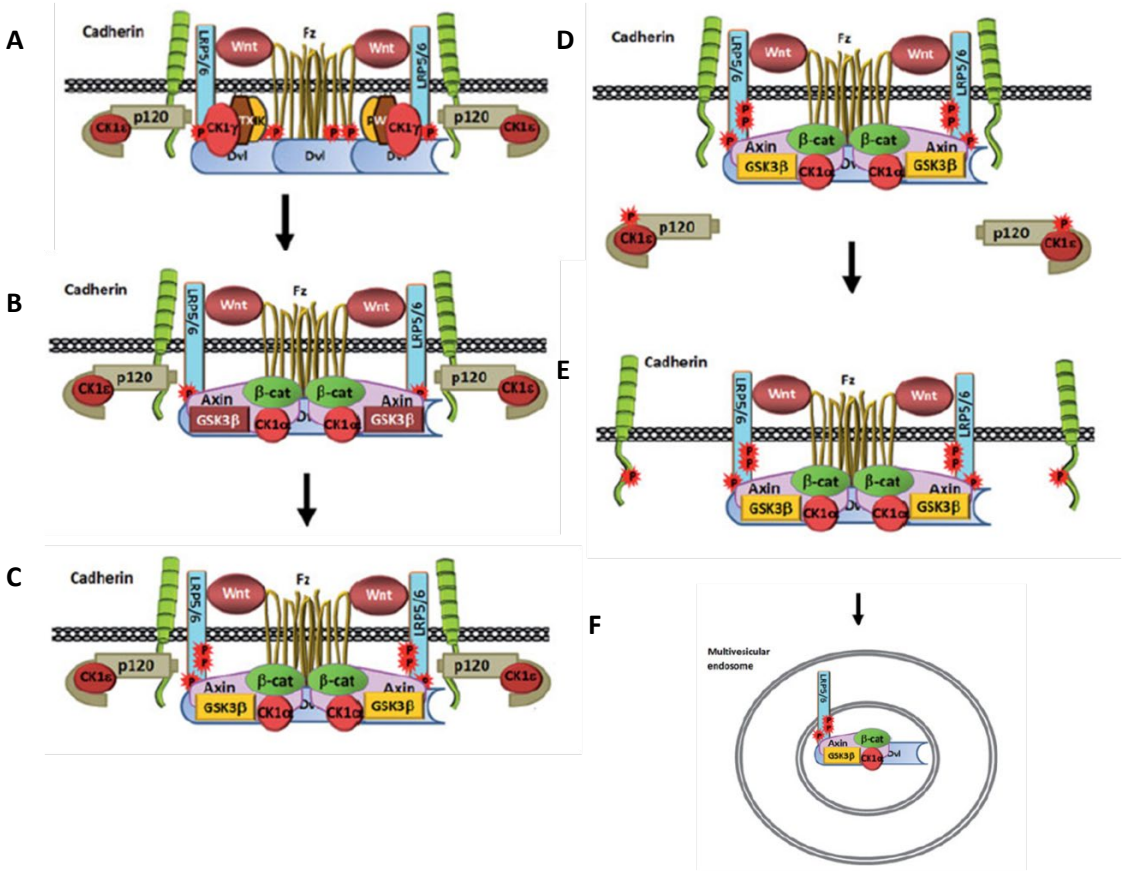

Figure 7: Canonical Wnt pathway activation. Representation of the principal events of the pathway from Dvl recruitment to the internalization of the degradation complex into MVB. Upon Dvl recruitment and formation of the signalosome, CK1γ phosphorylates Fz (A) inducing the recruitment of the β-catenin degradation complex to the signalosome (B-C). CK1α phosphorylates then p120-catenin disrupting its binding to E-Cadherin (D) and promoting its releasing from the signalosome (E). The signalosome is then internalized in MVB leading to β-catenin stabilization in the cytosol. Adapted from [40].

4. Non-canonical Wnt pathway

The non-canonical Wnt pathway or β-catenin-independent is a group of several routes that do not require the β-catenin transcriptional activity[45]. The two main non-canonical Wnt routes that have been described so far are the planar cell polarity (PCP) and the Wnt-Ca^{2+} pathway[18]. The non-canonical Wnt pathway shares common elements with the canonical pathway, such as the receptor Fz, Dvl2, p120-catenin or CK1ε, but differs in the co-receptor and in the final responses.

4.1. Non-canonical Wnt co-receptors

As mentioned above, a part from the Wnt ligands, the differential element between the canonical and the non-canonical pathways are the co-receptors that activate the signaling cascade. In the case of the non-canonical, these co-receptors are the Ror family of tyrosine kinase receptors, composed by Ror1 and Ror2[46,47], and Ryk[24].

All three co-receptors are essential during development. Mutations in any of the three produce severe malformations, but compared to the other two co-receptors, mutations in Ror2 produce malformations that are more similar to the ones generated when the Wnt5a gene is mutated. Moreover, Ror2 has been found upregulated in many cancers, becoming the most studied co-receptor[24]. For these reasons, although Wnt5a interacts with the three receptors[18], Ror2 has been considered the principal co-receptor involved in the activation of the non-canonical Wnt pathway.

4.1.1. Ror2

Ror2 is a tyrosine kinase receptor highly conserved in vertebrates. Structurally, it is formed by different domains. It has three extracellular domains: the Ig-like domain, the Frizzled-like cysteine-rich domain (CRD) and the Kringle domain, a single-pass transmembrane domain and the intracellular domains: a tyrosine kinase domain, a proline-rich domain and two serine/threonine-rich domains[48] (Figure 8).

The Ror2 CRD domain is similar to the one present in the Fz family of receptors and it is the responsible for the binding of Wnt ligands to the receptors[49]. Moreover, it has been described that Fz and Ror2 also interact through their CRD domains[50]. Another domain of Ror2 important for the signaling is the tyrosine kinase domain. Wnt5a stimulation induces Ror2 dimerization, which is necessary for its autophosphorylation and activation. In studies performed with a Ror2-KD mutant, the phosphorylation and activation of the receptor were partially decreased, demonstrating the

importance of Ror2 kinase domain[51]. Due to this partial reduction, some studies have suggested the presence of an associated kinase responsible for Ror2 phosphorylation.

In fact, it has been described that Src, an intracellular tyrosine kinase, interacts with Ror2 at the P-R domain and the first S/T-R domain, being responsible for Ror2 phosphorylation in tyrosine residues of the P-R domain upon Wnt5a stimulation[52].

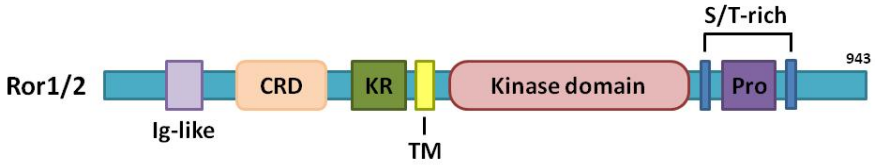

Figure 8: Representation of the Ror-family RTKs structure. The diagram shows the immunoglobulin-like (Ig-like) domain, the Frizzled-like cysteine-rich domain (CRD), the Kringle domain (KR), the transmembrane domain (TM), the tyrosine kinase domain, the proline-rich domain (Pro) and the two serine/threonine-rich domains (S/T-rich). Adapted from [18].

Other relevant factors for the activation of the non-canonical Wnt pathway, such as p120-catenin and CK1ε, have been described to interact with Ror2 in its intracellular part. p120-catenin binds to Ror2 in the juxtamembrane domain. Similarly to its role in the adherent junctions, p120-catenin controls the presence of Ror2 in the plasma membrane, protecting it from a clathrin-mediated internalization and subsequent degradation[53]. On the other hand, CK1ε binds to Ror2 in its C-terminal part and phosphorylates Ror2 on serine/threonine residues of the S/T-R domain allowing Ror2 autophosphorylation[54]. Besides this function in Ror2 activation, CK1ε also protects Ror2 from degradation, since in cells KO for CK1ε, the levels of Ror2 decreased[53] (Figure 9).

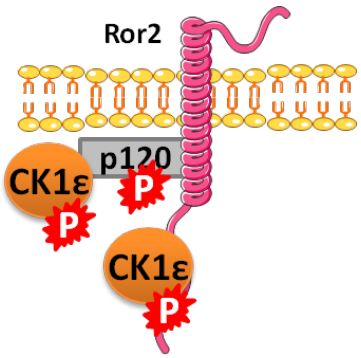

Figure 9: p120-catenin and CK1ε interact with Ror2. In basal conditions p120-catenin is constitutively bound to the Ror2 juxtamembrane domain. CK1ε interacts constitutively with the C-terminal part of Ror2. Adapted from [53].

As mentioned before, Ror2 is crucial during development. Mutations in the Ror2 gene generate pathological consequences due to failures during development. Deletion of the tyrosine kinase domain or endoplasmic reticulum retention and degradation of the protein causes a rare disorder called recessive Robinow Syndrome[55,56]. Moreover, nonsense mutations in the N or C-terminal of the tyrosine kinase domain of Ror2 causes Brachydactyly Type B syndrome[57].

Although Ror2 is critical during development, very low levels of the receptor have been detected in normal adult tissues[58]. In contrast, Ror2 has been found upregulated in some type of cancers, such as osteosarcoma, melanoma, renal carcinoma or neck and head squamous cell carcinoma[59].

4.1.2. Other non-canonical Wnt co-receptors: Ror1 and Ryk

Ror1 is the other member of the Ror family of tyrosine kinase receptors[47]. Ror1 has also been described as a Wnt5a co-receptor that activates the non-canonical pathway[20]. Structurally, Ror1 presents the same domains as Ror2[48], but it is still unknown if it also presents the same interactions in the intracellular part (Figure 8).

An alternative splice variant of Ror1 has been described, which lacks the extracellular part and the transmembrane domain[60]. Little is known about this truncated form of Ror1, but it was found in the nucleus and it has been reported to play a role in cell migration and cytoskeleton remodeling[61,62].

Ror1 has also a key role during development, maintaining neural progenitor cell fate[63] and, as Ror2, its expression in adult tissues is very low[64]. However, Ror1 has been found to be overexpressed and constitutively phosphorylated in B-cell chronic lymphocytic leukemia (CLL)[65,66]. These findings suggest that Ror1 could also have a role in regulating the non-canonical pathway and be important in cancer.

Ryk is a single-pass transmembrane receptor that, in contrast to the Ror family, presents an extracellular Wnt-binding WIF domain, a putative tetrabasic cleavage site, a γ-secretase cleavage site and an intracellular part containing the tyrosine kinase-related domain and a C-terminal part with a PDZ-binding domain[67] (Figure 10).

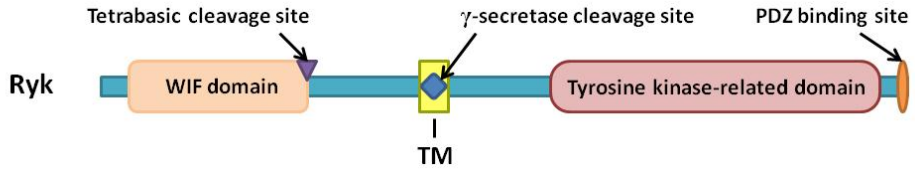

Figure 10: Representation of the Ryk receptor structure. The figure shows the different domains of the Ryk receptor: the Wnt-binding WIF domain, the putative tetrabasic cleavage site, the transmembrane domain with the γ-secretase cleavage site and the intracellular tyrosine kinase-related domain and the C-terminal PDZ-binding site. Adapted from [67].

Ryk has been described to participate in a complex with Wnt11 and Fz7 and to induce endocytosis of Dvl[68]. It has also been reported to regulate Wnt5a-induced axon growth and guidance[69]. Although

Ryk has been defined as a non-canonical Wnt co-receptor, it has been found activating the canonical pathway[70].

Little is known about how Ryk mediates Wnt signaling, although it seems that it can activate canonical and non-canonical pathways depending on the context[71]. Remarkably, upon Wnt stimulation, cleavage and nuclear translocation of the C-terminal fragment of Ryk occurs and it is essential for the neuronal Ryk-mediated responses[72].

4.2. Non-canonical Wnt pathway activation

The non-canonical pathway is activated when a non-canonical Wnt ligand, Wnt5a for instance, binds to the receptor Fz and the co-receptor Ror2[50,73] (Figure 11 B). This binding induces the phosphorylation of the receptors and the dimerization of Ror2[51], which is then activated[74]. Tyrosine phosphorylation of Ror2 increases its affinity to p120-catenin and CK1ε[53] (Figure 11 C).

As happens in the canonical pathway, when the receptor complex is formed, Ror2 constitutively-bound CK1ε interacts with Fz-bound PR61ε, which dephosphorylates CK1ε, thus activating it. CK1ε activation promotes Dvl2 recruitment to Fz, as occurs in the canonical pathway[41,53] (Figure 11 C). After Dvl2 recruitment to Fz, the clathrin adaptors associate to the complex and the clathrin-mediated endocytosis of the signaling complex is induced[75,76,77].

The internalization of the complex promotes the activation of Rac1 and RhoA that causes JNK2 phosphorylation and Rho-associated protein kinase activation, respectively. The activation of these two effectors leads to the polarization of the actin cytoskeleton to induce cell migration[77]. To go forward and activate invasion, the non-canonical pathway regulates the AP-1 target gene expression. Activated JNK2 induces c-Jun/ATF-2 binding to the AP-1 binding site of its target genes, among them metalloproteases (Mmp13, Mmp2 or Mmp9) that will degrade the ECM[46,78,79] (Figure 11 C).

Other final responses of the non-canonical pathway are the increased expression of Siah2[80], an E3 ubiquitin ligase that promotes β-catenin degradation, and the up-regulation of EMT-related genes[79] (Figure 11 C).

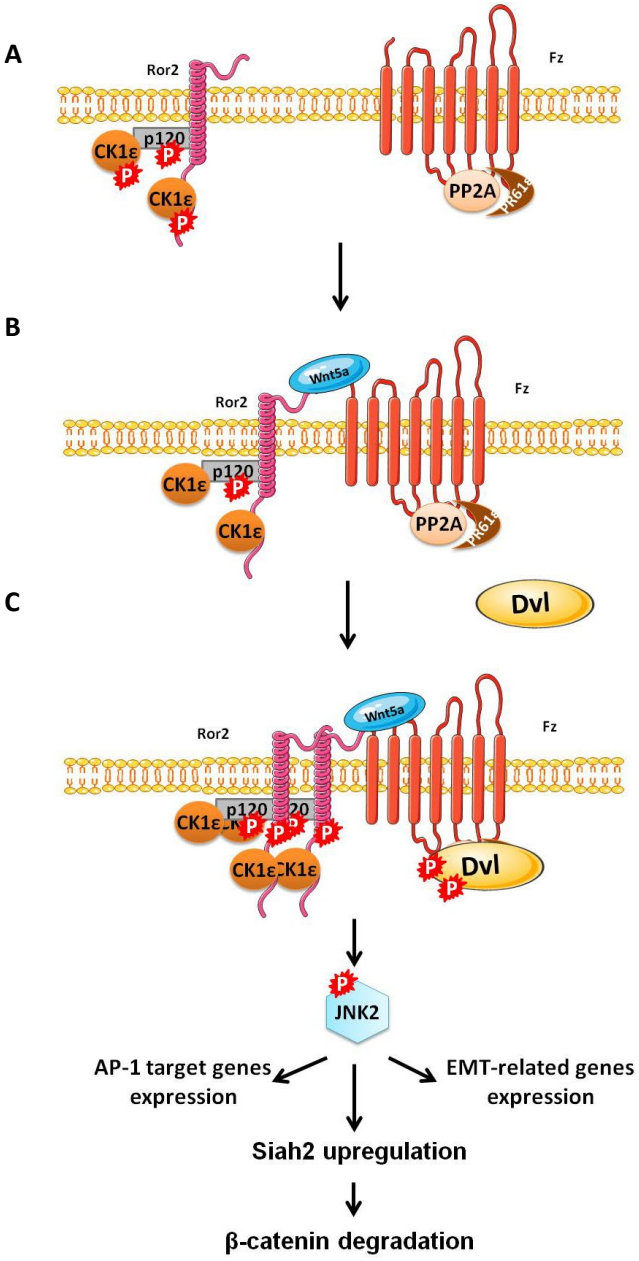

Figure 11: Non canonical Wnt pathway activation. Representation of the different steps in the activation of the pathway. In the absence of Wnt, Ror2-bound CK1ε is inactive(A). upon Wnt binding to Fz and Ror2, CK1ε is activated (B), leading to Dvl recruitment to Fz and Ror2 phosphorylation and dimerization. Dvl recruitment promotes JNK2 activation, which induces then the expression of AP-1 target genes, EMT-related genes and β-catenin degradation (C). Adapted from [53].

5. A common signaling axis in canonical and non-canonical Wnt pathway.

In 2014, Gujral et al described a new signaling branch for the non-canonical Wnt pathway that includes the activation of Fyn kinase, which phosphorylates the signal transducer and activator of transcription 3 (STAT3). In this study, performed in metastatic tumor-derived cells, after Wnt5a stimulation, Fz is tyrosine phosphorylated in the residue Tyr552 of its C-terminal part (Figure 12 E). Then, Fyn is recruited to this phosphorylated region of Fz, inducing Fyn activation. Activated Fyn phosphorylates STAT3 that promotes invasion by inducing the expression of invasion and EMT-related genes[79] (Figure 12 F).

Historically, canonical and non-canonical Wnt pathways have been separated due to the opposite effects produced on β-catenin stabilization[40,80]. However, both pathways induce similar final responses, such as migration or invasion[41,53]. These effects can be easily attributed to Rac1 and JNK2 signals that are commonly activated by both pathways, but in the recent years our group has described that canonical Wnts also activate the Fyn-STAT3 pathway[81].

Fyn-STAT3 activation, then, is commonly induced by canonical and non-canonical Wnts, Wnt3a and Wnt5a, respectively, but in a receptor-specific manner (Figure 12 A-F). DKK1, a specific inhibitor of the canonical LRP5/6 co-receptor, blocked Fyn-STAT3 Wnt3a-dependent activation and, on the other hand, specific Ror2 knock-down reduced Fyn-STAT3 activation by Wnt5a, but not by Wnt3a[81].

As mentioned above, Fz Tyr552 phosphorylation is required for Wnt-induced Fyn-STAT3 activation. Importantly, this residue is only found in Fz1, Fz2 and Fz7[79]. A kinase commonly involved in both pathways could perform this phosphorylation. Src is the tyrosine kinase responsible for Fz phosphorylation in this residue[81]. Indeed, Src is activated by canonical and non-canonical Wnts[76,81,82]. Moreover, Src has been found interacting with the canonical and non-canonical co-receptors, LRP5/6 and Ror2, respectively[52,29] (Figure 12 A-F). Ror2-Src interaction has been described to be necessary for Ror2 phosphorylation[52,83].

Interestingly, Dvl2 is not necessary for Wnt-STAT3 activation. In fact, Fz/Fyn interaction is incompatible with Dvl2 recruitment to Fz, which defines two distinct and antagonistic branches of Wnt signaling[44,81]. In this sense, previous results have suggested that Src inhibits canonical Wnt signaling by phosphorylating LRP5/6[29], but this inhibition could also be due to a Fz Tyr552 phosphorylation that induces Fyn activation and blocks the Dvl2-dependent branch[44].

Besides, p120-catenin and CK1ε, which are required for the activation of both canonical and non-canonical pathways, are not necessary for the canonical Wnt-STAT3 axis, while they are in the non-

canonical. This suggests that receptor polymerization and signalosome formation is not required for the canonical activation of the Wnt-STAT3 axis, although it is for the non-canonical[81]. CK1ε and p120-catenin play an essential role maintaining Ror2 levels and localization[53], respectively. Thus, as Ror2 is necessary for the non-canonical activation of the Wnt-STAT3 axis, these two factors are also required.

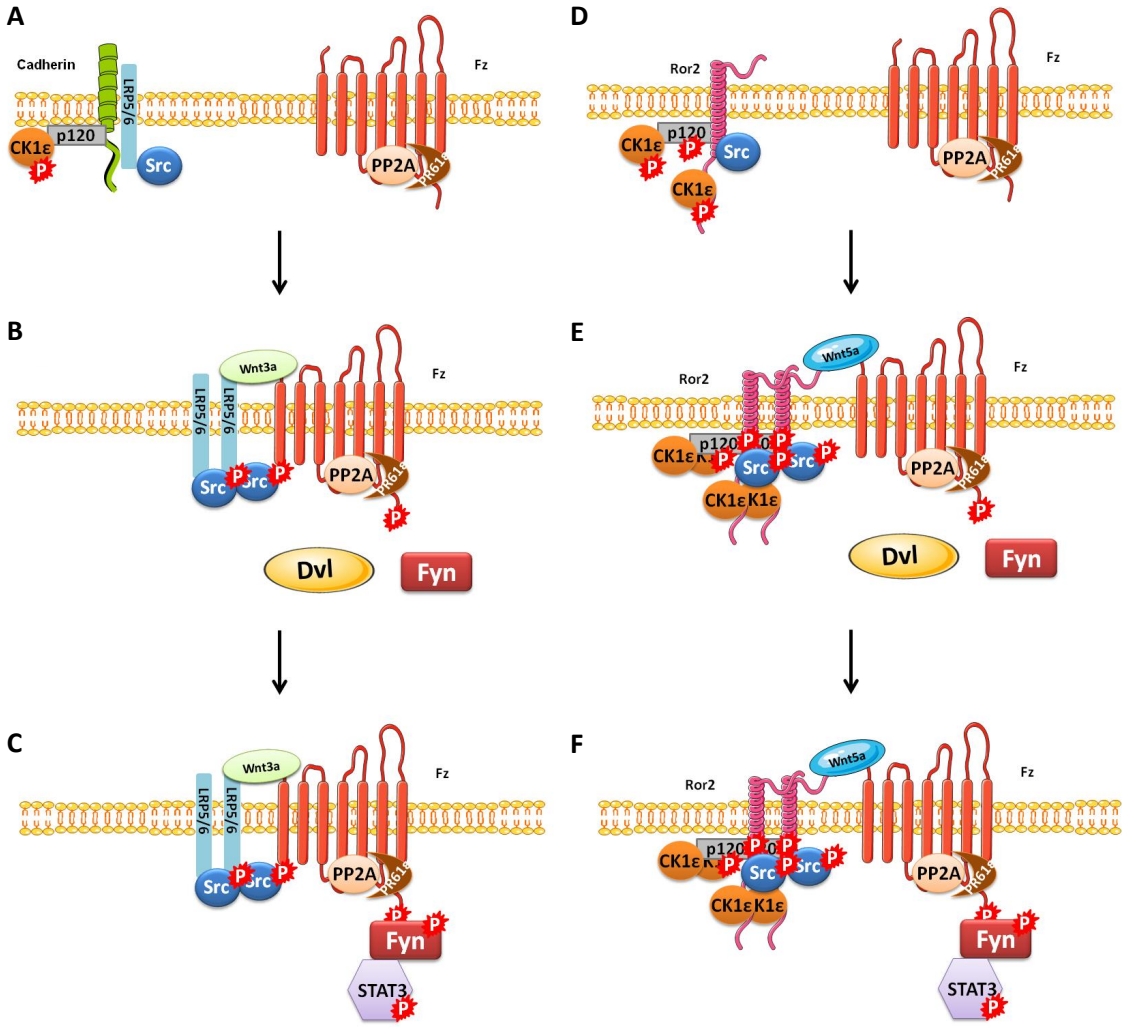

Figure 12: Canonical and non-canonical Wnt-STAT3 axis. Representation of the events in the activation of the Wnt-STAT3 axis. In the canonical pathway, Src is associated to LRP5/6 and is inactive in the absence of Wnt (A). Upon Wnt activation, Src is phosphorylated, promoting Fz Tyr552 phosphorylation (B). then, Fyn is recruited to Fz and STAT3 is activated (C). in the non-canonical, Src is also associated to Ror2 (D). Upon activation, Src phosphorylation also induces Fz Tyr552 phosphorylation (E) leading to Fyn recruitment and STAT3 activation (F). Adapted from [81].

5.1. Targets of the Wnt-STAT3 axis

Canonical and non-canonical Wnt signaling promotes the expression of several target genes needed for the Wnt-regulated processes during development and tissue homeostasis, but also during

tumorigenesis and metastasis[78,79,10]. These target genes are specifically activated by common or non-common signals between canonical and non-canonical pathway.

As explained before, STAT3 can be stimulated by canonical and non-canonical Wnts and through this activation, the expression of its target genes is induced. In a recent work of our group, a subset of genes related to EMT that were activated by both, canonical and non-canonical Wnt pathways, was found. As an example, Mmp7, Thbs1, CyclinD1 and Snail1. Interestingly, these genes were sensitive to Fyn depletion. This indicates that STAT3 activation is required for the expression of those EMT-related genes that are regulated by both, canonical and non-canonical Wnt pathways[81].

STAT3 has also been related with the expression of other important genes of the non-canonical Wnt pathway. For instance, Wnt5a has been described as a target gene of STAT3 in Chronic Lymphocytic Leukemia[84]. Moreover, STAT3 is also implicated in the gene regulation of DUB3, which is an key enzyme that regulates Snail1 protein stability[85,86].

5.2. Targets of the Wnt-JNK2 axis

In the canonical Wnt pathway, Fyn not only controls the expression of several target genes through the Wnt-STAT3 axis[81], it is also necessary for a full β-catenin gene transcription activation[40]. Fyn phosphorylates β-catenin in Tyr142 residue that mobilizes it from the adherent junctions allowing its nuclear translocation[38,40]. However, there are still some canonical Wnt target genes that do not depend on Fyn activation and just depend on the classical Wnt3a-Dvl2 axis, such as Axin2[81].

For the non-canonical Wnt pathway, there are target genes that are specific for the Wnt5a-Dvl2 axis. For instance, Siah2 and Mmp13 are activated by Wnt5a and do not depend on Fyn[78,81].

Interestingly, in general, those genes that are commonly activated by canonical and non-canonical Wnts are regulated through STAT3 and are related to EMT, while those genes that are only activated specifically just by canonical or non-canonical Wnts are not regulated through STAT3, those are regulated through Dvl[81]. However, Snail1 is not following this rule. Snail1 expression is induced by both canonical and non-canonical Wnts and it is regulated by both axis, STAT3 and Dvl[81,87,88].

6. The epithelial to mesenchymal transition (EMT)

The Epithelial to Mesenchymal Transition (EMT) is a reversible process in which cells lose the epithelial phenotype and acquire mesenchymal characteristics. On the other hand, Mesenchymal to Epithelial Transition (MET) is the opposite process through which mesenchymal cells become epithelial[89] (Figure 13).

Epithelial cells establish close contacts and present an apicobasal cell polarity through the formation of adherent junctions, desmosomes and tight junctions. This epithelial cell layer is separated from adjacent tissues by a basal lamina, acts as a barrier and has the capacity to mediate absorption. In contrast, mesenchymal cells organize in three-dimensional extracellular matrix and form the connective tissues adjacent to epithelia. EMT is an essential process during embryonic development[90].

During EMT, epithelial cells lose cell-cell contacts, including adherent junctions and desmosomes, modulate their cell polarity and reorganize their cytoskeleton by the expression of vimentin. Cells that have undergone EMT become isolated, resistant to apoptosis and acquire migratory and invasive properties[91] (Figure 13).

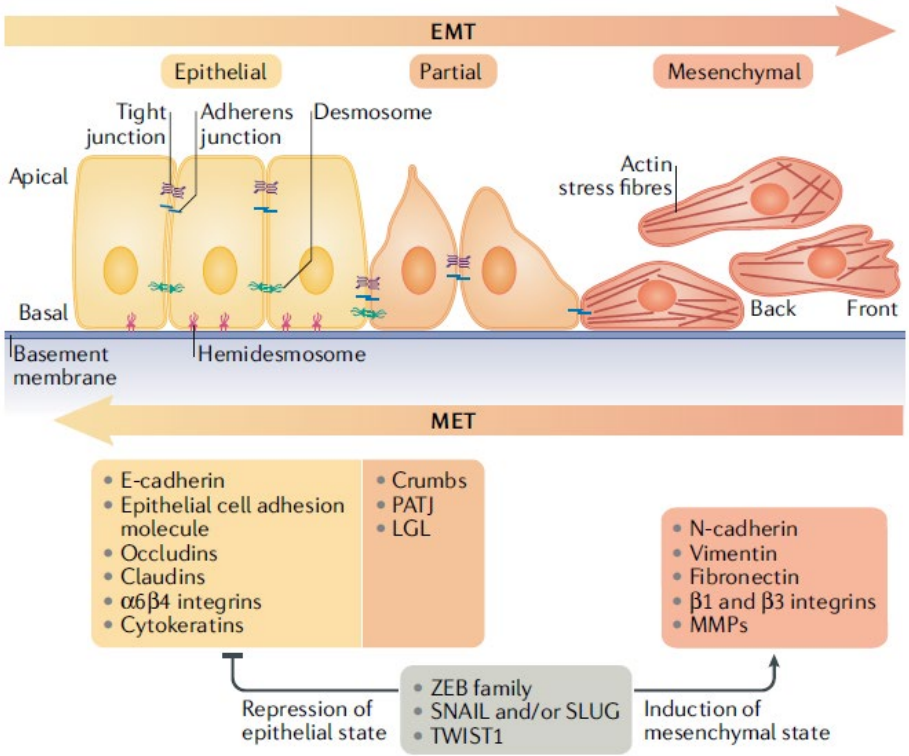

Figure 13: Markers of the Epithelial to Mesenchymal Transition. Representation of the EMT process and the principal markers of the epithelial and mesenchymal phenotypes. The main EMT drivers are also represented. Adapted from [89].

EMT plays a key role in regulating several of the hallmarks of cancer[92]. Besides its role in activating invasion and driving cancer metastasis, it is also involved in cell metabolism reprogramming, suppression of immune response, controlling telomere integrity and the acquisition of stem cell characteristics[93,94,95,96]. Therefore, EMT plays a central role in cancer regulation and progression.

EMT has been deeply studied allowing the characterization of several pathways that involve growth and differentiation factors, such as EGF, FGF, TGF-β and the Wnt pathway, and also transcriptional factors as Snail-family, Twist, Zeb and E47[89,91] (Figure 13).

6.1. EMT in cancer

EMT is a crucial process for the embryonic development and also in a physiological response to injury, but it has a high impact in pathological conditions such as fibrosis and cancer[97]. Mechanistically, signaling pathways and regulators participating in physiological EMT are similar to the ones orchestrating pathological EMT[90].

The relevance of EMT during cancer progression has been a matter of debate since many years because, although EMT processes have been observed in cancer in *in vitro* cancer cell models, convincing evidences of EMT in clinical samples are still lacking[90]. One explanation could be the difficulty to distinguish between cancer cells that have undergone EMT and cells from the tumor microenvironment or stroma, due to the similarity and the shared markers[98].

Interestingly, small aggregates of tumor cells have been found detaching from the tumor mass into the adjacent stroma providing evidences of EMT at the invasive fronts of human tumors[99]. In a similar way, EMT occurs at the invasive front producing single migratory cells that have lost E-Cadherin expression in colon carcinoma[100].

EMT and activation of stromal fibroblast, or cancer associated fibroblasts (CAFs), have been very related to Snail1 expression in both, tumor cells and fibroblasts[101]. A part from sustaining tumor growth, CAFs have also a very important role in metastasis[101]. It has been proposed that activated fibroblasts can induce tumor metastasis by physical interactions between them and tumor cells. This study claims that fibroblasts physically pull the tumor cells promoting invasion and metastasis[102]. CAFs have been also described to have an important role in Extracellular Matrix (ECM) remodeling[103]. ECM remodeling by CAFs has been proved to be an Snail1-dependent property and it has been related metastasis[104,105].

EMT has also been related to the acquisition of better chemoresistance properties[106]. Indeed, expression of Snail1 has been related to greater resistance to different drugs[107,108]. For instance, it has been proposed that Snail1 is stabilized upon DNA damage due to the downregulation of its ubiquitin ligase FBXL5[109]. This stabilization leads to Akt2 activation and association with Snail1[110,111], which enhances Akt activity on T45 in histone H3 that is associated with transcription termination after DNA damage[112]. Thus, Snail1 might confer resistance to DNA damaging agents through an association with Akt2.

Moreover, Snail1 knock-down treatments stopped tumor metastasis in melanoma[113] and sensitized tumor cells to cisplatin in lung adenocarcinoma, head and neck squamous and ovarian cancers[114,115,116].

6.2. EMT markers

As previously explained, during EMT, epithelial cells acquire a mesenchymal phenotype by losing cell-cell contacts due to a general repression of epithelial genes and an upregulation of mesenchymal genes. Among the epithelial genes that are repressed, the more representative are the occludins or the tight junctions[117], desmoplakins that are part of the desmosomes[118], and E-Cadherin, which is essential in the adherens junctions and is considered as the main epithelial marker. Therefore, loss of E-Cadherin is the principal feature of EMT[91].

6.2.1. EMT and E-Cadherin repression

E-Cadherin is considered the principal epithelial gene and its regulation drives EMT. Loss of this factor is associated to higher invasion and metastasis capacities[119], while its expression in tumor cells induces an epithelial phenotype and decreases migration[120]. Moreover, E-Cadherin ectopic expression reduces mesenchymal gene expression[121].

Several transcription repressors that are expressed in mesenchymal cells mediate E-Cadherin repression. These transcriptional factors bind to specific sequences in the CDH1 promoter containing a central region called E-boxes[91]. Snail family of transcriptional repressors, comprised mainly by Snail1 and Snail2, is one of the CDH1 gene repressors[122,123].

Furthermore, other EMT transcriptional repressors have been identified, such as the Zeb family, consisting in Zeb1 and Zeb2[124,125], the Twist family, where Twist1 and Twist2 are the most described[126,127] (Figure 13), and LEF1, which expression is regulated by the canonical Wnt

pathway[128]. Although all these transcriptional factors act as E-Cadherin repressors, only Twist is described to bind to E-boxes[129].

6.2.2. Mesenchymal gene expression

Besides the repression of epithelial genes, during EMT, mesenchymal genes are upregulated[90]. For instance, the upregulation of non-epithelial cadherins, such as N-Cadherin, replaces the loss of E-Cadherin. N-Cadherin expression is related with motility and invasive capacities[130,131].

Fibronectin (FN1) is another mesenchymal marker upregulated during EMT. FN1 is a dimeric extracellular protein that acts as a scaffold for the extracellular matrix (ECM). It is one of the main proteins in the ECM and has a key role in ECM remodeling[132].

Matrix metalloproteases (Mmp) are also involved in EMT and are upregulated in this process. These proteins degrade and modify the ECM and cell-cell contacts, forcing detachment of epithelial cells from the surrounding environment[133]. Moreover, other genes, such as vimentin, FSP1 or α-SMA are typical mesenchymal markers upregulated during EMT and their expression induces the acquirement of invasive properties[134].

However, the most important gene for this project that is upregulated during EMT is Snail1. Snail1 is considered the major driver of EMT[91] since it is upregulated during EMT and has a strong capability to repress E-Cadherin.

6.3. Wnt signaling and EMT

Wnt signaling has been historically related to EMT[135]. When the canonical Wnt pathway is triggered, β-catenin transcriptional activity is fully activated and promotes the expression of its target genes, such as Snail1[136]. Moreover, as previously explained, the canonical Wnt-STAT3 axis also induces Snail1 upregulation and the expression of mesenchymal markers such as Mmp7 and THBS1[81,137].

Besides β-catenin transcriptional activity, canonical Wnt induction indirectly promotes EMT through GSK3 inactivation. Snail1 is phosphorylated by GSK3 and this phosphorylation induces its degradation. Therefore, GSK3 inactivation induced in the canonical Wnt pathway, directly increases Snail1[138].

In a recent study it has been observed that p120-catenin, a key element in both canonical and non-canonical Wnt pathways, negatively regulates EMT by preventing an excessive canonical Wnt pathway activation[139].

Non-canonical Wnt signaling has also been related to EMT, although less than the canonical. The regulation of several Mmp by the non-canonical Wnt has been described, as well as other mesenchymal markers. For instance, Mmp2, Mmp7, Mmp9 and Mmp13 expression is induced by Wnt5a[78,81,140]. Snail1 upregulation by Wnt5a has been observed as well[79,81].

A crosstalk between non-canonical Wnt pathway and TGF-β pathway has been described. This study shows that Wnt11 promotes Fz8 activation, which then forms a complex with TGF-β receptors. This activation leads to migration, invasion and ATF2-dependent gene transcription and expression of EMT-related markers, N-Cadherin, Twist1, Zeb1 and Snail1[141].

7. Snail1 transcription factor

As previously mentioned, Snail family of transcription factors includes Snail1, Snail2 or Slug and a third less studied member, Snail3 or smug. Among these, Snail1 has been considered the main member of the family since its expression has been observed preceding the other EMT transcription factors (EMT-TF) and its ectopic expression induces other EMT-TF[101]. For this reason, Snail1 has been considered as a key marker of EMT[107].

Moreover, a part from inducing EMT in epithelial cells, Snail1 is also essential for fibroblasts activation[101], which have a crucial role in tumor invasion and metastasis[142].

7.1. Snail1 structure and function

Snail1 protein contains two different domains: the N-terminal part or regulatory domain and the C-terminal portion, which is de DNA-binding domain[122]. The regulatory domain comprises the SNAG domain, which is a short sequence relevant for the binding of co-repressors and plays a significant role in the repression function. Moreover, the serine-rich domain (SRD) and the nuclear export sequence (NES) are also localized in the regulatory domain[143]. These last two regions contain most of the Snail1 post-translational modifications that determine Snail1 localization or stabilization[107]. The DNA-binding domain comprises four Zinc finger regions (ZnF) of the C2H2 type[144] (Figure 14).

7.1.1. Snail1 repression function

Snail1 contains four ZnF domains of the C2H2 type in the C-terminal part. These domains are the responsible of E-Cadherin direct repression through the binding to tandem repeated E-boxes in the CHD1 promoter[145].

Besides its function, the ZnF domains also determine Snail1 nuclear localization due to the nuclear location signal (NLS) present there[144]. Several modifications in this region have been described. Phosphorylation by Lats2 and PAK1 induces Snail1 nuclear retention, which enhances its repressor function[146,147] (Figure 14).

7.1.2. Snail1 binding to co-repressors and transcriptional factors

Snail1 transcriptional function requires the interaction with other factors. These complex may differentially lead to Snail1 function as repressor or transcriptional activator of mesenchymal genes[148].

Through the SNAG domain, Snail1 binds to different co-repressors that have been associated with E-Cadherin repression[107] (Figure 14). Histone deacetylase (HDAC) complex Sin3a/HDAC1/HDAC2 associates with Snail1 through its SNAG domain[149]. Together with HCADs, it has been also described that Ajuba and FHL2, both containing LIM domains, also interact with Snail1 in the SNAG domain and promote E-Cadherin repression[150,151]. Ajuba mediates the interaction of Snail1 with the protein arginine methyltransferase 5 (PMRT5), a histone modifier that cooperates with E-Cadherin repression by transferring methyl groups to the CDH1 promoter[152]. Remarkably, Snail1 S11 phosphorylation by PKD1 blocks Ajuba binding, preventing Snail1 repression functions[153].

Another repressive regulator that binds to the Snail1 SNAG domain is the Polycomb complex 2 (PRC2), which also contributes to E-Cadherin repression[154]. In addition, through the SNAG domain, Snail1 binds to Lysyl oxidase-like 2 (LOXL2)[155]. LOXL2 oxidizes and demethylates K4 in histone 3, thus participating in Snali1-induced gene repression[156].

However, Snail1 not only acts as a transcriptional repressor, it activates transcription as well. For instance, during TGF-β-induced EMT, Snail1 interacts with the promoter of the mesenchymal gene FN1, activating its transcription[148]. In this case, activation is induced by

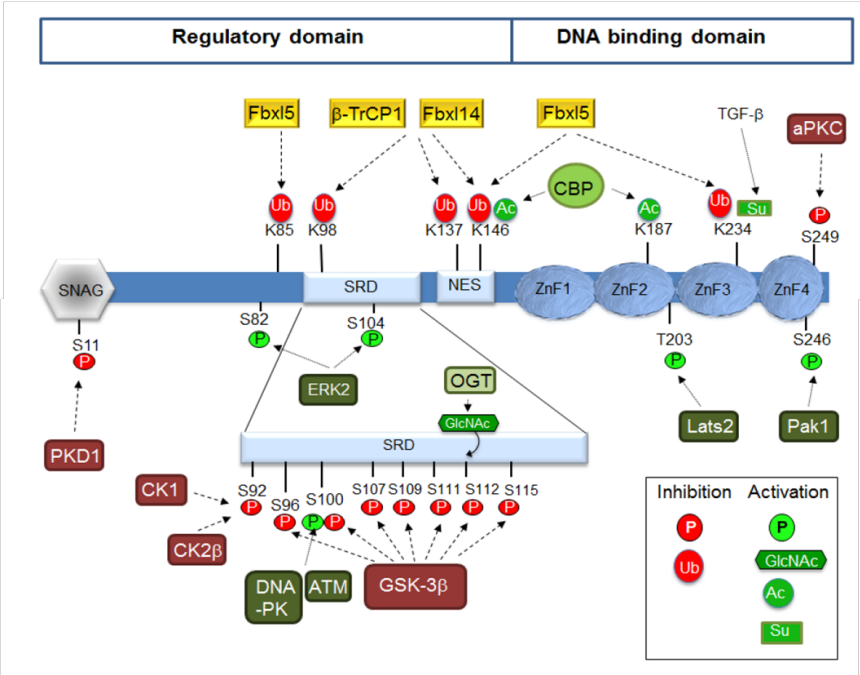

Figure 14: Snail1 protein structure and post-translational modifications controlling its function. The figure shows a representation of the different domains of Snail1. The modifications are depicted in green or red depending if they activate or inhibit Snail1, respectively. (Ub): Ubiquitination, (P): Phosphorylation, (Ac): Acetylation, (NAcGlc): Glycosylation and (Su); Sumoylation. Adapted from [107].

Snail1 association with the NFκB and PARP1 complex[148]. Snail1 interaction with β-catenin has also been described and stimulates β-catenin -induced transcription[157]. This interaction is not mediated by the SNAG domain[157]. Moreover, Snail1 induces transcriptional activation of Mmp9 and Zeb1 by direct binding to their promoters[158].

7.2. Snail1 target genes

Although the main function of Snail1 is the E-Cadherin repression, other targets have been identified. Besides repressing E-Cadherin, Snail1 also repress other epithelial markers, such as occludins, claudins or mucins, and upregulates mesenchymal markers. Snail1 also upregulates the expression of other co-repressors, to complete the EMT program[159].

Occludins and claudins are membrane proteins that are essential components of tight junctions[160,161]. Claudins expression has been inversely correlated with Snail1 in invasive breast tumors[162]. Snail1 has been described as a direct repressor of occludins and claudins, as E-box regions have been found in their promoters[163]. Mucin-1 is a transmembrane glycoprotein expressed at the apical membrane of epithelial cells. Interestingly, Mucin-1 is upregulated in some epithelial tumors[164]. However, two E-boxes have been found in the mucin-1 promoter, proving Snail1 direct repression[124].

Fibronectin and vimentin are mesenchymals markers and both are upregulated during EMT. As previously mentioned, Snail1 upregulates FN1 and vimentin during EMT[123]. The exact mechanism is not well understood yet. One possibility for FN1 is that upon E-Cadherin repression, the transcription factor NFκB becomes active and then is capable to induce FN1 upregulation[121]. Zeb1, like Snail1, is a zinc-finger transcription factor activated in EMT that also represses E-Cadherin and mucin-1, although less potent than Snail1[124,165]. It has been described that Snail1 cooperates with Twist1 in the regulation of Zeb1 during EMT[126]. Matrix metalloproteases (Mmps) have also an important during EMT and it has been shown that Snail1 expression increases the levels of Mmp2 and Mmp9[166,167].

Importantly, Snail1 has been demonstrated to regulate its own expression. This effect occurs due to the presence of an E-box in its promoter and through the direct binding of Snail1 to it[168]. Snail1 self-inhibition prevents aberrant activation of EMT and controls undesired effects[107].

7.3. Control of Snail expression, protein stability and gene transcription

Snail1 expression is highly controlled at different levels. *SNAI1* gene transcription is regulated by different transcription factors induced by several growth factors or cellular proteins, such as the TGF-β pathway, some cytokines, NFκB or the Wnt pathway. The stability of Snail1 mRNA is also controlled by some micro-RNAs and translation of Snail1 protein is regulated by some factors as well. Finally, the stability of Snail1 protein is highly controlled by E3 ubiquitin ligases and deubiquitinating enzymes, which promote Snail1 degradation and stabilization, respectively[107].

7.3.1. Regulation of protein stability

Snail1 is a short-lived protein with a half-life of about 25 minutes and it is highly regulated in epithelial cells[169]. In normal conditions, Snail1 is rapidly ubiquitinated and degraded by the proteasome[170]. Several E3 ubiquitin ligases of the multimeric SCF subtype have been described to participate in Snail1 ubiquitination, which suggests a highly redundant mechanism to maintain Snail1 protein levels low in non-pathological conditions[107] (Figure 15).

The SCF subtype of E3 ubiquitin ligases is a complex composed by a Skp1, Cullin1 and F-box, being this last protein the one that contributes to the substate specificity[170]. Recognition of Snail1 by the F-box module is normally associated with Snail1 phosphorylation, although it is not always a requisite[107] (Figure 14).

FBXW1 or β-TrCP1 recognize the Snail1 phosphorylated by GSK3 β in the SRD domain, curiously in a sequence also present in β-catenin [171,172]. In addition, GSK3β phosphorylation needs a previous phosphorylation of Snail1 in the S92 residue by CK1ε or CK2β[173,174] (Figure 14 and Figure 15).

However, it has been demonstrated that in many cells Snail1 degradation occurs independently of GSK3β, which implies that other E3 ubiquitin ligases may participate in this process[107].

FBXL14, another member of the SCF family, has been identified as phosphorylation-independent Snail1 E3 ubiquitin ligase[175]. Moreover, FBXL14 action has also been detected in other EMT transcription factors, such as Snail2, Twist1 and Zeb2[176]. In hypoxia conditions, FBXL14 expression is repressed, thus stabilizing Snail1[175] (Figure 14 and Figure 15).

FBXL5 has also been identified as a Snail1 E3 ubiquitin ligase. In particular, FBXL5 is a nuclear E3 ligase that binds to Snail1 C-terminal lysines[109] (Figure 14 and Figure 15). In this study, after blocking Snail1 nuclear export, FBXL5-mediated Snail1 degradation was inhibited, which suggests that Snail1 degradation occurs mainly in the cytosol[107,109].

Some of these FBX proteins controlling Snail1 stability are regulated by micro-RNAs. FBXL14 and FBXL5 are negatively regulated by miR-17/20a and miR-1306-3p, respectively. Therefore, the expression of these micro-RNAs induces Snail1 stabilization[177,178].

As Snail1 degradation is closely linked with its phosphorylation in specific residues, Snail1 dephosphorylation by specific phosphatases leads to Snail stabilization[170]. Snail1 stabilization has been observed due to dephosphorylation in the C-terminal domain by small phosphatases[179] and by poly-ubiquitinated PTEN[180]. Despite that, there are other kinases that phosphorylate Snail1 promoting its stabilization[107]. For instance, ATM phosphorylates Snail1 in the Ser100 residue inducing Snail1 stabilization[181] (Figure 14).

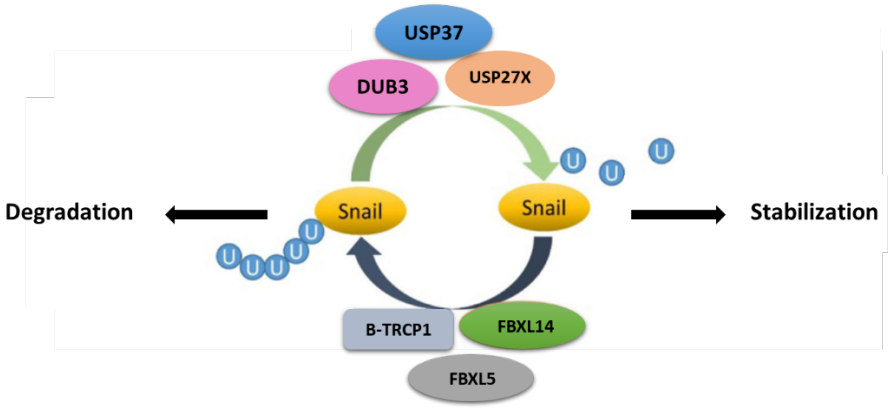

Figure 15: Snail1 protein stability regulation by E3 ubiquitin ligases and DUBs. Snail1 protein degradation is promoted by specific E3 ubiquitin ligases, such as β-TrCP1, FBXL14, FBXL5, Fbxo11 and Fbxo45 that induce Snail1 ubiquitination and subsequent degradation in the proteasome. In contrast, deubiquitinating enzymes or DUBs induce the opposite effect; DUBs eliminate the ubiquitin residues promoting Snail1 stabilization. Adapted from [182].

As mentioned before, other kinases that stabilize Snail1 are Lats2 and PAK1 that phosphorylate Snail1 in the C-terminal domain in the T203 and S246, respectively[146,147] (Figure 14). It has been described that in stress conditions, induced by gamma-irradiation or DNA damage, PAK1 and ATM phosphorylate Snail1 promoting its stabilization[147,181].

As explained above, Snail1 degradation requires ubiquitination and phosphorylation can prevent this process. In addition to this, ubiquitination can be reverted by the action of deubiquitinating enzymes (DUBs), which eliminate the ubiquitin residues in the Snail1 protein and, therefore, promote its stabilization[183]. Snail1 deubiquitination has been reported by several DUBs[107], being DUB3, UPS27X and USP37 the most relevant[184,108,185,186] (Figure 15). DUB3 and USP27X are induced by IL-6[86] and TGF-β[108], respectively, which are important modulators of EMT and suggest an important role for these DUBs during EMT. Moreover, DUB3 induction by IL-6 is thought to work through STAT3 activation, which is also important in regulating Snail1[85].

7.3.2. Snail1 transcription regulation

SNAI1 transcription regulation is another important level of Snail1 expression control. Since Snail1 is rapidly degraded, besides an increase in protein stability, upregulation of SNAI1 transcription is also necessary to observe changes in Snail1 expression. Some extracellular growth factors and cellular proteins have been related to Snail1. However, there are not so many extracellular factors that modulate Snail1 expression in tumors.

For instance, in humans, it was first described that *SNAI1* gene transcription is controlled by TGF-β and canonical Smads, thus TGF-β treatment induces *SNAI1* transcription[107]. However, most tumor cells do not respond to TGF-β and many of them are insensitive to it.

NFκB pathway also promotes *SNAI1* transcription, by directly binding the p50 and p65 subunits to the *SNAI1* promoter[187]. In a similar way, Akt also increases *SNAI1* transcription by activating NFκB and Smad3[188,189].

Remarkably, Wnt signaling effectors have been described to control *SNAI1* transcription. ERK or JNK participate in *SNAI1* regulation, since AP-1 sites are present in the *SNAI1* promoter[187,87]. In addition, STAT3 is another transcriptional factor that has been reported as a potent *SNAI1* activator. For instance, in cisplatin-resistant atypical teratoid/rhabdoid tumor cells, STAT3 was bound to *SNAI1* promoter region[88]. These findings corroborate data of our group that show that Snail1 is upregulated by both canonical and non-canonical Wnt pathways[81].

Finally, as mentioned before, Snail1 itself binds to the *SNAI1* promoter and inhibits its own transcription to avoid excessive EMT activation[168].

7.4. Snail1 expression in tumors

Snail1 expression occurs in many types of tumors. Snail1 role in EMT has been widely described and its overexpression correlates with increased migration, invasion and metastasis. Therefore, Snail1 expression in tumors has been associated to bad prognosis in many cancers[159].

However, Snail1 function is not restricted to tumor cells, but also to the stromal cells, mainly in CAFs[101]. The relevance of Snail1 in stromal cells was first determined when it was detected in the front of an invasive tumor, which leaded to think that Snail is important for stromal activation[190].

CAF activation is mainly induced by the TGF-β pathway and the activation is mediated by Snail1[191]. In CAFs, Snail1 induction by TGF-β, promotes the secretion of prostaglandin E2, which enhances the invasive and metastatic capacities of tumor epithelial cells, establishing a cross-talk between

stromal and cancer cells[192]. Moreover, Snail1 of the stromal cells regulates ECM remodeling facilitating tumor invasion[103].

Snail1 expression has also been detected in endothelial cells and it is required for tubulogenesis and neoangionesis[193]. Moreover, it has been proposed that Snail1 is necessary for TGF-β-induced endothelial to mesenchymal transition (EndMT)[194].

Although Snail1 expression in other types of stromal cells, such as immune system cells (macrophages or T lymphocytes), is still unknown, it has been proposed that Snail1-expressing cancer cells can modulate the immune response against the tumor[89]. For instance, in melanoma cells, Snail1 expression induced the formation of Treg lymphocytes and also decreased the antigen-presenting capacities of dendritic cells[113]. In addition, Snail1 expressed in tumor cells modulates the secretome of these cells and produces macrophage polarization towards a M2 phenotype, which has been associated to a more protumorigenic role[195].

OBJECTIVES

Objectives

Since Snail1 expression can be induced by canonical and non-canonical Wnts in fibroblasts, we wondered how Snail1 is controlled in tumor cells. So, the main objective of this thesis is to study the contribution of canonical and non-canonical Wnt pathways in controlling Snail1 expression in colon tumor cells and its role in tumor progression.

RESULTS

1. Ror2 is required for Snail1 expression in tumor cells

We centered this study in the regulation of Snail1 expression in tumors. It is known that Snail1 expression can be induced by several extracellular growth factors, such as TGF-β[107] and Wnt ligands[81]. However, it is not clear that these factors can regulate Snail1 expression in human colorectal tumors. For this reason, since we have previously observed that canonical and non-canonical Wnt pathways activate Snail1 expression in non-tumoral cells, we focused on studying how Wnt factors regulate Snail1.

1.1. Wnt and not TGF-β controls Snail1 expression in colorectal cancer cells

TGF-β signaling pathway has been described to control the expression of Snail1[107]. For instance, it is known that TGF-β treatment induces Snail1 expression in breast tumor cells and fibroblasts[108,191]. However, some elements of the TGF-β pathway machinery have been found mutated in human colorectal cancer[196]. Mutations in the TGF-β receptor have been found in human colorectal cancer samples[197]. Moreover, the SMAD family of proteins, which are the intracellular transducers of the TGF-β signaling, are also mutated or inactivated in many colorectal tumors[198], being the SMAD4 protein the one found mutated with higher frequency[199,200].

In order to study if TGF-β signaling controls Snail1 expression in colorectal cancer cells, we analyzed the levels of Snail1 in SW620 and HCT116 cell lines treated with the TGF-β receptor inhibitor (SB505124). As shown in Figure 16, the basal levels of Snail1 are not affected by the inhibition of TGF-β receptor, although SMAD2 phosphorylation is impaired at 5 μM and 20 μM (Figure 16). This effect suggests that TFG-β signaling is not controlling Snail1 expression in colorectal cancer cells.

As mentioned in the introduction, canonical and non-canonical Wnt pathways regulate Snail1 expression through JNK2 and STAT3[81,87]. We wondered if Wnt signaling could also be regulating Snail1 expression in colorectal cancer cell lines.

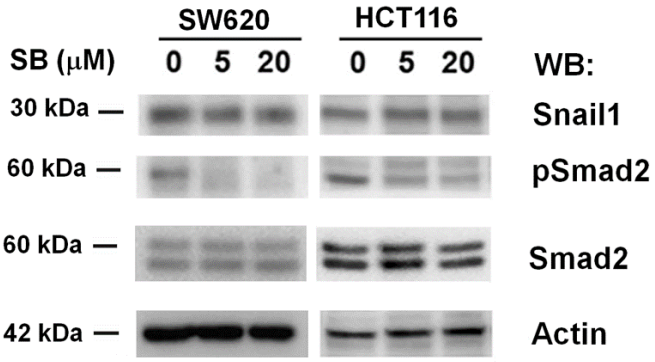

Figure 16: Inhibition of TGF-β receptor does not affect Snail1 expression in colorectal cancer cell lines. Colorectal cancer cells SW620 and HCT116 were treated for 24h with the inhibitor of TGF-β receptor, SB505124, at the indicated concentrations. After the treatment, cells were lysed and protein extracts were analyzed by WB with the indicated antibodies.

To study whether Wnt signaling regulates Snail1, we treated SW620 and HCT116 cells with LGK-974 inhibitor, which targets the Porcupine protein[17]. Porcupine is a key protein in the secretion and the activity of Wnt ligands[201] and its inhibition has been probed to impair Wnt signaling[16]. Inhibition of Wnt secretion by LGK decreased the expression of Snail1 in SW620 and HCT116 cells (Figure 17). To check that LGK is indeed blocking Wnt signaling, we confirmed that the phosphorylation of STAT3 is also affected. These results confirm that the basal expression of Snail1 is controlled by Wnt signaling and not by TGF-β signaling in colorectal cancer cells.

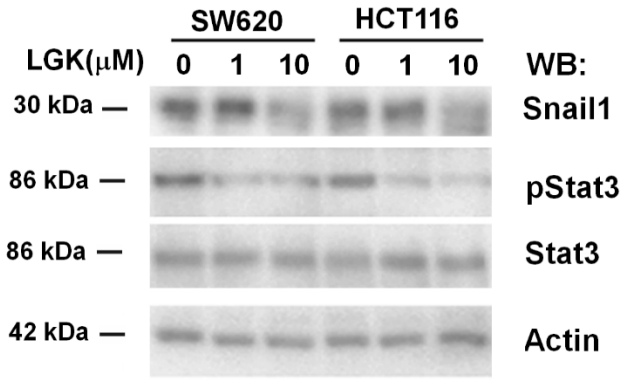

Figure 17: Inhibition of Wnt secretion decreases the expression of Snail1 in colorectal cancer cells. Colorectal cancer cells SW620 and HCT116 were treated for 24 h with the LGK-974 inhibitor at the indicated concentrations. After the treatment, cells were lysed and protein extracts were analyzed by WB with the indicated antibodies.

1.2. Ror2 correlates with Snail1 expression in colorectal tumors and colorectal tumor cell lines

Wnt signaling has been historically divided in canonical and non-canonical pathways depending on the capacity to promote β-catenin transcriptional activity[44]. Although this established classification, both pathways share common elements in the signaling cascade, such as the Fz receptor, Dvl recruitment to Fz, p120-catenin or CK1ε[41,53] and the activation of JNK2 and STAT3[81,53], but differ in the co-receptor used to activate the pathway[20].

Since we have observed in Figure 17 that Wnt signaling controls the expression of Snail1 in colorectal cancer cells, we decided to compare whether the expression of the canonical and non-canonical Wnt co-receptors, LRP5/6 and Ror2 respectively, correlates with Snail1 expression in human colorectal tumors. As explained in the introduction, two other co-receptors of the non-canonical Wnt pathway have been described, Ror1 and Ryk[24], but we centered the study in Ror2 because in our group we have previously described its importance in the activation of the non-canonical pathway[81,53]. We searched for colorectal cancer studies in the cancer database cBioPortal[202,203] and we used the TCGA Colorectal Adenocarcinoma Firehose Legacy database. This tool allowed us to analyze the mRNA expression of Snail1 and Wnt co-receptors in tumors.

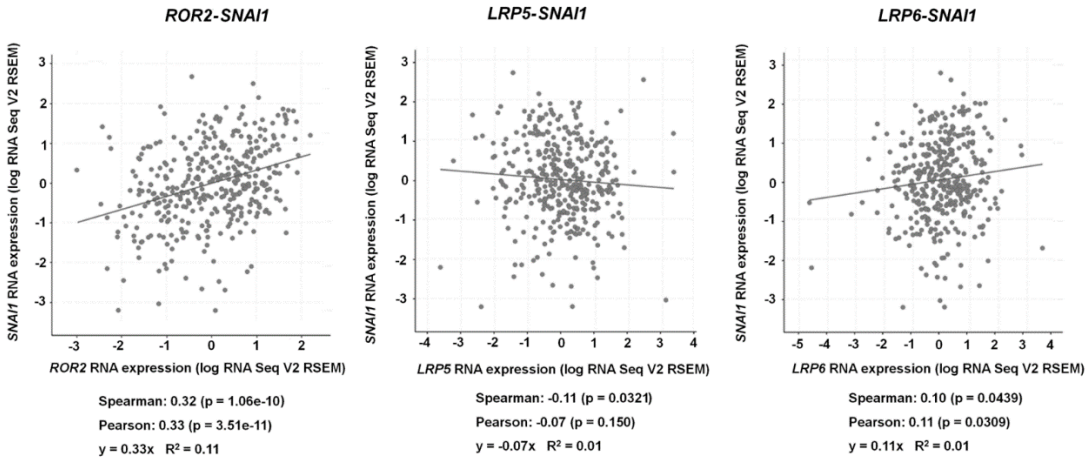

Figure 18: Snail1 expression correlates better with Ror2 than LRP5/6 in human colorectal cancer patients. The correlation between the RNA expression (log RNA seq V2 RSEM) from *SNAI1*, *ROR2*, *LRP5* and *LRP6* is represented. Data was obtained from the TCGA Colorectal Adenocarcinoma Firehose Legacy database and analyzed in the cBioPortal.

As shown in the graphs in Figure 18, the expression of Snail1 positively correlates with Ror2 in human colorectal adenocarcinoma samples. For the canonical co-receptors, the correlation between Snail1 and LRP5 is negative and for LRP6, although the correlation is still positive, there is

less correlation compared to Ror2. These results suggested that in colorectal cancer the non-canonical Wnt seems to be more involved in the control of Snail1 expression.

The correlation between Snail1 and Ror2 was also analyzed in eight different colorectal cancer cell lines (SW480, SW620, HCT116, HT29 M6, LS174, Caco2, DLD1 and LoVo). Consistent with the human colorectal tumor data, we found a general correlation between the protein levels of Snail1 and Ror2 (Figure 19). Those cell lines with higher Ror2 expression also showed higher levels of Snail1, while there is no correlation between Snail1 with the canonical co-receptors LRP5/6 nor with the other non-canonical co-receptor Ror1.

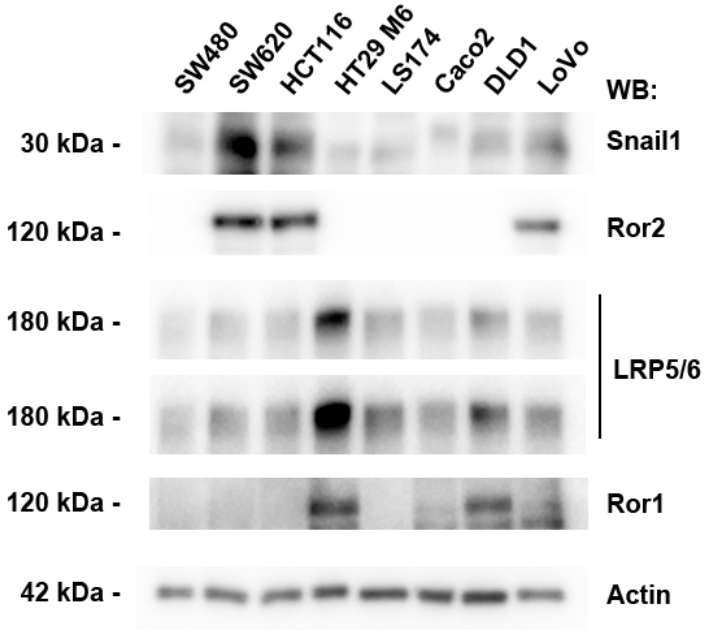

Figure 19: Snail1 protein levels correlate with Ror2 in colorectal cancer cell lines. Cells were lysed and protein extracts were analyzed by WB with the indicated antibodies.

This correlation was confirmed at mRNA levels (Figure 20). The mRNA expression of Ror2 and the other non-canonical co-receptors, Ror1 and Ryk, was analyzed from the colorectal tumor cell lines and compared with Snail1 mRNA expression. As observed in Figure 20, there is a general correlation between Ror2 mRNA levels and Snail1 (Figure 20 A and D), while no correlation was observed with Ror1 or Ryk, that are highly expressed in HT29 M6 cells (Figure 20 B, C and D).

Together, these results confirm that the expression of the non-canonical Wnt co-receptor Ror2 correlates with the expression of Snail1 in colorectal tumor cells. Therefore, the non-canonical Wnt pathway regulates the expression of Snail1 in colorectal cancer.

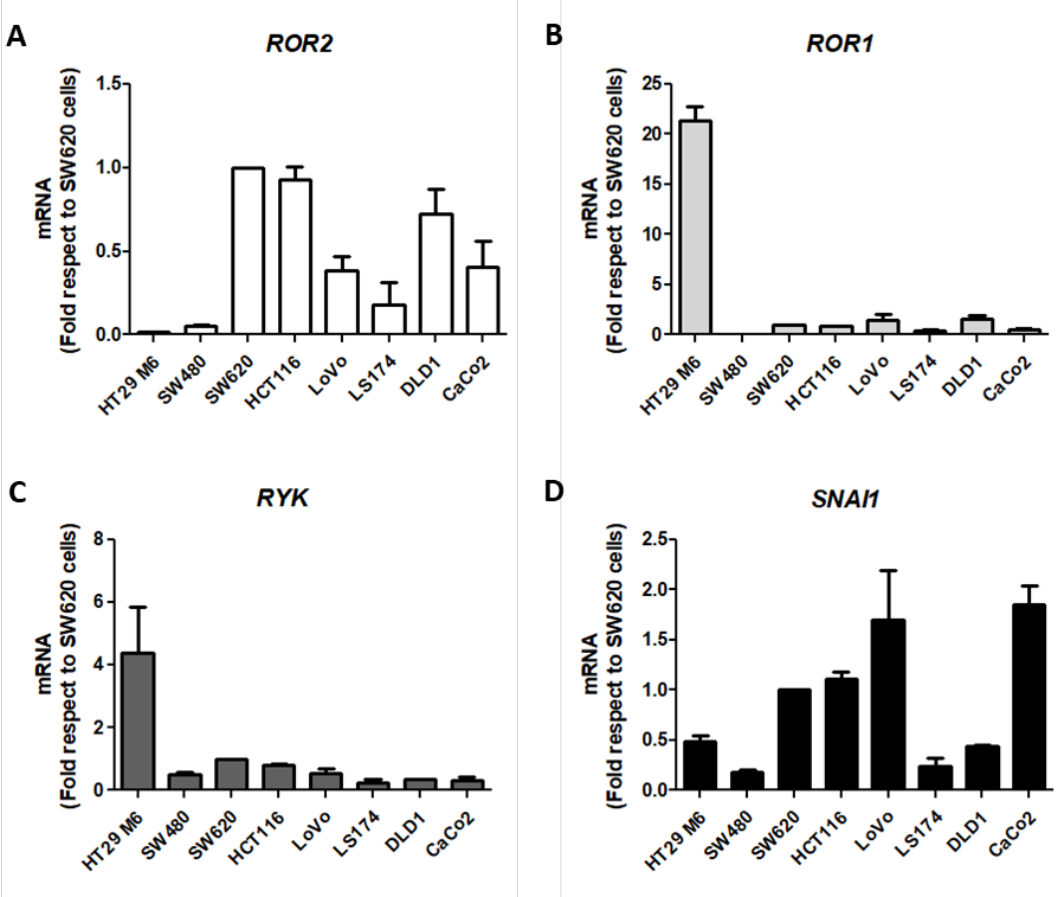

Figure 20: Ror2 and Snail1 mRNA levels correlate in colorectal tumor cell lines. mRNA was isolated from the colorectal cancer cell lines. A) *ROR2,* B) *ROR1,* C) *RYK* and D) *SNAI1* mRNA levels were quantified by quantitative RT-PCR (RT-qPCR). The mRNA levels are referred to the SW620 mRNA levels. Quantification of three different experiments is represented ± SD.

1.3. Frizzled2 receptor correlates with Snail1 expression

A part from Ror2, the receptor Frizzled (Fz) is also required for the activation of the non-canonical pathway[53,20]. We centered in Fz2 because it has been reported that the non-canonical Wnt pathway regulates EMT through Fz2[79]. We have also studied the expression of Fz2 in colorectal tumors from the TCGA Colorectal Adenocarcinoma Firehose Legacy on cBioPortal[202,203] database and compare it with Snail1 expression. As observed in the data from cBioPortal, the mRNA expression of Fz2 positively correlates with the expression of Snail1 (Figure 21).

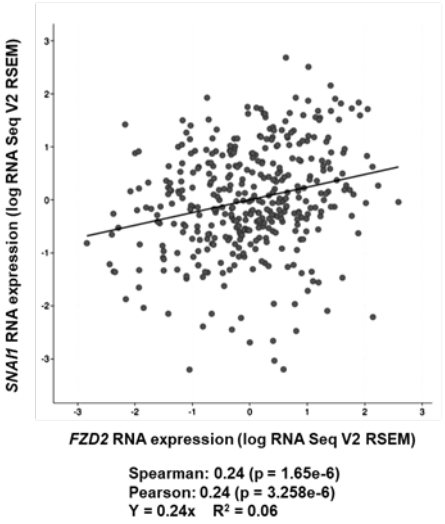

Figure 21: *SNAI1* mRNA expression correlates with *FZD2* in colorectal cancer patients. The correlation between the RNA expression (log RNA seq V2 RSEM) from *SNAI1*, and *FZD2* is represented. Data was obtained from the TCGA Colorectal Adenocarcinoma Firehose Legacy database and analyzed in the cBioPortal.

The protein levels of Fz2 were also analyzed in colorectal tumor cell lines and compared with Snail levels. In accordance with the human tumor database, Fz2 protein levels correlated with Snail1 protein levels (Figure 22 A-B). As shown in Figure 22 A, Fz2 expression is higher in those cell lines with also higher expression of Ror2. As expected, those cell lines with higher expression of Ror2 and Fz2 expressed higher levels of Snail1.

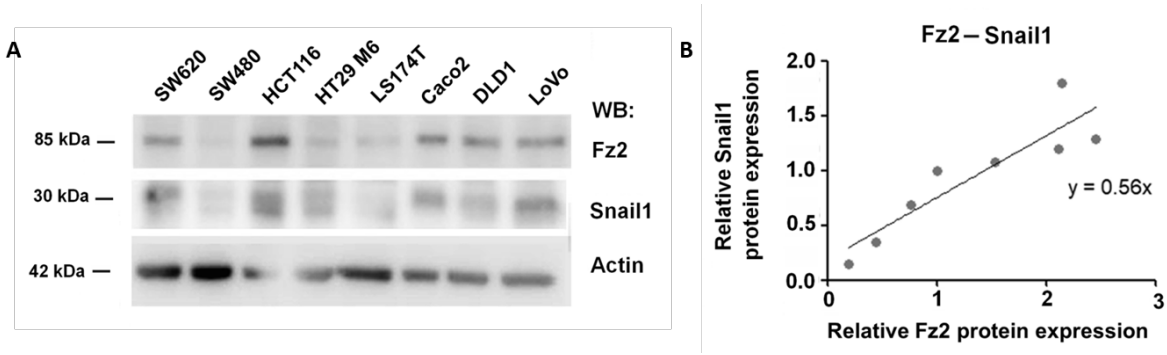

Figure 22: Fz2 protein levels correlate with Snail1 in colorectal cancer cell lines. A) Colon and breast cell lines were lysed and protein extracts were analyzed by WB with the specific antibodies. B) Correlation between Fz2 and Snail1 protein levels. Protein quantification from A.

1.4. Ror2 knock-down decreases Snail1 expression

To better understand how Ror2 controls the activation of the non-canonical pathway and the expression of Snail1, we decided to knock-down Ror2 from the SW620 and HCT116 cell lines, which expressed higher levels of Ror2. Thus, we carried out a stable knock-down of Ror2 in these cell lines. We achieved a very good efficiency in Ror2 depletion in both cell lines and, as shown in Figure 23, the levels of Ror2 were strongly decreased, both at protein and at mRNA levels.

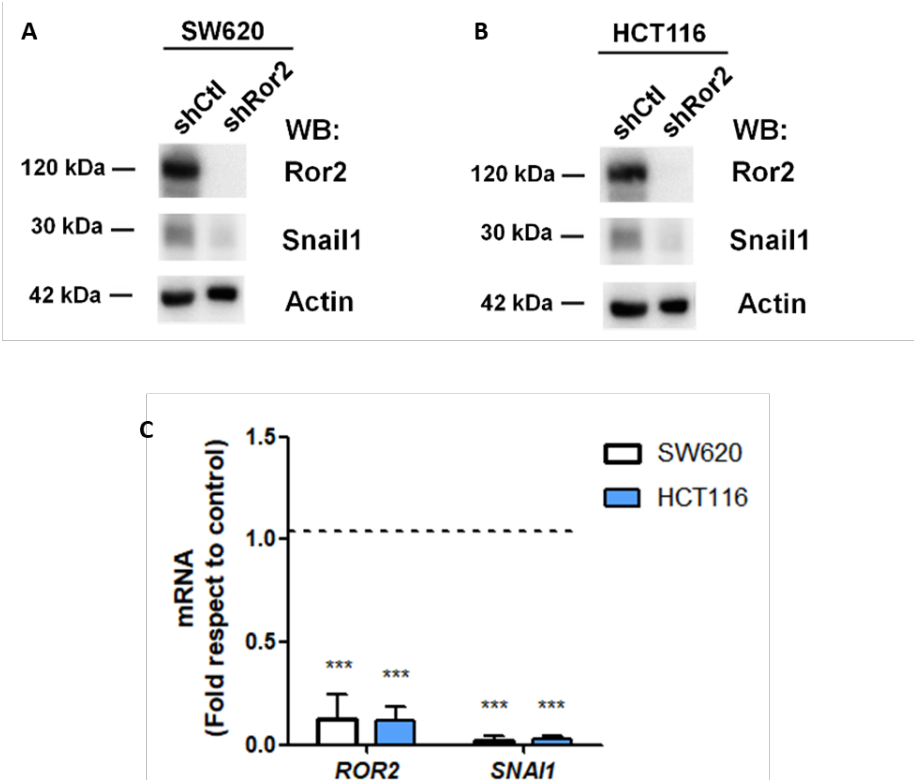

Figure 23: Ror2 knock-down decreases Snail1 protein levels. A-B) SW620 and HCT116 control and knock-down for Ror2 were lysed and protein extracts were analyzed by WB using the indicated specific antibodies. C) mRNA was isolated from the control and Ror2-depleted SW620 and HCT116 cells. *ROR2* and *SNAI1* mRNA levels were quantified by quantitative RT-PCR (RT-qPCR). The mRNA levels are referred to control cells mRNA levels. Quantification of three different experiments is represented ± SD.

As expected, when the non-canonical Wnt pathway was abrogated due to the decrease in Ror2 expression, Snail1 expression also decreased. The decrease in Snail1 expression was detected both at protein and mRNA levels (Figure 23). Together, these results support the idea that Ror2 controls the expression of Snail1 in colorectal tumors.

1.5. Ror1 and Ryk co-receptors are not affected by Ror2 depletion

As mentioned above, there are other co-receptors, Ror1 and Ryk, that can also activate the non-canonical Wnt pathway[24]. Although the expression of both co-receptors in SW620 and HCT116 cell lines is low (Figure 21; compared to HT29 M6 cells), we checked whether the knock-down of Ror2 in these cell lines affects the expression of Ror1 or Ryk.

We analyzed the mRNA expression of Ror1 and Ryk in the Ror2 depleted cells and no changes were observed (Figure 24). This result confirms that Ror2 depletion is not affecting the expression of the other two non-canonical Wnt co-receptors.

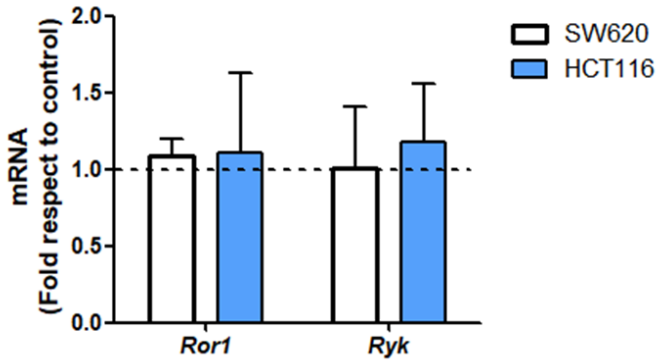

Figure 24: *ROR1* and *RYK* expression is not affected by Ror2 depletion. mRNA was isolated from the control and Ror2-depleted SW620 and HCT116 cells. *ROR1* and *RYK* mRNA levels were quantified by quantitative RT-PCR (RT-qPCR). The mRNA levels are referred to control cells mRNA levels. Quantification of three different experiments is represented ± SD.

1.6. Ror2 depletion affects JNK2 and STAT3 basal phosphorylation

As previously described, the non-canonical Wnt pathway activates different branches. Among these, the ones promoting JNK2 and STAT3 phosphorylation upon Wnt5a stimulation are the most representative and studied in our group[81,53]. Thus, we analyzed the basal phosphorylation levels by WB.

As expected, the basal levels of JNK2 and STAT3 phosphorylation were decreased in the SW620 and HCT116 Ror2 knock-down cells, whereas their total levels did not change (Figure 25), as a consequence of a less capacity to activate the pathway due to Ror2 depletion. This result, with the concomitant Snail1 down-regulation, confirms the idea that Ror2 is the co-receptor controlling the activation of the non-canonical Wnt pathway in colorectal tumor cells.

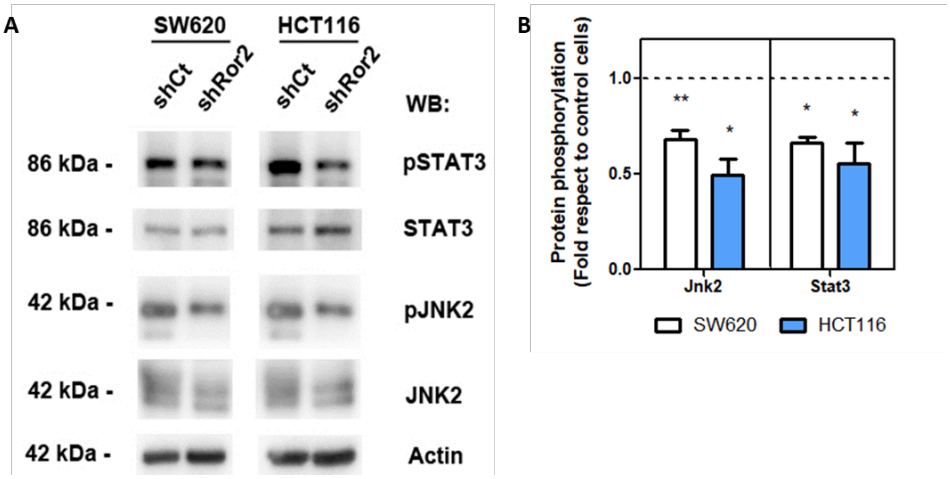

Figure 25: Ror2 knock-down decreases JNK2 and STAT3 basal phosphorylation. A) SW620 and HCT116 control and Ror2 knock-down cells were lysed and protein extracts were analyzed by WB with the specific antibodies. B) Quantification of phosphorylated JNK2 and STAT3 levels. Mean ± SD of three different experiments is represented.

1.7. The expression of non-canonical Wnt target genes decreases in Ror2-depleted cells

Activation of canonical or non-canonical Wnt signaling, promotes the expression of several target genes that are essential during embryonic development and tissue homeostasis, but also during cancer progression and metastasis[81,79]. In order to determine the relevance of Ror2 in the expression of the non-canonical Wnt target genes, several of these genes were analyzed in the SW620 and HCT116 Ror2 knock-down cells. In addition, the expression of canonical Wnt target genes was also assessed.

Siah2, Mmp9 and Mmp13 were selected as well-documented non-canonical Wnt5a-target genes[79,78,80]. As expected, the basal mRNA levels of Siah2, Mmp9 and Mmp13 were decreased in the Ror2 knock-down cells (Figure 26), as a consequence of the poor activation of the pathway. This result confirms that Ror2 is also necessary for the expression of non-canonical target genes in tumor cells.

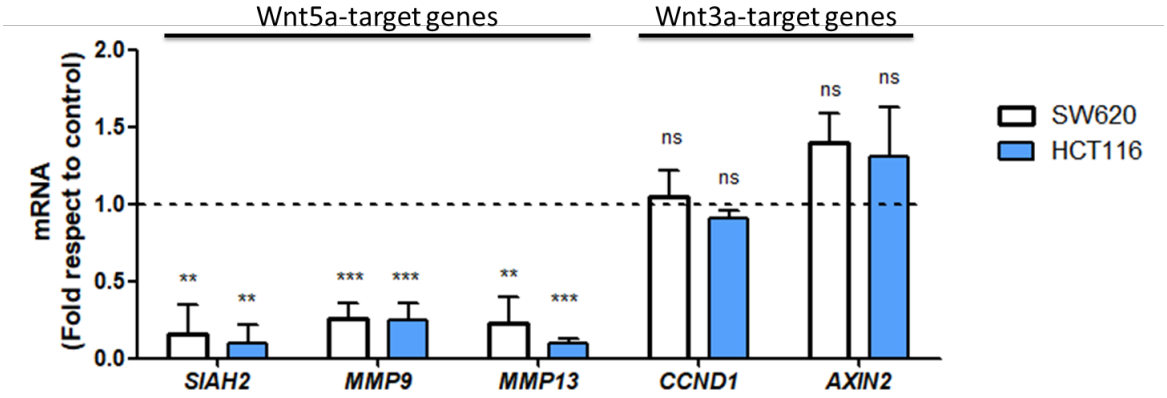

Figure 26: Ror2 knock-down decreases Wnt5a target genes, but not Wn3a target genes. mRNA was isolated from SW620 and HCT116 control and Ror2 knock-down cells and *SIAH2, MMP9, MMP13, CCND1* and *AXIN2* mRNA was analyzed by RT-qPCR. The mRNA levels are referred to the control cells levels. Quantification of three different experiments is represented ± SD.

On the other hand, target genes of the canonical Wnt pathway, such as Axin2 (AXIN2) and CyclinD1 (CCND1)[81], were also analyzed in the SW620 and HCT116 Ror2 knock-down cells. As shown in Figure 26, mRNA expression of Axin2 and CyclinD1 was not modified by Ror2 knock-down, suggesting that the canonical Wnt pathway is not affected by the depletion of Ror2 in colorectal cancer cells.

1.8. Ror2 depletion decreases mesenchymal gene expression whereas epithelial genes increase

Snail1 is one of the main EMT transcriptional factors and controls one of the most important steps in EMT, the E-Cadherin repression[122,123]. Besides, Snail1, directly or indirectly, also regulates the expression of several mesenchymal markers[121,126]. For these reasons, as Ror2 depletion causes a dramatically decrease in Snail1 levels, we have studied how Ror2 down-regulation could affect epithelial and mesenchymal markers.

The protein levels of E-Cadherin were analyzed in the Ror2 knock-down SW620 and HCT116 cells and both cell lines presented an increase in E-cadherin (Figure 27A and B). This rise in E-Cadherin, which is an epithelial marker, was accompanied by a decrease in αSMA protein levels (Figure 27A and B), which is a mesenchymal marker that increases during EMT and fibroblast activation[204]. Interestingly, the protein levels of Fz2 receptor were strongly reduced in the Ror2 knocked-down cells (Figure 27A and B). This finding is in accordance with previous works in which Fz2 has been closely related with EMT[79,205].

We corroborated these changes in epithelial and mesenchymal markers at mRNA level as well. As observed in Figure 27C, the mRNA levels of Fz2 were decreased in the Ror2-depleted cells and, inversely, E-Cadherin mRNA increased. Moreover, the expression of two other EMT transcription factors, such as Zeb1 and Twist1, decreased also in Ror2-depleted cells. These results suggested that the reduction in Snail1 expression, due to Ror2 depletion, promotes a mesenchymal to epithelial transition (MET) that induces the expression of epithelial markers and decreased the mesenchymal ones in colorectal tumor cells.

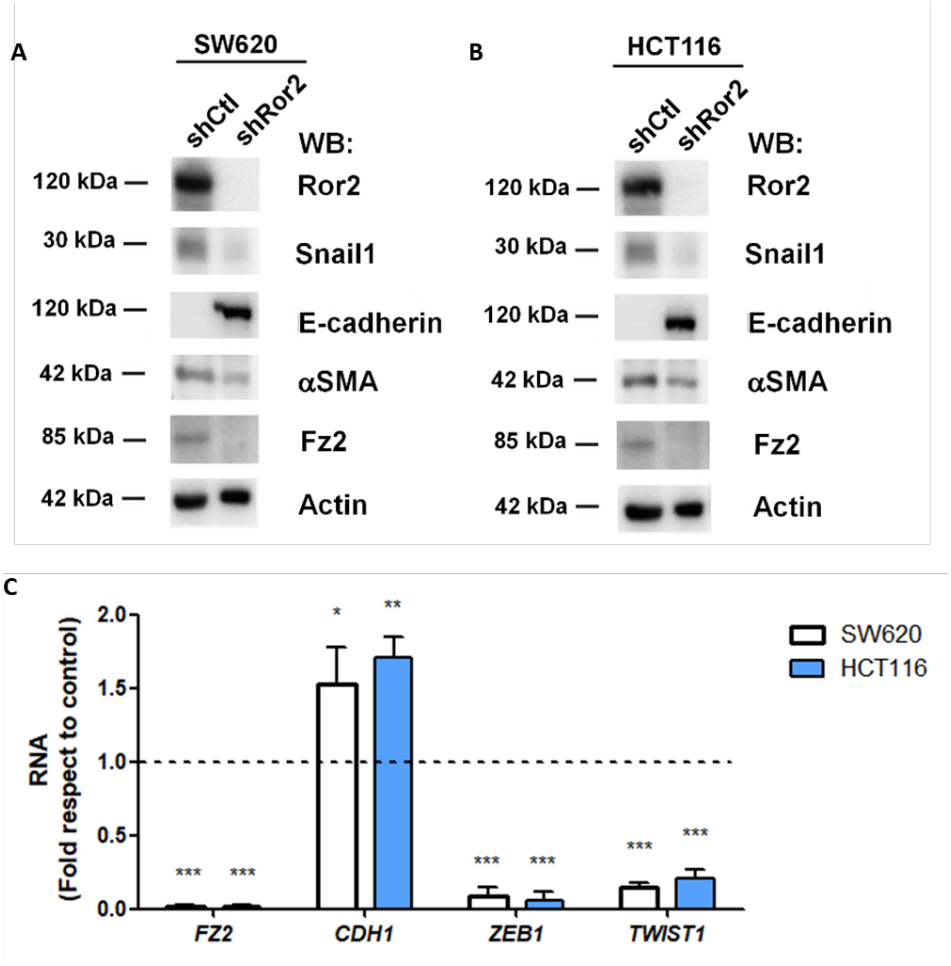

Figure 27: Ror2 knock-down decreases mesenchymal genes expression whereas increases epithelial genes. A-B) SW620 and HCT116 control and knock-down for Ror2 were lysed and protein extracts were analyzed by WB using the indicated specific antibodies (Note that Ror2, Snail1 and Actin panels are the same that in Figure 23, because all the panels showed in this figure came from the same experiment). C) mRNA was isolated from the control and Ror2-depleted SW620 and HCT116 cells. *FZD2, CDH1, ZEB1* and *TWIST1* mRNA levels were quantified by quantitative RT-PCR (RT-qPCR). The mRNA levels are referred to control cells mRNA levels. Quantification of three different experiments is represented ± SD.

2. Mechanism of Snail1 regulation by Ror2

In the previous experiments we have observed that non-canonical Wnt pathway controls Snail1 expression through Ror2. However, the complete mechanism of Snail1 regulation by Ror2 is not studied. Indeed, Snail1 expression is controlled by multiple mechanisms[107]. For this reason, we wanted to assess whether Ror2 and the non-canonical pathway is controlling Snail1 expression at different levels.

2.1. Snail1 transcription is controlled by Ror2 through Jnk2 and Stat3 activation

Since the non-canonical Wnt pathway induces Snail1 expression and Ror2 depletion decreases the mRNA levels of Snail1, we first analyzed if Ror2 knock-down is affecting the transcription of the *SNAI1* gene. We assessed the *SNAI1* promoter activity with a Luciferase assay. For this experiment, two different fragments of the *SNAI1* promoter were used; a short fragment -194/+59 and a long fragment -869/+59 (Figure 28). The constructs were overexpressed in the Ror2 knock-down SW620 and HCT116 cells and the Luciferase activity was determined.

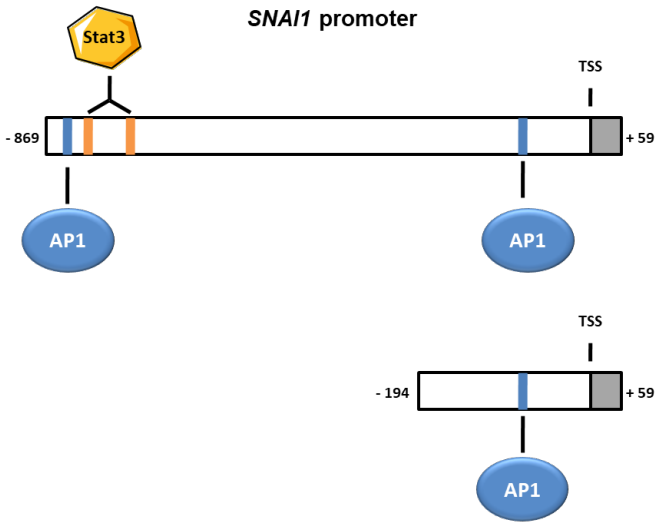

Figure 28: Representation of the -869/+59 and -194/+59 *SNAI1* promoter fragments. The fragments of the *SNAI1* promoter used for the promoter activity experiments are represented. STAT3 and AP1 putative binding sequences are represented in orange and blue, respectively. Base pairs distance is represented relative to the transcription starting site (TSS).

As shown in Figure 29, the activity of both, the short and the long fragment of the *SNAI1* promoter, was lower in the Ror2 knock-down cells compared to the control cells. This result suggested that Snail1 transcription is controlled by the non-canonical Wnt pathway in a Ror2-dependent manner.

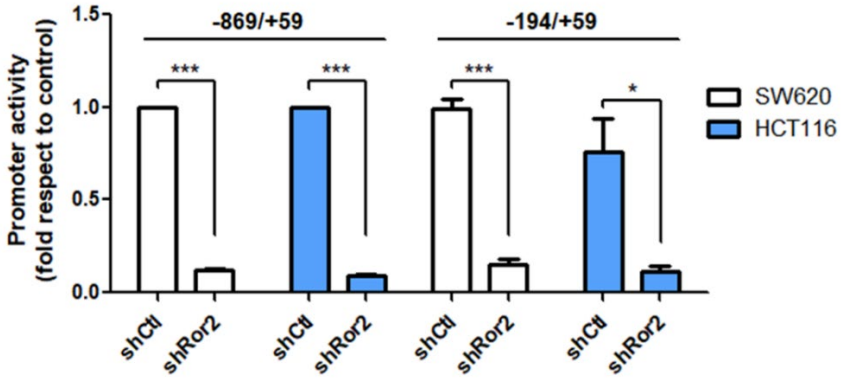

Figure 29: *SNAI1* promoter activity is decreased in Ror2-depleted cells. SW620 and HCT116 control and Ror2 knock-down cells were transfected with pGL3*prSNA P900 or pGL3*prSNA P300 and pTK-Renilla. After 48 hours the basal activity of the *SNAI1* promoter was analyzed. The graph shows the quantification of three independent experiments ± SD.

Interestingly, when we looked to the fragments of the *SNAI1* promoter region that we used, we found two putative AP1 and STAT3 binding motifs (Figure 28). These binding motifs are present in the long fragment, whereas in the short only one AP1 binding motif is found (Figure 28).

Then, the *SNAI1* promoter activity was also analyzed with specific inhibitors of STAT3 and JNK2. The promoter fragments were overexpressed in wildtype SW620 and HCT116 cells and cells were treated with the inhibitors. After treatment, the *SNAI1* promoter activity was analyzed as before. As observed in the graph, the long fragment of the promoter containing the AP1 and STAT3 binding motifs showed a reduced activity with both inhibitors (Figure 30). By contrast, the activity of the short form, which only presents one AP1 motif (Figure 28), was just affected by JNK2 inhibitor and not by STAT3 inhibitor (Figure 30).

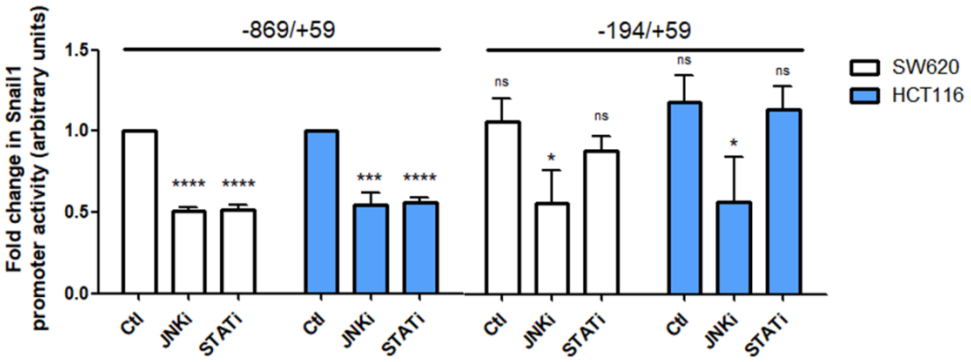

Figure 30: Inhibition of JNK2 and STAT3 decreases *SNAI1* promoter activity. SW620 and HCT116 control and Ror2 knock-down cells were transfected with pGL3*prSNA P900 or pGL3*prSNA P300 and pTK-Renilla. After 32 hours cells were treated with the indicated inhibitors for 16 hours and after a total of 48 hours the basal activity of the *SNAI1* promoter was analyzed. The graph shows the quantification of three independent experiments ± SD.

This effect of JNK2 and STAT3 inhibition on Snail1 expression was also confirm at protein level. SW620 cells were treated with both inhibitors and the protein expression of Snail1 was analyzed. As shown in Figure 31, Snail1 protein levels decreased by both, JNK2 and STAT3 inhibition.

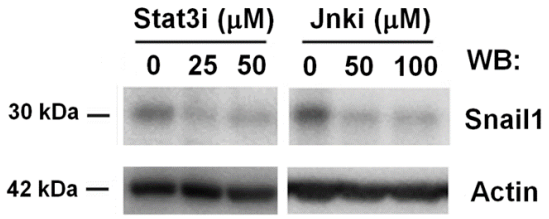

Figure 31: Inhibition of JNK2 and STAT3 decreases Snail1 protein levels. SW620 and HCT116 cells were treated for 24h with the STAT3 or JNK2 inhibitor at the indicated concentrations. After the treatment, cells were lysed and protein extracts were analyzed by WB with the indicated antibodies.

These findings confirmed that Snail1 expression is controlled by JNK2 and STAT3 activation.

2.2. Ror2 increases Snail1 protein stability

Besides Snail1 transcription regulation, Snail1 is a very unstable protein and it is widely known that regulation of Snail1 protein stability is also highly controlled by different mechanisms[107]. Thus, Snail1 protein stability was investigated in the Ror2 knock-down SW620 cells. As Snail1 protein levels in Ror2-depleted cells are very low, ectopic Snail1-HA was overexpressed to study protein stability. 24 hours after Snail1-HA overexpression, cells were treated with cycloheximide at different times and the Snail1-HA levels were analyzed by WB.

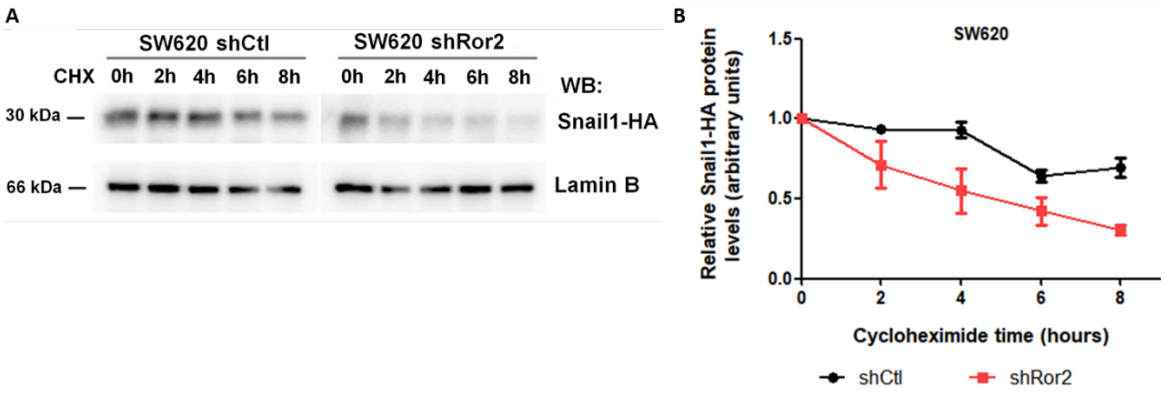

Figure 32: Ror2 increases Snail1 protein stability. Control and Ror2 knock-down SW620 cells were transfected with ectopic Snail1-HA and treated with 50 µg/mL cycloheximide for the indicated times. A) Cells were lysed and Snail1-HA protein levels were analyzed by WB with the indicated antibodies. B) The graph shows the quantification of three independent experiments using the ImageJ software and represented as the mean of each time point ± SD.

As shown in Figure 32, Snail1 protein stability was decreased in the Ror2 depleted cells, compared to control cells. Therefore, Ror2 also modulates Snail1 protein stability, although the mechanism is still not known.

2.3. Ror2 depletion modulates the expression of DUB3

Snail1 protein levels are controlled by specific ubiquitin E3 ligases that ubiquitinate Snail1 promoting its proteasomal degradation[170]. However, ubiquitination can be reverted by the action of the DUBs. As explained in the introduction, different Snail1 E3 ligases and DUBs have been described[107]. Whether Ror2 can affect the E3 ligases or DUBs activity is not known, but as a decrease in Snail1 protein stability has been observed in the Ror2-depleted cells, the expression of the different Snail1 E3 ligases and DUBs was also analyzed in these cells.

The expression of the three main Snail1 ubiquitin E3 ligases was analyzed and no clear changes were observed in the Ror2-depleted cells compared to control cells (Figure 33). Just the expression of FBXL5 seems to be increased when Ror2 is knocked-down. On the other hand, when we studied the expression of Snail1 DUBs, we observed a clear decrease in DUB3 (Figure 33). Together, the increase in FBXL5, but mainly the strong decrease in DUB3 expression, explains why Snail1 protein stability decreases in Ror2-depleted cells.

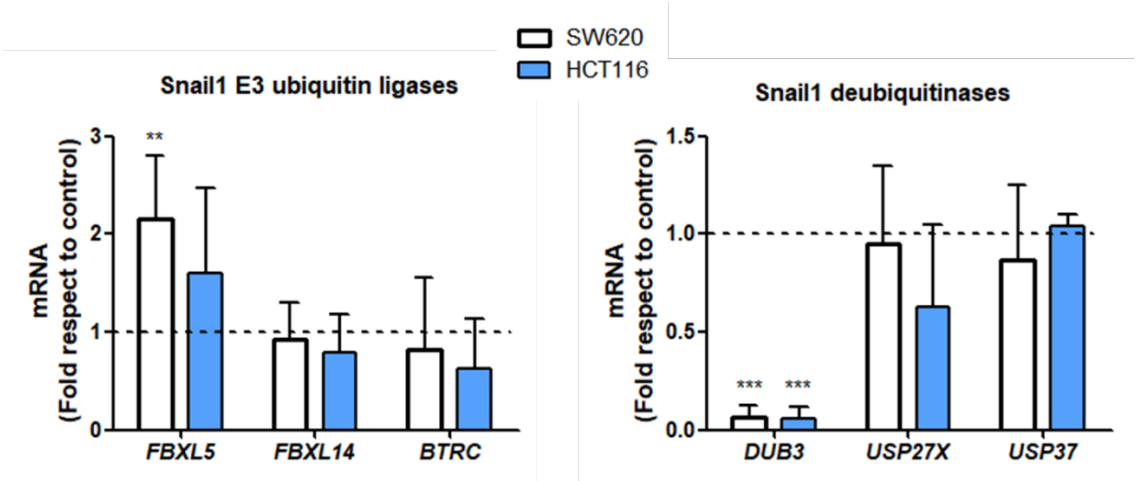

Figure 33: Ror2 modulates DUB3 expression. mRNA was isolated from SW620 and HCT116 control and Ror2 knock-down cells. *FBXL5, FBXL14, BTRC* (β-TRCP1), *USP17L2* (DUB3), *USP27X* and *USP37* mRNA was analyzed by RT-qPCR. The mRNA levels are referred to the control cells levels. Quantification of three different experiments is represented ± SD.

3. Wnt5a – Ror2 – Snail1 define a positive feedback loop

It has been previously described that Wnt5a is upregulated in a STAT3-dependent manner. Indeed, evolutionary conserved STAT3-binding sites have been found in the *WNT5A* gene[206] and downregulation of STAT3 induces a decrease in Wnt5a expression[84] in lymphocytic leukemia cells. Moreover, it has been found that Ror2 knock-down induced downregulation of Wnt5a[207].

For these reasons, we wondered if in our Ror2-depleted SW620 and HCT116 cells, Wnt5a expression is affected.

3.1. Ror2 depletion decreases the production of Wnt5a ligand

First, we analyzed the expression of Wnt5a in Ror2 knock-down cells. As observed in Figure 34A, the mRNA levels of Wnt5a were decreased in both Ror2-depleted SW620 and HCT116 cells. This result confirms previously reported data and suggests that Wnt5a is a gene target of the non-canonical Wnt pathway. Moreover, conditioned medium from Ror2-depleted SW620 cells was also analyzed to confirm that these cells also produce less Wnt5a ligand. Wnt5a protein levels in the conditioned medium were analyzed by WB and it was confirmed that Ror2-depleted cells produce and secrete less Wnt5a ligand (Figure 34B).

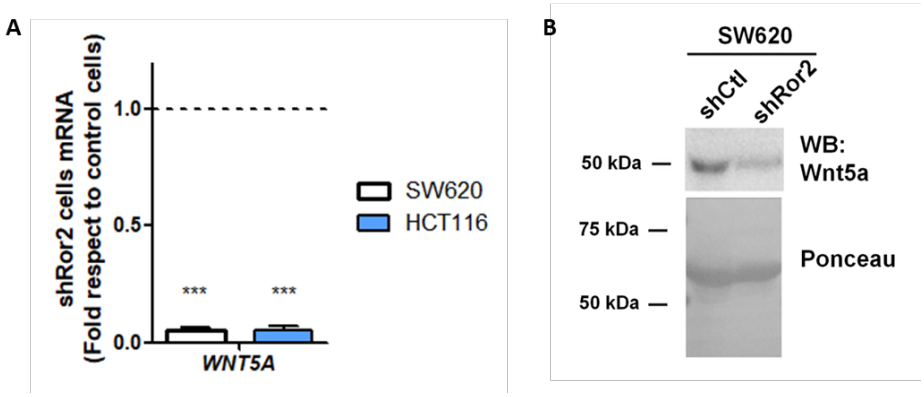

Figure 34: Ror2 depletion decreases Wnt5a production. A) mRNA was isolated from SW620 and HCT116 control and Ror2 knock-down cells. *WNT5A* mRNA was analyzed by RT-qPCR. The mRNA levels are referred to the control cells levels. Quantification of three different experiments is represented ± SD. B) Conditioned medium from SW620 control and Ror2-depleted cells was analyzed by WB with the indicated antibodies. Ponceau was used as loading control.

To study whether this decrease in the production of Wnt5a in the Ror2-depleted cells is affecting the capacity to activate the non-canonical Wnt pathway, we collected conditioned medium from control and Ror2 knock-down cells and mMSCs were treated at different time points. The capacity to induce STAT3 phosphorylation was analyzed. STAT3 phosphorylation was induced with the control conditioned medium from SW620 and HCT116 cells at 30', while conditioned medium from

Ror2-depleted cells failed to induce STAT3 phosphorylation or induced less. This effect was observed both in SW620 and HCT116 cell lines (Figure 35).

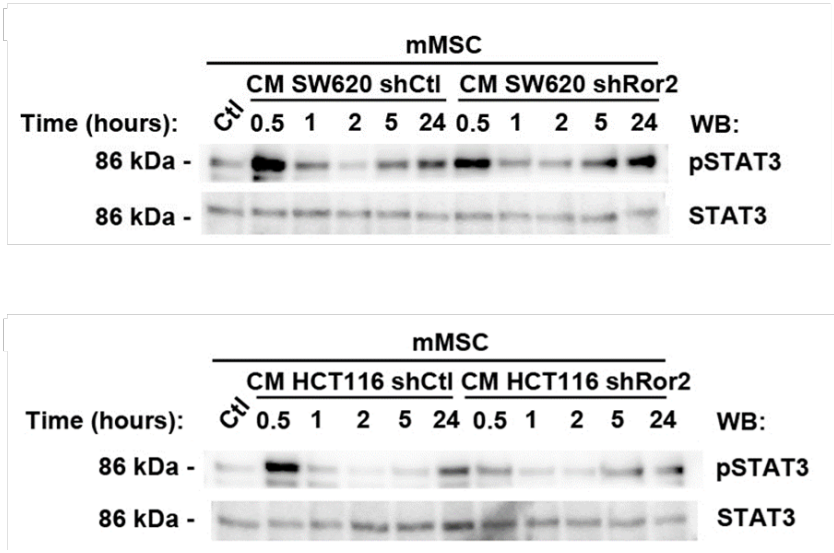

Figure 35: Conditioned medium from SW620 and HCT116 control cells induces STAT3 phosphorylation in mMSCs. mMSCs were treated at the indicated times with conditioned medium from SW620 and HCT116 control and Ror2-depleted cells. After the treatment, cells were lysed and protein extracts were analyzed by WB with the indicated antibodies.

As previously described, STAT3 phosphorylation can also be induced through the canonical Wnt pathway by the action of Wnt3a[81]. To test whether STAT3 phosphorylation induced by SW620 and HCT116 conditioned medium is due to the action of Wnt5a, we knocked-down Ror2 in HEK293T cells and treated them with control or Ror2-depleted HCT116 cells conditioned medium. As expected, Ror2 depletion in HEK293T cells caused a decrease in the induction of STAT3 phosphorylation (Figure 36), which suggested that Wnt5a and the activation of the non-canonical Wnt pathway mainly cause this induction.

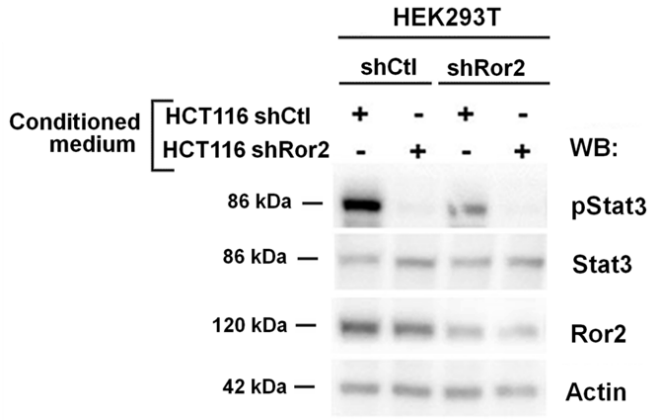

Figure 36: Depletion of Ror2 in HEK293T cells decreased STAT3 phosphorylation after treatment with HCT116 control cells conditioned medium. HEK293T cells were knocked-down for Ror2 and were treated at the indicated with conditioned medium from HCT116 control and Ror2-depleted cells. After the treatment, cells were lysed and protein extracts were analyzed by WB with the indicated antibodies.

As observed, Ror2 depletion caused a decrease in the expression and production of Wnt5a, which in turn cannot induce activation of STAT3 in other cell types. Therefore, with these findings we hypothesized that Wnt5a induces the expression of itself through Ror2 and the non-canonical Wnt pathway, generating a kind of autoactivation loop.

3.2. Inhibition of Wnt secretion mimics Ror2 depletion

After determining that Ror2 depletion affected the expression of Wnt5a in SW620 and HCT116 cells and Wnt5a seems to be regulating its own expression, we wondered if blocking the secretion of Wnt factors could mimic the depletion of Ror2. For that, we used the LGK-974 inhibitor. Although LGK blocks the function of Porcupine and it may affect all Wnt factors, canonical and non-canonical, we have centered in the analysis of Wnt5a expression.

We treated the SW620 and HCT116 wildtype cells with 10 µM LGK and analyzed the expression of Wnt5a at mRNA level. As observed in Figure 37A, when the secretion of Wnt factors was blocked with LGK, the mRNA levels of Wnt5a decreased compared to cells that were not treated. Moreover, the conditioned media from SW620 cells treated or not with LGK was also analyzed to determine the secretion of Wnt5a and it was also decreased compared to control conditioned medium (Figure 37B). These results reinforced our hypothesis that Wnt5a is regulating its own expression, because when its secretion is blocked and there is less Wnt5a in the medium, a smaller activation of the non-canonical Wnt pathway occurs and, therefore, less Wnt5a expression is observed.

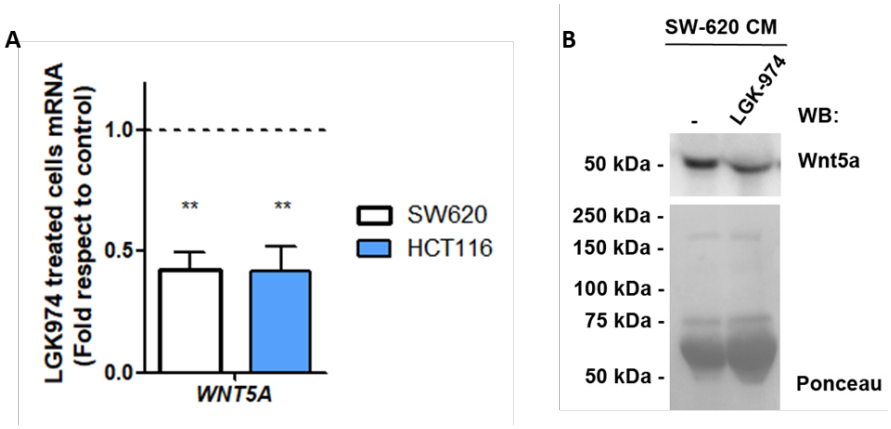

Figure 37: LGK974 decreases Wnt5a production. A) mRNA was isolated from SW620 and HCT116 cells treated with LGK974 for 24 hours. *WNT5A* mRNA was analyzed by RT-qPCR. The mRNA levels are referred to the control cells levels. Quantification of three different experiments is represented ± SD. B) Conditioned medium from SW620 control and LGK974 treated cells was analyzed by WB with the indicated antibodies. Ponceau was used as loading control.

Although LGK is reducing the expression and secretion of Wnt5a, it should be affecting the secretion of all Wnt factors. We checked whether LGK treatment was reducing the expression of both,

canonical and non-canonical Wnt target genes and, indeed, it was. As shown in Figure 38A, the expression of canonical target genes *AXIN2* and *CCND1* was decreased compared to not treated cells and the same happened with the non-canonical target genes *MMP9* and *SIAH2.* Moreover, in accordance with Figure 17, the cells treated with LGK expressed decreased mRNA levels of *SNAI1* as well (Figure 38A).

Interestingly, inhibition of Wnt secretion with LGK also promoted a decrease in the protein levels of Ror2 and Fz2 (Figure 38B). This effect was similar to the effect observed with Ror2 depletion (Figure 27). Thus, we hypothesized that Ror2 and Fz2 expression is also controlled by the non-canonical Wnt pathway, although LGK is inhibiting canonical Wnt signaling too and it cannot be discarded that it could have an effect on its expression.

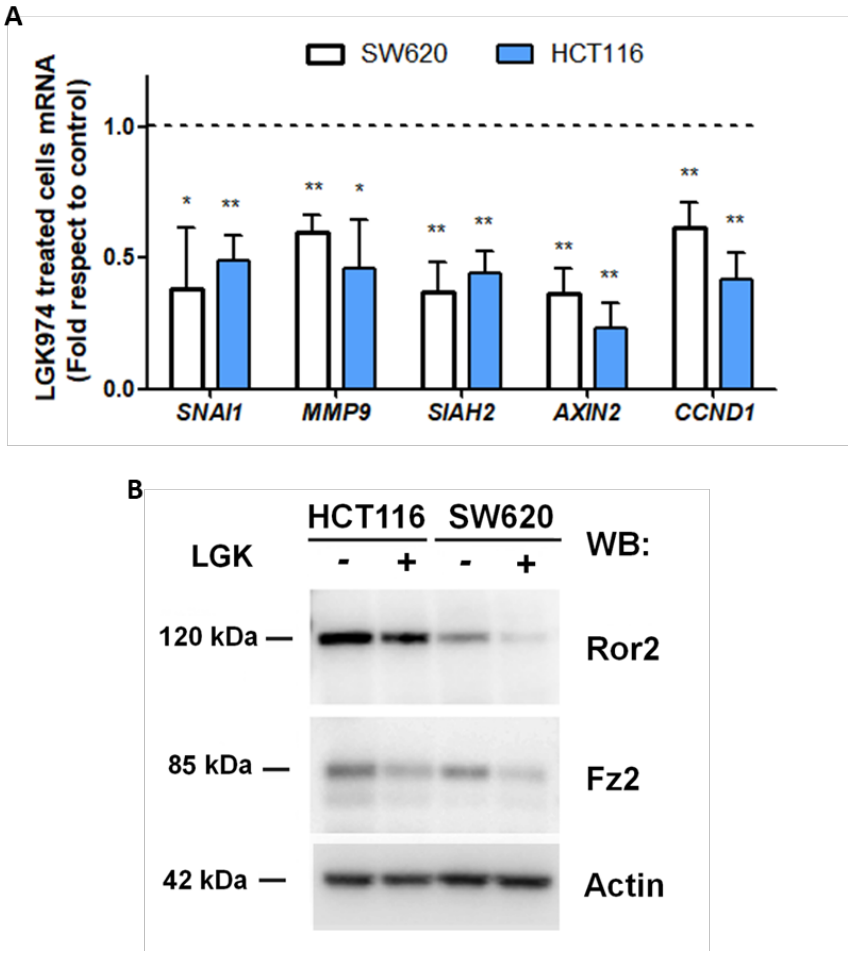

Figure 38: LGK974 decreased canonical and non-canonical target genes expression. SW620 and HCT116 cells were treated with LGK974 for 24 hours. A) mRNA was isolated from the control and treated cells. *SNAI1, MMP9, SIAH2, AXIN2* and *CCND1* mRNA levels were quantified by quantitative RT-PCR (RT-qPCR). The mRNA levels are referred to control cells mRNA levels. Quantification of three different experiments is represented ± SD. B) After treatment, cells were lysed and protein extracts were analyzed by WB using the indicated specific antibodies.

3.3. Wnt5a induces the expression of Snail1, Wnt5a, Ror2 and Fz2

As mentioned, these previous findings suggested that Wnt5a – Ror2 generate an autoactivation feedback loop that regulates the expression of Wnt5a, Ror2 and Fz2. In order to study this hypothesis, we treated SW620 and HCT116 cells with Wnt5a conditioned medium for 16 hours and analyzed the levels of all these factors. Wnt5a treatment induced an increase in the protein levels of Ror2 and Fz2 in HCT116 cells, besides increasing Snail1 expression (Figure 39A).

The upregulation of Ror2, Fz2 and Snail1 was also confirmed at mRNA levels in both cell lines (Figure 39B). Moreover, an increase in Wnt5a expression was also detected (Figure 39B), confirming that Wnt5a is a target gene of itself, as suggested by previous experiments. The mRNA levels of Mmp13 were analyzed as a positive control for the non-canonical Wnt pathway activation and as expected, Mmp13 expression increased with Wnt5a treatment. Interestingly, DUB3 expression was also increased upon Wnt5a treatment (Figure 39B). This was an interesting result because we have previously seen that DUB3 expression was strongly downregulated in Ror2-depleted cells. These results, together with previous studies showing that DUB3 is related to STAT3 activation, suggested us that DUB3 is a target gene of the non-canonical Wnt pathway.

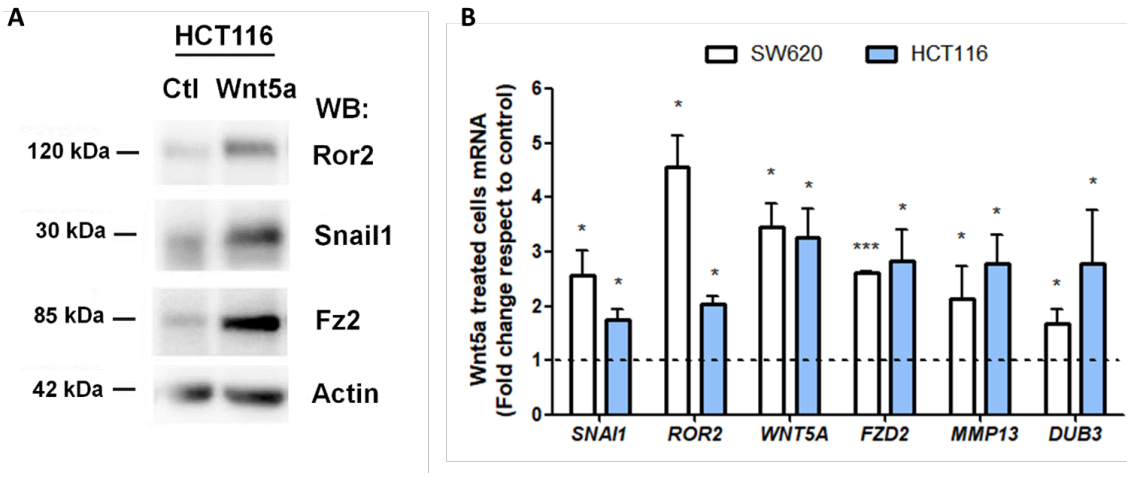

Figure 39: Wnt5a treatment induces the expression of Snail1, Ror2, Fz2 and Wnt5a. SW620 and HCT116 cells were treated with Wnt5a conditioned medium for 16 hours. A) After treatment, cells were lysed and protein extracts were analyzed by WB using the indicated specific antibodies. B) mRNA was isolated from the control and treated cells. *SNAI1, ROR2, WNT5A, FZD2, MMP13* and *USP17L2* (DUB3) mRNA levels were quantified by quantitative RT-PCR (RT-qPCR). The mRNA levels are referred to not treated cells mRNA levels. Quantification of three different experiments is represented ± SD.

3.4. Overexpression of Ror2 or Snail1 induces the activation of the non-canonical Wnt signaling

At that point, we have observed that the non-canonical Wnt pathway defines an autoactivation loop in which the expression of all the proteins that participate in its activation is regulated by themselves. Another way to activate the non-canonical Wnt signaling that we have previously reported is the overexpression of Ror2[81]. Then, we wondered if Ror2 overexpression in colorectal cancer cells could also activate the pathway.

For that, we chose the SW480 cell line due to its low expression of Ror2 and Snail1 (see Figure 20). Ror2-HA was overexpressed in SW480 cells and Ror2-overexpressing clones were selected. The expression of Snail1, Fz2 and Wnt5a was analyzed in two different Ror2-overexpressing SW480 clones. As shown in Figure 40, in both clones, Ror2 overexpression induced an increase in the expression of Snail1, Fz2 and Wnt5a. This result confirms that Ror2 overexpression is sufficient to activate the non-canonical Wnt pathway and induce the expression of the different partners involved. Moreover, it also reinforces our previous results and highlights the importance of Ror2 in the regulation of the non-canonical Wnt pathway.

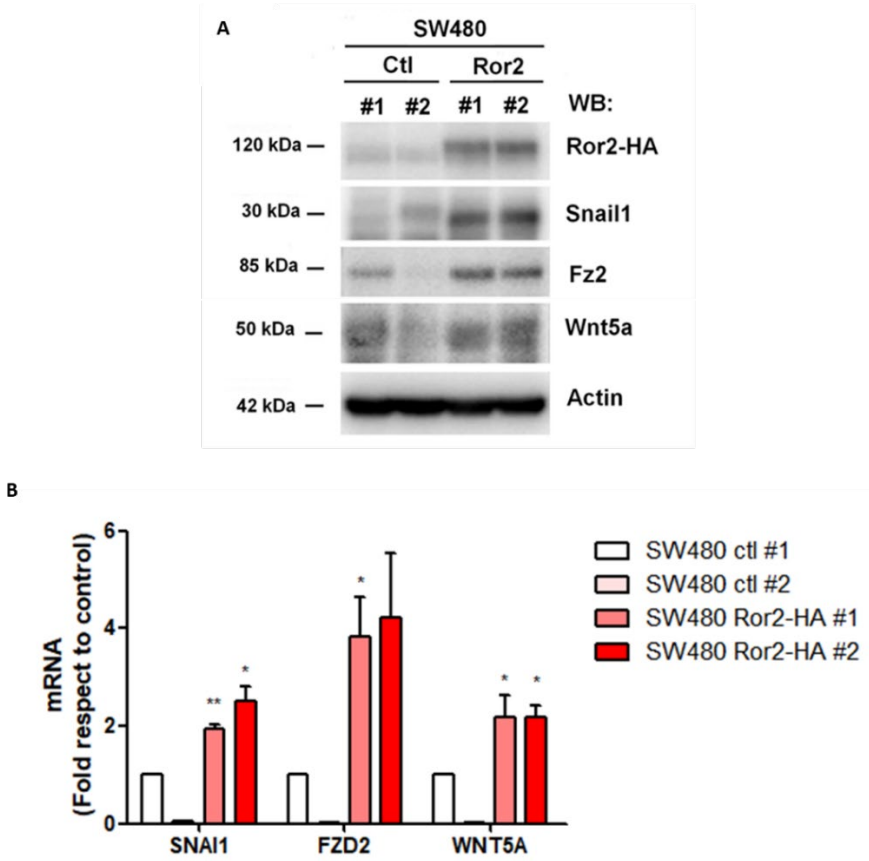

Figure 40: Overexpression of Ror2 in SW480 increases Snail1, Fz2 and Wnt5a expression. A) SW480 control and overexpressing Ror2 clones were lysed and protein extracts were analyzed by WB using the indicated specific antibodies.

B) mRNA was isolated from SW480 cells. *SNAI1, FZD2* and *WNT5A* mRNA levels were quantified by quantitative RT-PCR (RT-qPCR). The mRNA levels are referred to not treated cells mRNA levels. Quantification of three different experiments is represented ± SD.

The most important effect observed upon non-canonical Wnt pathway stimulation is the upregulation of Snail1. It is known that upregulation of Snail1 is one of the main steps in EMT[91]. Our findings also have revealed that upon Ror2 knock-down, Snail1 is down-regulated together with different mesenchymal markers and, epithelial markers such as E-Cadherin, are upregulated (Figure 27). These results suggested that Ror2 expression and non-canonical Wnt pathway activation correlate with a more mesenchymal phenotype of the cell. Therefore, we wanted to study how the non-canonical Wnt pathway elements are affected when cells are forced to undergo EMT.

To do that, we used HT29 M6 and SW480 cells overexpressing Snail1 or not. These colorectal cells are very epithelial, but when Snail1 is overexpressed they are forced to undergo EMT and loss E-Cadherin expression, while mesenchymal markers are upregulated[122]. In those cells, we analyzed the expression of Ror2 and Fz2. As observed in Figure 26, both, HT29 M6 and SW480 overexpressing Snail1 cells presented higher levels of Ror2 and Fz2 than control cells. Moreover, these cells also expressed more FN1 and αSMA, which are well known mesenchymal markers (Figure 41).

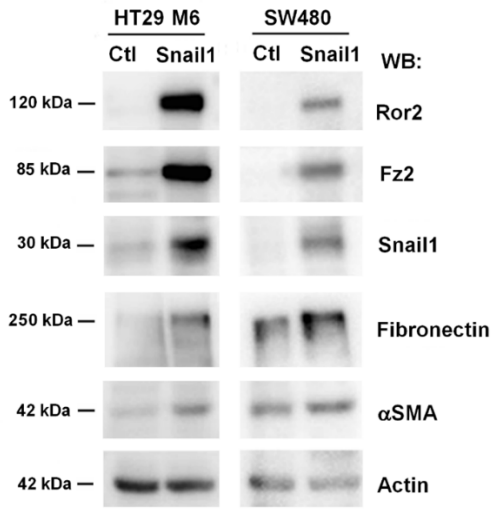

Figure 41: Overexpression of Snail1 in HT29 M6 and SW480 cells increases Ror2, Fz2, FN1 and αSMA protein levels. HT29 M6 and SW480 control and overexpressing Snail1 cells were lysed and protein extracts were analyzed by WB using the indicated specific antibodies.

These changes were longer confirmed at mRNA levels. In Snail1 overexpressing HT29 M6 and SW480 cells, the mRNA levels of Wnt5a, Ror2 and FZ2 were increased compared to control cells (Figure 42). These findings confirmed that the non-canonical Wnt pathway activation and,

therefore, the expression of Ror2, Fz2 and Wnt5a are related to a more mesenchymal state of the cell.

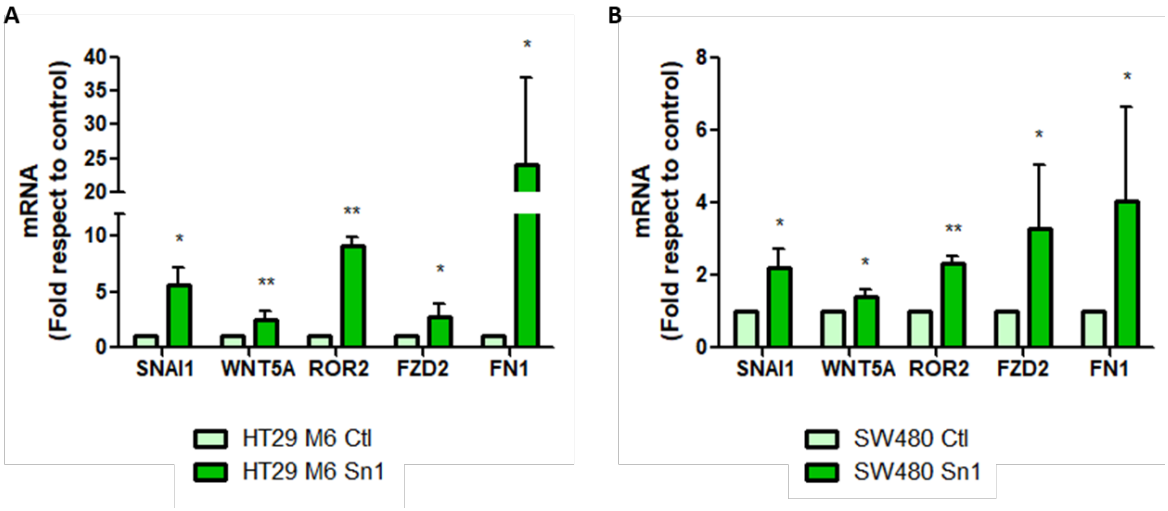

Figure 42: Overexpression of Snail1 in HT29 M6 and SW480 cells increases *SNAI1*, *WNT5A*, *ROR2*, *FZD2* and *FN1* expression. mRNA was isolated from A) HT29 M6 and B) SW480 control and overexpressing Snail1 cells. *SNAI1, WNT5A, ROR2, FZD2* and *FN1* mRNA levels were quantified by quantitative RT-PCR (RT-qPCR). The mRNA levels are referred to not treated cells mRNA levels. Quantification of three different experiments is represented ± SD.

3.5. Snail1 knock-down decreases the expression of Wnt5a, Ror2 and Fz2

In the previous experiments, we have seen that Ror2, Fz2 and Wnt5a are upregulated when epithelial cells undergo EMT induced by Snail1 overexpression. We wondered then how the expression of these proteins is affected upon Snail1 knocked-down in the colorectal cells that express high levels of Snail1 and Ror2.

Snail1 was knocked-down from SW620 and HCT116 cells with a specific siRNA. After 48 hours of transfection, the mRNA levels were analyzed. As expected, Snail1 knock-down decreased the expression of Ror2, Fz2, Wnt5a and FN1 (Figure 43A). However, when we analyzed the cells knocked-down for Snail1 by WB, no changes were observed in Ror2 or Fz2 protein levels, while Snail1 and FN1 levels decreased (Figure 43B).

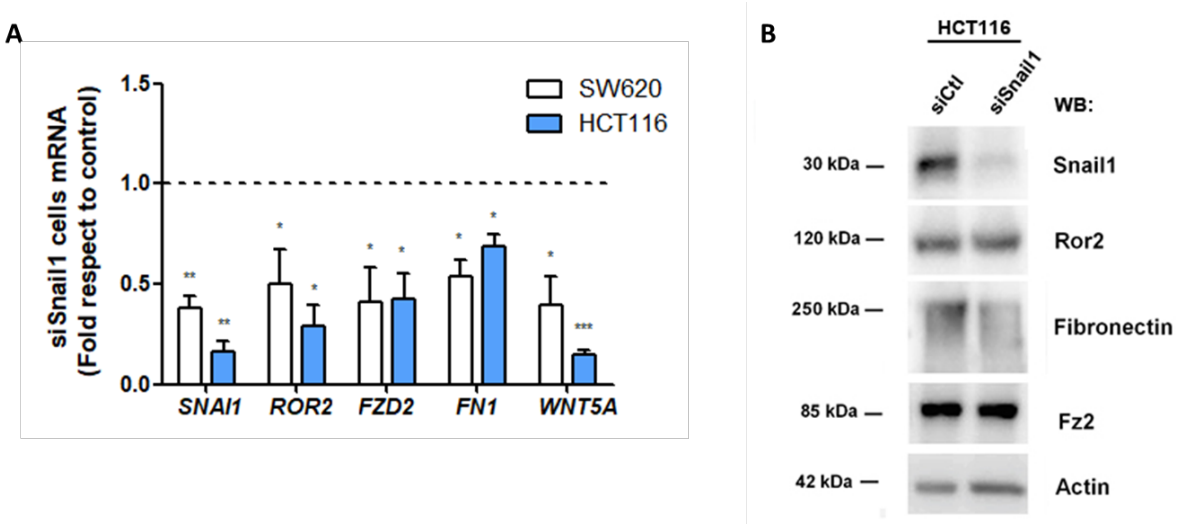

Figure 43: Snail1 knock-down decreases Ror2, Fz2 and Wnt5a expression. A) mRNA was isolated from SW620 and HCT116 control and Snail1-depleted cells. *SNAI1, ROR2, FZD2, FN1* and *WNT5A* mRNA levels were quantified by quantitative RT-PCR (RT-qPCR). The mRNA levels are referred to not treated cells mRNA levels. Quantification of three different experiments is represented ± SD. B) SW620 and HCT116 control and Snail1-depleted cells were lysed and protein extracts were analyzed by WB using the indicated specific antibodies.

All together these results confirmed that Wnt5a – Ror2 – Snail1 generate a positive feedback loop of activation, since Wnt5a induces the expression of itself, Ror2 and Snail1. Moreover, the overexpression or downregulation of Ror2 or Snail1 modulates the expression of themselves and the other partners of the non-canonical Wnt pathway.

4. Ror2-depletion inhibits CAF activation by tumor cells

Cancer associated fibroblasts (CAFs) are one of the main components of the tumor stroma[101]. It is well described the crosstalk between the tumor stromal cells and cancer cells and its importance for tumor progression[192]. Indeed, several reports have described that the cancer cells activate CAFs by the secretion of different cytokines, such as TGF-β[192,191]. It is also known that Snail1 plays an essential role in CAF activation[191,192]. Thus, in this part we investigated whether the Ror2 knockdown SW620 and HCT116 tumor cells maintain their capacity to promote fibroblast activation.

4.1. Conditioned medium from Ror2-depleted tumor cells decreases mMSCs activation

To investigate whether Ror2 depletion in the tumoral cells affect the capacity to induce fibroblast activation, we used mMSCs as a fibroblast model. We chose this cell line due to its lower basal activation state compared to other fibroblasts, such as MEFs or CAFs. mMSCs were treated with conditioned medium from control and Ror2-depleted SW620 cells at different time points and fibroblast activation markers were analyzed. As observed, upon control conditioned medium treatment, FN1 and Snail1 protein levels were increased over time, while less increase was observed in the Ror2-depleted cells conditioned medium (Figure 44). This suggested that upon Ror2 depletion the tumor cells lost the capacity to promote fibroblast activation.

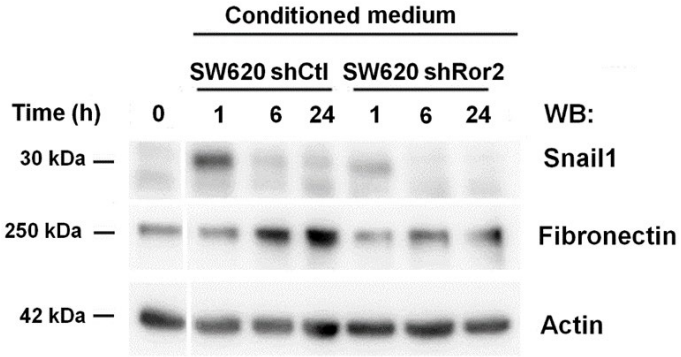

Figure 44: Conditioned medium from SW620 Ror2-depleted cells does not induce fibroblast activation. mMSCs were treated at the indicated times with conditioned medium from SW620 control and Ror2-depleted cells. After the treatment, cells were lysed and protein extracts were analyzed by WB with the indicated antibodies.

One of the main cytokines described to promote fibroblast activation is TGF-β. To study if TGF-β is implicated in the reduced capacity of the Ror2-depleted tumor cells to activate fibroblasts, we analyzed TGF-β signaling activation in the mMSCs. We treated mMSCs with control or Ror2-depleted cells conditioned medium at different times and analyzed SMAD2 phosphorylation. Control conditioned medium induced SMAD2 phosphorylation starting at 30' and with a peak at 1h.

By contrast, SMAD2 phosphorylation was totally abrogated with Ror2-depleted cells conditioned medium (Figure 45).

These findings suggested that depletion of Ror2 in the tumoral cells impairs its capacity to induce fibroblast activation. This seems to be due to a lower ability to activate the TGF-β signaling pathway in fibroblasts.

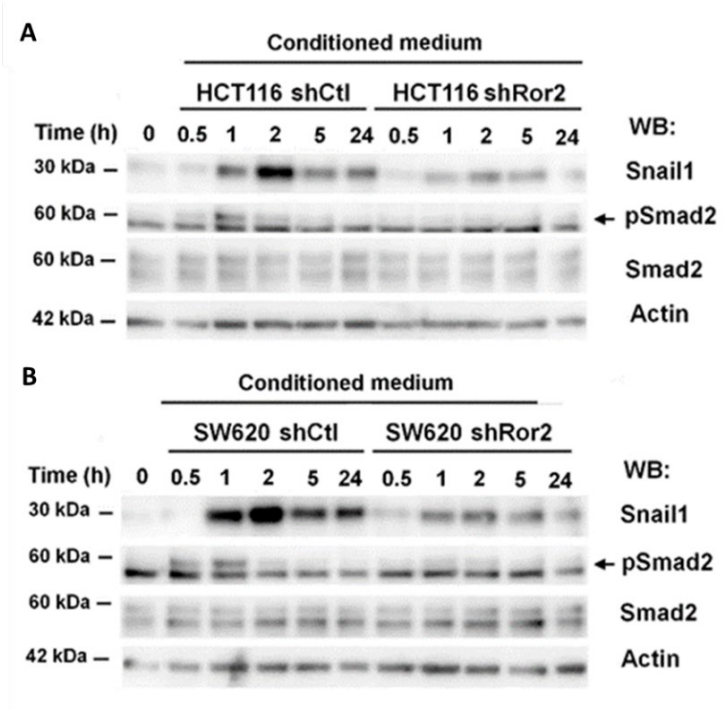

Figure 45: Conditioned medium from SW620 and HCT116 Ror2-depleted cells does not induce fibroblast activation. mMSCs were treated at the indicated times with conditioned medium from SW620 and HCT116 control and Ror2-depleted cells. After the treatment, cells were lysed and protein extracts were analyzed by WB with the indicated antibodies.

4.2. Ror2-depleted cells produced less TGF-β

The preceding results suggested that Ror2 depletion could be affecting the capacity of these cells to produce TGF-β. So, we analyzed the expression of TGF-β in the Ror2-depleted HCT116 and SW620 cells. As observed in Figure 46A, TGF-β expression was dramatically decreased in the cells knocked-down for Ror2. We wondered if these changes in TGF-β expression were accompanied by changes in the production of the cytokine to the medium. To study that, we used a HEK293T cell line that express a TGF-β reporter system. These cells were treated with the control and Ror2-depleted conditioned medium from HCT116 cells and the luciferase activity was measured. In concordance with the previous result, Ror2-depleted cells induced lower activation of the TGF-β reporter (Figure 46B). Therefore, these cells produced less TGF-β.

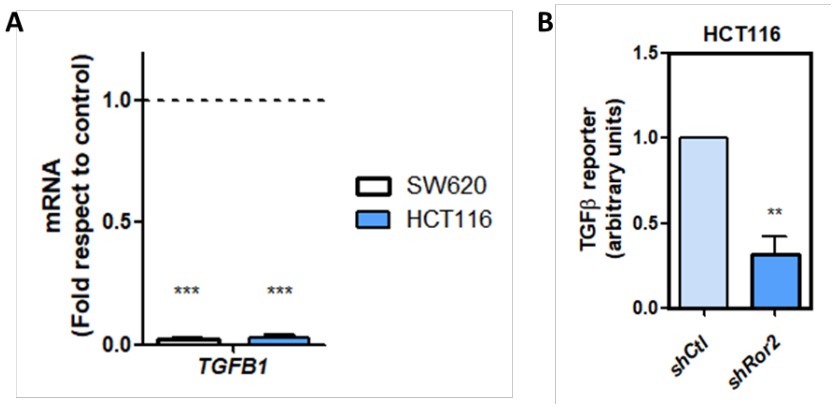

Figure 46: Ror2-depleted cells produce less TGF-β. A) mRNA was isolated from SW620 and HCT116 control and Ror2-depleted cells. *TGFB1* mRNA was analyzed by RT-qPCR. The mRNA levels are referred to the control cells levels. Quantification of three different experiments is represented ± SD. B) HEK293T cells overexpressing TGF-β reporter system were treated with HCT116 control and Ror2-depleted cells conditioned medium. Luciferase activity was measured. Quantification of three different experiments is represented ± SD.

The decrease observed in TGF-β production in the Ror2-depleted cells is not only important for the capacity of these cells to promote fibroblast activation; it is also relevant for the autocrine activation of the TGF-β pathway, which is required for several functions of the tumoral cells. So, we analyzed also the basal activation state of the TGF-β pathway in the Ror2-depleted cells. As observed in Figure 47, SMAD2 phosphorylation decreased in the cells lacking Ror2.

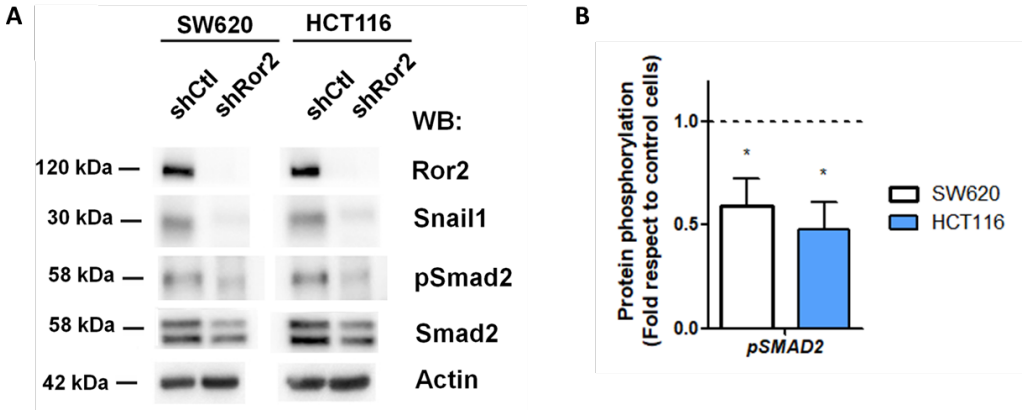

Figure 47: Ror2 depletion affects the basal phosphorylation level of SMAD2. A) SW620 and HCT116 control and Ror2 knock-down cells were lysed and protein extracts were analyzed by WB with the specific antibodies. B) Quantification of phosphorylated SMAD2 levels referred to control cells. Quantification of three different experiments is represented ± SD

4.3. Ror2-depleted cells decrease mMSC-induced invasion in co-culture

As explained before, it has been largely described that tumor cells-induced fibroblast activation also promote fibroblast migration and invasion[192]. We have previously observed that Ror2 depletion

compromises the capacity of the tumoral cells to induce fibroblast activation and we wanted to further analyze whether the capacity to promote fibroblast invasion is also reduced.

We performed an invasion assay using transwells. The transwells were previously coated with Matrigel and the cells were seeded on top. We analyzed the capacity of the mMSCs to invade towards the Matrigel alone or in co-culture with control or Ror2-depleted SW620 and HCT116 cells. As shown inFigure 48, incubation with control cells induced fibroblast invasion, while no invasion was observed when the fibroblasts were co-cultured with Ror2-depleted cells. Moreover, the TGF-β receptor inhibitor SB prevented the induction of fibroblast invasion by control tumor cells.

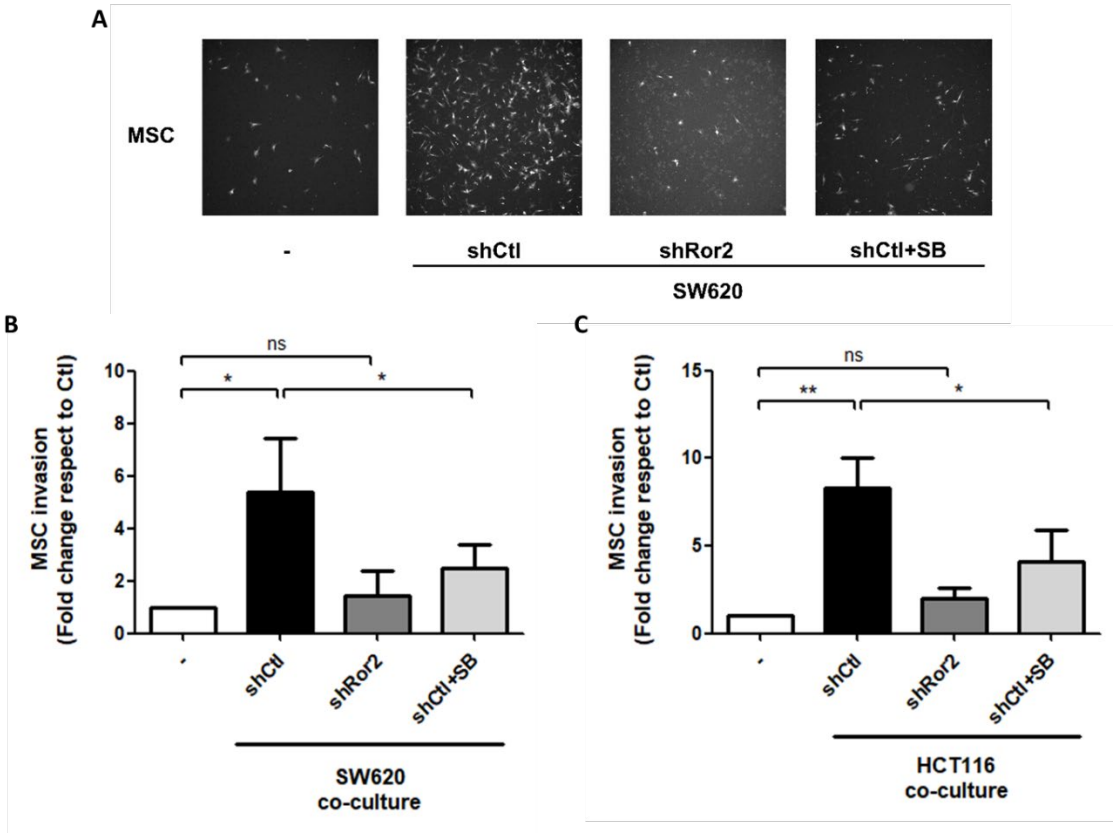

Figure 48: Ror2 depletion impairs tumor cells-induced mMSC invasion. mMSCs were seeded on top of Matrigel-coated transwells alone or in co-culture with SW620 and HCT116 control (treated or not with SB) or Ror2-deplted cells. A) Representative images of invaded mMSCs. B-C) Quantification of three different experiments is represented ± SD.

4.4. TGF-β inhibition decreases Fibronectin but not Snail1 activation in mesenchymal cells upon addition of conditioned medium from tumor cells

As observed in Figure 48, a TGF-β receptor inhibitor decreased invasion of fibroblast induced by the tumor cells. An explanation could be that inhibition of TGF-β signaling is preventing fibroblast activation. To study the effect of TGF-β inhibition on fibroblast activation, we treated mMSCs with

conditioned medium from control SW620 and HCT116 cells alone or with SB and analyzed fibroblast activation markers Snail1 and FN1 at early and late times. FN1 and pSMAD2 protein levels increased at 16 and 1 hours of treatment, respectively, and both were prevented by addition of SB inhibitor (Figure 49). However, Snail1 induction was not affected. Control cell conditioned medium induced a rapid increase in Snail1 protein levels at 1 hour and was not affected by SB (Figure 49). These results suggested that, although TGF-β inhibition seems to be sufficient to prevent fibroblast invasion, other factors secreted by tumor cells are also responsible of fibroblast activation.

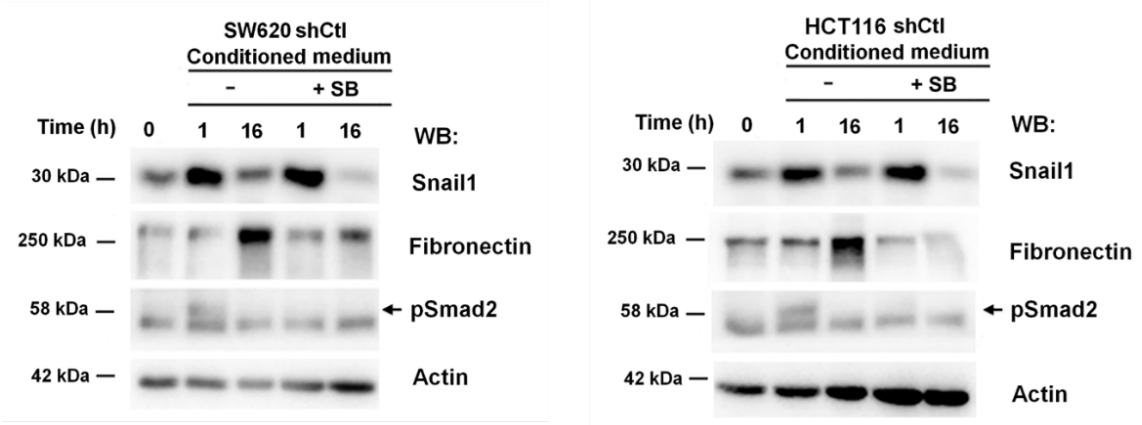

Figure 49: TGF-β inhibition decreases Fibronectin1 and pSMAD2 induction but not Snail1 in mMSCs. mMSCs were treated at the indicated times with conditioned medium from SW620 and HCT116 control cells alone or with SB. After the treatment, cells were lysed and protein extracts were analyzed by WB with the indicated antibodies.

4.5. Wnt secretion inhibition mimics the effect of Ror2-deleted tumor cells conditioned medium

As previously observed, inhibition of Wnt signaling by LGK mimicked the effect of Ror2 depletion. Interestingly, LGK treatment in SW620 and HCT116 cells strongly decreased the mRNA expression of *TGFB1*, *TGFB2* and *TGFB3* (Figure 50A). This effect was similar than the one triggered by Ror2 depletion.

Once we have observed that LGK decreased TGF-β production, we investigated the capacity of tumor cells, previously treated with LGK, to activate fibroblasts. Therefore, we treated mMSCs with conditioned medium from control SW620 and HCT116 cells previously incubated or not with LGK during 24 hours. As observed in Figure 50B and C, conditioned medium from cells treated with LGK was much less efficient in inducing Snail1 and FN1 expression in fibroblasts.

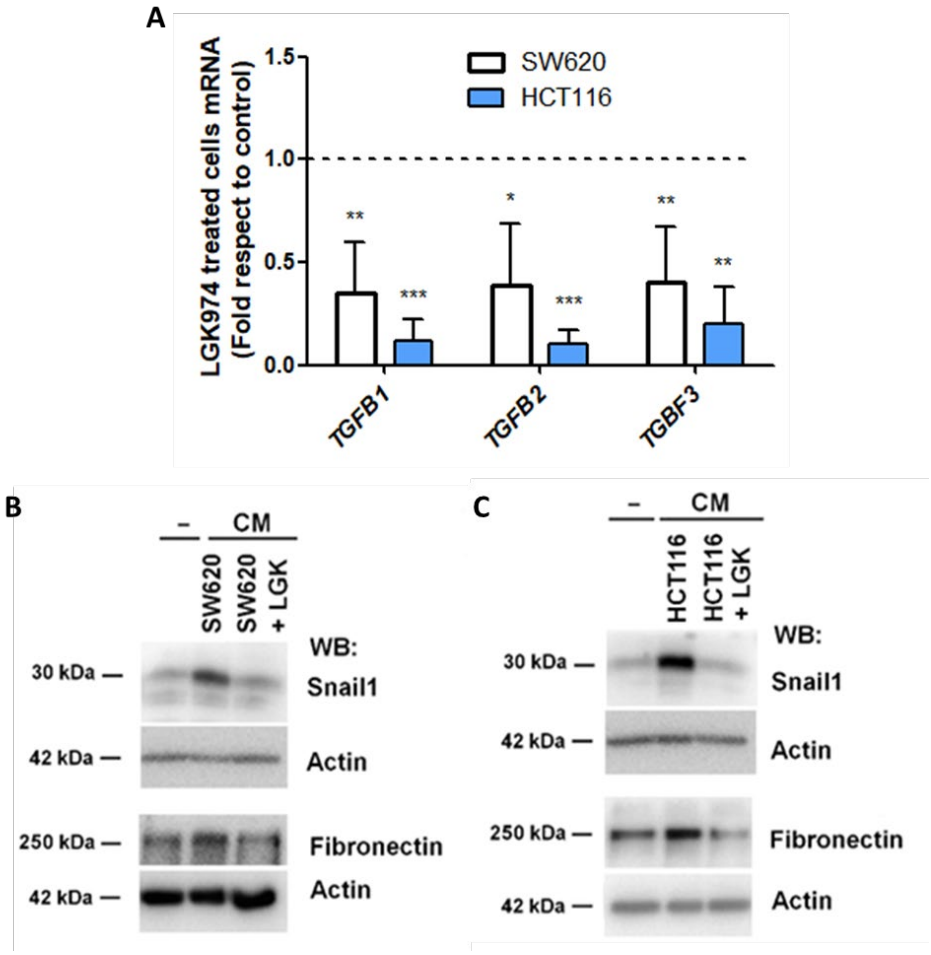

Figure 50: LGK974 decreases TGF-β production and fibroblast activation. A) mRNA was isolated from SW620 and HCT116 cells treated or not with LGK974. *TGFB1, TGFB2* and *TGFB3* mRNA was analyzed by RT-qPCR. The mRNA levels are referred to the control cells levels. Quantification of three different experiments is represented ± SD. B-C) mMSCs were treated for 16 hours with conditioned medium coming from SW620 or HCT116 cells previously treated with LGK974 or not. After the treatment, cells were lysed and protein extracts were analyzed by WB with the indicated antibodies.

4.6. Snail1-overexpression in tumor cells increase their ability to activate fibroblasts

Contrary to Ror2 depletion or Wnt inhibition, Snail1 overexpression in colorectal tumor cells showed increased levels of Wnt5a, Ror2 among others. Then, it is reasonable to think that the Snail1 overexpressing cells could also produce more TGF-β and have an increased capacity to promote fibroblast activation than control cells. To check that, we treated mMSCs with conditioned medium from control and Snail1-overexpressing HT29 M6 cells and analyze Snail1 and FN1 activation in fibroblasts. As shown in Figure 51, conditioned medium from HT29 M6 overexpressing Snail1 cells presented an enhanced capacity to activate fibroblasts compared to control cells.

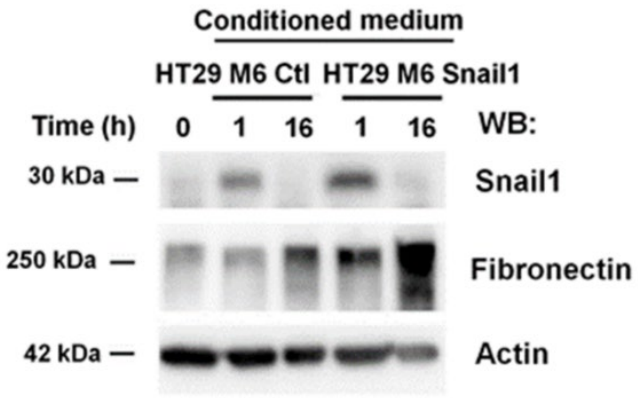

Figure 51: HT29 M6 overexpressing Snail1 induces fibroblast activation. mMSCs were treated at the indicated times with conditioned medium from HT29 M6 control or overexpressing Snail1 cells. After the treatment, cells were lysed and protein extracts were analyzed by WB with the indicated antibodies.

Together, all these results indicated that Wnt5a and the non-canonical Wnt pathway is controlling TGF-β production by colorectal cancer cells and the capacity of these cells to promote fibroblast activation and invasion.

5. Ror2 controls tumorigenesis

The requirement of Ror2 for non-canonical Wnt activation in non-tumoral cell lines has been previously described by our group[81,53]. Moreover, since Ror2 knock-down decreased Snail1 expression in colorectal cell lines, we wanted to investigate whether Ror2 depletion affects cellular properties depending on Snail1.

5.1. Ror2 depletion impairs proliferation and colony formation capacities

Ror2-depleted cells showed slower proliferation than control cells *in vivo* when injected in mice. Thus, we wanted to assess if Ror2 depletion also provokes changes in proliferation *in vitro*. Ror2 depletion did not affect SW620 or HCT116 cell proliferation when the cells were cultured in normal culture conditions, in DMEM medium with 10% FBS (Figure 52A). However, when these cells were cultured just with 1% FBS, in more physiologic conditions, Ror2-depleted cells showed a slightly decrease in proliferation (Figure 52B).

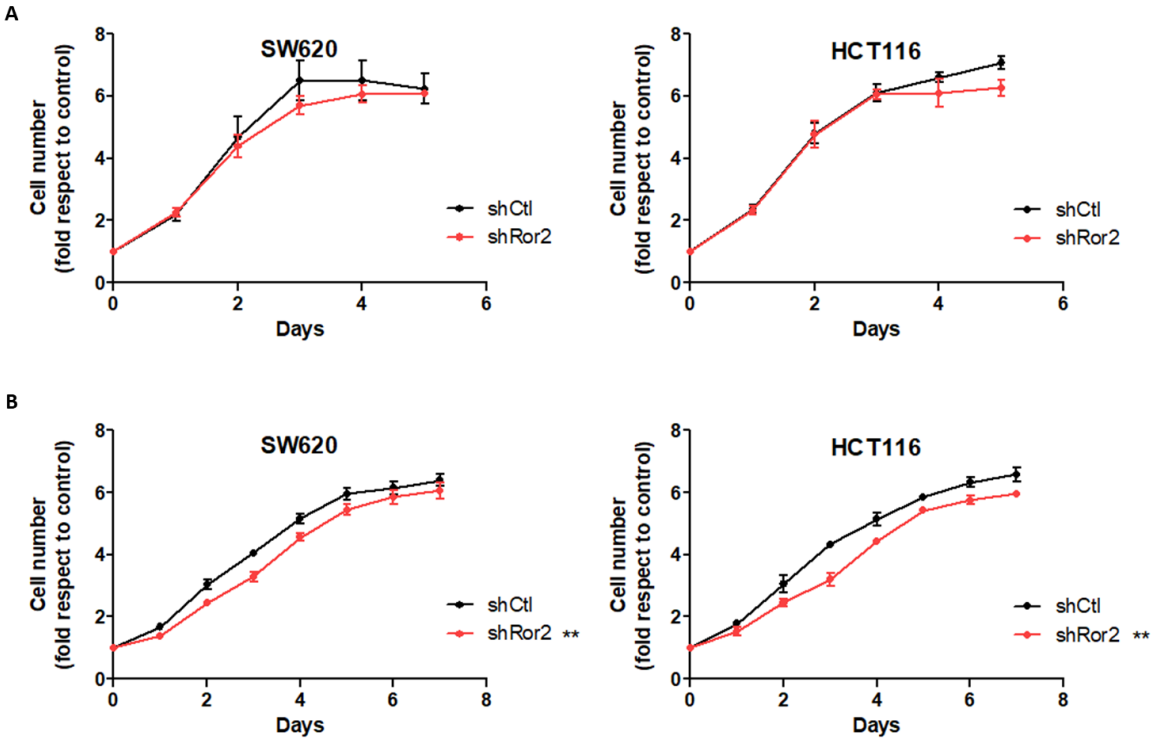

Figure 52: Ror2 depletion decreases cell proliferation at 1% FBS. The graph shows the proliferation capacity of SW620 and HCT116 control and Ror2-depleted cells in A) 10% FBS and B) 1% FBS conditions. Cell proliferation was measured every day and the representation shows the average of three independent experiments ± SD.

This change in cell proliferation was confirmed with clonogenic assays. These experiments were carried out seeding a few numbers of cells, 1000 cells/well, and we let them grow forming colonies for 10 days. As observed in Figure 53A, the difference in the clonogenic capacities of the Ror2-depleted SW620 and HCT116 cells was easily visible. The clonogenic capacity was clearly lower in the Ror2-depleted cells, especially when cells were growing at 1% FBS (Figure 53A and B). Finally, colony formation capability was also analyzed in soft agar. For that, control and Ror2 knock-down cells were mixed with soft agar at 0.6% and seeded on top of a layer of soft agar at 1%. Colonies were left to grow during 21 days. As shown inFigure 54, the colony formation capacities of these cells were also compromised when cells were depleted of Ror2.

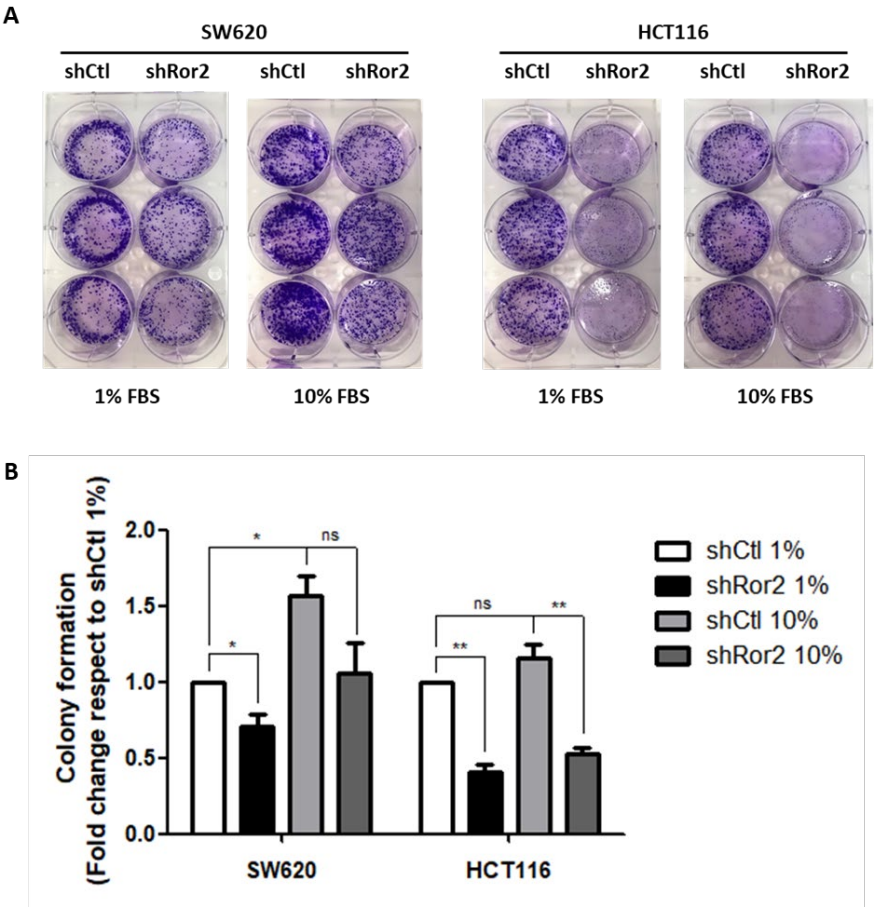

Figure 53: Ror2 depletion impairs the clonogenic capacities in 2D culture. A) Representative images of the clonogenic assay. 1000 cells/well of control and Ror2-depleted SW620 and HCT116 cells were seeded and after stopping the experiment at day 10 pictures were obtained. B) Quantification of three independent experiments ± SD is represented.

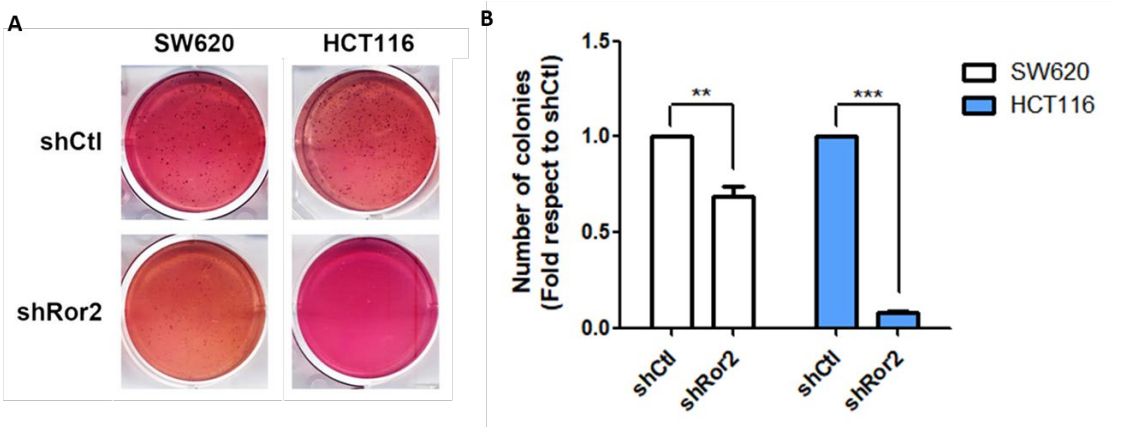

Figure 54: Ror2 knock-down decreases the clonogenic capacities in soft agar 3D culture. A) Representative images of the clonogenic assay in soft agar. Control and Ror2-depleted SW620 and HCT116 cells were seeded in soft agar and after stopping the experiment at day 21 pictures were obtained. B) Quantification of three independent experiments ± SD is represented.

Colony formation capacity was also assessed in SW480 overexpressing Ror2 cells. The assay was performed as in Figure 53 at 1% FBS. Confirming the role of Ror2 in controlling tumor formation, SW480 cells overexpressing Ror2 presented higher colony formation capacities (Figure 55). This finding is in accordance to previous results and clearly demonstrates that Ror2 expression confers to the cell a more stem phenotype and, thus, is controlling tumor formation.

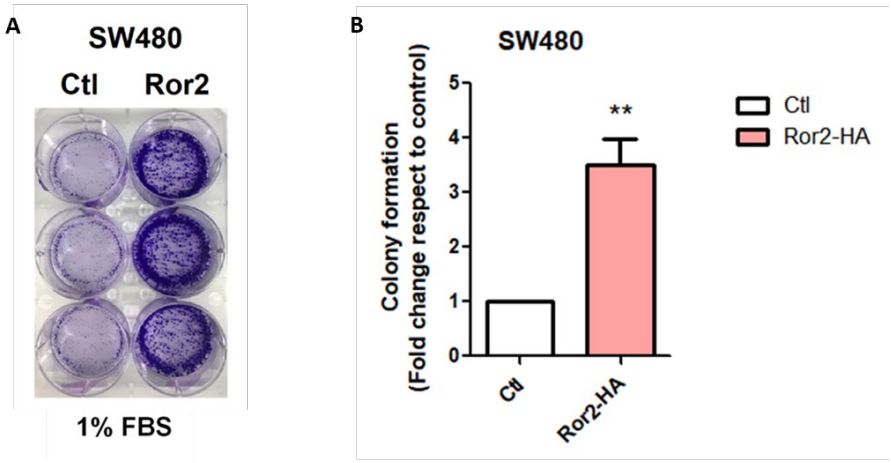

Figure 55: Ror2 overexpression increases clonogenic capacities in 2D culture. A) Representative image of the clonogenic assay. 1000 cells/well of control and Ror2-overexpresing SW480 cells were seeded and after stopping the experiment at day 10 pictures were obtained. B) Quantification of three independent experiments ± SD is represented.

5.2. Ror2 depletion decreases migration and invasion

Non-canonical Wnt pathway has been described to promote migration and invasion[53,208], two processes that are dependent on Snail1[108,192]. To test whether Ror2 depletion is affecting these Snail1-dependent processes, migration and invasion assays were performed with the Ror2 knock-

down SW620 and HCT116 cells. Both experiments were done in a Boyden chamber seeding the cells in the upper compartment and using 10% FBS DMEM as a chemoattractant in the lower compartment.

In the migration assay, Ror2 knock-down cells were directly seeded on the upper compartment and were left to migrate overnight (16 hours) towards the other side of the membrane. For the invasion assay, cells were seeded on the upper chamber previously coated with Matrigel 0.5 µg/µL and were left to invade for 48 hours. Ror2 knock-down cells showed less migration and invasion capacities than control cells (Figure 56). Therefore, Ror2 controls Snail1-dependent tumor migration and invasion.

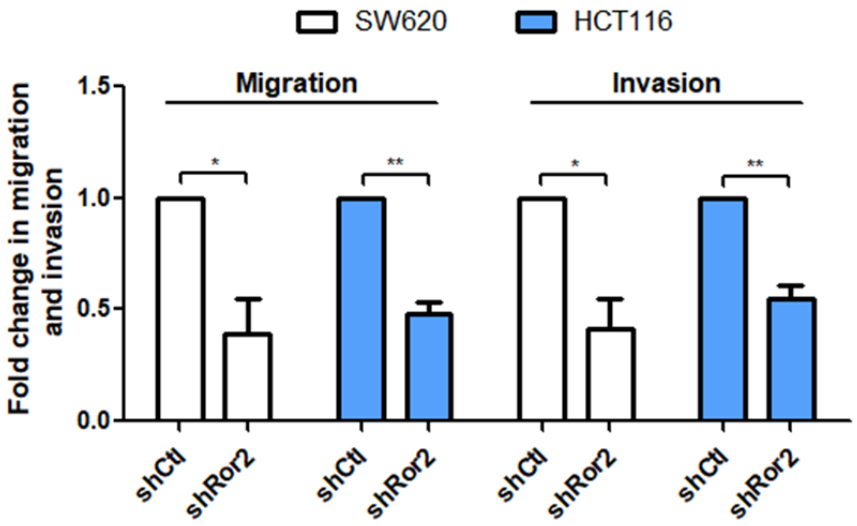

Figure 56: Ror2 knock-down decreases cell migration and invasion. Control and Ror2 knock-down SW620 and HCT116 cells were seeded in Transwell chambers, coated or not with Matrigel 0.5µg/µL. Culture medium containing 10% FBS was added to the lower chamber and after 16 hours for migration or 48 hours for invasion, cells were fixed and stained with crystal violet and optical density was quantified at 590 nm. The graph shows the quantification of three independent experiments ± SD.

5.3. Ror2 depletion impairs tumor growth

The tumorigenic capability of the Ror2 knock-down cells was also assessed. For this, control and Ror2-depleted SW620 cells were subcutaneously injected in immune-deficient NOD scid gamma mice. Tumors were left to grow until they reached a detectable volume at day 15. Then, tumor growth was quantified every 2-3 days. As observed in the graph, control tumors grew faster than Ror2-depleted tumors, which took more time start to grow. At day 28, when those mice injected with the control SW620 cells have to be euthanized, control tumors presented a bigger average size compared to those generated from Ror2-depleted SW620 cells (Figure 57).

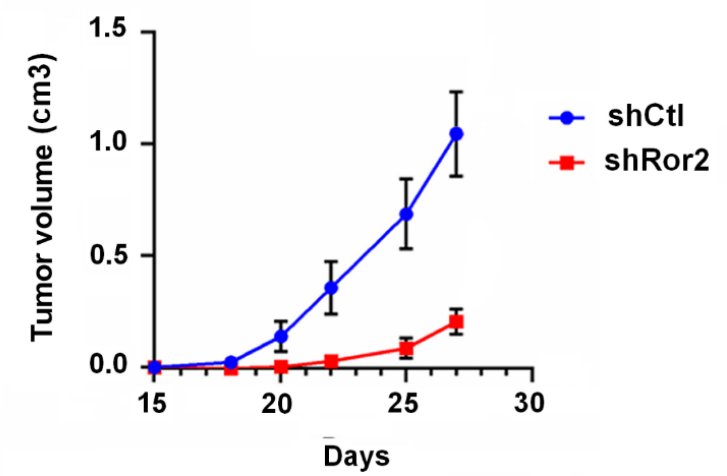

Figure 57: Ror2 depletion decreases tumor growth. SW620 control and Ror2-depleted cells were injected in NOD SCID gamma mice. Once tumors were detectable at day 15, tumor growth was measured. The graph shows the mean volume of tumors at each time point ± SD.

The tumor phenotype was analyzed at early time point, 15 days, when they started to be detectable. At that time, control tumors presented higher infiltration by stromal cells than Ror2-depleted tumors. Control tumors showed higher Fibronectin and Vimentin levels in the stroma than Ror2-depleted tumors. Moreover, these tumors also presented higher phosphorylated STAT3, a marker of Wnt signaling (Figure 58).

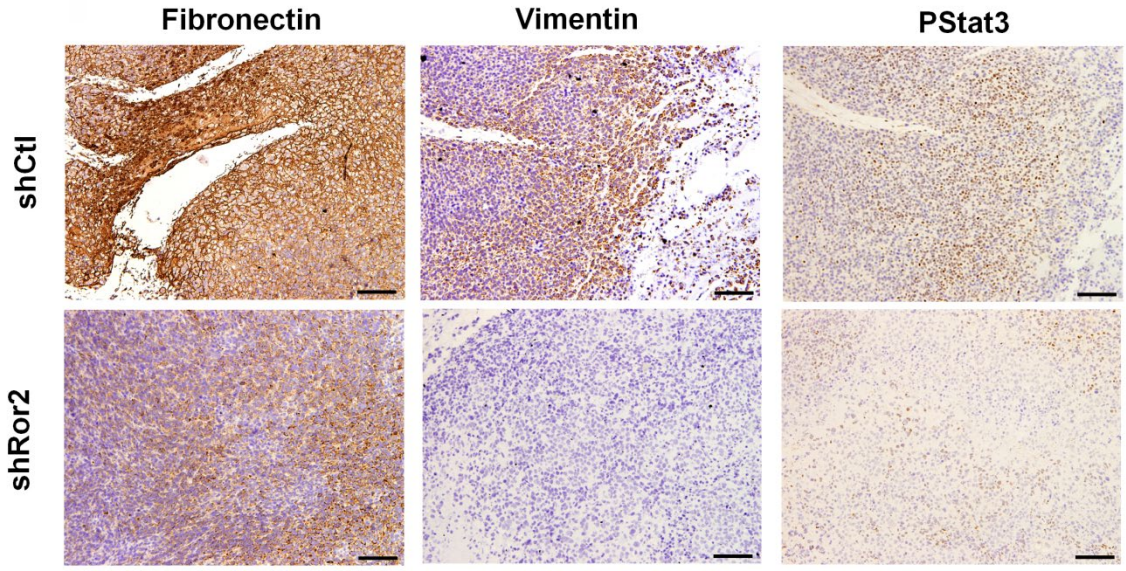

Figure 58: Ror2-depleted tumors show a lower CAF infiltration. Representative images of the tumors generated by shCtl and shRor2 SW620 cells in mice. Fibronectin, Vimentin and pSTAT3 analysis of the tumors.

6. Ror2 – Snail1 axis promotes resistance to cisplatin

Cell resistance to apoptosis is one of the hallmarks of cancer[92] and EMT has been described to play a role in the acquisition of resistance to chemotherapeutic drugs[89]. Interestingly, Snail1 has been related to the acquisition of chemoresistance properties[89,209]. In this part of the study, the resistance of Ror2 knock-down SW620 and HCT116 cells to different chemotherapeutic drugs was assessed, since we have previously determined that Ror2 is a modulator of Snail1 expression.

6.1. Ror2 knock-down decreases cisplatin resistance

Cisplatin and platin-based agents, such as oxaliplatin, are widely studied chemotherapeutic agents used for treatment of numerous types of human cancers, such as colorectal, bladder, ovarian or lung cancers. Cisplatin induces apoptosis by causing DNA damage[210]. In fact, it has been previously proposed that Snail1 expression enhances chemoresistance[115,116]. We wondered whether Ror2 depletion could also affect cisplatin resistance.

To determine the cell sensitivity towards cisplatin, MTT assay was performed treating control and Ror2-depleted SW620 and HCT116 cells with increasing concentrations of cisplatin for 24 hours. As observed in the graph, SW620 and HCT116 Ror2 knock-down cells showed higher sensitivity to cisplatin compared to control cells that were more resistant (Figure 59). This result suggested that Snail1 expression is promoting resistance to cisplatin.

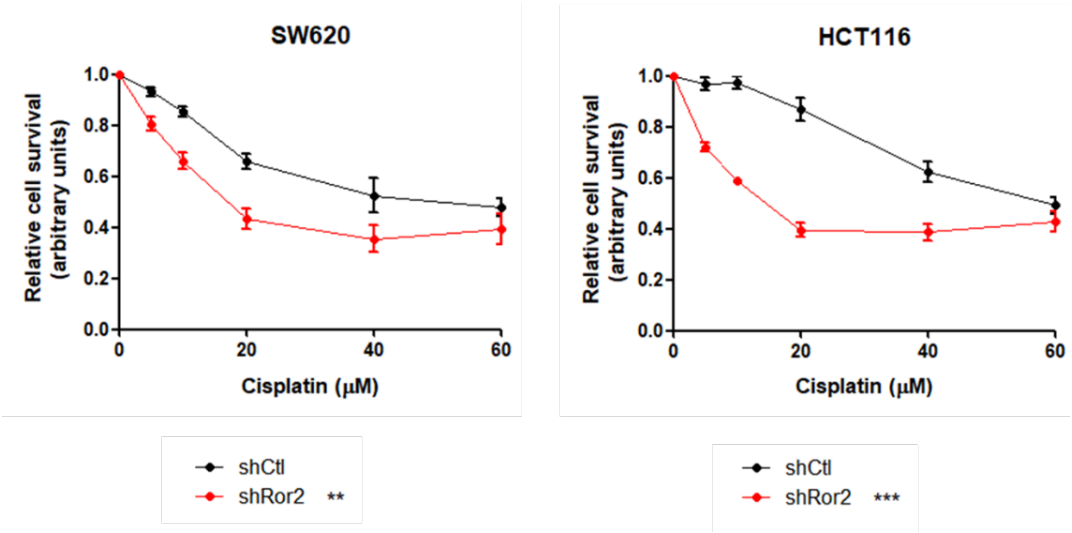

Figure 59: Ror2 modulates cell resistance to cisplatin. Control and Ror2 knock-down SW620 and HCT116 cells were treated with the indicated increasing concentrations of cisplatin for 24 hours. Then, MTT assay was performed and after solubilization, optical density was quantified at 590 nm. Results are represented as the mean of three independent experiments ± SD.

6.2. Snail1 overexpression increases cisplatin resistance in the Ror2-depleted cells

According to the results obtained with the Ror2 knock-down cells, Snail1 expression seems to be very related to cisplatin resistance. To further study that, we overexpressed Snail1 in Ror2-depleted cells and analyzed the sensitivity of these cells to cisplatin. As observed in Figure 60, Snail1 overexpression in Ror2-depleted cells rescued cisplatin resistance.

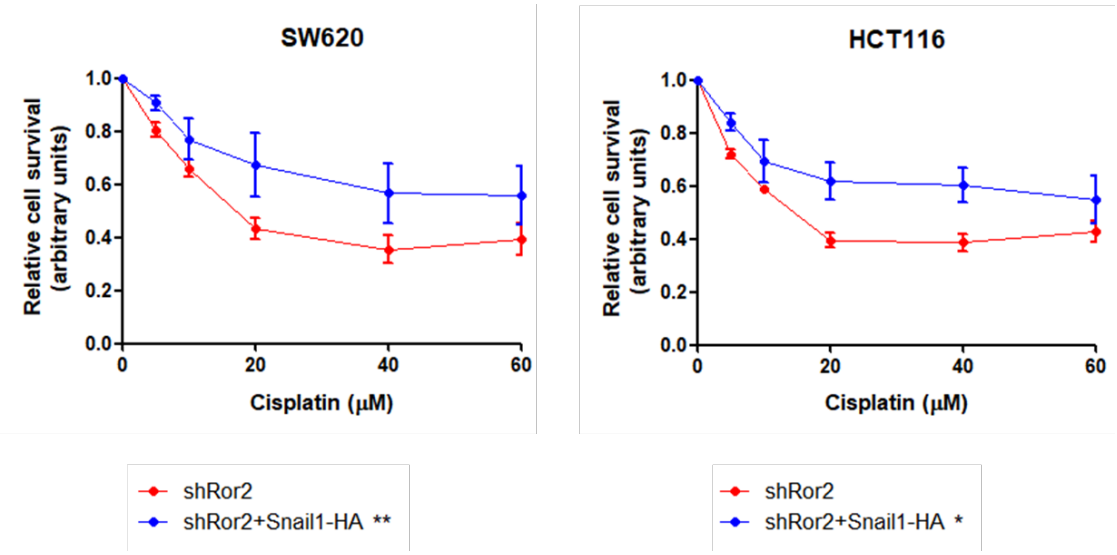

Figure 60: Snail1 overexpression increases Ror2-depleted cells resistance to cisplatin. Ror2 knock-down SW620 and HCT116 cells overexpressing Snail1 or not were treated with the indicated increasing concentrations of cisplatin for 24 hours. Then, MTT assay was performed and after solubilization, optical density was quantified at 590 nm. Results are represented as the mean of three independent experiments ± SD.

In accordance with these last results, a higher resistance to cisplatin was also observed in HT29 M6 cells overexpressing Snail1 compared to control cells (Figure 61). These results confirmed that Snail1 expression directly confers resistance to cisplatin in colorectal cancer cells.

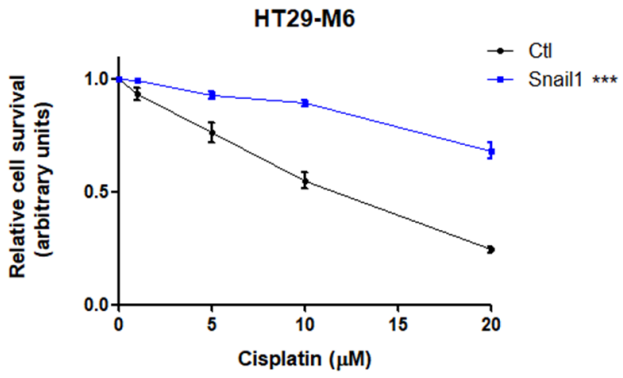

Figure 61: Snail1 overexpression induces resistance to cisplatin. Control and Snail1-HA overexpressing HT29-M6 cells were treated with the indicated increasing concentrations of cisplatin for 24 hours, respectively. Then, MTT assay was

performed. After solubilization, optical density was quantified at 590 nm. Results are represented as the mean of three independent experiments ± SD.

6.3. Cisplatin increases Snail1 protein stability and Snail1 transcription

The higher sensitivity of the Ror2 knock-down cells is clearly due to the low levels of Snail1 in these cells. But not only that, interestingly, upon cisplatin treatment Snail1 protein levels increased in the control cells and this effect does not occur in the Ror2 knock-down cells (Figure 62). pCHK1 was used as a maker of DNA damage generated upon cisplatin.

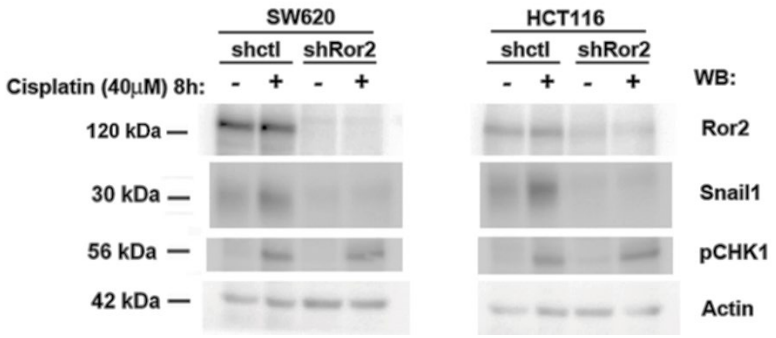

Figure 62: Ror2 knock-down impairs Snail1 increase upon cisplatin. SW620 and HCT116 control and knock-down for Ror2 were treated with 40 μM cisplatin for 8 hours. After treatment, cells were lysed and protein extracts were analyzed by WB using the indicated specific antibodies.

In addition, a time course experiment with cisplatin increased Snail1 protein levels in control cells with a peak at 16 hours, although the increase is maintained until 48 hours (Figure 63). Cisplatin induced a higher increase of cleaved Caspase 3, an apoptosis marker, in the cells depleted of Ror2, while little increase was observed in the control cells (Figure 63). This confirmed that higher Snail1 levels reduces the cisplatin-induced apoptosis.

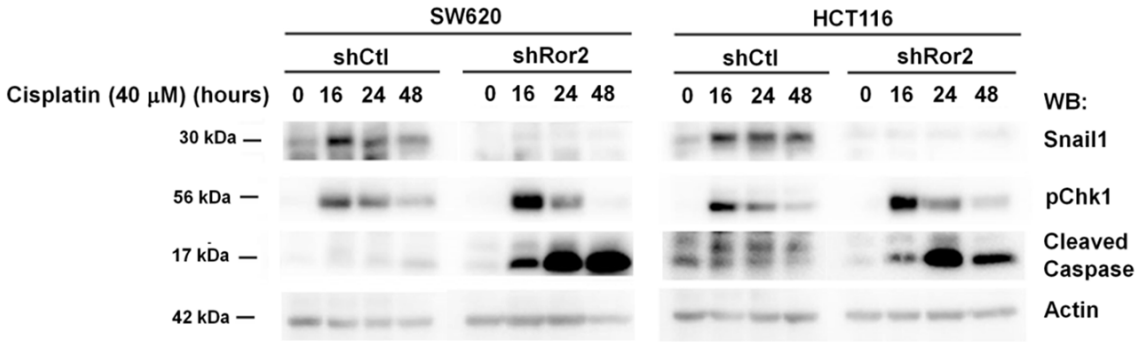

Figure 63: Cisplatin induces higher cell death in Ror2-depleted SW620 and HCT116 cells than in control cells. SW620 and HCT116 control and Ror2-depleted cells were treated with cisplatin at the indicated times. After treatment, cells were lysed and protein extracts were analyzed by WB using the indicated specific antibodies.

Although the increase in Snail1 expression is clear at protein level, we also evaluated Snail1 mRNA expression upon cisplatin treatment in SW620 and HCT116 cells. As observed in the graph, cisplatin increased Snail1 mRNA levels as well (Figure 64).

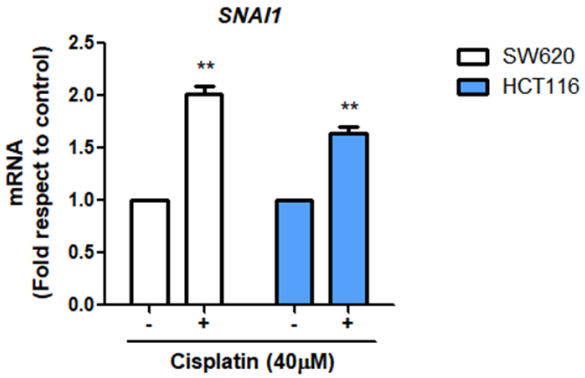

Figure 64: Cisplatin induces higher Snail1 mRNA expression. mRNA was isolated from SW620 and HCT116 cells treated or not with cisplatin for 16 hours. *SNAI1* mRNA was analyzed by RT-qPCR. The mRNA levels are referred to not treated cells levels. Quantification of three different experiments is represented ± SD.

Besides these changes observed in Snail1 mRNA, it has been described that the Snail1 E3 ubiquitin ligase FBXL5 is downregulated in DNA damage conditions, such as doxorubicin or γ-irradiation treatments[109]. This effect leads to Snail1 protein stabilization. Thus, Snail1 protein stabilization was also assessed upon cisplatin treatment. For this purpose, we used control and Snail1 HT29-M6 cells. Cisplatin treatment induced an increase of the ectopic Snail1-HA protein levels, which indicates that cisplatin promotes higher Snail1 protein stability (Figure 65).

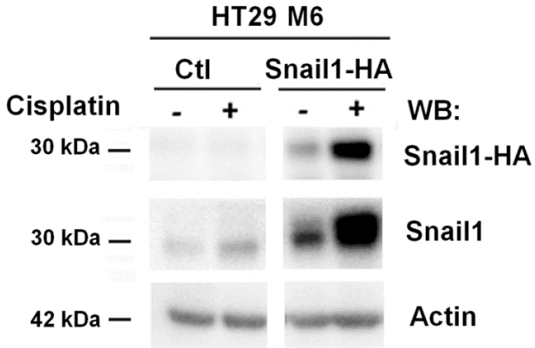

Figure 65: Cisplatin promotes Snail1 protein stabilization. HT29 M6 control and overexpressing Snail1 cells were treated with cisplatin for 16 hours. After treatment, cells were lysed and protein extracts were analyzed by WB using the indicated specific antibodies.

6.4. Ror2 also modulate the response to other DNA-damage agents

As Snail1 is inducing higher resistance to cisplatin in colorectal cancer cells, we wondered if this effect is also promoted by other chemotherapy drugs, such as oxaliplatin and 5-Fluorouracil (5-FU), which are current treatments for colorectal cancer patients[211].

We assessed whether Ror2-depleted cells were also more sensitive to oxaliplatin and 5-FU. As observed in the graph (Figure 66), control HCT116 cells were also more resistant to oxaliplatin and 5-FU than Ror2-depleted cells, as happened with cisplatin. This result suggested that Snail1-induced resistance seems to be a general effect to DNA-damaging drugs.

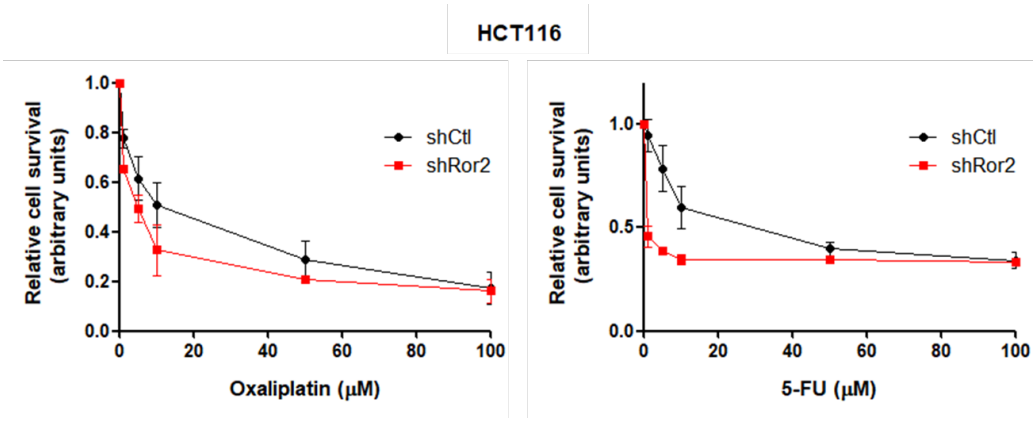

Figure 66: Ror2-depleted HCT116 cells are more sensitive to oxaliplatin and 5-FU than control cells. Control and Ror2 knock-down SW620 and HCT116 cells were treated with the indicated increasing concentrations of oxaliplatin and 5-FU for 24 hours. Then, MTT assay was performed and after solubilization, optical density was quantified at 590 nm. Results are represented as the mean of three independent experiments ± SD.

Moreover, Snail1 protein levels also increased when cells were treated with other DNA-damaging drugs, such as doxorubicin, oxaliplatin and 5-FU (Figure 67). These results confirmed the role of Snail1 in promoting better resistance towards DNA-damaging chemotherapy drugs, such as cisplatin, oxaliplatin, 5-FU and doxorubicin.

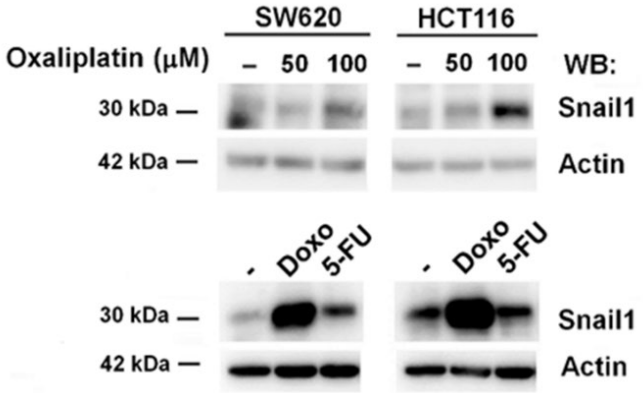

Figure 67: DNA-damaging drugs induce Snail1 increase. SW620 and HCT116 cells were treated with doxorubicin, 5-FU and oxaliplatin for 16 hours. After treatment, cells were lysed and protein extracts were analyzed by WB using the indicated specific antibodies.

6.5. Inhibition of Wnt secretion decreases cisplatin resistance

After finding that Snail1 expression promoted higher resistance of colorectal cancer cells to cisplatin, we wondered how this effect could be reverted. As confirmed in previous results, the non-canonical Wnt pathway is controlling Snail1 expression through Wnt5a and Ror2 and when the signaling was blocked with LGK inhibitor, the expression of Snail1 decreased considerably.

For this reason, the sensitivity of control SW620 and HCT116 cells to cisplatin and LGK co-treatment was assessed in a MTT assay. Addition of LGK increased the toxicity of cisplatin. Wnt inhibition by LGK reversed the Snail1 protective effect to a similar level than Ror2 depletion (Figure 68).

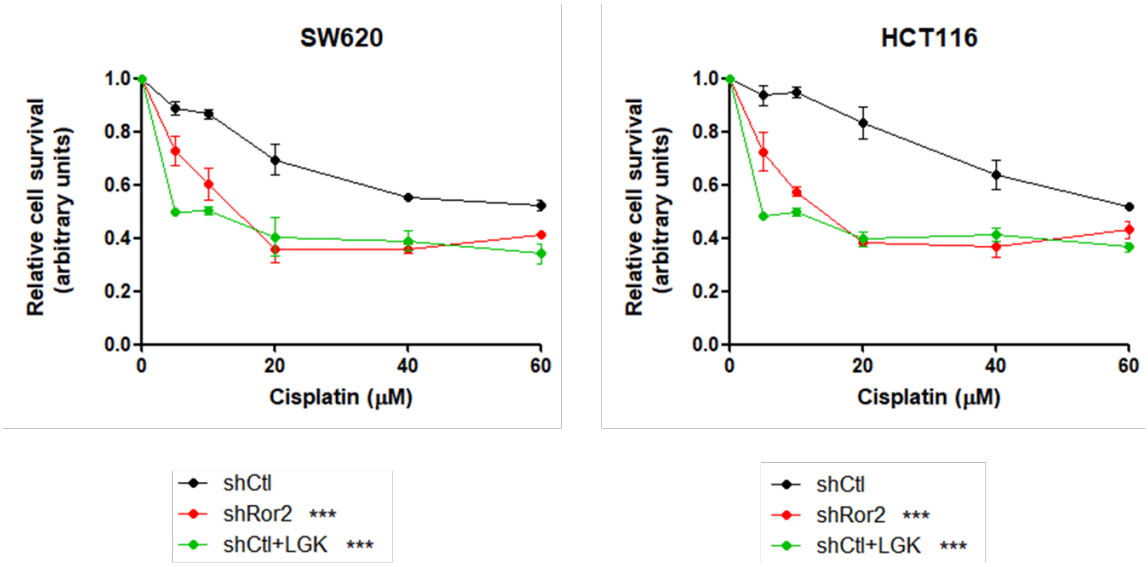

Figure 68: LGK enhances cisplatin toxicity. SW620 and HCT116 control, co-treated or not with LGK974 10µM, and Ror2-depleted cells were treated with the indicated increasing concentrations of cisplatin for 24 hours. Then, MTT assay was performed and after solubilization, optical density was quantified at 590 nm. Results are represented as the mean of three independent experiments ± SD.

6.6. Non-canonical Wnt pathway controls Snail1 upregulation upon cisplatin

Since LGK is preventing the protective effect of Snail1 to cisplatin, we wanted to study this effect at molecular level. LGK co-treatment blocked the Snail1 increase in response to cisplatin (Figure 69). This result suggested that Wnt signaling is required for Snail1 upregulation upon cisplatin, because when Wnt signaling is blocked, the expression of Snail1 is maintained at very low levels and cisplatin cannot induce Snail1 increase.

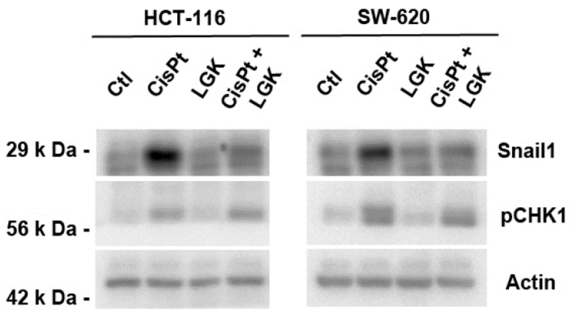

Figure 69: LGK974 prevents Snail1 upregulation upon cisplatin. SW620 and HCT116 cells were treated with 40 µM cisplatin, LGK974 10µM or the combination of both for 16 hours. After treatment, cells were lysed and protein extracts were analyzed by WB using the indicated specific antibodies.

Then, as we have previously demonstrated that Snail1 expression is controlled by non-canonical Wnt, we wanted to elucidate whether it is also regulating the cisplatin-induced increase of Snail1. As observed in Figure 70A, addition of recombinant Wnt5a reversed the effect of LGK of blocking cisplatin-induced Snail1 upregulation. Moreover, when recombinant DKK1, which is a specific inhibitor of the canonical Wnt pathway, was added together with cisplatin, no effect was observed and Snail1 levels increased in response to cisplatin (Figure 70A). Same results were obtained with another Porcupine inhibitor, the WntC59[212]. Similar than LGK, WntC59 also blocked the cisplatin-induced Snail1 upregulation and this effect was rescued by addition of recombinant Wnt5a (Figure 70B).

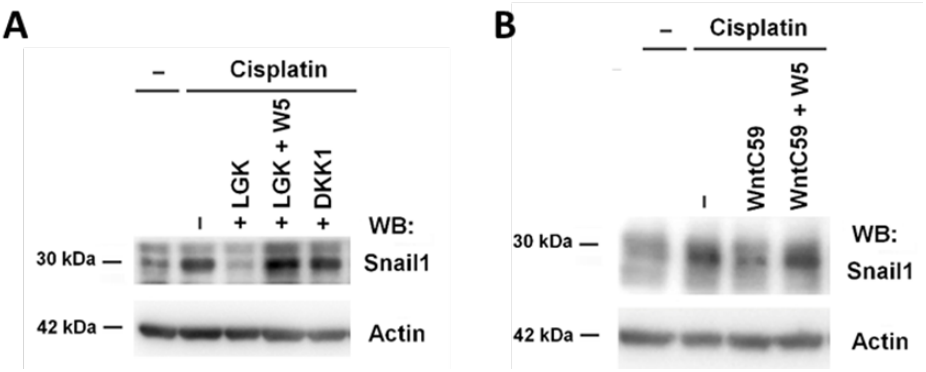

Figure 70: Non-canonical Wnt is required for cisplatin-induced Snail1 upregulation. A) SW620 and HCT116 cells were treated with the indicated treatments (40 µM cisplatin and LGK974 10µM) for 16 hours. B) SW620 and HCT116 cells were treated with the indicated treatments (40 µM cisplatin and WntC59 100nM) for 16 hours. A-B) After treatment, cells were lysed and protein extracts were analyzed by WB using the indicated specific antibodies.

Moreover, this effect was confirmed in other colorectal cancer cell lines. Similar results were obtained in LoVo, DLD1 and CaCo2 cells. In these cells, cisplatin increased the levels of Snail1 and

LGK prevented this increase (Figure 71). Together, these results confirmed that the non-canonical Wnt pathway is required for cisplatin-induced Snail1 upregulation in colorectal cancer cells.

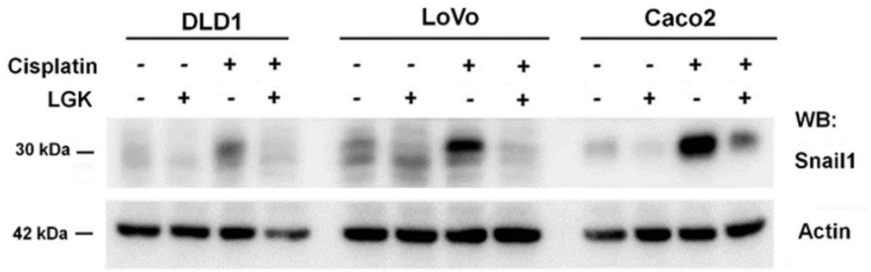

Figure 71: LGK974 prevents cisplatin-induced Snail1 increase in other colorectal cancer cells. DLD1, LoVo and CaCo2 cells were treated with 40 µM cisplatin, LGK974 10µM or the combination of both for 16 hours. After treatment, cells were lysed and protein extracts were analyzed by WB using the indicated specific antibodies.

6.7. Wnt inhibition blocks cisplatin-induced Snail1 increase in Mouse Tumor Organoids

To extend our findings to a more physiologic model, we used cells derived from tumors generated in mice with APC, Kras, TGF-β receptor 2 and p53 mutations (LAKTP mice)[213]. These murine cells when grown in Matrigel reorganize themselves to generate organoids. Therefore, we used these mouse derived tumor organoids (MTOs) to confirm our previous results *in vitro*.

MTOs were treated with cisplatin and Snail1 levels were analyzed. As expected, cisplatin increased Snail1 protein levels also in MTOs (Figure 72). Moreover, both, LGK and WntC59 co-treatment blocked the cisplatin-induced Snail1 upregulation (Figure 72). STAT3 phoshorylation was also blocked by Wnt inhibitors.

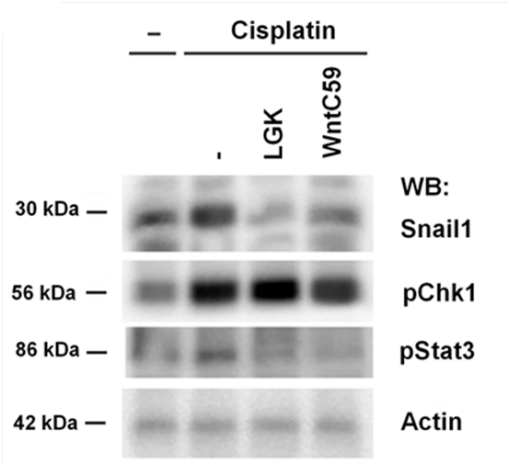

Figure 72: LGK974 and WntC59 block cisplatin-induced Snail1 increase in MTOs. MTOs grown in Matrigel were treated with 40 µM cisplatin, WntC59 100nM or the combination of both for 16 hours. After treatment, cells were lysed and protein extracts were analyzed by WB using the indicated specific antibodies.

With this result, we have corroborated that cisplatin also induced Snail1 in MTOs and inhibition of Wnt signaling prevented this upregulation as happened in the colorectal SW620 and HCT116 cell lines.

6.8. Wnt inhibition reduces MTOs increases sensitivity to cisplatin

We have previously observed that Wnt inhibition with LGK increases cisplatin sensitivity in SW620 and HCT116 cells and as cisplatin-induced Snail1 upregulation was impaired by LGK and WntC59 in MTOs, the sensitivity to cisplatin was analyzed. For this experiment, we used higher concentrations of cisplatin and let the treatments for 72 hours. In accordance to the results obtained from Ror2-depleted cells, Wnt inhibition by both, LGK and WntC59, augmented MTOs sensitivity to cisplatin (Figure 73).

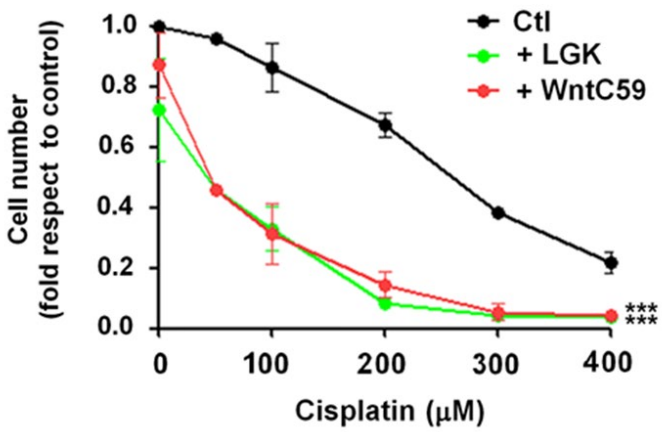

Figure 73: LGK974 and WntC59 induced higher sensitivity to cisplatin in MTOs. MTOs were treated with increasing concentrations of cisplatin alone or in combination with LGK974 (10µM) or WntC59 (100nM). After 72 hours of treatment cell viability was quantified using CellTiter-Glo. Results are represented as the mean of three independent experiments ± SD.

7. Paclitaxel and other microtubule-affecting drugs promote a Snail1-modification that prevents Snail1-induced resistance

7.1. Snail1 does not promote higher resistance to paclitaxel

Paclitaxel is another drug commonly used in different types of cancers, such as ovarian, lung or breast cancer. Paclitaxel is a microtubule-stabilizing drug that induces mitotic arrest leading to cell death[214]. It has been proposed that Snail1 promotes resistance to paclitaxel in ovarian cancer[159]. As happened before with cisplatin, we speculated that probably the Ror2-depleted cells would also show less resistance to paclitaxel.

In order to measure the cell resistance to paclitaxel, an MTT assay was performed. In this case, the Ror2 knock-down SW620 and HCT116 cells were treated with increasing concentrations of paclitaxel for 24 hours. Interestingly, no differences were observed in the resistance to paclitaxel when comparing the Ror2 knock-down and control cells (Figure 74).

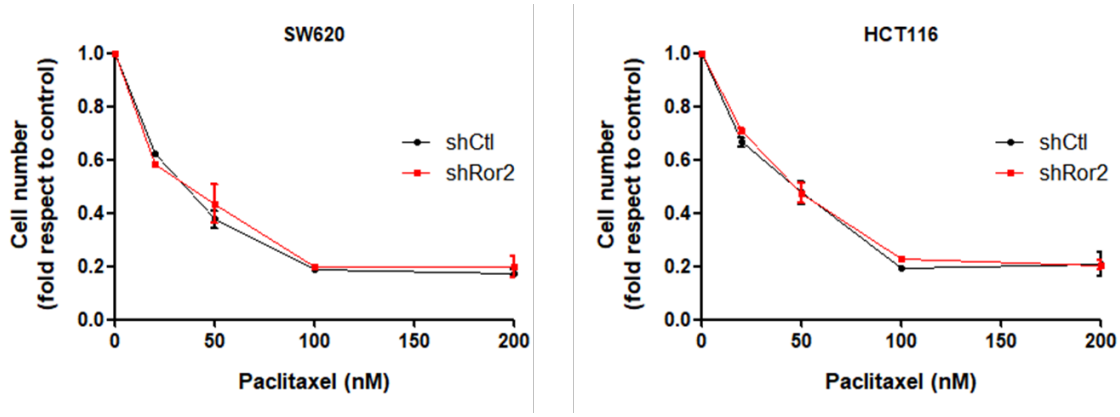

Figure 74: Ror2 expression does not affect resistance to paclitaxel. Control and Ror2 knock-down SW620 and HCT116 cells were treated with the indicated increasing concentrations of paclitaxel for 24 hours. Then, MTT assay was performed and after solubilization, optical density was quantified at 590 nm. Results are represented as the mean of three independent experiments ± SD.

According to this result, when HT29 M6 cells overexpressing Snail1 or not, were challenged to paclitaxel no differences in cell survival were observed (Figure 75). Therefore, Snail1 expression is not inducing any change in the sensitivity of colorectal cancer cells to paclitaxel.

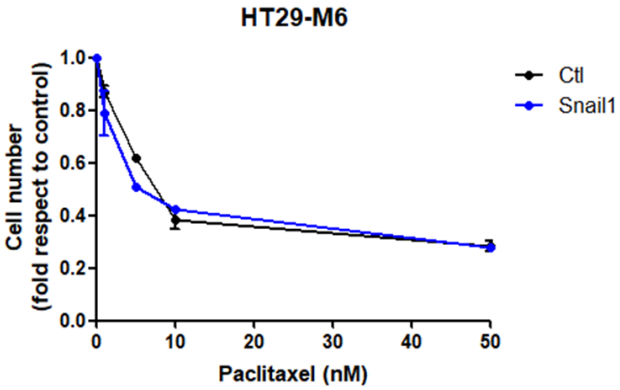

Figure 75: Snail1 overexpression does not induce higher resistance to paclitaxel. Control and Snail1 overexpressing HT29-M6 cells were treated with the indicated increasing concentrations of paclitaxel for 24 hours, respectively. Then, MTT assay was performed. After solubilization, optical density was quantified at 590 nm. Results are represented as the mean of three independent experiments ± SD.

7.2. Paclitaxel induces an increase in Snail1 expression

In contrast to cisplatin, Ror2 knock-down cells showed similar resistance to paclitaxel than control cells. However, Snail1 protein levels also increased upon paclitaxel treatment in the control cells, while less is observed in the Ror2-depleted cells due to its low Snail1 expression (Figure 76).

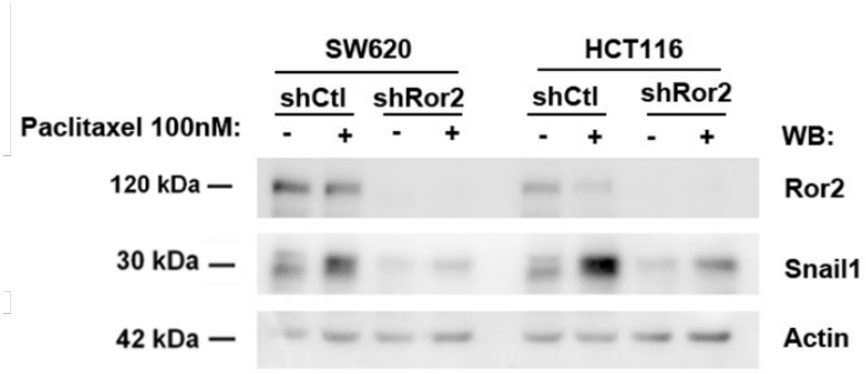

Figure 76: Paclitaxel induces an increase on Snail1 protein levels. SW620 and HCT116 control and knock-down for Ror2 were treated with 100 nM paclitaxel for 16 hours. After treatment, cells were lysed and protein extracts were analyzed by WB using the indicated specific antibodies.

Since paclitaxel induced similar molecular effects on Snail1 protein levels than cisplatin, we investigated whether it also induced higher Snail1 mRNA expression. As shown in the graph, paclitaxel also induced Snail1 mRNA increase (Figure 77). Therefore, although cisplatin and paclitaxel were inducing higher Snail1 expression, Snail1 was only promoting better resistance to cisplatin.

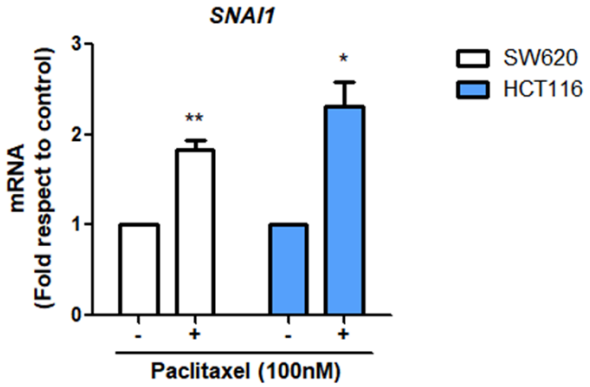

Figure 77: Paclitaxel induces higher Snail1 mRNA expression. mRNA was isolated from SW620 and HCT116 cells treated or not with paclitaxel for 16 hours. *SNAI1* mRNA was analyzed by RT-qPCR. The mRNA levels are referred to not treated cells levels. Quantification of three different experiments is represented ± SD.

7.3. Microtubule-affecting drugs induce Snail1 phosphorylation

Cisplatin and paclitaxel induced similar effects on Snail1 expression, although only Snail1-expressing cells showed better resistance to cisplatin. We questioned the reason of this differential effect from a molecular point of view.

When comparing Snail1 protein levels upon cisplatin and paclitaxel treatment in SW620 and HCT116 cells, we realized that in paclitaxel-treated cells, Snail1 showed a shift in the molecular weight of the band in the WB, which could correspond to phosphorylation (Figure 78).

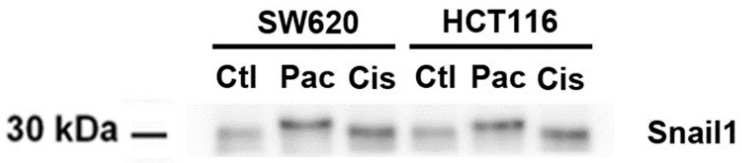

Figure 78: Paclitaxel induces a change on Snail1 protein molecular weight. SW620 and HCT116 cells were treated with 40 µM cisplatin and 100 nM paclitaxel for 16 hours. Cells were lysed and protein extracts were analyzed by WB using the Snail1 antibody.

It is described that Snail1 can suffer several post-translational modifications, including phosphorylation, which modify its function, activating or inhibiting it[107] (see introduction Figure 14). In order to investigate whether Snail1 is phosphorylated when the cells are treated with paclitaxel, a dephosphorylation assay was performed. SW620 and HCT116 wildtype cells were treated with paclitaxel for 16 hours and the cell extracts were divided in 3 different conditions. In one condition, calf intestinal phosphatase (CIP) was added in order to dephosphorylate. As shown in Figure 79, paclitaxel-induced shift in Snail1 molecular weight was abrogated in the CIP-treated condition. A

general anti-pTyr was used as a control for unspecific dephosphorylation (Figure 79). This result confirmed that Snail1 is phosphorylated upon paclitaxel treatment.

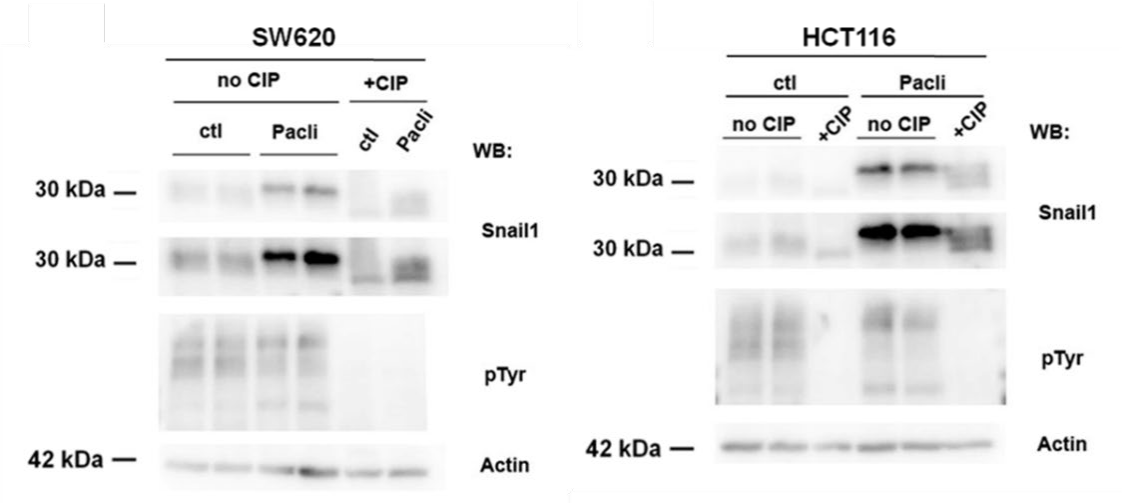

Figure 79: Paclitaxel induces Snail1 phosphorylation. SW620 and HCT116 cells were treated with 100 nM paclitaxel for 16 hours. Cells were lysed and cell extracts were used for a dephosphorylation assay with CIP. After incubating 1 hour at 37°C protein extracts were analyzed by WB with the indicated antibodies.

It is important to remark that Snail1 phosphorylation was only observed in paclitaxel treatment but not in cisplatin treatment. We asked then, if Snail1 phosphorylation is just specific for paclitaxel or if it is a general effect of the microtubule-affecting drugs. To assess this issue, SW620 and HCT116 cells were treated with nocodazole[215], vinblastine[216] and colcemid[217], which, as paclitaxel, are microtubule-affecting drugs used to treat some types of cancers[218]. After a 16 hours treatment, Snail1 protein levels were analyzed by WB. Nocodazole, vinblastine and colcemid also induced Snail1 phosphorylation (Figure 80), suggesting that this is a general effect for the microtubule-affecting drugs.

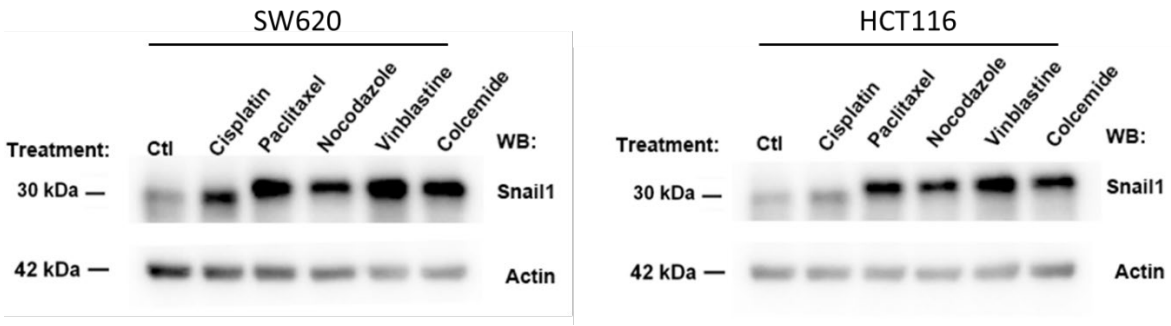

Figure 80: Microtubule-affecting drugs induce Snail1 phosphorylation. SW620 and HCT116 cells were treated with 40 µM cisplatin, 500 nM paclitaxel, 500 nM nocodazole, 500 nM vinblastine and 500 nM colcemid for 16 hours. Cells were lysed and protein extracts were analyzed by WB using the specific antibodies.

8. Non-canonical Wnt signaling factors correlate with Snail1 in human colorectal tumors

8.1. Non-canonical Wnt factors, but not canonical ones, correlate with Snail1 in colorectal tumors

In addition to the positive correlation between Ror2 and Snail1 expression in human colorectal cancers previously presented, other interesting correlations were observed in the TCGA database. For instance, a positive correlation is observed between Snail1, Wnt5a and TGF-β (Figure 80). As we have reported, Snail1 is a target gene of the non-canonical Wnt pathway. Therefore, Snail1 expression positively correlates with other Wnt5a target genes, such as MMP9 or MMP13, while a negative correlation is observed between Snail1 and β-catenin or Axin2, classical components of the canonical Wnt pathway (Figure 81).

Moreover, expression of other members of the non-canonical Wnt pathway also correlated. For instance, the expression of Fz2 positively correlates with Snail1, Ror2 and Wnt5a expression (Figure 81). Together, these results highlighted the importance of the non-canonical Wnt pathway in the regulation of Snail1 expression and provided strong evidences that Wnt5a, Ror2, Fz2 and Snail1 participate in a common axis in colorectal tumors.

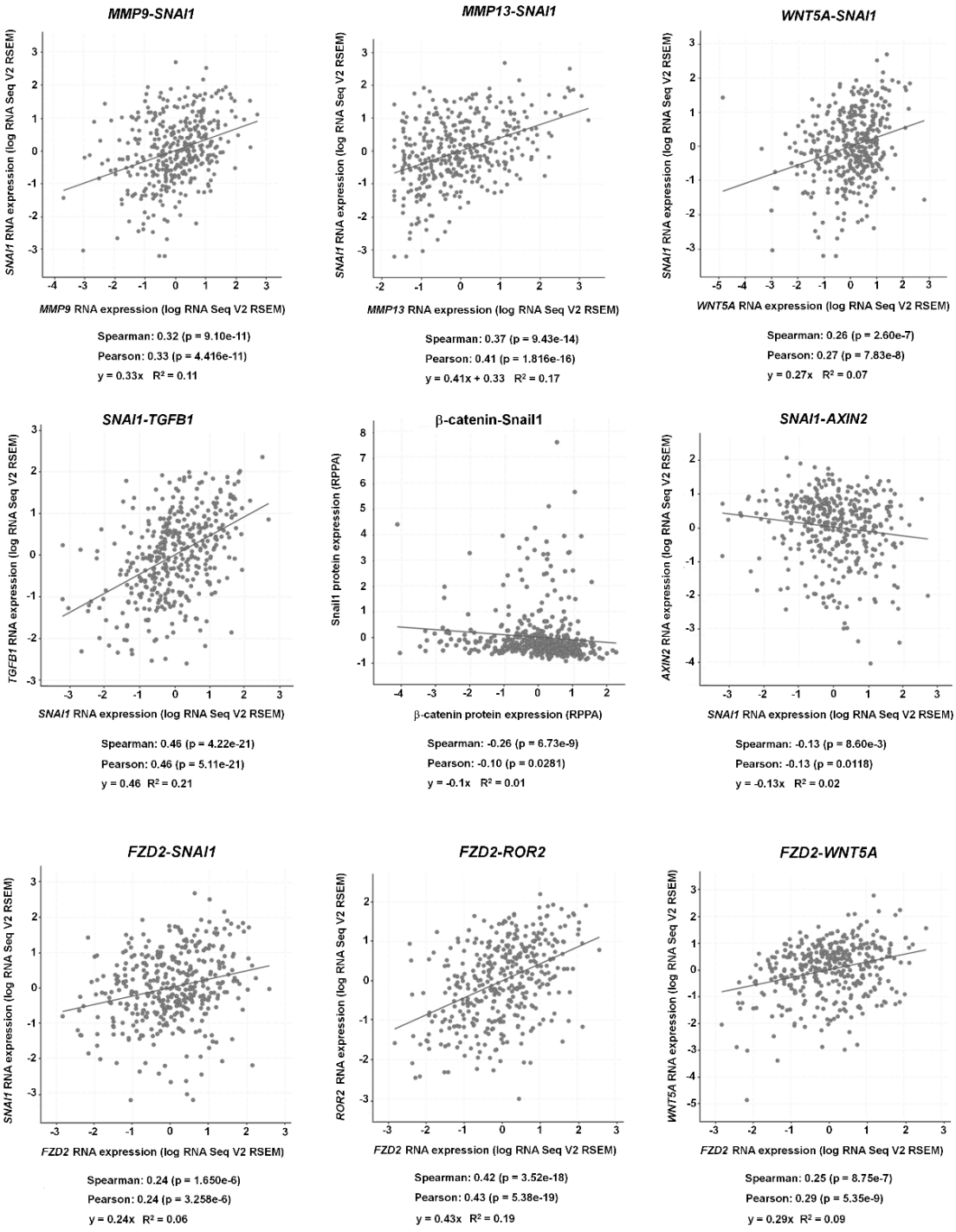

Figure 81: Expression of Snail1, Ror2, Fz2 and Wnt5a correlates with non-canonical genes. The correlation between the RNA expression (log RNA seq V2 RSEM) from *SNAI1*, *ROR2*, *FZD2*, *MMP9*, *MMP13*, *WNT5A*, *AXIN2* and *TGFB1* is represented. Correlation between Snail1 and β-catenin protein expression is also represented. Data was obtained from the TCGA Colorectal Adenocarcinoma Firehose Legacy database and analyzed in the cBioPortal.

8.2. Wnt5a and Ror2 correlate with Snail1 in other human cancers

The expression of Wnt5a, Ror2 and Snail1 was also investigated in the TCGA database of other human tumors, apart from colorectal. The correlation between Wnt5a and Ror2 with Snail1 expression was compared to the correlation between Snail1 and the canonical Wnt3a and LRP5. As observed in Figure 82, a better correlation is observed between Snail1 and the non-canonical Wnt5a and Ror2 than with the canonical Wnt3a and LRP5 in most of the different human cancer types that were studied. This result further corroborates that the non-canonical Wnt pathway is regulating Snail1, but not only in colorectal cancer; it seems that this regulation is also taking place in other type of cancers.

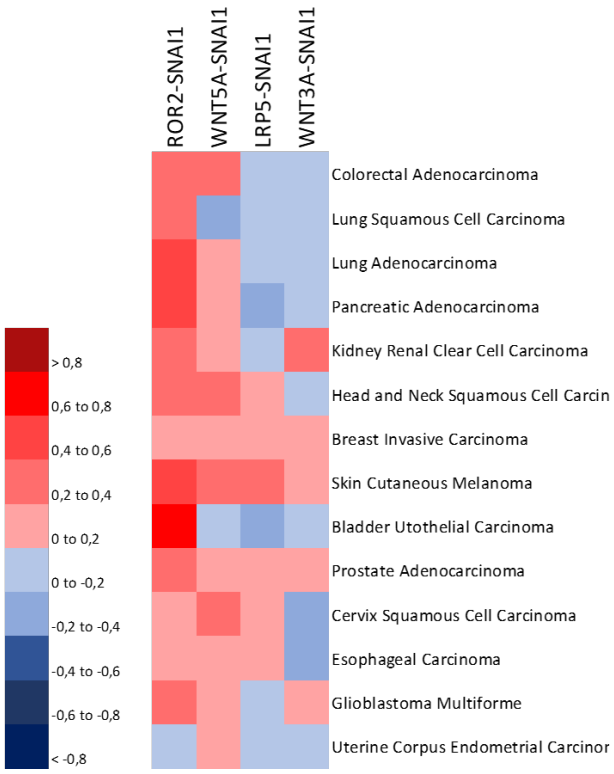

Figure 82: Wnt5a/Ror2 and not Wnt3a/LRP5 correlate with Snail1 in human cancers. The correlation between the RNA expression (log RNA seq V2 RSEM) of *SNAI1* and *ROR2-WNT5A*, or *LRP5-WNT3A* is represented in the heatmap. Spearman coefficient was represented in a color scale. Data was obtained from the TCGA Colorectal Adenocarcinoma Firehose Legacy database and analyzed in the cBioPortal.

DISCUSSION

1. Ror2 controls Snail1 expression in colon tumor cells

Snail1 is a transcriptional factor with a key role during EMT as it directly represses E-Cadherin expression and other epithelial genes and induces the expression of several mesenchymal genes[219,159]. The regulation of Snail1 expression has been widely studied. Different factors have been implicated in *SNAI1* transcription, such as SMADs, STAT3, NFκB or different MAPK pathway downstream effectors, among others[107].

TGF-β signaling pathway has been considered the classical regulator of Snail1 expression. Different studies have reported that TGF-β upregulates Snail1 mainly in fibroblasts, but also in tumoral cells[104,191,108]. However, in colorectal cancer, some of the effectors of the TGF-β signaling pathway are generally mutated, which makes these cells insensitive to TGF-β. Mutations in the TGF-β receptor and SMADs proteins have been found frequently in colorectal tumors[197,198,199]. Particularly, SW620 cells have mutation in the SMAD4 gene, while HCT116 cells present mutations in TGF-β receptor II (TGFβRII) lacking its expression[220,221]. Since HCT116 cells have mutated TGFβRII, the decrease in SMAD2 phosphorylation observed upon SB treatment was surprising. However, basal phosphorylation of SMAD2 has been previously observed in HCT116 cells, being also increased upon TGF-β treatment[222], suggesting that other mechanisms may be regulating it. Despite of that, inhibition of TGF-β receptor with SB in SW620 and HCT116 cells did not change Snail1 expression. Therefore, Snail1 expression is not controlled by TGF-β in colon cancer cells.

Other factors that have been described to control Snail1 expression are Wnt ligands. Canonical and non-canonical Wnts regulate Snail1 expression in fibroblasts through a common signaling pathway involving STAT3[81]. In colon cancer cells, Snail1 expression is affected by Porcupine inhibitors that blocks both, canonical and non-canonical Wnt signaling. However, specific inhibition of the non-canonical Wnt pathway by depletion of the co-receptor Ror2 shows that Snail1 expression is controlled by the non-canonical Wnt pathway through the receptor Fz2 and Ror2, since knock-down or Ror2 strongly affected the expression of Snail1. Analyzing data from the TCGA database revealed that in human colorectal tumors the expression of Ror2 correlates with the expression of Snail1. Moreover, the expression of the non-canonical Wnt factor Wnt5a also correlates with Snail1, not only in colorectal, also in other type of tumors, while no correlation, or less, is observed between the canonical Wnt3a or the canonical co-receptor LRP5/6 and Snail1.

Interestingly, depletion of Ror2 in colon cancer cells seems to induce a MET process. This process leads to an increase in epithelial markers such as E-Cadherin, whereas mesenchymal markers decrease probably as a consequence of the downregulation of Snail1. Remarkably, in breast cancer

cells, Ror2 downregulation also increased E-Cadherin expression and reduced mesenchymal genes expression, such as Snail1 or Vimentin, and the contrary effect was observed when Ror2 was overexpressed[140]. This confirms that Ror2 is related with EMT not only in colon, but also in other cancers, such as breast cancer.

How the non-canonical Wnt pathway is controlling Snail1 expression through Ror2 was also studied. The increase in *SNAI1* mRNA levels due to Wnt stimulation has been previously described and it has been attributed to STAT3 activation that is common for canonical and non-canonical Wnts[81,79]. However, analysis of the *SNAI1* promoter region showed STAT3 putative binding motifs, but also for AP1, which is a downstream effector of JNK2[223]. Therefore, since inhibition of JNK2 and STAT3 decreases the SNAI1 promoter activity, the non-canonical Wnt signaling is controlling *SNAI1* transcription through JNK2 and STAT3 activation in a Ror2-dependent manner (Figure 83). However, no differences were observed in promoter activity between the two fragments in both cell lines and inhibition of JNK2 or STAT3 was sufficient for decreasing promoter activity to the same level in the long fragment. This suggests that, probably this assay is not the best to know what is really happening *in vivo*, but is useful to analyze *in vitro* how Snail1 transcription is controlled. ChIP experiments should be done to finally confirm that both AP1 and STAT3 are in fact directly binding to the *SNAI1* promoter region.

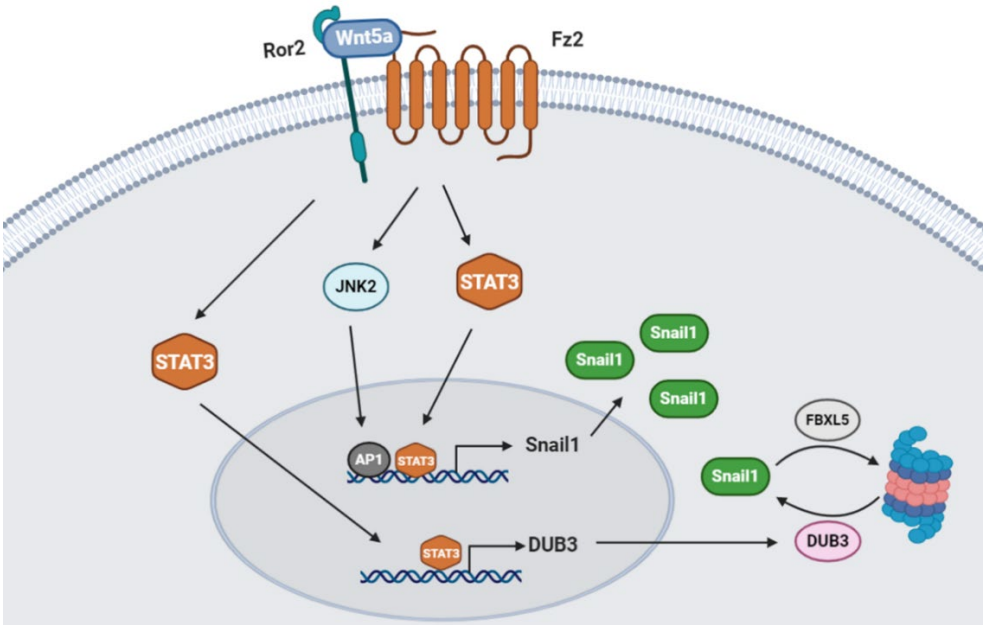

Figure 83: The non-canonical Wnt pathway controls SNAI1 transcription and Snail1 protein stability. Upon Wnt5a binding to Ror2 and Fz2, JNK 2 and STAT3 are phosphorylated and activated. Then, in the nucleus AP1 and STAT3 promote

the transcription of SNAI1. Wnt5a, though STAT3 activation, also promotes the expression of DUB3, which induces higher Snail1 protein stability.

Another important step in Snail1 regulation is the regulation of its protein stability. Snail1 protein stability is specifically regulated by different E3 ubiquitin ligases that target Snail1 for proteasomal degradation and other enzymes called DUBs that remove ubiquitin to promote Snail1 stabilization. Snail1 protein stability is also affected by Ror2 depletion. This is caused by a decrease in the expression of a specific DUB, DUB3, that is down-regulated upon knock-down of Ror2 and, more importantly, that is upregulated upon Wnt5a stimulation in a STAT3-dependent manner, as it has been described before[86]. Whether Ror2 is controlling the expression of the Snail1 E3 ubiquitin ligases is not clear, although a significant increase in FBXL5 expression was observed in Ror2-depleted SW620 cells.

Although Snail1 expression can be controlled at multiple levels by many different mechanisms in different cell types, we have demonstrated that in colon cancer cells it is regulated by the non-canonical Wnt pathway in a Ror2-dependent manner. This regulation seems to affect mainly transcription and protein stabilization, although it cannot be discarded that other levels of regulation could be also affected. Moreover, the contribution of other signaling pathways, such as canonical Wnt pathway or TGF-β pathway, may be also playing a role in controlling Snail1 expression and cannot be discarded in a low percentage of colorectal tumors in which no mutations are found in these pathways. For instance, the colon cancer cell lines that were mostly used in this work, SW620 and HCT116 cells, presented mutations in canonical Wnt pathway elements. SW620 cell line has mutations in the *APC* gene, while HCT116 cell line presents a heterozygous mutation consisting of three base deletion in the *CTNNB1* gene in the region in which the protein is phosphorylated by GSK3β[224]. Thus, in both cell lines β-catenin transcriptional activity is high, although not maximal, since inhibition of Porcupine with LGK decreases the expression of several Wnt3a target genes.

We have centered our study in the contribution of Ror2 in the regulation of Snail1 in cells with elevated expression of Ror2, because these cells present also higher levels of Snail1. However, there is a cell line, DLD1, that express medium levels of Snail1 while no expression of Ror2 was observed. In this case, DLD1 express high levels of Ror1, another co-receptor of the non-canonical Wnt pathway. All our results indicate that the non-canonical Wnt pathway is controlling Snail1 expression in a Ror2-dependent manner in colon cancer cells.

2. Ror2/Snail1 define a positive loop of autoactivation

Besides impairing Snail1 expression, blockade of the non-canonical by Ror2 knock-down and Porcupine inhibition prevented the secretion of Wnt5a, suggesting that Wnt5a is a target gene of itself. This decrease in the production of Wnt5a affects not only the capacity to activate the pathway in an autocrine manner, but also the ability to activate other cells in a paracrine manner. The initial hypothesis was that Wnt5a is promoting an autoactivation loop in which an initial Wnt5a signal, through Ror2, promotes the activation of more Wnt5a production that spread the signal and maintains the higher expression of Snail1. This idea was confirmed since treating colon cancer cells with exogenous Wnt5a, as expected it upregulates Snail1, but also induces an increase in the expression of Wnt5a.

Apart from Snail1 and itself, Wnt5a signaling also modulates the expression of two other very important proteins that participate in the pathway as Fz2 and Ror2: inhibition of the non-canonical Wnt pathway with LGK decreased the expression of both. Similarly, in the Ror2-deleted cells the expression of Fz2 was also dramatically affected. Accordingly, Wnt5a stimulation induces the expression of Fz2 and Ror2. Therefore, activation of this pathway self-amplifies its response increasing the levels of the ligand, receptor and co-receptor that initiate it (Figure 84).

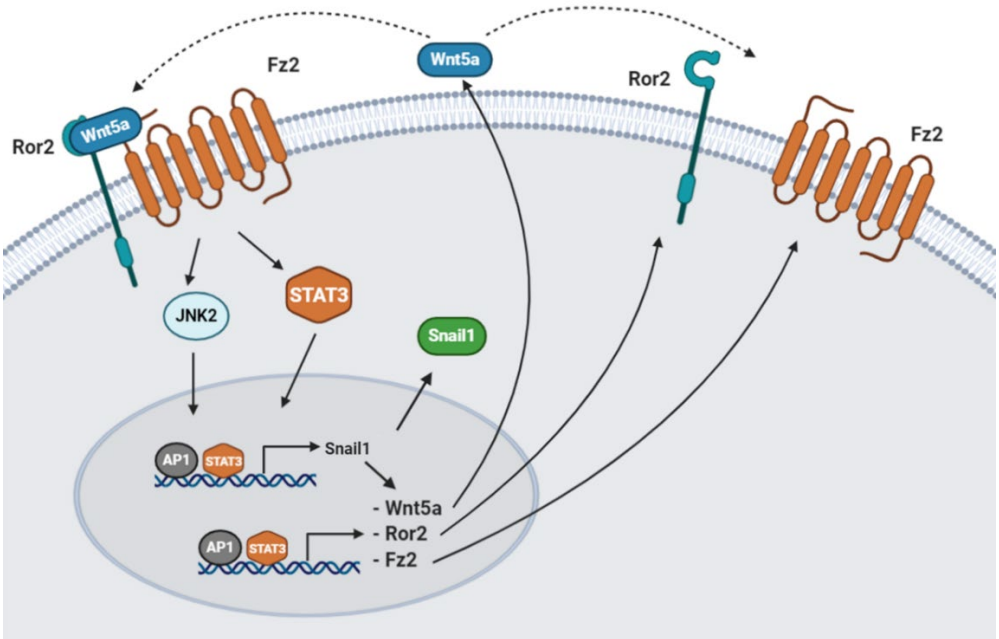

Figure 84: Wnt5a/Ror2/Snail1 define a loop of autoactivation. Upon stimulation of the pathway by Wnt5a binding to the receptors, Snail1 is expressed, but it also promotes the expression of Wnt5a, Ror2 and Fz2. Therefore, all these factors that participate in the activation of the pathway can self-perpetuate the signaling.

Importantly, Fz2 has been previously related to EMT in different studies[79,205]. In a recent study, the authors have demonstrated that Fz2 correlated with an EMT signature in patients with poor survival from different type of cancers. In the same study, it is also possible to see that Fz7, another receptor of the Fz family, also correlates with the EMT signature in colon cancer[205]. Interestingly, Fz2 and Fz7, together with Fz1, are the only Fz members that present the Tyr552 in the C-terminus. The phosphorylation of this Tyr552 by Src is required for Fyn binding to Fz, activation of STAT3 and, thus, for the expression of Snail1[81,79]. In our analysis, Fz2 also correlates with Snail1, Ror2 and Wnt5a in human colorectal tumors, giving a robust confirmation that all these proteins are implicated in the signaling.

Another important effect that we have observed is that the overexpression of Ror2 and Snail1 is sufficient to activate the self-stimulatory loop. Ror2 and Snail1 overexpression induces an increase in Wnt5a that, probably, activates the pathway and, later, induces the upregulation of Ror2 and Fz2. Snail1 overexpression also increases other mesenchymal markers, such as Fibronectin and αSMA that are sensitive to Snail1 depletion. However, Ror2 and Fz2 expression is not sensitive to Snail1 depletion, suggesting that their induction by Snail1 is more indirect that the one involving Fibronectin or αSMA.

Together, our findings have demonstrated that Snail1 expression is controlled by the non-canonical Wnt pathway in a Ror2-dependent manner in colorectal cancer cells. Moreover, a self-stimulatory loop involving Wnt5a, Ror2 and Snail1 that is associated to EMT have been revealed.

3. Ror2 is required for tumor progression, metastasis and fibroblast activation

Ror2 and the non-canonical Wnt pathway have been implicated in many types of cancers and correlate with poor overall survival of the patients[225,226]. For instance, in gastric cancer the expression of non-canonical Wnt proteins correlated with poor survival[227]. Moreover, in general, the expression of Ror2 correlates with a worse prognosis in colorectal cancer[226,228]. In accordance to that, our results clearly demonstrated that Ror2 is required for tumor growth, since tumors derived from Ror2 knock-down cells appeared later and grew more slowly than controls.

When proliferation was analyzed *in vitro* in normal culture conditions (DMEM 10% FBS) no differences were observed between control and Ror2-depleted cells. However, when it was assessed reducing the FBS percentage at 1%, statistically significant differences were observed. In this condition, Ror2 depletion reduced cell proliferation. This observation matches with the reduction in tumor growth showed *in vivo*, probably because reducing the percentage of FBS conferred a more real condition, much similar to the condition in which cells are when injected to the mice. The reduction in cell proliferation after Ror2 depletion has been previously reported in ovarian cancer[229].

The knock-down of Ror2 also affected the colony formation capacities in 2D and 3D. Similar to the results observed in proliferation, the differences in colony formation were more evident at 1% FBS, although at 10% the depletion of Ror2 also prevented colony formation, in contrast to proliferation. The clonogenic capacities in soft agar were also reduced after Ror2 depletion. The capacity of the cells to grow in this assay has been related to the acquisition of CSCs properties. Moreover, some authors have linked EMT with the acquisition of stemness properties in different tumors[95]. Therefore, since Ror2 depletion induces a MET process by decreasing mesenchymal markers and increasing epithelial, it is likely that Ror2-depleted cells have less CSCs properties compared to Ror2 expressing cells.

It has been described that Ror2 promotes migration and invasion in different types of cancer[226,229]. In addition, Ror2 is essential for Wnt5a-induced invasion[53]. In our model, the knock-down of Ror2 decreases migration and invasion of colon cancer cells *in vitro*. Both processes have been related to EMT and particularly to Snail1[230]. *In vivo*, the tumors generated from control cells were more invasive to the subjacent tissue than those coming from Ror2-depleted cells, confirming that Ror2 expression is correlated with higher migrative and invasive properties. Thus, it is possible that upon

reduction of Ror2 levels, the expression of Snail1 is affected and then it reduces the proliferative, migrative and invasive capacities due to a MET process.

Another important component of the tumor is the stroma[101]. The activation of stromal CAFs by the tumoral cells is a key step for tumor cells invasion and metastasis and is a process in which Snail1 has an important role. CAF activation has been described to be dependent on Snail1 expression, since CAFs KO for Snail1 are not capable to be activated and induce tumor cells invasion[192]. Moreover, activated CAFs express higher levels of Snail1, FN1 or αSMA, among other markers[103]. This crosstalk between cancer cells and CAFs in which cancer cells activate fibroblasts, by secreting cytokines such as TGF-β or others, and fibroblasts induce and support cancer cells invasion is very important for cancer progression and metastasis (Figure 85). Interestingly, Ror2-depleted colon cancer cells have a reduced capacity to activate fibroblasts. Therefore, Ror2-depleted cells did not induce fibroblast invasion, whereas Ror2-expressing cells did. This reduced capacity is due to a lower TGF-β production, since the expression of this cytokine is dependent of the Wnt5a/Ror2/Snail1 axis. Importantly, although FN1 upregulation is impaired with a TGF-β inhibitor, Snail1 is not. This suggests that TGF-β is not the only cytokine responsible to induce fibroblast activation. Other factors secreted by tumor cells, such as Wnts, might also participate in this effect. Moreover, when the pathway is activated by Snail1 overexpression in HT29 M6 cells, these cells have the ability to better induce fibroblast activation. This result indicates that cells that have undergone EMT have increased capacities to activate the tumor microenvironment and induce a more invasive phenotype of the tumor (Figure 85).

4. Ror2/Snail1 regulate resistance to cisplatin and DNA-damage drugs

Cisplatin or platin-based drugs, such as oxaliplatin, are common chemotherapy treatments used to treat colorectal cancer, among other types of cancers. These agents induce cell death by causing DNA damage[210]. EMT and Snail1 have been related to the acquisition of chemoresistance to some drugs[89,231]. In colon cancer cells, Ror2 depletion affects the sensitivity to cisplatin since Ror2-expressing cells showed higher resistance to cisplatin. The role of Snail1 in promoting chemoresistance was confirmed, since Snail1 overexpression restored the resistance to cisplatin in the cells depleted of Ror2 and HT29 M6 cells. The levels of Snail1 not only promote higher resistance to cisplatin but to other drugs that also produce DNA damage, such as oxaliplatin and 5-FU, while no effect of Snail1-induced chemoresistance is observed to paclitaxel.

Interestingly, cisplatin treatment increased the protein stability and transcription of Snail1. This increase does not occur in cells that lack Ror2, due to the low levels of Snail1 expression in these cells. Accordingly, the apoptosis marker cleaved Caspase 3 increased in Ror2-depleted cells upon cisplatin treatments while in the Ror2-expressing cells the increase was lower or undetectable. Importantly, as mentioned above, this effect of Snail1-induced chemoresistance seems to be not only restricted to cisplatin since other drugs, such as oxaliplatin, 5-FU and doxorubicin, also elevated Snail1 expression.

Surprisingly, Snail1 expression did not promote higher resistance to paclitaxel. However, paclitaxel treatment also upregulated Snail1 expression as happens with the DNA damaging drugs, but this increase was accompanied by a phosphorylation of Snail1. Interestingly, different drugs that affect microtubule stability, such as paclitaxel, nocodazole, vinblastine and colcemid, promote Snail1 phosphorylation. Snail1 is a protein that can be highly modified post-translationally and phosphorylation is one of these modifications[107]. We have not identified the kinase responsible of this phosphorylation associated to Snail1 inactivation.

The Snail1 function in cisplatin chemoresistance is relevant for cancer treatment. As Snail1 is one of the main drivers of EMT and EMT has been related with the acquisition of CSCs properties[231], it is reasonable to think that Snail1-expressing cells that survive after chemotherapy present stemness properties that induce the appearance of new tumors or metastasis. So, a way to inhibit Snail1-induced chemoresistance would be important for treating cancer. We have demonstrated that the non-canonical Wnt pathway controls Snail1 expression in colon cancer cells and Wnt inhibition with Porcupine inhibitors decreases Snail1 expression. Thus, Porcupine inhibitors were

the principal candidates to block Snail1-induced chemoresistance since Wnt inhibition enhanced cisplatin sensitivity of Ror2-expressing cells to similar levels than Ror2-depleted cells.

This reduced chemoresistance is associated to a decrease in Snail1 expression after blocking the non-canonical Wnt pathway. In fact, the cisplatin-induced upregulation of Snail1 is not observed when cells are co-treated with cisplatin and LGK. As Porcupine inhibition blocks both canonical and non-canonical Wnt signaling, the implication of one or the other pathways in the upregulation of Snail1 upon cisplatin is a matter of debate. However, the addition of recombinant Wnt5a to cells co-treated with cisplatin and LGK or WntC59 completely reversed the effect of Wnt inhibition. This suggests that, indeed, it is the non-canonical Wnt pathway the one relevant in controlling cisplatin-induced Snail1 upregulation. Moreover, addition of DKK1, a specific canonical Wnt inhibitor, did not affect the upregulation of Snail1 caused by cisplatin, confirming that the canonical Wnt pathway is not relevant in these cells. Whether cisplatin induces activation of the non-canonical Wnt pathway and then, Snail1 is expressed is still not clear and more experiments should be performed in order to determine this. However, it is clear that the non-canonical Wnt pathway in colon cancer cells controls Snail1 expression and activation of this pathway is required for cisplatin-induced Snail1 increase.

The chemoresistance inhibitory effect of Porcupine inhibitors was also confirmed in the MTOs model. LGK and WntC59 blocked the upregulation of Snail1 upon cisplatin also in these cells. In accordance to that, LGK and WntC59 increased the toxicity of cisplatin and promoted more MTOs death. Porcupine inhibitors are in preclinical assays for their use in colon cancer. Their effect has been mainly attributed to the block of the canonical Wnt pathway, since β-catenin upregulation is a common characteristic of colon cancer, due to mutations mainly in APC. However, we have demonstrated that the effect of Porcupine inhibitors in colon cancer should be more related to the capacity to inhibit the non-canonical Wnt pathway and the inhibition of Snail1 expression. Actually, the expression of Snail1, and also Ror2, is increased in colorectal tumors that do not responded to oxaliplatin treatment, compared to patients that do. Therefore, inhibitors of Porcupine might be useful in combination with chemotherapeutic drugs in colon cancer treatment.

Based on our results and the literature in the field, the following model is proposed for the control of Snail1 expression by the non-canonical Wnt signaling and its role in colorectal cancer progression and metastasis. Ror2-expressing colon cancer cells produce Wnt5a that induces a positive loop of autoactivation. Wnt5a, through Ror2, activates STAT3 and JNK2 that promote the expression of Snail1, but also Wnt5a, Ror2, Fz2 and TGF-β. Snail1 expression also induces the expression of

Fibronectin 1 and other genes related to cell survival. These genes may contribute to the chemoresistance effect to cisplatin. Colon cancer cells-produced TGF-β induces the activation of the stromal fibroblasts and leads to fibroblast invasion, cancer progression and metastasis (Figure 85).

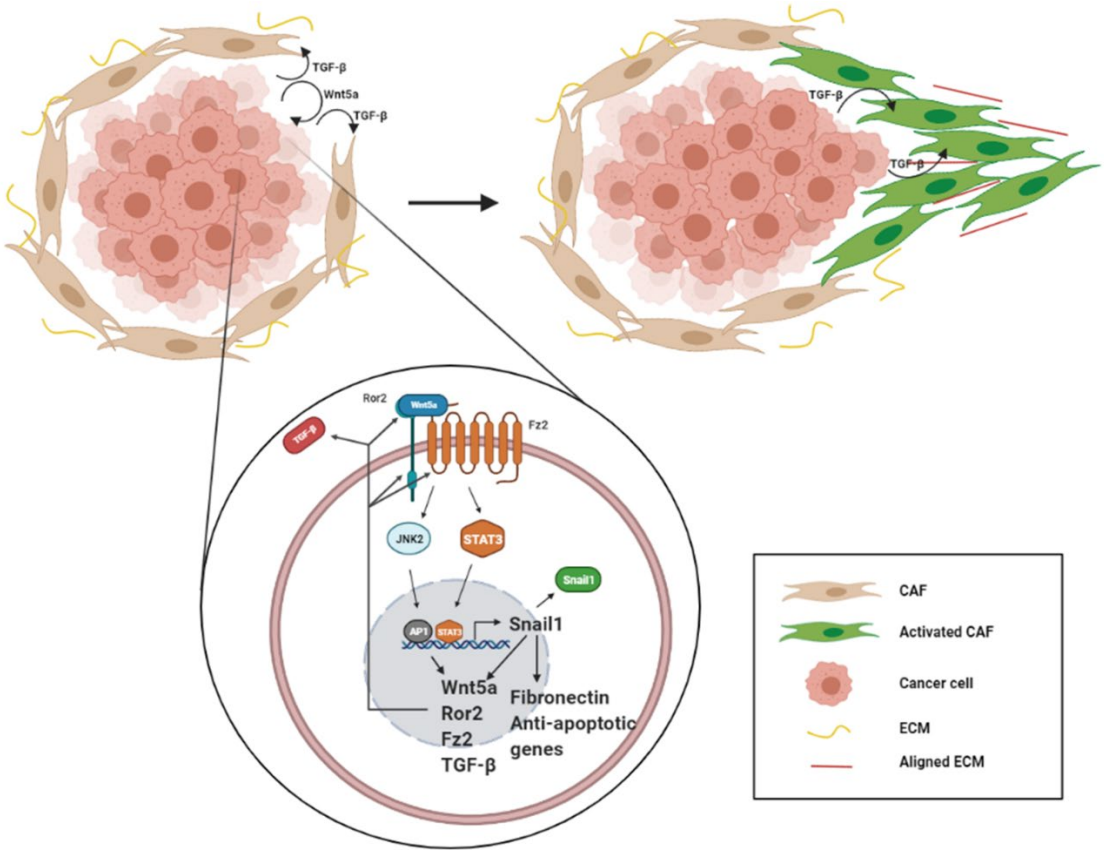

Figure 85: Role of the non-canonical Wnt signaling in the control of Snail1 expression and cancer progression. Ror2-expressing colon cancer cells produce Wnt5a that self-amplifies the signaling and induces Snail1 expression and TGF-β that promote fibroblast activation. Upon fibroblast activation, fibroblast invasion is promoted and leads cancer cell metastasis. Snail1 expression in the cancer cells induces the expression of anti-apoptotic genes that help the cells to resist chemotherapy.

CONCLUSIONS

CONCLUSIONS

- ✓ Ror2 and Wnt5a expression correlates with Snail1 expression in colon cancer cells and human colorectal tumors.
- ✓ Ror2 is necessary for Snail1 expression in colon cancer cells.
- ✓ The non-canonical Wnt signaling controls *SNAI1* transcription and Snail1 protein stability in a Ror2-dependent manner.
- ✓ Wnt5a, Ror2 and Snail1 define an autoactivation feedback loop in tumor cells.
- ✓ Ror2 controls migration, invasion and tumorigenesis in colon cancer model.
- ✓ Ror2 regulates the secretion of Wnt5a and TGF-β by cancer cells and promotes fibroblast activation.
- ✓ Cisplatin promotes higher expression of Snail1.
- ✓ Ror2/Snail1 axis promotes higher resistance to cisplatin, but not to paclitaxel.
- ✓ Porcupine inhibitors block Snail1-induced cisplatin resistance and impair colon cancer metastasis.

MATERIALS AND METHODS

1. Cell culture

1.1. Eukaryotic cell lines

The different cell lines used in the thesis were growth and maintained in Dulbecco's modified Eagle's Medium (DMEM) high glucose supplemented with 10% Fetal Bovine Serum (FBS), 4,5 g/L of Sodium Pyruvate, 2 mM Glutamine, 56 U/L Penicillin, 56 U/L Streptomycin and non- essential amino acids. Cells were maintained at 37°C and 5% of CO_2. The cell lines used during this thesis were the following:

Table 1: Enumeration and characteristics of the cell lines used in the thesis.

Cell line	Origin	Characteristics
SW620	Human colorectal adenocarcinoma	Epithelial morphology
HCT116	Human colorectal adenocarcinoma	Epithelial morphology
SW480	Human colorectal adenocarcinoma	Epithelial morphology
HT29 M6	Human colorectal adenocarcinoma	Epithelial morphology. Resistant to 10^{-6}M methotrexate.
HT29 M6 Snail1	Human colorectal adenocarcinoma	Epithelial morphology. HT29 M6 that constitutively overexpress Snail1[122].
LS174	Human colorectal adenocarcinoma	Epithelial morphology
Caco2	Human colorectal adenocarcinoma	Epithelial morphology
DLD1	Human colorectal adenocarcinoma	Epithelial morphology
LoVo	Human colorectal adenocarcinoma	Epithelial morphology
HEK 293T	Human embryonic kidney	Non tumoral. Epithelial morphology. Transformed with Adenovirus 5.
HEK 293T TGF-β reporter	Human embryonic kidney	Non tumoral. Epithelial morphology. Express a TGF-β reporter luciferase

mMCS	Mesenchymal stem cells from mouse bone marrow	Mesenchymal phenotype.
L cells	Subcutaneous mouse connective tissue	Fibroblastic phenotype
L cells Wnt3a	Subcutaneous mouse connective tissue	Fibroblastic phenotype. Contains Wnt3a plasmid. G418 selection
L cells Wnt5a	Subcutaneous mouse connective tissue	Fibroblastic phenotype. Contains Wnt5a plasmid. G418 selection

Stock cells were grown in treated plastic flasks of 75 cm^2. Before reaching confluence, medium was removed, cells were washed two times with PBS and allowed to detach with 0.5% trypsin (Gibco). Exposure time to trypsin depends on every cell line, going from 2 to 5 minutes at 37ºC. Then, cells were seeded again in 75 cm^2 flasks for stock cells or in treated plastic plates of different sizes depending on the experiment that was performed.

For experiments, cells were counted with an optical microscope and a Neubauer chamber. Addition of trypan blue to the mix of resuspended cells allowed to determine cell integrity and viability. Number of seeded cells depended on the performed experiment.

1.2. Obtention of conditioned medium

1.2.1. Wnt3a and Wnt5a conditioned medium

Control and Wnt3a or Wnt5a conditioned media (CM) were obtained from parental L, Wnt3a-L or Wnt5a-L cells respectively. Cell maintenance was performed with DMEM 10% FBS for L cells and supplemented with G418 at 0.4mg/mL for Wnt3a-L and Wnt5a-L, to avoid losing plasmid expression. To obtain CM, the three cell lines were seeded in 75 cm^2 flasks with DMEM 10% FBS without G418. After 4 days, CM first batch was recovered and fresh DMEM was added to the cells. After 3 more days, second batch was obtained and mixed with the first. CM was then centrifuged to eliminate cells that could be present. Supernatant was stored at 4ºC and used as CM.

1.2.2. Ror2-depleted and Snail1 overexpressing cells conditioned medium

Control and Ror2-depleted cells CM were obtained from SW620 and HCT116 shCtl or shRor2 cells, respectively. Control and Snail1-overexpressing cells CM were obtained from HT29 M6 control or Snail1-overexpressing cells. To obtain CM, all cell lines were seeded in 60 cm^2 plates with DMEM

10% FBS. After 24 hours, CM was recovered and centrifuged to eliminate cellular rests. Supernatant was stored at 4ºC and used as CM.

1.2.3. DKK1 conditioned medium

DKK1 is an antagonist of co-receptor LRP5/6. DKK1 CM is produced by overexpressing the pcDNA3-DKK1-HA in HEK293T cells that secret the protein to the medium. 24 hours after transfection CM first batch was recovered and fresh DMEM is added. After 24 hours more, second batch was obtained and mixed with the first. CM is then centrifuged and supernatant is used.

1.3. Cellular treatments

During this work, cells were treated with specific components in the different experiments performed. The different treatments are specified in Table 2.

Table 2: Cell culture treatments

Treatment	supplier	Concentration
SB-505124	Sigma	5 – 20 µM
LGK974	Sellechchem	1 – 10 µM
Wntc59	PeproTech	100 nM
Jnk inhibitor II	Calbiochem	25 – 50 µM
S3I-201	Selleckchem	50 – 100 µM
Cycloheximide (CHX)	Sigma	50 µg/mL
Recombinant Wnt5a	R & D System	50 ng/mL
Cisplatin	Calbiochem	40 – 400 µM
Doxorubicin	Sigma	5 nM
5-Fluorouracil	Accord	1 – 100 µM
Oxaliplatin	Teva	1 – 100 µM
Paclitaxel	Teva	1– 500 nM
Nocodazole	MedChemExpress	500 nM
Vinblastine	Stada	500 nM
Colcemid	Gibco	500 nM

2. Cell transfection and selection of transfectants

For expression or down-regulation of the different proteins of interest, the different cell lines were seeded in plates of different sized depending on the experiment performed. After seeding, cells were left 24 hours to properly adhere and grown until confluence reach about 50-60%. Then, cells were transfected differently depending on the transfected DNA.

2.1. DNA transfection with PEI

Transfection is performed mixing the DNA of interest with DMEM (without serum and antibiotics) and 10 µL of Polyethylenimine (PEI, Plysciences Inc.) was added per each µg of DNA. The mix was vortexed for 10 seconds and incubated for 20 minutes at RT before adding it over the plate. After 6 hours of transfection, cell medium was removed and fresh DMEM 10% FBS was added to avoid PEI toxicity.

For the stable Ror2 knock-down cells generation, SW620 and HCT116 cells were co-transfected with the vectors pLKO shRNA-Ror2 or pLKO shRNA-Control together with phrGFP. After 2 weeks, GFP positive cells were selected and the Ror2 knock-down efficiency was analyzed. Selection was maintained by adding puromycin.

For SW480 overexpressing Ror2-HA cells, SW480 cells co-transfected with the vectors pcDNA3 Ror2-HA or pcDNA3 empty vector together with phrGFP. After 2 weeks, GFP positive cells were selected and the Ror2-HA overexpression efficiency was analyzed.

1.1. siRNA transfection

Transient Snail1 knock-down was performed using small interfering RNA (siRNA). 24 hours after seeding cells, culture medium was changed and DMEM without antibiotic was added. Cells were transfected using DharmaFECT transfection agent (Dharmacon) with 0.2 nmoles of siRNA control (siCtl) or Snail1 siRNA (siSnail1), according to manufacturer's instructions. Experiments were performed after 48 hours of transfection.

2.2. DNA constructs

Table 3: DNA constructs employed during the thesis.

Vector	Origin
pcDNA3	
pcDNA3.1 Ror2-HA	Given by Dr. Y. Minami.
pcDNA3.1 Snail1-HA	Cloned in the lab. Described previously[122].
pcDNA3 DKK1-HA	Given by Dr A. Muñoz.
phrGFP	Empty vector.
pLKO	
pLKO shRNA non-targeting	#SHC001, SIGMA.
pLKO shRNA-Ror2 #1	#TRCN1492, SIGMA.
pLKO shRNA-Ror2 #2	#TRCN10625, SIGMA.
siRNA	
Control siRNA	D-001810-02-50, Dharmacon.
Snail1 siRNA	L-010847-01-0005, Dharmacon.
pGL3*	
pGL3* *SNAI1* -869/+59	Cloned in the lab. Described previously[187].
pGL3* *SNAI1* -194/+59	Cloned in the lab. Described previously[187].

2.3. Plasmid DNA purification

Escherichia coli cells were used to produce and purify plasmid DNA constructs. E. coli cells were grown in the rich medium Luria Broth (LB) in liquid. The medium is sterilized in an autoclave machine for 20 minutes at 120ºC. All the plasmid DNA constructs used in this study were previously transformed into E. coli. These, are maintained at -80ºC with 15% glycerol in liquid LB. When needed, transformed stock E. coli were grown in LB at 37ºC and agitation supplemented with the specific antibiotic for selection, depending on the transformed plasmid, but usually Ampicillin at 150mg/L. After an overnight culture, plasmid DNA isolation is performed with the commercial Wizard Plus SV Minipreps (Promega) or Maxipreps (Qiagen) kits, following manufacturer's protocols.

3. Techniques for protein analysis

3.1. Cell extracts preparation:

To obtain protein extracts, culture medium was first removed from the culture plates and cells were washed two times with cold PBS (4°C). Then, cells were scrapped with lysis buffer, 1% SDS lysis buffer or RIPA buffer depending on the experiment. Volume of lysis buffer depends on the cell type, the size of the culture plate and the cell confluence. After cell lysis, protein concentration was determined using the DC Protein Assay kit.

- 1% SDS lysis buffer: Used to solubilize all cellular components including the nucleus. To avoid SDS precipitation, protein extracts were collected at RT. Samples were boiled at 95°C until they were perfectly homogenized and were centrifuged at 16000g for 5 minutes.
- RIPA lysis buffer: Used to solubilize almost all cellular compartments and keeping protein interactions. After scrapping, samples were centrifuged at 16000g for 10 minutes at 4ªC.

3.2. Electrophoresis and Western blot:

Western blot was the method used to analyze the results from the experiments performed at protein level. Protein analysis was performed in denaturing conditions by adding SDS in the polyacrilamide electrophoresis gels. Used gels were 8-12% polyacrilamide. Loading buffer 5x was added to the samples and boiled at 95°C for 5 minutes before loading samples into the gel. The Mini-Protean System (Bio-Rad) was used to run the gels in Tris-Glycine-SDS buffer (TGS).

After gel electrophoresis, proteins were transferred to a Polyvinylidene difluoride (PVDF) membrane in a wet system at 100 V for 60 minutes with cold transference buffer. Before immunoblotting, the PDVF membrane was blocked with TBS-Tween 20 (Tris-buffered Saline) 1% BSA for 45 minutes at RT. Then the membrane was incubated with the primary antibody diluted in TBST 0.1% BSA for 2 hours at RT or overnight at 4°C.

The following antibodies were used in this thesis:

Table 4: List of antibodies used in the thesis.

Protein	Host	Dilution	Reference	Provider
c-Caspase3	Rabbit	1/1000	9661S	Cell Signaling
E-Cadherin	Mouse	1/1000	610182	BD Transduction Labs
Fibronectin1	Rabbit	1/1000	ab2413	Abcam

Fz2	Rabbit	1/1000	ab52565	Abcam
HA tag	Mouse	1/1000	H9658	Sigma
JNK2	Rabbit	1/1000	178953	Abcam
Lamin B	Rabbit	1/1000	ab16048	Abcam
LRP5/6	Rabbit	1/1000	2560S	Cell Signaling
pChk1	Rabbit	1/1000	2348	Cell Signaling
pJNK2	Rabbit	1/1000	07-175	Millipore
pSMAD2	Rabbit	1/1000	3108	Cell Signaling
pSTAT3	Rabbit	1/1000	9145S	Cell Signaling
pTyrosine general	Mouse	1/1000	4370	Cell Signaling
Ror1	Rabbit	1/1000	4102S	Cell Signaling
Ror2	Mouse	1/1000	Sc-374174	Santa Cruz
SMAD2/3	Rabbit	1/1000	8685	Cell Signaling
Snail1	Rabbit	1/1000	3879	Cell Signaling
STAT3	Mouse	1/1000	Sc8019	Santa Cruz
Wnt5a	Goat	1/1000	AF645	R&D Systems
αSMA	Mouse	1/1000	A2547	Sigma
β-Actin	Mouse	1/10000	A5441	Sigma

After incubation, the primary antibody was removed from the membrane. Then, the membrane was washed three times with TBST. After washes, the membrane was incubated with the specific secondary antibody for 1 hour at RT. Finally, the membrane was washed three times again with TBST. Membrane was developed with Immobilon Western Chemiluminiscent HRP Substrate (Millipore) in Alliance Q9 (UVITEC) chemiluminescence imager. When indicated, protein levels were quantified using ImageJ software.

3.3. Dephosphorylation assay

This assay was performed to analyze the phosphorylation level of Snail1 after treatment with paclitaxel. After overnight treatment with paclitaxel cells were lysed with RIPA buffer (with protease inhibitors) and cellular extracts were separated in three different conditions. In two conditions, cellular extracts were incubated for 1 hour with phosphatase inhibitors at 4ºC or 37ºC in order to maintain protein phosphorylation. In the third condition, cellular extracts were

incubated for 1 hour with calf intestinal phosphatase (CIP) at 37ªC. After incubation, protein concentration was determined and samples were analyzed by WB.

4. RNA analysis

4.1. RNA extraction

Before starting RNA extraction protocol, cells were washed two times with cold PBS. Then, cells were lysed in TRIzol reagent (Invitrogen). The volume of the reagent used depends on the culture plate surface. The lysates were mixed with 200 µL chloroform and 300 µL water and tubes were vortexed and incubated for 2 minutes. Then, tubes were centrifuged at 12000 g for 20 minutes at 4°C. The aqueous phase of the samples was transferred to a new eppendorf tube and mixed with 500 µL RNase free isopropanol and tubes were incubated for 20 minutes at RT to precipitate RNA. Next, tubes were centrifuged at 12000g for 20 minutes at 4°C. Supernatant was discarded and the pellet was washed with RNase free 70% ethanol and centrifuged at 12000g for 15 minutes at 4°C. Ethanol was completely removed and the RNA pellet was resuspended in 30-50 µL RNase free water and incubated for 5 minutes at 55°C.

4.2. Quantitative Real Time PCR (RT-qPCR)

RNA was retrotranscribed using First Strand cDNA Synthesis KIT (Roche) following manufacturer's protocol. Triplicates of 40-100 ng cDNA were amplified using the SYBR Green LightCycler 480 Real Time System (Roche). The primers used fo RT-qPCR in this Thesis are listed in Table 5.

Table 5: Primer sequences used in quantitative RT-PCR.

Gene	Forward (5'-3')	Reverse (5'-3')
AXIN2	TGTCTTAAAGGTCTTGAGGGTTGAC	CAACAGATCATCCCATCCAACA
BTRC	CACTCACAGCTTTCCAGACAT	TGCTGCAAGAGAAGGCACTC
CDH1	CCTGGGCAGAGTGAATTTTG	GGCGTAGACCAAGAAATGGA
CCND1	CTCAGACTTGCGCGTCACAG	CAGAACACGGCTCACGCTTA
FBXL5	CTTACCCAGACTGACATTTCAGATTC	GAAGACTCTGGCAGCAACCAA
FBXL14	AACGGATCCACCATGGAGACCCACA TCTCATGC	CAAGATATCCCCTTCTGGAGCTTCCC
FZD2	GTGCCGCTCTATCTGTGAGC	CTCCGTCCTCGGAGTGGTT
HPRT	GGCCAGACTTTGTTGGATTTG	TGCGCTCATCTTAGGCTTTGT
MMP9	TTCAGGGAGACGCCCATTTC	TGGGTGTAGAGTCTCTCGCT

MMP13	AAGGACCCTGGAGCACTCAT	CCTGGACCATAGAGAGACTGGA
ROR1	AGTGCCTACCTCATCATGGAAC	GAGCCATAGATGGTGGACCG
ROR2	CTCGAGTAAAAGGGACACCAGAGTG GC	AAGCTTCCCTTATATTCCTCAGGCTTC AA
RYK	AGCTCCAACCACTTCTACGC	AACCACTTCTACGCGTGTGT
SIAH2	TTTGCCATCGTCCTGCTCAT	AACCACTTCTACGCGTGTGT
SNAI1	GTGCCTCGACCACTATGCC	GCTGCTGGAAGGTAAACTCTGG
TGFB1	AAGTGGAGCAGCACGTGGAG	CAGCCGGTTGCTCAGGTATC
TGFB2	TACTACGCCAAGGAGGTTTACAAA	TTGTTCAGGCACTCTGGCTTT
TGFB3	CTGGCCCTTTACAACAGC	CACTGAGGACACATTGAAGC
TWIST1	CAGCTACGCCTTCTCGGTCT	CTCGGACAAGCTGAGCAAGA
USP17L2	GGTCTTTGTCTCCAGAGGGC	GGTCTTTGTCTCCAGAGGGC
USP27X	CGTCCCTGGCCCATCTTATC	CAGGGCTTAAGGGTCTTGGG
USP37	TCCTGCCTGTAGCCAGAAG	GGCTTTGTTTCGCTCCACTG
WNT5A	GCTCCCCGACAGCCTGGTCT	AGGACATGGCCGCTGGGTGT
ZEB1	CGGCGCAATAACGTTACAAA	TTTCACTGCTCCTCCCTGGT

5. Cell proliferation and viability assays

5.1. MTT assay

Cell proliferation and cell viability in cell lines were analyzed by MTT assay. For cell viability assays, 6000 cells were seeded in 96 well plates and after 24 hours cells were treated with the drugs at different concentrations for 24 hours. For cell proliferation assays, 4000 cells were seeded. Cell viability and proliferation was measured by adding 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) in DMEM 0% FBS for 3-4 hours at 37°C. After incubation, cells were solubilized in a solution of DMSO and isopropanol (1:4). The absorbance of the resulting solution was quantified in an Infinite M200 microplate reader (Tecan). The absorbance value at 590 nm was equivalent to the number of viable cells.

5.2. Celltiter glo assay

For MTOs viability experiments celltiter glo (Promega) was used. 4000 MTO cells were seeded in Matrigel (normal MTOs growing conditions) in 96 well plates and after 72 hours cells were treated with the different treatments. 72 hours later, the assay was performed by adding 15µL of celltiter

glo in each well. After incubation, luminescence was analyzed with FB-12 luminometer (Berthold Detection Systems). The luminescence value was equivalent to the number of viable cells.

5.3. Colony formation assays

For the 2D colony formation assays, 1000 cells were seeded in 6 well plates and allowed to grow for 10 days in DMEM 1% or 10% FBS. After 10 days of incubation, cells were fixed and stained with a buffer containing 30% methanol and 0.5% Crystal violet for 20 minutes. After incubation, cells were washed with PBS and the number of colonies were quantified with ImageJ software.

For the 3D soft agar colony formation assays, 6 well plates were firstly coated with a mix of DMEM (supplemented with Sodium Bicarbonate, Sodium Chloride, FBS and antibiotics to a final concentration of 2x) and incubated for 30 minutes at room temperature to allow agar polymerization. After incubation, a mix of 0.6% agar and DMEM 10% FBS containing 5000 cells/well was added on top of the 1% agar. The mix was incubated 30 minutes again to allow agar polymerization. Finally, fresh DMEM 10% was added twice weekly on the top to avoid agar desiccation. After 21 days of grow, the experiments were stopped and MTT assay was performed. Micrographs were taken and colonies were quantified with ImageJ software.

6. Migration and invasion assays

Migration and invasion assays were performed using the Boyden chamber system. For both assays, 40-100x10^3 cells were resuspended in DMEM 0.1% FBS in a final volume of 150 µL. For invasion assays, the transwells (3442, Costar) were first coated with 50 µL Matrigel 0.5 µg/µL (354230, Corning) and incubated for 2h at 37°C. Then, cells were seeded on top of the coating and incubated for 48 hours. For migration assays, cells were directly seeded on the transwells and incubated for 16 hours. DMEM 10% FBS was used as chemoattractant added in the lower compartment. After stopping the assay, cells were fixed with 100% MetOH for 20 minutes and the non-invading cells were removed from the transwell. Invading cells were stained with Crystal Violet (30% in MetOH) for 15 minutes. After three washes with PBS, cells were eluted with 30% Acetic Acid and absorbance was analyzed at 590 nm with the Infinite M200 microplate reader (Tecan).

For co-culture invasion assays, 80x10^3 tumoral cells and 20x10^3 GFP+ mMSCs were seeded on top of Matrigel-coated transwells. FBS gradient was used as chemoattractant as in single culture invasion assay. The assay was stopped at 24h to analyze fibroblast invasion. Cells were washed with PBS and fixed with 4% p-formaldehyde during 20 minutes. Non-invading cells were removed with a

cotton swap and transwell membranes were mounted for microscopy analysis. Five random photos (4x) of each membrane were taken to analyze invasion of GFP+ fibroblasts. Number of invading cells was quantified using ImageJ software.

7. *SNAI1* promoter activity assay

Reporter assays were carried out seeding cells in 20 cm^2 plates and transfected with 50ng of two *SNAI1* promoter fragments cloned in pGL3* basic vector (Promega), pGL3* *SNAI1* -869/+59 and pGL3* *SNAI1* -194/+59. They were co-transfected with 1ng of pTK-Renilla, the plasmid used as control that contains the Renilla Luciferase gene under the control of a constitutively active promoter. After 24 hours, cells were trypsinized and seeded in 96-well plates. 24 hours later, cells were lysed with the kit Dual Luciferase Reporter Assay System (Promega) and the promoter activity was quantified using the FB-12 luminometer (Berthold Detection Systems). The relative Firefly/Renilla Luciferase activity determines the Snail1 promoter activity. The mean ± SD of three independent experiments is represented.

8. TGF-β reporter assay

The TGF-β reporter assay was used to determine the production of TGF-β by Ror2-depleted cells. HEK 293T TGF-β activity reporter cells were generated after infection with TGF/SMAD Luciferase Reporter Lentivirus (Kerafast). HEK 293T cells were treated with conditioned medium from control and Ror2-depleted HCT116 cells. After overnight treatment with conditioned medium, cells were lysed with the kit Dual Luciferase Reporter Assay System (Promega) and luciferase activity was measured with FB-12 luminometer (Berthold Detection Systems). The mean ± SD of three independent experiments is represented.

9. Human tumor data analysis

The co-expression data of mRNA and protein was obtained from TCGA Colorectal Adenocarcinoma (Firehose Legacy) data in the cBioPortal public database (http://www.cbioportal.org/) on May, 2020[202,203]. In this study we assessed the mRNA expression of *ROR2, SNAI1, LRP5, LRP6, MMP9, MMP13, WNT5A, TGFB1, AXIN2* and *FZD2*. Protein expression of Snail1 and β-catenin was also analyzed.

The expression of *SNAI1, ROR2* and *WNT5A* in colorectal cancer patients treated with oxaliplatin was assessed using the public available Gene Expression Omnibus dataset GSE83129. Gene

expression data were downloaded from the GEO (https://www.ncbi.nlm.nih.gov/geo) and analyzed using the Geo2R function from NCBI (https://www.ncbi.nlm.nih.gov/geo/geo2r) on April, 2021.

10.Statistical analyses

All the results shown were representative from at least three independent experiments. Data are represented 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 < 0.05 are symbolized with one asterisk, $p < 0.01$ with two asterisks and $p < 0.001$ with three asterisks.

11.Reagents and solutions

1% SDS lysis buffer: 1% SDS,

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

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.

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

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

TGS buffer: 25 mM Tris-HCl pH 8.3, 192 mM glycine, 5% SDS.

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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