

Noncanonical Wnt signaling promotes colon tumor growth, chemoresistance and tumor fibroblast activation

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Abstract

Colon tumors of the mesenchymal subtype have the lowest overall survival. Snail1 is essential for the acquisition of this phenotype, characterized by increased tumor stemness and invasion, and high resistance to chemotherapy. Here, we find that Snail1 expression in colon tumor cells is dependent on an autocrine noncanonical Wnt pathway. Accordingly, depletion of Ror2, the co-receptor for noncanonical Wnts such as Wnt5a, potently decreases Snail1 expression. Wnt5a, Ror2, and Snail1 participate in a self-stimulatory feedback loop since Wnt5a increases its own synthesis in a Ror2- and Snail1-dependent fashion. This Wnt5a/Ror2/Snail1 axis controls tumor invasion, chemoresistance, and formation of tumor spheres. It also stimulates TGF β synthesis; consequently, tumor cells expressing Snail1 are more efficient in activating cancer-associated fibroblasts than the corresponding controls. Ror2 downmodulation or inhibition of the Wnt5a pathway decreases Snail1 expression in primary colon tumor cells and their ability to form tumors and liver metastases. Finally, the expression of *SNAIL1*, *ROR2*, and *WNT5A* correlates in human colon and other tumors. These results identify inhibition of the noncanonical Wnt pathway as a putative colon tumor therapy.

Keywords cancer stem cell; chemoresistance; metastasis; noncanonical Wnt; Snail1

Subject Categories Cancer; Signal Transduction

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Introduction

The canonical Wnt signaling pathway is involved in the development and progression of many human cancers (Nusse & Clevers, 2017). The main consequence of the activation of this pathway is the stabilization of β -catenin, although other reactions such as JNK2 activation are also required for β -catenin translocation to the nucleus and for the transcription β -catenin/Tcf4 target genes (MacDonald & He, 2013; García de Herreros & Duñach, 2019). However, not all Wnt ligands act through the canonical signaling; some, such as Wnt5a, activate a different pathway not involving β -catenin that has been called noncanonical Wnt signaling (Van Amerongen *et al*, 2008). Molecularly, canonical and noncanonical Wnts use a common receptor, Fz, but different co-receptors: LRP5/6, the canonical, and Ror2, the noncanonical pathway (Van Amerongen *et al*, 2008; Grumolato *et al*, 2010; MacDonald & He, 2013). They also differ in the elements acting downstream in both pathways although some, such as CK1 ϵ , Dvl2, and Fyn, are activated by both types of ligands (Kikuchi *et al*, 2012; MacDonald & He, 2013; García de Herreros & Duñach, 2019).

Besides activating specific genes, both canonical and noncanonical Wnt pathways stimulate a common set of genes associated with epithelial-to-mesenchymal transition (EMT) and cell invasion, including Snail1 (Yook *et al*, 2005; Dissanayake *et al*, 2007; Gujral *et al*, 2014; Villarreal *et al*, 2020; Feng *et al*, 2022). This is dependent on the common activation of Stat3, which requires the Src-catalyzed phosphorylation of Fz proteins such as Fz2 at Tyr552. Modification of this residue promotes Fyn binding to Fz2, Fyn stimulation, and Stat3 phosphorylation at Tyr705 (Gujral *et al*, 2014; Villarreal *et al*, 2020). In addition to other specific responses, canonical and noncanonical Wnts also stimulate another pathway involving Dvl2 and JNK2 (Yamanaka *et al*, 2002; Wu *et al*, 2008; Villarreal *et al*, 2020).

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Snail1 is among the genes transcriptionally activated by the Wnt/Fyn/Stat3 axis in fibroblasts (Villarreal *et al.*, 2020). Snail1 is a transcriptional factor essential for triggering EMT, a process leading to the acquisition of fibroblastic features by epithelial cells (Baulida *et al.*, 2019; Yang *et al.*, 2020). Snail1 expression confers higher invasion to tumor cells and also other traits associated with EMT such as greater chemoresistance and cancer stem cell properties (Yang *et al.*, 2020). The precise agents controlling Snail1 expression in colon tumor cells have not been identified although several extracellular factors promote Snail1 expression in different tumor cells; FGF2, HGF, TGF β , and few others (Peinado *et al.*, 2003; Grotegut *et al.*, 2006). These proteins enhance binding to *SNAIL1* promoter of NF κ B, Stat3, Myc, HMGA2, AP-1, and Egr-1 transcriptional factors activating *SNAIL1* transcription (Baulida *et al.*, 2019). However, increased transcription by itself is not sufficient to enhance Snail1 since this protein is very unstable and Snail1 upregulation needs always to be associated with increased protein stability (Díaz & García de Herreros, 2016). For instance, Snail1 expression is totally dependent on the activity of specific deubiquitinases (Dubs) such as Dub3, Usp27X, and Usp37 (Wu *et al.*, 2017; Lambies *et al.*, 2019; Xiao *et al.*, 2019). The precise regulation of Snail1 Dubs by Snail1-inducing factors has not been assessed other than the upregulation of Usp27X and Dub3 by TGF β and TNF α , respectively (Wu *et al.*, 2017; Lambies *et al.*, 2019).

The molecular classification of colon tumors has shown that the mesenchymal (CMS4) subtype has the lowest overall survival (Guinney *et al.*, 2015). These tumors show an upregulation of genes implicated in EMT. Accordingly, Snail1 presence in tumor cells has also been associated with poor prognosis and an increased resistance to chemotherapeutic agents (Kaufhold & Bonavida, 2014). Therefore, to inhibit Snail1 and EMT would be particularly relevant to decrease colon tumor lethality. When analyzing Snail1 expression sensitivity to different drugs in colon tumor cell lines, we have observed that it is markedly repressed by Porcupine inhibitors, compounds that block the secretion of Wnt factors (Liu *et al.*, 2013). This suggests that the autocrine production of Wnt factors controls Snail1 expression. The cell lines used in our study were generated from β -catenin/APC-mutated tumors and harbor ligand-independent transcriptional activity of β -catenin. However, inhibitors of Wnt secretion act on APC wild-type and also on APC-mutated tumors (Liu *et al.*, 2013; Madan *et al.*, 2016). Our results indicate that Snail1 is controlled by β -catenin-independent noncanonical Wnt signaling. Accordingly, Ror2 depletion severely downregulates Snail1 in tumor cells and affects growth in colon spheres, tumor invasion, and chemosensitivity.

Results

Ror2 is necessary for Snail1 expression in colon tumor cells

As TGF β and Wnt factors induce Snail1 expression in fibroblasts (see Introduction), we assessed the relative contribution of the autocrine secretion of these factors on Snail expression in SW620 and HCT116, two tumor cells with high endogenous expression. These two cells presented an altered canonical Wnt signaling due to mutations in *APC* (SW620) or in *CTNNB1* (HCT116) genes (Rowan *et al.*, 2000; Huang *et al.*, 2021). The TGF β receptor I inhibitor

SB505124 (SB; DaCosta Byfield *et al.*, 2004) did not modify Snail1 levels in these cells although it decreased Smad2 phosphorylation (Fig 1A). Similar results were obtained with LY2109761 (Bandyopadhyay *et al.*, 2006), a compound that inhibits TGF β receptor I and II at different doses (Appendix Fig S1A). Moreover, supplementation of HCT116 and SW620 cells with TGF β did not modify Snail1 levels, in contrast to what happens in fibroblastic mesenchymal stem cells (MSC) (Appendix Fig S1B), indicating that TGF β does not control Snail1 expression in these intestinal tumor cells.

Instead, Snail1 was downregulated by the addition of LGK974 (LGK; Fig 1A), a Porcupine (PRCN) inhibitor that blocks acylation of Wnt factors, both canonical and noncanonical, preventing their secretion (Liu *et al.*, 2013). LGK reduced Stat3 phosphorylation, a modification produced by activation of both pathways (Fig 1A). This result suggests that Wnt signaling is constitutively activated in these cells by the autocrine expression of Wnt factors that control Snail1 expression. Besides repressing *SNAIL1* RNA levels (Appendix Fig S2A), LGK also decreased the expression of *AXIN2* and *CCND1*, two targets of the canonical Wnt pathway, and also of *MMP9* and *SIAH2*, two RNAs induced by noncanonical Wnts.

Since canonical and noncanonical Wnt pathways share a common receptor but differ in the co-receptor, we determined the relative expression of these co-receptors. As seen in Appendix Fig S2B and C, the noncanonical co-receptor Ror2 correlated much better with Snail1 expression than the canonical one, LRP5/6, in a small panel of colon tumor cell lines. The TCGA database also showed a significant positive correlation between *SNAIL1* and *ROR2* mRNA, better than that obtained with *LRP5* or *LRP6* (Table 1). Other noncanonical co-receptors, such as Ror1 or Ryk, were not expressed in colon tumor cells or do not show a positive correlation with Snail1 (Appendix Fig S2B and C).

More definitive conclusions were obtained downregulating Ror2 in both SW620 and HCT116 by stable expression of a specific shRNA (shRor2 #1). Ror2 depletion potently decreased Snail1 protein (Fig 1B) and RNA (Fig 1C). No effects of shRor2 transfection were observed on other noncanonical Wnt co-receptors such as Ror1 or Ryk (Appendix Fig S3A). Downregulation of Ror2 in SW620 and HCT116 cells was also reproduced using a different shRNA (shRor2 #2) that also decreased Snail1 protein (Appendix Fig S3B). Ectopic expression of murine Ror2 cDNA, not sensitive to this shRNA, increased Snail1 protein levels in shRor2 HCT116 cells (Appendix Fig S3C). The effect of Ror2 downregulation was not limited to *SNAIL1* since expression of other mesenchymal gene products such as α SMA, TWIST, ZEB1, MMP9, or MMP13 was also decreased (Fig 1B and C). Conversely, and likely as consequence of the inactivation of transcriptional factors associated with EMT as Snail1 and Twist, E-cadherin protein and RNA were upregulated (Fig 1B and C). As expected, expression of the noncanonical Wnt target genes *SIAH2* and *MMP9* was reduced by Ror2 depletion whereas the canonical Wnt targets *AXIN2* and *CCND1* were not affected (Fig 1C). Remarkably, the expression of Fz2, a receptor for both canonical and noncanonical Wnts, was also sensitive to Ror2 depletion both as protein and RNA (Fig 1B and C). Expression of Fz2 also showed a close correlation with Snail1 in the different cell lines analyzed (Appendix Fig S4A and B). Ror2 depletion also decreased other signals associated with Wnt5a activation such as Stat3 or Jnk2 phosphorylation (Fig 1D; Appendix Fig S3D).

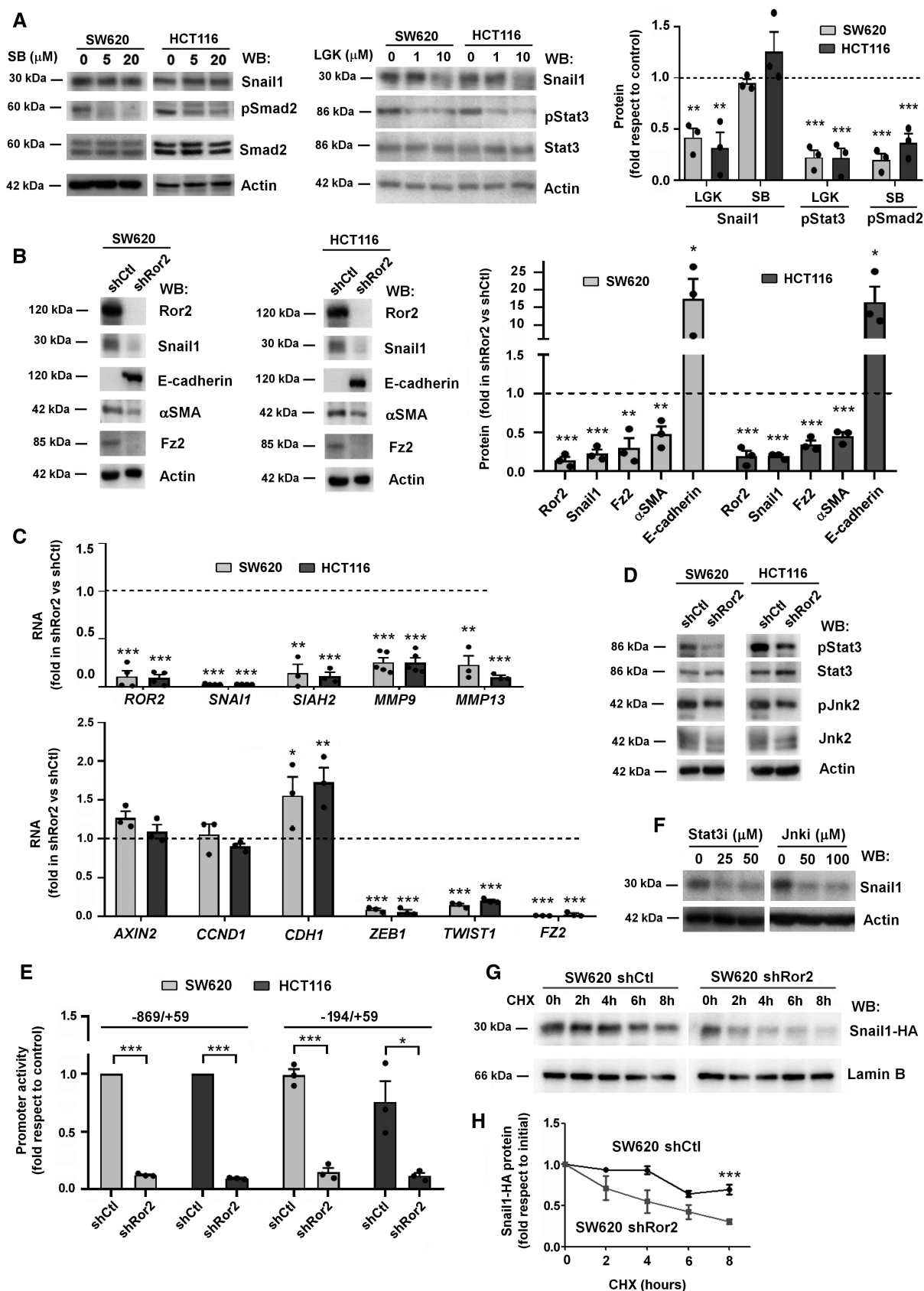


Figure 1.

Figure 1. Inactivation of noncanonical Wnt signaling decreases the expression of Snail1 and other mesenchymal markers in colon tumor cell lines.

- A SW620 and HCT116 cells were treated with the indicated concentrations of SB505124 (SB) or LGK974 (LGK) for 16 h. Total cell extracts were analyzed by WB. Protein quantification was analyzed by densitometry of three different experiments and represented (mean $\pm$ SD; right panel).
- B SW620 and HCT116 cells expressing shCtl or shRor2 vectors were analyzed by WB. Quantification of the WBs from three biological replicates is presented in the right panel. Values correspond to fold variation in shRor2 versus shCtl cells (mean $\pm$ SD).
- C RNA was isolated from shCtl or shRor2 SW620 and HCT116 cells, and the expression of the indicated genes was assessed by qRT-PCR. Values correspond to fold variation in shRor2 versus shCtl cells. Results are mean $\pm$ SD of at least three biological replicates performed in triplicate.
- D SW620 and HCT116 cells expressing shCtl or shRor2 vectors were analyzed by WB. Quantification of the WBs is presented in Appendix Fig S3D as in (B).
- E *SNAIL1* promoter activity was analyzed in HCT116 and SW620 cells and represented with respect to the activity of the $-869/+59$ promoter for each cell line (mean $\pm$ SD from three biological replicates performed in triplicate).
- F HCT116 cells were treated with the indicated concentrations of Stat3 inhibitor S3I-201 or JNK inhibitor II for 16 h. Cells were lysed and Snail1 levels determined by WB. Quantification of three biological replicates is presented in Appendix Fig S3E.
- G, H In (G), shCtl or shRor2 SW620 cells were transiently transfected with a plasmid encoding Snail1-HA. Cells were treated with cycloheximide (CHX) (50 μ g/ml) for the indicated times, and ectopic Snail1 was analyzed by WB. A quantification of four biological replicates (mean $\pm$ SD) is presented in (H).

Data information: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (unpaired t-test). The dashed line in A, B, and C corresponds to the protein or RNA value in untreated or shCtl-transfected cells.

Source data are available online for this figure.

We also investigated the molecular basis for the Ror2-dependent Snail1 expression. Ror2 downmodulation in both SW620 and HCT116 cells markedly decreased the activity of a $-869/+59$

Table 1. Expression of *WNT5A*, *SNAIL1* and *ROR2* and *Wnt5A*-target genes correlates in colon tumors.

Gene expression association	Spearman	Pearson
RNA		
ROR2/ <i>SNAIL1</i>	0.32 ($P = 1.06 \text{ e-}10$)	0.33 ($P = 3.51 \text{ e-}11$)
<i>SNAIL1</i> /LRP5	-0.11 ($P = 0.032$)	-0.07 ($P = 0.150$)
<i>SNAIL1</i> /LRP6	0.10 ($P = 0.0439$)	0.11 ($P = 0.0309$)
MMP9/ <i>SNAIL1</i>	0.32 ($P = 9.1 \text{ e-}11$)	0.33 ($P = 4.41 \text{ e-}11$)
MMP13/ <i>SNAIL1</i>	0.37 ($P = 9.43 \text{ e-}14$)	0.41 ($P = 1.816 \text{ e-}16$)
WNT5A/ <i>SNAIL1</i>	0.26 ($P = 2.60 \text{ e-}7$)	0.27 ($P = 7.83 \text{ e-}8$)
WNT3A/ <i>SNAIL1</i>	0.06 ($P = 0.220$)	0.07 ($P = 0.151$)
AXIN2/ <i>SNAIL1</i>	-0.13 ($P = 8.60 \text{ e-}13$)	-0.13 ($P = 0.0118$)
TGFB1/ <i>SNAIL1</i>	0.46 ($P = 4.22 \text{ e-}21$)	0.46 ($P = 5.11 \text{ e-}21$)
FZ2/ <i>SNAIL1</i>	0.24 ($P = 1.650 \text{ e-}6$)	0.24 ($P = 3.258 \text{ e-}6$)
WNT5A/FZ2	0.25 ($P = 8.75 \text{ e-}7$)	0.29 ($P = 5.35 \text{ e-}9$)
FZ2/ROR2	0.42 ($P = 3.52 \text{ e-}18$)	0.43 ($P = 5.38 \text{ e-}19$)
ROR2/FN1	0.62 ($P = 2.62 \text{ e-}41$)	0.61 ($P = 5.52 \text{ e-}40$)
AXIN2/WNT5A	-0.08 ($P = 0.115$)	-0.06 ($P = 0.23$)
WNT5A/MMP9	0.29 ($P = 8.60 \text{ e-}9$)	0.32 ($P = 1.61 \text{ e-}10$)
WNT5A/MMP13	0.42 ($P = 1.88 \text{ e-}17$)	0.43 ($P = 1.22 \text{ e-}18$)
WNT5A/TWIST1	0.43 ($P = 9.39 \text{ e-}19$)	0.46 ($P = 2.03 \text{ e-}21$)
USP17L2/WNT5A	0.45 ($P = 2.46 \text{ e-}3$)	0.42 ($P = 5.6 \text{ e-}3$)
USP17L2/MMP13	0.22 ($P = 0.158$)	0.18 ($P = 0.238$)
USP17L2/AXIN2	-0.06 ($P = 0.769$)	-0.06 ($P = 0.687$)
Protein		
β -catenin/Snail1	-0.26 ($P = 6.73 \text{ e-}9$)	-0.10 ($P = 0.0281$)

The correlation between the RNA expression (log RNA seq V2 RSEM) of the indicated genes is represented. Spearman coefficients higher than 0.4 were highlighted in dark gray and higher than 0.2, in light gray. Data were obtained from the TCGA Colorectal Adenocarcinoma Firehose Legacy database and analyzed in the cBioPortal. Scatter plots are shown in Appendix Figs S10 and S11.

fragment of *SNAIL1* human promoter (Barberà *et al*, 2004; Fig 1E). This DNA fragment contains binding sites for Stat3 and AP1, two transcriptional factors activated by Wnt5a. Stat3 binding sites were located at $-772/-766$ and $-732/-725$ whereas those of AP1, at $-824/-821$ and $-95/-92$ (Fig EV1A). A shorter promoter ($-194/+59$) was also repressed by Ror2 depletion (Fig 1E). This shorter fragment does not contain Stat3 binding sites; accordingly, only the longer promoter was repressed by a Stat inhibitor whereas the activity of both fragments was decreased by a JNK inhibitor (Fig EV1B). Binding of Stat3 and the AP1-complex member c-Jun to *SNAIL1* promoter was also decreased by Ror2 downmodulation in HCT116 and SW620 cells, as determined by ChIP assays (Fig EV1C). In line with these results, inhibition of Stat3 and JNK2 activities diminished Snail1 expression (Fig 1F; Appendix Fig S3E).

Another prominent point of control of Snail1 expression is protein stability. Snail1 protein half-life is very finely regulated by a complex set of E3 ubiquitin ligases and Dubs (see Introduction). Snail1 protein stability was lower in Ror2-depleted than in control cells (Fig 1G and H). We analyzed the main E3 ubiquitin ligases controlling Snail1 expression (Díaz & García de Herreros, 2016): only *FBXL5* RNA, and not *BTRC* (corresponding to β TrCP1) or *FBXL14*, was slightly upregulated by shRor2 (Fig EV1D). Regarding Snail1 Dubs, *USP17L2* (the gene encoding Dub3) RNA was considerably decreased in Ror2-depleted cells (Fig EV1D), suggesting that the lower expression of this Snail1-stabilizing enzyme is relevant for the reduced Snail1 levels in Ror2-depleted cells.

Wnt5a, Ror2, and Snail1 form a feedback loop in tumor cells

The results presented above indicated that Wnt5a and Ror2 control Snail1 expression in tumor cells. Remarkably, inhibition of this non-canonical Wnt pathway, either depleting Ror2 or with LGK also decreased *WNT5A* RNA (Fig 2A) and Wnt5a secreted protein (Fig 2B). Expression of other Wnt proteins, corresponding to canonical (Wnt1 and 3a) and noncanonical (Wnt5b and 11) ligands, was also downregulated in Ror2-depleted cells (Appendix Fig S5A). The conditioned medium from Ror2-depleted cells showed a lower capability to activate the noncanonical pathway in fibroblasts than the conditioned medium from control cells, as assessed determining Stat3 phosphorylation 30 min after addition of the medium (Fig 2C; Appendix Fig S5B). As seen in Appendix Fig S5C, the effect of the

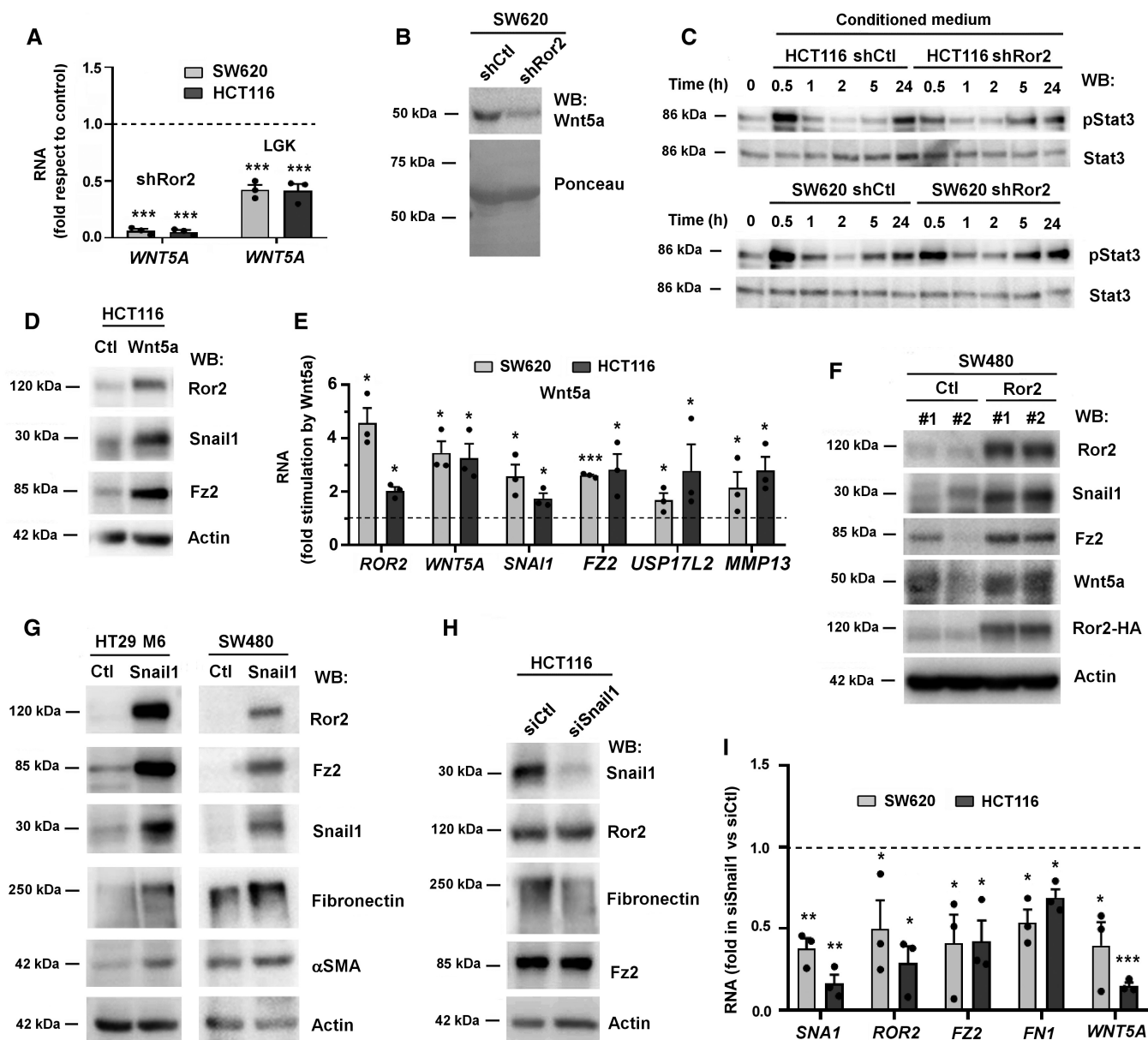


Figure 2. Wnt5a, Ror2, and Snail1 form a self-stimulatory signaling pathway.

- A** RNA was isolated from shCtl or shRor2 HCT116 and SW620 cells, treated with LGK (10 μM) for 16 h when indicated and expression of WNT5A assessed by qRT-PCR. Results are presented relative to control (mean ± SD of three biological replicates performed in triplicate).
- B** Wnt5a protein levels were determined by WB in the conditioned medium of shCtl or shRor2 SW620 cells.
- C** MSC were treated with the specified conditioned media for the indicated times, and pStat3 was analyzed by WB. Results from two different experiments are represented in Appendix Fig S5B.
- D, E** HCT116 or SW620 cells were treated with the conditioned medium from control or Wnt5a-producing cells for 16 h and expression of the indicated genes assessed by WB (D) or qRT-PCR (E) (mean ± SD of three biological replicates performed in triplicate in E). Results from three different experiments corresponding to D were densitometered and represented in Appendix Fig S5E.
- F** SW480 cells were stably transfected with Ror2-HA or the empty vector phrGFP. Two independent clones of control and Ror2 were lysed and analyzed by WB. Quantification of the results is presented in Appendix Fig S5G.
- G** WB analysis of HT29 M6 and SW480 overexpressing Snail1. Quantification is presented in Appendix Fig S5I.
- H, I** SW620 and HCT116 cells were transfected with control or Snail1 siRNA and analyzed by WB (H) or qRT-PCR (I, mean ± SD of three biological replicates performed in triplicate). Quantification of the results from H is presented in Appendix Fig S5K.

Data information: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (unpaired t-test). The dashed line in A, E, and I corresponds to the RNA value in control cells.

Source data are available online for this figure.

conditioned medium of tumor cells on Stat3 phosphorylation in fibroblasts was dependent on the expression of Ror2 also in these cells.

These results suggest the existence of a feedback loop where Wnt5a activates its own synthesis. Moreover, addition of LGK not only decreased Snail1 expression (as seen in Fig 1A) but also that of Ror2 and Fz2 (Appendix Fig S5D). Conversely, stimulation of HCT116 and SW620 by Wnt5a increased Ror2, Fz2, Snail1, and Wnt5a proteins or RNAs (Fig 2D and E; Appendix Fig S5E). This Wnt5a-induced Snail1 upregulation was not sensitive to TGF β inhibition by SB (Appendix Fig S5F). *USP17L2* (Dub3) RNA was also stimulated as well as *MMP13*, a *bona fide* target of the noncanonical Wnt pathway (Fig 2E). These results suggest that Wnt5a, besides activating its own expression, also increases other elements of the pathway as Ror2 and Fz2, showing the existence of a feedback loop.

Other experiments confirmed the existence of this self-stimulation. Ror2 overexpression in SW480, cells with low basal levels of this co-receptor, also increased Snail1 expression as well as that of Fz2 and Wnt5a (Fig 2F; Appendix Fig S5G and H).

Snail1 was also involved in the loop. Besides stimulating classical mesenchymal markers, such as fibronectin or α SMA, Snail1 ectopic expression in HT29 M6 or SW480 cells increased expression of Ror2, Fz2, Wnt5a, and even endogenous human Snail1 that could be discriminated from ectopic murine Snail1 by RT-PCR (Fig 2G; Appendix Fig S5I and J). A transient transfection of a Snail1 siRNA, which potently downregulated Snail1 protein and RNA, decreased fibronectin expression, but only affected *ROR2* and *FZ2* RNA and not protein (Fig 2H and I; Appendix Fig S5K), suggesting that Snail1 effect on these gene products requires more time than on fibronectin and indicating a different extent of involvement of Snail1 in the expression of noncanonical Wnt target genes.

Wnt5a/Ror2/ modulates TGF β production and fibroblast activation

Another relevant consequence of the inhibition of the Wnt5a/Ror2/Snail1 pathway is a decreased capability for tumor cells to promote fibroblast activation. Besides affecting Wnt5a levels (see above), Ror2 depletion also decreased TGF β production. First, endogenous Smad2 phosphorylation was reduced by Ror2 depletion in SW620 and HCT116 cells (Fig 3A). *TGFB1* RNA was potently repressed by Ror2 depletion or LGK addition (Fig 3B); similar results were obtained with *TGFB2* and *TGFB3* (Fig EV2A). Moreover, Ror2 depletion in SW620 or HCT116 decreased the capability of the conditioned medium produced by these cells to stimulate a TGF β -sensitive reporter (Fig EV2B) or to increase Smad2 phosphorylation in fibroblasts (Fig EV2C; Appendix Fig S6A). Conditioned medium from cells depleted in Ror2 or treated with LGK was much less efficient than control conditioned medium in activating Snail1 or fibronectin expression in MSC, two proteins that are induced during fibroblast activation but with different kinetics (Figs 3C and EV2C and D; Appendix Fig S6A and B). Stimulation of fibronectin by the conditioned medium of SW620 or HCT116 cells was considerably decreased by the addition of the TGF β receptor inhibitor SB (Figs 3D and EV2E); by contrast, Snail1 was not affected, suggesting that different factors secreted by tumor cells are responsible for the upregulation of both proteins. Accordingly, Snail1 upregulation in MSC

caused by the conditioned medium from HCT116 cells was sensitive to Ror2 elimination in the receiving MSC cells and not by SB (Fig EV2F), indicating that Snail1 was likely stimulated by non-canonical factors such as Wnt5a expressed by HCT116 cells.

Fibroblast activation also stimulates invasion. Therefore, as shown in Fig 3E, incubation with control SW620 or HCT116 cells increased fibroblast invasion through Matrigel; this stimulation was not observed when these cells were co-cultured with Ror2-depleted cells and was prevented by the addition of SB. Finally, overexpression of Snail1 in HT29 M6 cells enhanced the capability of the conditioned medium derived from these cells to stimulate MSC activation, as assessed analyzing Snail1 and fibronectin expression (Fig 3F; Appendix Fig S6C). Altogether, these results indicate that noncanonical Wnt signaling controls TGF β production by cancer cells and their capability to activate fibroblasts.

Noncanonical Wnt signaling is required for tumorigenesis

Snail1 expression confers the capability to form tumor spheres or tumor organoids (Mani *et al*, 2008). Elimination of Ror2 did not alter the proliferation of either SW620 or HCT116 cells when these cells were cultured in DMEM plus 10% FCS but slowed down cell growth when they were maintained in 1% FCS (Fig 4A). LGK also inhibited cell growth at both FCS concentrations (Appendix Fig S7A). The effects of the inhibition of Wnt5a/Ror2 signaling were more evident when cells were grown at limit dilution: Ror2 depletion decreased the clonogenic capability, especially when it was assayed at low FCS (Fig 4B; Appendix Fig S7B), and colony formation in soft agar (Fig 4C; Appendix Fig S7C). Conversely, SW480 cells overexpressing Ror2 were more efficient in generating colonies than the corresponding controls (Appendix Fig S7B). shRor2 cells did not form tumor spheres in Matrigel when cells were grown in tumoroid culture medium, different to shCil SW620 cells, and remained attached to the plate (Fig 4D and E). Addition of LGK also reduced the formation of colonies and tumor spheres (Fig 4E; Appendix Fig S7B and D).

Since some of these properties (as formation of tumor spheres) are associated with stemness, we analyzed the expression of genes characteristic of tumor colon stem cells (Merlos-Suárez *et al*, 2011). In SW620 cells, Ror2 interference and LGK decreased the expression of *LGR5*, *ASCL2*, and *SMOC2*, RNAs associated with stemness, whereas increased the differentiation-related gene *KRT20* (Fig 4F). Snail1 depletion also promoted a similar decrease in *LGR5*, *ASCL2*, and *SMOC2* RNAs (Fig 4F).

The tumorigenic capability of these cells was also affected. At day 28, when most of the mice bearing the control SW620 cells have to be euthanized, these tumors presented an average size considerably bigger than those generated from SW620 Ror2-depleted cells (Fig 5A). Tumors from these cells show a delayed onset compared with those formed by SW620 control cells. The initial lesions from both types of tumors (obtained at day 18 from the control and day 22 from shRor2-generated tumors) exhibited a different infiltration by activated fibroblasts, as shown by the lower expression of fibronectin and vimentin in the stroma of shRor2 tumors (Fig 5B and C). Ror2-depleted cells also generated tumors with lower muscle invasion (Fig 5D and E). Accordingly, Ror2 depletion significantly diminished migration and invasion of these cells through Matrigel (Fig 5F).

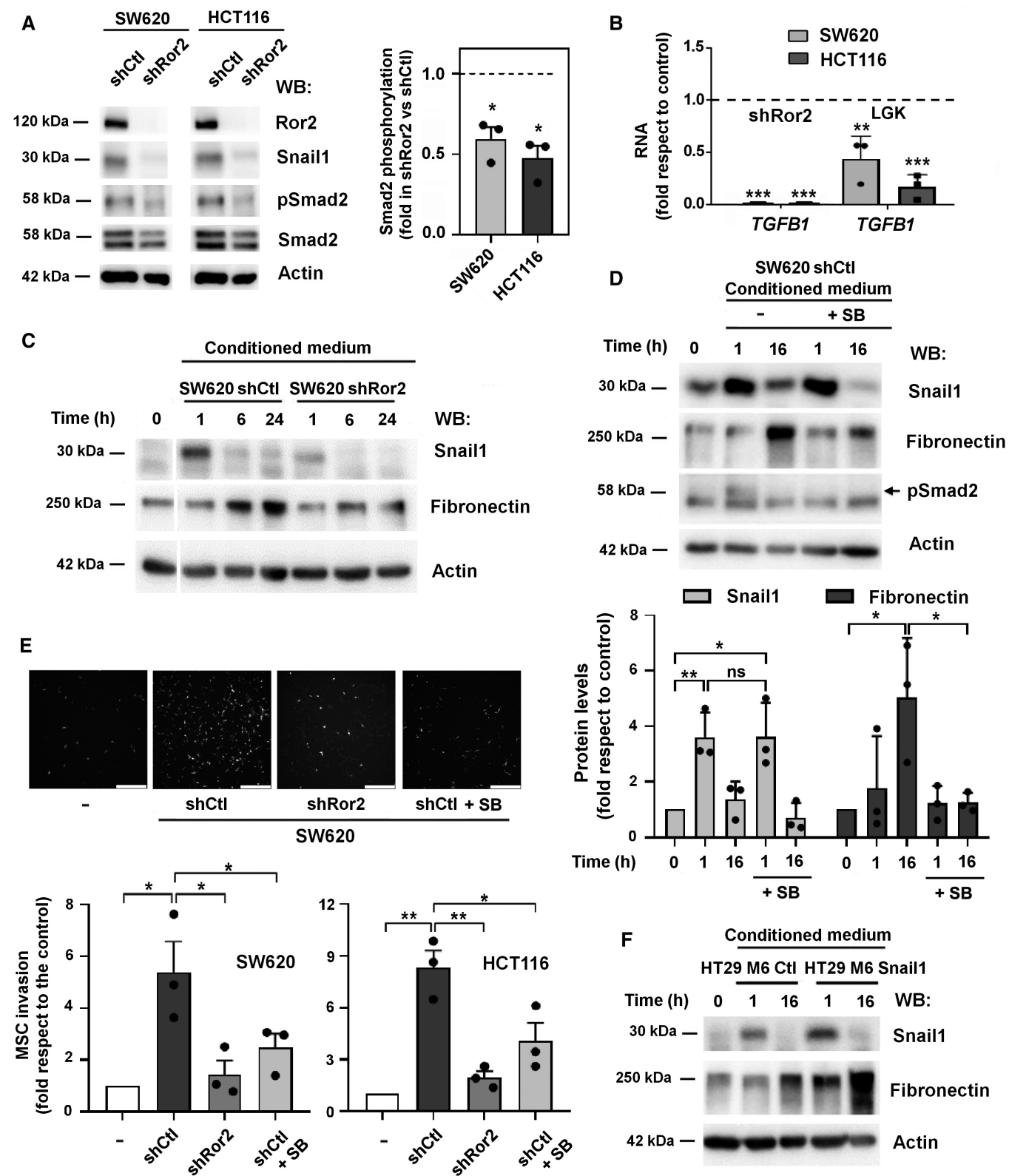


Figure 3.

Figure 3. Noncanonical Wnt stimulates TGF β production by tumor cells and fibroblast activation.

- A Smad2 phosphorylation was determined in extracts from shCtl or shRor2 SW620 and HCT116 cells. The quantification is presented in the right panel (mean $\pm$ SD or three biological replicates).
- B RNA was isolated from HCT116 and SW620 control, stably depleted of Ror2 or treated with LGK for 16 h. Expression of *TGF β 1* was assessed by qRT-PCR (mean $\pm$ SD of three biological replicates performed in triplicate).
- C, D MSC were treated with the conditioned media from shCtl or shRor2 SW620 (C, D) for the indicated times. TGF β receptor inhibitor SB505124 (SB, 5 μ M) was added when indicated in D. Cells were lysed and proteins analyzed by WB. Quantification of the protein levels from D is represented at the lower panel (mean $\pm$ SD of three biological replicates); from C, in Appendix Fig S6B.
- E GFP-labeled MSC were seeded in Matrigel-coated transwells with SW620 and HCT116 cells and supplemented with SB when indicated. After 48 h, noninvading cells were removed from the upper surface of the membrane and cells present at the lower surface were fixed. GFP-labeled cells were counted in five different fields per filter by ImageJ software and represented (bottom). A representative picture of SW620 cells in each condition is shown (top). The bar corresponds to 500 μ m. The figure shows mean $\pm$ SD of three experiments.
- F MSC were treated with the conditioned media from Ctl or Snail overexpressing HT29 M6 cells for the indicated times. Cells were lysed and proteins analyzed by WB. Quantification of protein levels is represented in Appendix Fig S6C.

Data information: ns, not significant; * P < 0.05; ** P < 0.01; *** P < 0.001 (unpaired t-test). The dashed line in B corresponds to the RNA value in control cells.

Source data are available online for this figure.

Interference with the Wnt5a/Snail1 axis decreases metastasis of primary colon tumor cells

To extend the conclusions of our study, we analyzed cells derived from tumors generated in mice with APC, Kras, TGF β -receptor 2, and p53 mutations (Tauriello *et al*, 2018; LAKTP mice). These murine cells form tumor organoids (MTO) when grown embedded in Matrigel, and liver metastasis when implanted in the spleen (Tauriello *et al*, 2018). MTO cells expressed Snail1 and Ror2 and other genes associated with this axis (Fig 6A). Snail1 expression was decreased by LGK and by another porcupine inhibitor, WntC59 (Chen *et al*, 2009; Fig 6A and B). These compounds downregulated Ror2 and Fz2 whereas increased E-cadherin (Fig 6A and B). WntC59 significantly reduced proliferation of tumor spheres, to a greater extent when it was added before the spheres were formed (Fig 6C and D). Both WntC59 and LGK decreased the expression of genes associated with stemness and upregulated *Krt20*, related to differentiation (Fig 6E).

To specifically inhibit noncanonical Wnt signaling in these cells, we generated a dominant-negative mutant Ror2 consisting in the extracellular domain of this protein (Appendix Fig S8A). This Ror2 extracellular domain (R-ECD) was produced in HEK293 cells and purified from the conditioned medium (Appendix Fig S8B and C). Addition of R-ECD to MTO cells specifically inhibited noncanonical Wnt targets such as *Siah2* and others without affecting canonical target genes (*Axin2*, *Ccnd1*) (Appendix Fig S8D). *Snail1*, *Wnt5a*, and

Ror2 RNAs were also decreased by R-ECD (Appendix Fig S8D) as well as Snail1 protein (see below). Ror2-ECD also reduced tumor sphere formation (Fig 6D).

The *in vivo* effect of the inhibition of the Wnt5a/Snail1 axis was assessed using WntC59. This compound was used in these experiments with preference to LGK since it presents a better *in vivo* delivery. WntC59 decreased tumor growth when MTO were subcutaneously grafted in nude mice (Fig 6F and G). WntC59-treated tumors also showed a lower expression of mesenchymal markers (Fig 6H). MTO cells were also injected in immune-competent mice; in these experiments, WntC59 prevented the appearance of tumors (Appendix Fig S9A and B).

Similar results were obtained using MTO cells with downmodulated Ror2. In addition to Ror2, these cells presented lower Snail1 protein than controls (Fig 6I). Expression of noncanonical gene targets (*Siah2*) or genes associated with a mesenchymal phenotype was also downregulated; on the contrary, *Cdh1* expression was stimulated and the canonical Wnt target *Axin2*, not affected (Fig 6J). Ror2-depleted MTOs were less efficient in generating tumors when grafted in immunocompetent mice (Fig 6K and L; Appendix Fig S9C and D). Ror2-depleted tumors also showed a lower expression of mesenchymal markers (Fig 6M).

WntC59 also prevented the formation of metastasis produced by MTO cells in immune-competent mice (Fig 6N and O). Three weeks after injection in the spleen, these cells originated liver metastasis in

Figure 4. Tumor sphere formation is prevented by noncanonical Wnt inhibition.

- A Cell number was determined by the MTT assay for SW620 and HCT116 cells cultured in DMEM plus 1% FCS or 10% FCS (mean $\pm$ SD of three biological replicates).
- B Clonogenic assay carried out with HCT116 cells cultured in DMEM plus 1% or 10% FCS for 10 days. Colonies were stained with crystal violet and counted using ImageJ software. Results are presented as mean $\pm$ SD of triplicate samples of three independent experiments. The micrograph shows the results of a representative experiment.
- C Representative micrographs of the effects of Ror2 depletion on HCT116 cells soft-agar colony formation (21 days; left). Representative micrographs of SW620 cells are shown in Appendix Fig S7B. Results show mean $\pm$ SD of the number of colonies in triplicate samples of three independent experiments (right).
- D, E Cells were seeded for tumor sphere formation in tumor organoid complete medium. In D, representative micrographs of shCtl and shRor2 SW620 cells. The scale bar corresponds to 25 μ m. In E, SW620 cells were supplemented with LGK (10 μ M), starting the day of seeding. After 4 days, the wells were stained (top) and the number of colonies counted and represented (bottom) (mean $\pm$ SD of three biological replicates). A more detailed micrograph of the colonies is shown in Appendix Fig S7D.
- F RNA was isolated from SW620 control, stably depleted of Ror2, treated with LGK974 for 16 h or transfected with siSnail1 and the expression of the stem cell gene markers *LGR5*, *ASCL2*, *SMOC2* and the differentiation marker *KRT20* was assessed by qRT-PCR. The figure shows mean $\pm$ SD of at least three biological replicates (performed in triplicate) represented with respect to the corresponding control (dashed line).

Data information: * P < 0.05; ** P < 0.01; *** P < 0.001 (unpaired t-test).

Source data are available online for this figure.

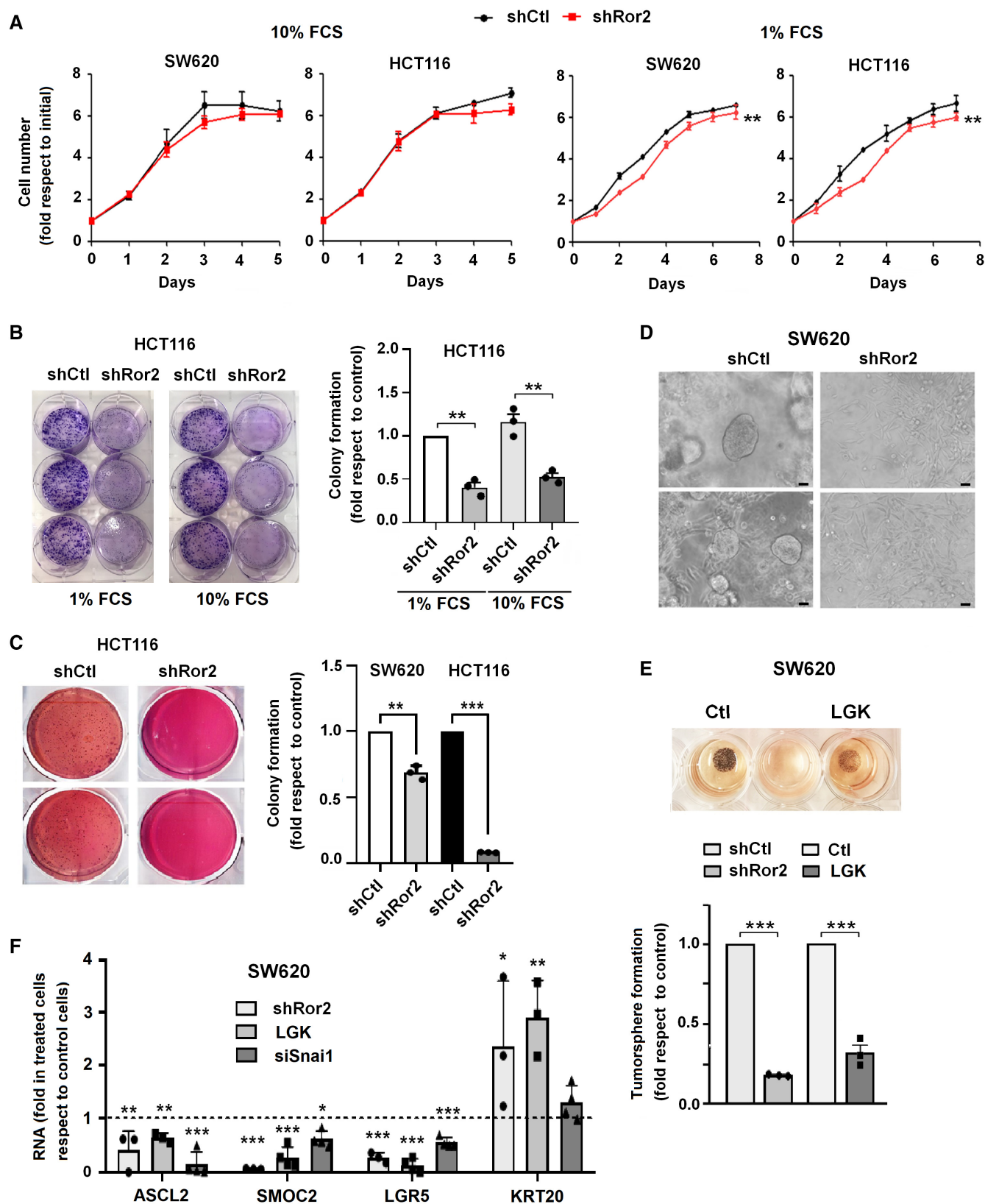


Figure 4.

approximately 60% of the cases (Fig 6O). Treatment with WntC59 starting the same day of implantation totally inhibited the appearance of metastases (Fig 6O; Appendix Fig S9E). The postmortem analysis revealed the absence of tumors in WntC59-treated mouse livers (Appendix Fig S9F).

The Wnt5a/Ror2/Snail1 axis modulates cisplatin resistance of colon cancer cells

Another cellular property associated with Snail1 expression and EMT is an enhanced chemoresistance. In accordance with the low

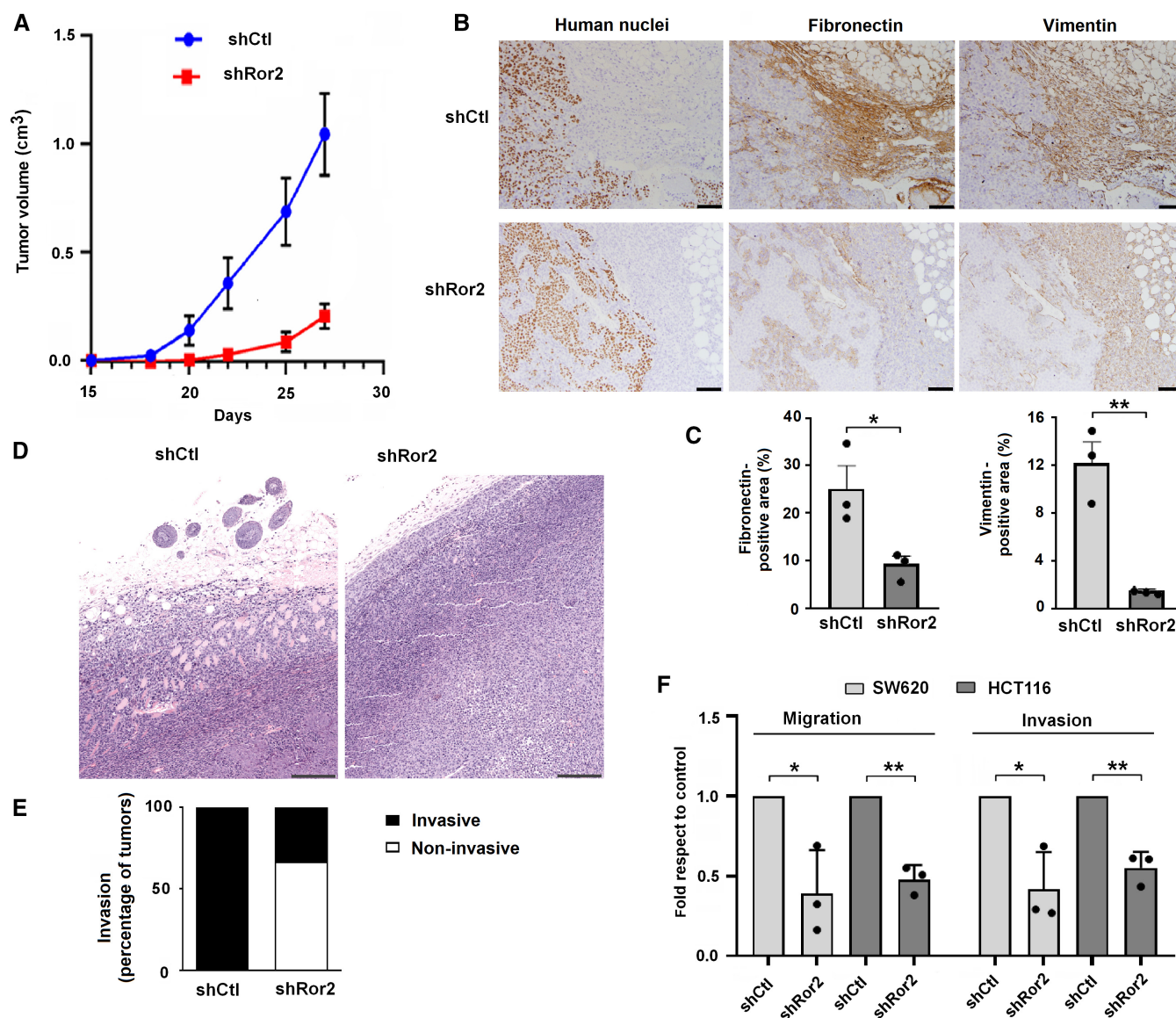


Figure 5. Noncanonical Wnt controls tumorigenesis of colon cancer cells.

A *In vivo* tumor growth of shCtl or shRor2 SW620 cells subcutaneously injected in NOD/SCID mice (10⁵ cells/flank; eight tumors per condition; mean ± SD).
B, C Representative images of fibronectin and vimentin staining in consecutive sections from paraffin-embedded tumors obtained when tumors were initially detected (days 18 or 22). Staining with an anti-human antibody is also enclosed. The scale bar corresponds to 10 μm (B). Fibronectin and vimentin staining was quantified as described in Methods (six tumors, three areas per tumor) and represented in (C; mean ± SD).
D Representative images of the invading front of the tumors. The scale bar corresponds to 4 μm.
E Percentage of tumors showing muscle invasion.
F shCtl and shRor2 SW620 and HCT116 cells were seeded in transwell chambers coated with Matrigel when appropriate. After 48 h, cells were removed from the upper surface of the membrane and cells present at the lower surface were fixed and stained with Crystal Violet. Results are presented as mean ± SD from three biological replicates.

Data information: **P* < 0.05; ***P* < 0.01; (unpaired *t*-test).

Source data are available online for this figure.

Snail1 expression in Ror2-depleted HCT116 and SW620, these cells showed a higher sensitivity to cisplatin (Fig 7A), oxaliplatin, and 5-fluorouracil (FU; Fig EV3A) than control cells. Addition of the Wnt inhibitor LGK also increased cisplatin cytotoxicity (Fig 7A). Snail1 ectopic expression in Ror2-depleted cells rescued cisplatin resistance (Fig 7B). A higher resistance was also observed upon ectopic Snail1 expression in HT29 M6 cells (Fig 7C).

Cisplatin increased Snail1 expression in both SW620 and HCT116 cells (Figs 7D and E, and EV3B) as it has been previously described for DNA damaging insults (Sun *et al*, 2012; Viñas-Castells *et al*, 2014; Sonogo *et al*, 2019). This effect was abrogated by Ror2 depletion, since shRor2 cells expressed very little Snail1. Cisplatin increased cleaved caspase 3, an apoptosis marker, to a higher extent in shRor2 than in control cells, further corroborating their higher sensitivity (Fig 7D). Other results confirmed that noncanonical Wnt signaling is required for cisplatin-induced Snail1 upregulation. LGK prevented the Snail1 raise in response to cisplatin, an effect that was not produced by the addition of recombinant DKK1, a specific inhibitor of canonical Wnt signaling (Fig 7E). This LGK-induced Snail1 drop was compensated by addition of recombinant Wnt5a (Fig 7E). WntC59 also prevented the increase in Snail1 caused by cisplatin (Fig EV3C and D), an effect also rescued by supplementation with recombinant Wnt5a (Fig EV3D). The cisplatin action on Snail1 expression seems to be mostly post-translational since cisplatin increased *SNAIL1* mRNA to a much lesser extent than protein (Fig EV3E) and remarkably boosted the levels of ectopically expressed Snail1 in HT29 M6 cells (Fig EV3F). Oxaliplatin and doxorubicin, but not FU also elevated Snail1 protein (Fig EV3G). Similar results were obtained with other cell lines; Snail1 was increased by cisplatin and downregulated by LGK in LoVo, DLD1, and Caco2 colon tumor cells (Fig EV3H).

These results were also validated in MTO cells. Snail1 expression was stimulated by cisplatin and decreased by LGK or WntC59 (Figs 7F and EV3I). Chk1 phosphorylation, a signal of DNA damage, was also elevated by cisplatin but not affected by these two Wnt inhibitors (Fig 7F). Both LGK and WntC59 increased MTOs

sensitivity to cisplatin (Fig 7G). These results were also reproduced downmodulating Ror2; shRor2 MTO cells presented a decreased upregulation in Snail1 protein in response to cisplatin (Fig 7H), enhanced levels of cleaved caspase 3 and higher cisplatin toxicity (Fig 7H and I). Similar results were also obtained in cells supplemented with R-ECD (Fig EV3I, J and K).

Expression of *SNAIL1*, *ROR2*, *FZD2*, and *WNT5A* strongly correlates in human tumors

Finally, we analyzed the expression of the relevant genes in public databases. Besides the *ROR2-SNAIL1* positive relationship previously mentioned, the TCGA database of colon tumors showed positive correlations between the expression of *SNAIL1* and *WNT5A* or the noncanonical target genes *MMP9* or *MMP13* and not with the canonical ligand *WNT3A* or the canonical target *AXIN2* (Table 1; Appendix Fig S10). Snail1 protein did not correlate with β -catenin protein levels. Expression of other members of the Wnt5a/Ror2/Fz2/Snail1 pathway was also connected; for instance, expression of *SNAIL1* associated with that of *TGFB1* or *FZ2* (Table 1; Appendix Fig S11) and *FZ2* also correlated with *ROR2* or *WNT5A*. As expected, *WNT5A* presented a positive relationship with the targets of the noncanonical pathway *MMP9* and *MMP13*, and also with *TWIST1*, but not with the canonical target gene *AXIN2* (Table 1; Appendix Fig S11). Although its expression in colon tumors was limited, *USP17L2* (Dub3) RNA also associated with *WNT5A* and targets of the noncanonical Wnt but not with those of canonical Wnts. The positive correlation between the expression of *SNAIL1* and *WNT5A* or *ROR2* RNAs was also observed in other different tumors (Fig EV4A); by contrast, *SNAIL1* did not associate with *WNT3A* or *LRP5*. Finally, we analyzed the prognosis significance of a non-canonical Wnt signature in colorectal cancer. Patients with tumors displaying higher expression of *ROR2*, *SNAIL1*, *WNT5A*, *MMP13*, *ZEB1*, *TWIST1*, *ACTA2*, and *FN1* presented a lower overall survival than controls (Fig EV4B).

Figure 6. Inhibition of noncanonical Wnt decreases the tumorigenic and metastatic potential of primary colon tumor cells.

- A, B LAKTP murine tumor (MTO) cells were seeded in the indicated culture conditions and supplemented with LGK (10 μ M) or WntC59 (100 nM). Total protein extracts (A) or RNA (B) were obtained and analyzed after 16 h of treatment. Quantification of the WB is also presented (A, right). Results are mean $\pm$ SD of at least three biological replicates.
- C Cell number was analyzed by CellTiter-Go assay in MTO cells supplemented with WntC59 (100 nM) every 3 days (mean $\pm$ SD of three biological replicates).
- D MTO were seeded in the presence of WntC59 or recombinant R-ECD (0.2 μ g/ml) and 4 days later the wells were stained and the number of cells represented respect the corresponding control (mean $\pm$ SD of three biological replicates).
- E Effect of LGK and WntC59 on the expression of the stem cell gene markers and the differentiation marker *KRT20* was determined by qRT-PCR (mean $\pm$ SD of three biological replicates performed in triplicate).
- F, G *In vivo* tumor growth of MTO cells subcutaneously injected in NOD/SCID mice (F) (5×10^5 cells/flank; eight tumors per condition) and treated with WntC59 (10 mg/kg/day in the drinking water) or vehicle. Tumor weight of NOD/SCID mice at day 26 is presented in (G). In F and G, mean $\pm$ SD of the eight tumors is shown.
- H Representative images of Hematoxylin/Eosin (H/E), fibronectin and vimentin staining in sections from paraffin-embedded tumors obtained at day 26 in NOD/SCID mice. The scale bars correspond to 50 μ m (H/E, fibronectin) or 100 μ m (vimentin).
- I, J WB (I) and RNA analysis (J) of MTO cells infected with shRor2 or shCtl. Quantification of the WB is also presented (I, right). Results are mean $\pm$ SD of three biological replicates (performed in triplicate in J).
- K, L C57BL/6J mice (eight tumors per condition) were injected subcutaneously with luciferase-expressing LAKTP MTO (shCtl or shRor2). The kinetics of *in vivo* luminescence are shown (mean $\pm$ SD of the values of eight tumors). Tumor weight at day 38 is presented in (L).
- M Representative images of fibronectin and vimentin staining in sections from paraffin-embedded tumors from LAKTP MTO cells obtained at day 38. The scale bars correspond to 50 μ m.
- N, O C57BL6 mice were injected in the spleen with MTO cells and treated since day 1 with WntC59 or vehicle. In (N), kinetics of *in vivo* luminescence (mean $\pm$ SD of the values in five mice); in (O), the percentage of mice showing *in vivo* luminescence.

Data information: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (unpaired t-test). The dashed line in A, B, E, I and J corresponds to the protein or RNA value in control cells. Source data are available online for this figure.

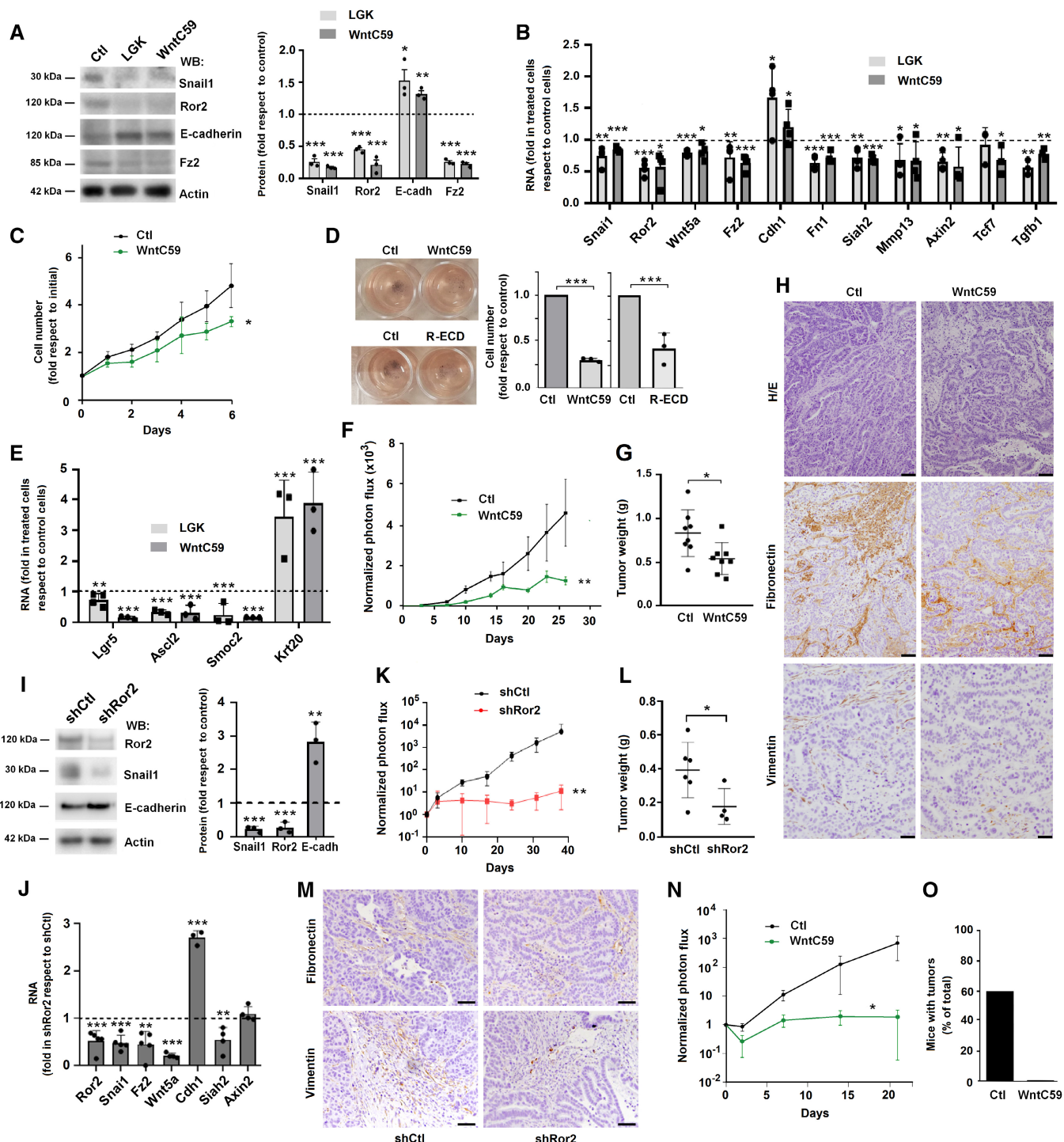


Figure 6.

Discussion

The relevance of noncanonical Wnt signaling in colon cancer cell proliferation and growth has been shown by several authors (Gujral *et al*, 2014; Voloshanenko *et al*, 2018; Pashirzad *et al*, 2021). In this article, we demonstrate the existence of an autocrine noncanonical

Wnt signaling pathway that controls the expression of Snail1 and other mesenchymal markers in colon tumor cells. Snail1 is a transcriptional factor critically involved in EMT that confers to tumor cells higher invasion and increased resistance to chemotherapeutic drugs. After the initial activation of Snail1 expression (likely by a combination of oncogenic mutations, extracellular factors, and

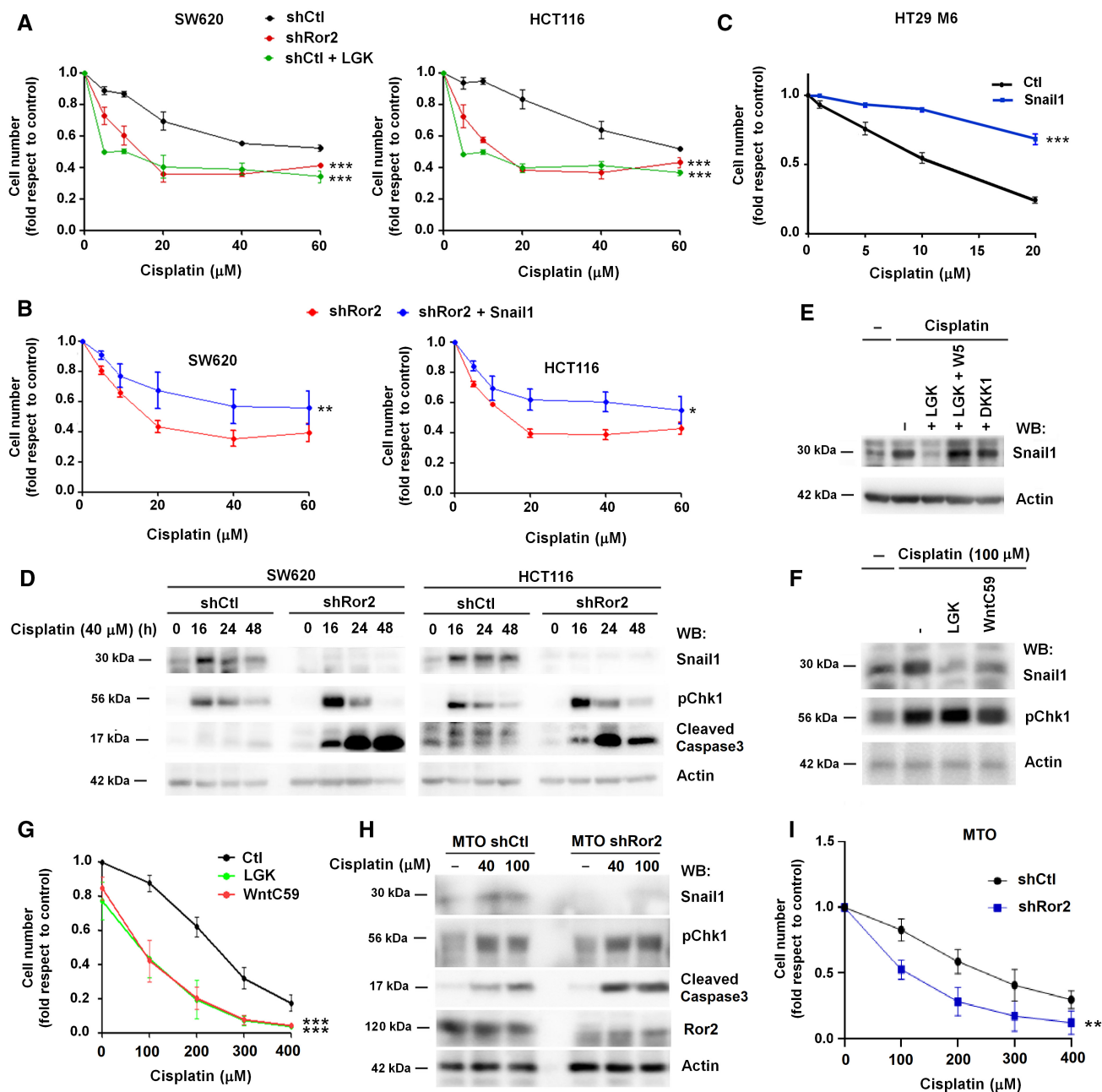


Figure 7. Wnt5a and Ror2 modulate cisplatin resistance of tumor cells.

A–C Viability was assayed by MTT in the indicated cells treated with LGK when specified and supplemented with cisplatin for 24 h. In (B), cisplatin resistance was assessed in shRor2 cells transfected with a Snail1 expression plasmid or the corresponding empty plasmid. The figure shows mean $\pm$ SD of the results of three experiments.

D Cells were treated with cisplatin for the indicated times. Total extracts were lysed and analyzed by WB.

E HCT116 cells were treated with LGK (10 μ M), recombinant Wnt5a (50 ng/ml) and control or DKK1 conditioned medium (obtained as described in Methods), combined with cisplatin (40 μ M) for 16 h. Total extracts were obtained and analyzed by WB.

F, H MTO cells, either not-transfected or infected with shCtl or shRor2 were treated with LGK (10 μ M), WntC59 (100 nM) or the corresponding controls, supplemented with cisplatin when indicated and analyzed by WB. Quantification of the results of Snail1 analysis from (F and H) is presented in Fig EV3I.

G, I MTO cell viability was determined as above (mean $\pm$ SD of the results of three-four experiments).

Data information: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (unpaired t-test).

Source data are available online for this figure.

stress conditions; Fig EV5a), Snail1 increases the synthesis of Wnt5a and other proteins that participate in this pathway, as Fz2 and Ror2 (Fig EV5b). Once activated, this pathway self-amplifies its response since Wnt5a also increases the synthesis of Snail1 and consequently, of Fz2, Ror2, and Wnt5a itself (Fig EV5b). The existence of self-stimulatory loops has been documented in the pathways controlling Snail1 expression and EMT in different cells (García de Herreros & Baulida, 2012; Zhang *et al*, 2014). Thus, when activated, the elevated levels of Wnt5a, Ror2, Fz2, and Snail1 self-perpetuate the signaling and maintain a tumoral phenotype characterized by higher invasion and chemoresistance.

Another consequence of our results is the involvement of non-canonical Wnt signaling in Snail1-induced EMT in colon cancer cells. Stimulation of Wnt5a by Snail1 has also been observed in epidermoid carcinomas (Ren *et al*, 2011). As our results and this work demonstrate, Snail1 activation of mesenchymal genes does not just involve a higher activity of β -catenin transcriptional activity, as it has been repeatedly described (Solanas *et al*, 2008; Stemmer *et al*, 2008; Freihen *et al*, 2020) but the stimulation of noncanonical Wnt pathway. It remains to be stabilized how Snail1 promotes Ror2, Wnt5a, and Fz2 expression. It is noteworthy that, although Fz2, Ror2, and Wnt5a are induced by Snail1 in tumor cells, as other mesenchymal markers such as fibronectin and α SMA, they are not as sensitive as these to Snail1 depletion, suggesting that their induction by Snail1 is more indirect. Snail1 directly stimulates mesenchymal genes such as FN1 by direct binding to their promoters (Stanisavljevic *et al*, 2011; Hsu *et al*, 2014). It is likely that this is not the case for Fz2 or Ror2 and their expression is consequence of the stimulation of signaling pathways by Snail1 that, although dependent on this factor for its initiation, do not require it for the maintenance, as it has been described for other mesenchymal genes (Baulida & García de Herreros, 2015).

In this article, we also show that Ror2 or Snail1 depletion decreases stem cell markers, indicating a positive relationship between Snail1 and stemness, similarly to what has been reported by other authors (see Mani *et al*, 2008 or Morel *et al*, 2008), although some data from the Hecht laboratory are discrepant (Beyes *et al*, 2019). According to these authors, Snail1 ectopic expression in LS174T colon tumor cells represses the expression of stemness genes. It is possible that, as these authors indicate in their article, “the relationship between EMT and stemness varies in different tumor entities” although it is more likely that the contrary effects depend on the extent of Snail1 expression in the cell lines used. As it has been commented in a recent consensus review (Yang *et al*, 2020), a very extensive EMT as that caused by a high overexpression of Snail1 or other EMT factors might produce different and opposite effects than a partial one, since it decreases cell growth. According to this idea, it is now accepted that a partial EMT is that related to metastasis since it simultaneously increases invasion and stemness features.

Moreover, our results unveil another contribution of EMT to tumor invasion. As presented, tumor cells with high Snail1 and a more mesenchymal phenotype display a higher capability to promote fibroblast activation. Besides producing Wnt5a, these cells also synthesize more TGF β ; accordingly, the levels of active TGF β in the conditioned medium are decreased by Ror2 depletion in HCT116 or SW620 cells and stimulated by Snail1 in HT29 M6 or SW480 cells. This result indicates that EMT, as caused by Snail1 in HT29 M6 cells

or reversed by Ror2 depletion in HCT116 or SW620, confers to tumor cells a greater capability to activate the neighbor microenvironment (Fig EV5c). This has a considerable impact on tumor development since activated fibroblasts enhance epithelial tumor invasion by remodeling the matrix and secreting specific factors (Fig EV5d; Gaggioli *et al*, 2007; Alba-Castellón *et al*, 2016). Moreover, they also affect other tumoral traits, for instance, they prevent tumor infiltration by cytotoxic T-cells (Tauriello *et al*, 2018). An implication of this result is that EMT, even if limited to few cells in a tumor might stimulate invasion through a nonautonomous effect; thus, promoting the activation of CAFs in the tumor microenvironment.

We also investigated the possibility that this secreted TGF β might have an autocrine effect since this factor can stimulate Snail1 transcription in several cellular contexts (Peinado *et al*, 2003; Zavadil *et al*, 2004; Gal *et al*, 2008). However, we have not been able to activate Snail1 expression in the colon cancer cell lines used in our studies adding exogenous TGF β (see Appendix Fig S1B), although Smad2 phosphorylation was detected. This might be related to the deficient TGF β signaling observed in colon tumors, particularly in those most advanced, due to SMAD4 or TGFBR2 loss (Taketo & Edelmann, 2009).

Another finding is the specific stimulation of Fz2 expression by Wnt5a and Snail1. Fz2 is a member of the Fz receptor family capable to stimulate Stat3 phosphorylation through its binding to and the activation of Fyn tyrosine kinase (Gujral *et al*, 2014). This requires the Src-catalyzed phosphorylation of Fz2 at Tyr552 (Gujral *et al*, 2014; Villarreal *et al*, 2020). Among the members of the Fz family, this tyrosine is only conserved in Fz1 and Fz7, suggesting that only these three receptors are capable to activate Stat3. Fz2 is raised by Wnt5a and Snail1 in tumor cells and correlates with the expression of these two genes in colon tumors. It is likely that the elevated expression of this receptor, together with that of Ror2, is essential to self-perpetuate the expression of Snail1 and mesenchymal markers in colon tumors.

PORCN inhibitors such as LGK are in preclinical assays for their use in colon tumors and other neoplasms (Kim *et al*, 2020; Bugter *et al*, 2021). Although many tumor cells with APC mutations increase β -catenin stability independent of Wnt action, canonical factors also promote other responses required for a full β -catenin transcriptional effect, such as those required for β -catenin translocation to the nucleus (García de Herreros & Duñach, 2019). Therefore, inhibitors of canonical Wnt secretion might affect APC-mutated tumors as reported previously (Liu *et al*, 2013; Madan *et al*, 2016). However, our data suggest that their main impact, at least in a subset of APC-mutated tumors, the most mesenchymal ones, is related to their capability to inhibit the action of noncanonical Wnts and to prevent the expression of Snail1, with subsequent effects on tumor initiation, chemoresistance, and stroma activation. Therefore, PORCN inhibitors might be combined with chemotherapeutic agents in colon tumor treatment.

Materials and Methods

Antibodies and reagents

The following antibodies were used in this study: Ror2 and Stat3 (from Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-374174

and sc-8019, respectively); phospho-Stat3 (Tyr705) (pStat3), Snail1, Ror1, phosphor-Smad2 (Ser465/467) (pSmad2), Smad2/3, phosphor-Chk1 (Ser345) (pChk1), Caspase 3 and LRP5/6 (from Cell Signaling, Danvers, MA, USA; 9145S, 3879, 4102S, 3108, 8685, 23489661S and 2560S); Fz2, Lamin B, Fibronectin and Jnk2 (from Abcam, Cambridge, UK; 52565, 16048, 2413 and 178953); β -actin, α -Smooth Muscle Actin (α SMA) and human nuclei (clone 3E1.3) (from Sigma, St. Louis, MO, USA; A5441, A2547, and MAB4383); Vimentin (from BD-Pharmingen, San Diego, CA, USA; 550513); HA (Roche, Basilea, Switzerland; 11867423001, clone 3F10); phospho-Jnk2 (Thr183/Tyr185, Thr221/Tyr223) (pJnk2) (Millipore, Billerica, MA, USA; 07-175), Wnt5a (from R&D System, Minneapolis, MN, USA; AF645). Other reagents: SB505124 (from Sigma; S4696), S31-201, LGK974, and LY2109761 (from Selleckchem, Houston, TX, USA; S1155, S7143, and S2704), JNK inhibitor II (from Calbiochem, Darmstadt, Germany; 420119); WntC59 (from MedChemExpress, Monmouth Junction, NJ, USA; HY-15659).

Cell culture

Authenticated cell lines (HEK293, SW480, SW620, HCT116, LS174, Caco2, DLD12, LoVo, and HT29 M6) were obtained from the European Collection of Authenticated Cell Cultures or the American Type 1 Culture Collection (ATCC) and supplied by the Cancer Cell Line Repository from IMIM. The generation and use of HT29 M6 and SW480 cells transfected with Snail1 has been reported (Batlle *et al*, 2000; Solanas *et al*, 2008). Murine mesenchymal stem cells (MSC) were obtained as indicated (Alba-Castellón *et al*, 2016). All cell lines were used for no more than 20 passages and routinely tested by PCR to verify that they remained Mycoplasma-free. Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 4.5 g/l glucose, 1 mM sodium pyruvate, 2 mM glutamine, 100 IU/ml penicillin, 100 mg/l streptomycin, and 10% fetal calf serum (FCS; Gibco) and maintained at 37°C in a humid atmosphere containing 5% CO₂. Assays were performed with cells at 60–70% confluency.

Stimulation of the noncanonical pathway by Wnt5a was performed adding conditioned medium from L fibroblasts control or stably transfected with a plasmid encoding Wnt5a (both obtained from the ATCC, ref. CRL-2648 and CRL-2814, respectively; Manassas, VA, USA). Alternatively, cells were treated with recombinant Wnt5a (from R&D; 645-WN) at 50 ng/ml. DKK1 was prepared from cells transfected with pcDNA3-DKK1, gently provided by Dr. A. Muñoz, CIB-CSIC, Madrid, Spain.

Mouse tumor organoids (MTO) derived from colon tumors with APC, TGFB receptor 2 and p53 deletions and a Kras-Gly12Asp activating mutation (LAKTP cells), and transfected with Luciferase were kindly provided by Dr. E Batlle (Institut de Recerca Biomèdica de Barcelona; Tauriello *et al*, 2018). These cells were cultured embedded in Matrigel in MTO culture medium (advanced DMEM/F12, GIBCO), supplemented with B27 without retinoic acid (Thermo Fisher 12587010) and 10 mM HEPES pH 8 (Sigma), 2 mM L-Glutamine, 50 ng/ml recombinant human EGF (MedChemExpress), 100 ng/ml recombinant murine Noggin (MedChemExpress) and 1 μ M galunisertib (LY2157299, Selleckchem). For MTO passaging, Matrigel drops were washed twice with Hanks' Balanced Salt Solution (HBSS) (137 mM NaCl, 5.4 mM KCl, 0.25 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 0.1 g/l D-Glucose, 4.2 mM NaHCO₃) and treated

with Cell Recovery Solution (Corning, 354253) for 10 min at 37°C. Cells were centrifuged at 500 g and treated with Trypsin–EDTA (Sigma) for 10 min at 37°C, followed by mechanical disaggregation of organoid fragments (by pipetting) until a single-cell suspension was obtained. Cells were centrifuged at 500 g and seeded in Matrigel. After polymerization at 37°C, MTO culture medium was added to the drops and cells cultured at 37°C with 5% CO₂.

Cell transfection and selection of transfectants

Human shRNAs specific for *ROR2* (TRCN0000001492, 5'-CGACAA GCTGAACGTGAAGAT-3'; and TRCN00000010625, 5'-GCACAG CCCAAATCATAACTT-3'; considered #1 and #2, respectively, in this article) and shRNA control (5'-CGCTGAGTACTTCGAAATGTC-3') were obtained from Mission shRNA, Sigma St. Louis, MO, USA. For stable expression of shRNA in HCT116 and SW620 cells, the indicated shRNAs were co-transfected with phrGFP using PEI (Polyethylenimine, Polysciences, Inc; Niles, IL, USA); cells were grown for 2 weeks and sorted by BD FACS Aria Fusion Cell Sorter (BD Biosciences, San Jose, CA, USA) at the Parc de Recerca Biomèdica de Barcelona (PRBB) FACS facility. Most of experiments were performed with shRor2 #1, otherwise indicated. Downregulation of the Ror2 was analyzed after cell sorting by Western blot (WB). Ror2 overexpression was achieved by transfecting pcDNA3-Ror2-HA (murine, kindly provided by Dr. Y. Minami, Kobe University, Japan). Snail1 downregulation was performed using SNAIL1-specific small interfering RNA (siRNA) (Dharmacon, Lafayette, CO, USA, L-010847-01-0005) or siRNA control (D-001810-02-50). Cells were transfected using DharmaFECT transfection agent (Dharmacon) with 0.2 nmols of siRNA control (siCtl) or Snail1 siRNA (siSnail1), according to the manufacturer's instructions. Experiments were performed 48 h after transfection. For Snail1 overexpression experiments, ectopic pcDNA3-Snail1-HA was transiently overexpressed in shCtl and shRor2 SW620 and HCT116 cells using PEI. Cells were analyzed 48 h after transfection.

For the generation of MTOs deficient in shRor2 expression, MTOs were infected with lentiviral particles containing an shRNA targeting Ror2 (or a nontargeting shRNA) and mCherry. Infected cells were selected with puromycin at 1 mg/ml. Cells were grown for 2 weeks, sorted by BD FACS Aria Fusion Cell Sorter (BD Biosciences, San Jose, CA, USA) at the Parc de Recerca Biomèdica de Barcelona (PRBB) FACS facility and analyzed by WB.

Human tumor bioinformatic analysis

The co-expression data of mRNA and protein and survival curves were obtained from TCGA Colorectal Adenocarcinoma (Firehose Legacy) data in the cBioPortal public database (<http://www.cbioportal.org/>) on May 2020 (Cerami *et al*, 2012; Gao *et al*, 2013). In this study, we assessed the mRNA expression of *ROR2*, *SNAIL1*, *LRP5*, *LRP6*, *MMP9*, *MMP13*, *WNT5A*, *TGFB1*, *AXIN2*, *FZD2*, *TWIST1*, and *USP17L2* (DUB3). Protein expression of Snail1 and β -catenin was also studied. For the survival curves, a patient group with high expression of *ROR2*, *SNAIL1*, *FZD2*, *WNT5A*, *MMP13*, *ZEB1*, and *ACTA2* was generated. The correlation of *SNAIL1* association with *ROR2*, *WNT5A*, *LRP5*, or *WNT3A* was also studied in different human tumors from TCGA (Firehose Legacy) data in the cBioPortal public database (<http://www.cbioportal.org/>) on May

2020. Spearman coefficient was represented in a heatmap graph using GraphPad software.

Mouse tumorigenesis experiments

Animals were maintained in a specific pathogen-free area at the PRBB Animal Facility. All the procedures were approved by the Animal Research Ethical Committee from the PRBB and by the Generalitat de Catalunya (CEEAGH-19-0028) following the EU Directive 2010/63/EU. For heterotopic xenografting, 8-week-old NOD/SCID mice or C57BL/6J (males, from Charles River Laboratories, Wilmington, MA, USA) were subcutaneously inoculated on their hind leg flanks with 5×10^5 cells LAKTP grown as MTOs or 10^5 SW620 cells and monitored daily for tumor growth. When tumors got a prestabilized size, mice were euthanized and tumors fixed by 4% formaldehyde and embedded in paraffin for hematoxylin and eosin staining and immunohistochemistry. For metastases analysis, 5×10^5 LAKTP cells, expressing luciferase and grown as MTOs, were diluted in cold 3:1 HBSS-Matrigel (growth factor reduced) (100 μ l) and injected in the spleen of C57BL/6J mice (Charles River Laboratories). Mice were analyzed for *in vivo* bioluminescent determination by using the IVISTM SD Imaging System Xenogen Imaging Technologies and the Living Image software 2.60.2. Bioluminescent quantification was performed after intraperitoneal inoculation of 100 μ l of luciferin (Perkin Elmer) solution (25 mg/ml). When indicated WntC59 (10 mg/kg/day) or vehicle were added to the drinking water.

Preparation and purification of Ror2-extracellular domain (R-ECD)

The extracellular domain of Ror2 (amino acids 1–404, R-ECD) was amplified from pcDNA-mRor2, using the primers 5'-GGTGGAAAGCTTATGCTCGGGGC-3' (forward) and 5'-GGTGGTGCGGCCGCAACAGAATCC-3' (reverse), containing HindIII and NotI restriction sites, respectively. The PCR product was purified, digested with HindIII and NotI and inserted into pcDNA6 myc-His eukaryotic expression vector (Invitrogen, Waltham, MA, USA). For R-ECD production, HEK293 cells were transfected with pcDNA6-R-ECD-myc-His using polyethylenimine. After the transfection, the HEK293 medium was replaced by DMEM plus FBS (10%) and the conditioned medium was collected after 24 h. Conditioned medium was adjusted to 20 mM Tris-HCl pH 8.2 and 300 mM NaCl (binding buffer), and R-ECD was purified by affinity chromatography on Nickel-NTA beads (Qiagen, Hilden, Germany). After binding for 1 h at 4°C, beads were washed with binding buffer plus 5 mM imidazole and eluted in the same buffer plus 250 mM imidazole. R-ECD concentration and purity in the eluate was assessed by SDS-gel electrophoresis and Coomassie staining.

Cell viability assays

Cell proliferation and cell viability in cell lines were routinely analyzed by MTT assay. For cell viability assays, 6,000 cells were seeded in 96-well plates, and after 24 h, cells were treated with the drugs at different concentrations for 24 h. For cell proliferation assays, 4,000 cells were seeded. Cell viability and proliferation was measured 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 h at 37°C. After incubation, cells were solubilized in DMSO: isopropanol (1:4) and the absorbance at 590 nm of the solution was quantified in an Infinite M200 microplate reader (Tecan).

Alternatively, MTOs viability experiments were performed with Celltiter Glo (Promega). Four thousand MTO cells were seeded in Matrigel in standard MTOs culture conditions in 96-well plates. Three days later, the assay was performed by adding 15 μ l of Celltiter Glo in each well and analyzing luminescence in a FB-12 luminometer (Berthold Detection Systems).

For 2D colony formation assays, 1,000 cells were seeded in 6-well plates and allowed to grow for 10 days in DMEM plus 1% or 10% FBS. Then, cells were fixed and stained with a buffer containing 30% methanol and 0.5% Crystal violet for 20 min. After incubation, cells were washed with PBS and the number of colonies was quantified with ImageJ software (Schindelin *et al*, 2012). For 3D soft agar colony formation assays, 6-well plates were firstly coated with a mix of agar (1%) in DMEM (supplemented with NaHCO₃, NaCl, FBS and antibiotics) and incubated for 30 min at room temperature to allow agar polymerization. After incubation, a mix of 0.6% agar and DMEM (10% in FBS) containing 5,000 cells/well was added on top of the 1% agar. The mix was incubated for 30 min again to allow agar polymerization. Fresh DMEM 10% was added twice weekly on top to avoid agar desiccation. After 21 days of growth, the experiments were stopped and the MTT assay was performed. Pictures of the colonies were taken, and colonies were quantified with ImageJ software.

Colon tumor sphere formation was assessed by growing cells in mouse tumor organoid culture medium as indicated for MTO cells.

Migration and invasion assays

HCT116 and SW620 cells (10^5) were resuspended in 150 μ l DMEM plus BSA (0.1%) and FBS (0.1%). Cells were seeded on a Transwell filter chamber (Costar 3422, Thermo Fisher Scientific, Waltham, MA, USA) without coating (migration) or coated with 0.5 mg/ml Matrigel (BD Biosciences, 354230). After 30 min at 37°C, the lower chamber was filled with DMEM plus FCS (10%), which was used as chemoattractant. Assays were stopped at 48 h; cells that have not penetrated in the lower chamber were then removed from the upper surface of the membrane, while cells that adhered to the lower surface were fixed with 100% methanol for 20 min and stained with Crystal Violet. Cells were eluted with 30% acetic acid, and the OD was measured at 590 nm. In co-culture invasion experiments, GFP-labeled MSC (2×10^4) were seeded with HCT116 or SW620 cells (8×10^4). Assays were performed as above, and invasion was stopped at 16 h; cells were washed with PBS and fixed with p-formaldehyde (4%) for 20 min. Five random photographs (10 $\times$) of each membrane were taken to analyze the area of invasion. Only GFP-labeled cells were analyzed with ImageJ software.

Immunohistochemical analysis of tumors

Samples were fixed with p-formaldehyde (4%) at room temperature. Fixed samples were dehydrated and paraffin-embedded according to standard procedures. Sections (2.5 μ m) were prepared and stained with hematoxylin and eosin for histological evaluation. After

standard deparaffinization and rehydration of the samples, antigen retrieval was carried immersing the sections in Tris-EDTA buffer pH 9 or citrate buffer pH 6 and boiling for 15 min. Samples were blocked during 2 h in Tris-buffered saline (TBS) plus FBS (1%) and BSA (1%) and incubated with anti-human nuclei (1/100), anti-Vimentin (1/200) or anti-fibronectin (1/100) primary antibodies overnight. Signal was amplified with EnVision+ System HRP Labelled Polymer (anti-mouse or anti-rabbit, DAKO) and visualized with the DAB kit (DAKO).

To evaluate vimentin and fibronectin staining, a minimum of three different areas per tumor were imaged and stromal area was manually delimited. Percentage of vimentin- or fibronectin-positive area within stroma was calculated with ImageJ and represented. For evaluation of invasion, the medial section of each tumor, six per condition, was obtained and HE stained. Invasion of tumor epithelial cells toward adjacent muscle was determined. No blinding was done in these determinations.

RNA isolation and analysis

RNA was obtained using RNeasy Mini Kit (Qiagen, 74106), retro-transcribed, and analyzed by quantitative PCR in triplicate as in Solanas *et al* (2008) using *HPRT* as control in a SYBR Green LightCycler 480 Real Time System (Roche) at the PRBB Genomic Facility. The list of the primers used is provided in Appendix Table S1.

Promoter activity assays

Reporter assays were carried out by transfecting 50 ng of two *SNAI1* promoter fragments (Barberà *et al*, 2004) cloned in pGL3* basic vector (Promega). Cells were co-transfected with 1 ng of SV40-Renilla luciferase plasmid as control for transfection efficiency. Expression of Firefly and Renilla luciferases was analyzed 48 h after transfection, according to the manufacturer's instructions in a FB-12 luminometer (Berthold Detection Systems, Bad Wildbad, Germany). Alternatively, for the determination of TGF β response, HEK293T cells were infected with TGF β /SMAD Luciferase Reporter Lentivirus (Kerafast, Boston MA, USA). The mean $\pm$ SD of the results of three independent transfections is given.

Chromatin immunoprecipitation (ChIP) assays

1×10^7 cells were crosslinked with 1% formaldehyde and incubated with 0.125 M glycine to stop the reaction. Cells were lysed in 50 mM Tris-HCl pH 8, 2 mM EDTA, 0.1% NP40 and 10% glycerol supplemented with a protease inhibitor cocktail and centrifuged at 800 g for 5 min. Pellets were resuspended in nuclear lysis buffer (50 mM Tris-HCl pH 8, 1% SDS, 10 mM EDTA, and protease inhibitor cocktail) and sonicated to generate 200 to 1,500 bp DNA fragments. Samples were diluted 1:10 (16.7 mM Tris-HCl pH 8, 1.2 mM EDTA, 167 mM NaCl, 1.1% Triton X-100 and 0.01% SDS), pre-cleared with Protein-G-agarose for 3 h, and immunoprecipitated with antibodies against Stat3 (Santa Cruz Biotechnology), c-jun (Cell Signaling) and irrelevant mouse or rabbit IgGs as control. DNA-protein complexes were pelleted with protein-G-agarose. Then, samples were treated with elution buffer (100 mM NaCO₃ and 1% SDS) and incubated at 37°C 1 h. Then, 200 mM NaCl was added and immunoprecipitates de-crosslinked by incubation at 65°C

overnight followed by digestion with proteinase K for 1 h at 55°C. DNA was purified using the MinElute PCR purification kit (Qiagen), and promoter regions were detected by qPCR amplification using two pairs of oligonucleotides corresponding to the distal (−659/+569; forward: 5'-AAAGGCCGTGGCATTTCAG-3'; reverse: 5'-CAT TGACGAGGGAACGCAC-3') or proximal (−231/−155; forward: 5'-CACCTGCTCGGGGAGTGG-3'; reverse: 5'-TATCTGCCACGCCCT TTGT-3') regions of *SNAI1* human promoter.

Cell lysis and protein analysis by western blot

Cell extracts were obtained in SDS lysis buffer (Tris-HCl pH 7.5, 25 mM; NaCl, 150 mM and SDS 1%) and analyzed by WB using the indicated primary antibodies and HRP-conjugated secondary antibodies. WB quantification was performed using ImageJ software. Protein levels were quantified and corrected by actin levels, or by total protein levels in case of phosphorylated proteins, and referred to control cells or control treatment depending on the experiment. Quantification is shown as the mean of at least three different experiments $\pm$ SD.

Statistical analysis

All results shown were representative from at least three independent experiments. Data are presented as mean $\pm$ SD. When appropriate, statistical analyses were conducted using Excel software (Microsoft, Redmond, WA, USA) and data were analyzed for significance using the 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.

Data availability

No primary datasets have been generated and deposited.

Expanded View for this article is available [online](#).

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Author contributions

Guillem Fuertes: Conceptualization; data curation; formal analysis; validation; investigation; visualization. **Beatriz DelValle-Pérez:** Conceptualization; formal analysis; validation; investigation; visualization. **Javier Pastor:** Investigation; visualization. **Evelyn Andrades:** Investigation. **Raúl Peña:** Formal analysis; investigation; methodology; project administration. **Antonio García de Herreros:** Conceptualization; resources; formal analysis; supervision; funding acquisition; visualization; writing—original draft; writing—review and editing. **Mireia Dunach:** Conceptualization; resources;

formal analysis; supervision; funding acquisition; writing—original draft; writing—review and editing.

Disclosure and competing interests statement

The authors declare that they have no conflict of interest.

References

- Alba-Castellón L, Olivera-Salguero R, Mestre-Farrera A, Peña R, Herrera M, Bonilla F, Casal JI, Baulida J, Peña C, García de Herreros A (2016) Snail1-dependent activation of cancer-associated fibroblast controls epithelial tumor cell invasion and metastasis. *Cancer Res* 76: 6205–6217
- Bandyopadhyay A, Agyin JK, Wang L, Tang Y, Lei X, Story BM, Cornell JE, Pollock BH, Mundy GR, Sun LZ (2006) Inhibition of pulmonary and skeletal metastasis by a transforming growth factor- β type I receptor kinase inhibitor. *Cancer Res* 66: 6714–6721
- Barberà MJ, Puig I, Domínguez D, Julien-Grille S, Guaita-Esteruelas S, Peiró S, Baulida J, Francí C, Dedhar S, Larue L et al (2004) Regulation of snail transcription during epithelial to mesenchymal transition of tumor cells. *Oncogene* 23: 7345–7354
- Batlle E, Sancho E, Francí C, Domínguez D, Monfar M, Baulida J, García de Herreros A (2000) The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cell. *Nat Cell Biol* 2: 84–89
- Baulida J, García de Herreros A (2015) Snail1-driven plasticity of epithelial and mesenchymal cells sustains cancer malignancy. *Biochim Biophys Acta* 1856: 55–61
- Baulida J, Díaz VM, García de Herreros A (2019) Snail1: a transcriptional factor controlled at multiple levels. *J Clin Med* 8: 757
- Beyes S, Andrieux G, Schrempp M, Aicher D, Wenzel J, Antón-García P, Boerries M, Hecht A (2019) Genome-wide mapping of DNA-binding sites identifies stemness-related genes as directly repressed targets of SNAIL1 in colorectal cancer cells. *Oncogene* 38: 6647–6661
- Bugter JM, Fenderico N, Maurice MM (2021) Mutations and mechanism of WNT pathway tumour suppressors in cancer. *Nat Rev Cancer* 21: 5–21
- Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson L et al (2012) The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov* 2: 401–404
- Chen B, Dodge ME, Tang W, Lu J, Ma Z, Fan CW, Wei S, Hao W, Kilgore J, Williams NS et al (2009) Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. *Nat Chem Biol* 5: 100–107
- DaCosta Byfield S, Major C, Laping NJ, Roberts AB (2004) SB-505124 is a selective inhibitor of transforming growth factor- β type I receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* 65: 744–752
- Díaz VM, García de Herreros A (2016) F-box proteins: keeping the epithelial-to-mesenchymal transition (EMT) in check. *Semin Cancer Biol* 36: 71–79
- Dissanayake SK, Wade M, Johnson CE, O'Connell MP, Leotlela PD, French AD, Shah KV, Hewitt KJ, Rosenthal DT, Indig FE et al (2007) The Wnt5A/protein kinase C pathway mediates motility in melanoma cells via the inhibition of metastasis suppressors and initiation of an epithelial to mesenchymal transition. *J Biol Chem* 282: 17259–17271
- Feng Y, Ma Z, Pan M, Xu L, Feng J, Zhang Y, Shao C, Guo K, Duan H, Zhang Y et al (2022) Wnt5a promotes the metastasis of esophageal squamous cell carcinoma by activating the HDAC7/SNAIL signaling pathway. *Cell Death Dis* 13: 480
- Freihen V, Rönsch K, Mastroianni J, Frey P, Rose K, Boerries M, Zeiser R, Busch H, Hecht A (2020) SNAIL1 employs β -catenin-LEF1 complexes to control colorectal cancer cell invasion and proliferation. *Int J Cancer* 146: 2229–2242
- Gaggioli C, Hooper S, Hidalgo-Carcedo C, Grosse R, Marshall JF, Harrington K, Sahai E (2007) Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. *Nat Cell Biol* 9: 1392–1400
- Gal A, Sjöblom T, Fedorova L, Imreh S, Beug H, Moustakas A (2008) Sustained TGF β exposure suppresses Smad and non-Smad signalling in mammary epithelial cells, leading to EMT and inhibition of growth arrest and apoptosis. *Oncogene* 27: 1218–1230
- Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E et al (2013) Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal* 6: pl1
- García de Herreros A, Baulida J (2012) Cooperation, amplification, and feedback in epithelial-mesenchymal transition. *Biochim Biophys Acta* 25: 223–228
- García de Herreros A, Duñach M (2019) Intracellular signals activated by canonical Wnt ligands independent of GSK3 inhibition and β -catenin stabilization. *Cell* 8: E1148
- Grotegut S, von Schweinitz D, Christofori G, Lehenbre F (2006) Hepatocyte growth factor induces cell scattering through MAPK/Egr-1-mediated upregulation of snail. *EMBO J* 25: 3534–3545
- Grumolato L, Liu G, Mong P, Mudbhary R, Biswas R, Arroyave R, Vijayakumar S, Economides AN, Aaronson SA (2010) Canonical and noncanonical Wnts use a common mechanism to activate completely unrelated receptors. *Genes Dev* 24: 2517–2530
- Guinney J, Dienstmann R, Wang X, de Reyniès A, Schlicker A, Soneson C, Marisa L, Roepman P, Nyamundanda G, Angelino P et al (2015) The consensus molecular subtypes of colorectal cancer. *Nat Med* 21: 1350–1356
- Gujral TS, Chan M, Peshkin L, Sorger PK, Kirschner MW, MacBeath GA (2014) A noncanonical Frizzled2 pathway regulates epithelial-mesenchymal transition and metastasis. *Cell* 159: 844–856
- Hsu DSS, Wang HJ, Tai SK, Chou CH, Hsieh CH, Chiu PH, Chen NJ, Yang MH (2014) Acetylation of snail modulates the cytokinome of cancer cells to enhance the recruitment of macrophages. *Cancer Cell* 26: 534–548
- Huang TC, Peng KC, Kuo TT, Lin LC, Liu BC, Ye SP, Chu CC, Hsia SM, Chang HY (2021) Predicting agents that can overcome 5-FU resistance in colorectal cancers via pharmacogenomic analysis. *Biomedicine* 9: 882
- Kaufhold S, Bonavida B (2014) Central role of Snail1 in the regulation of EMT and resistance in cancer: a target for therapeutic intervention. *J Exp Clin Cancer Res* 33: 62
- Kikuchi A, Yamamoto H, Sato A, Matsumoto S (2012) Wnt5a: its signalling, function and implications in disease. *Acta Physiol* 204: 17–33
- Kim MJ, Huang Y, Park IL (2020) Targeting Wnt signaling for gastrointestinal cancer therapy: present and evolving views. *Cancer* 12: 3638
- Lambies G, Miceli M, Martínez-Guillamon C, Olivera-Salguero R, Peña R, Frías CP, Calderón I, Atanassov BS, Dent SYR, Arribas J et al (2019) TGF β -activated USP27X Deubiquitinase regulates cell migration and chemoresistance via stabilization of Snail1. *Cancer Res* 79: 33–46
- Liu J, Pan S, Hsieh MH, Ng N, Sun F, Wang T, Kasibhatla S, Schuller AG, Li AG, Cheng D et al (2013) Targeting Wnt-driven cancer through the inhibition of porcupine by LGK974. *Proc Natl Acad Sci U S A* 110: 20224–20229
- MacDonald BT, He X (2013) Frizzled and LRP5/6 receptors for Wnt/ β -catenin signaling. *Cold Spring Harb Perspect Biol* 4: a007880

- Madan B, Ke Z, Harmston N, Ho SY, Frois AO, Alam J, Jeyaraj DA, Pendharkar V, Ghosh K, Virshup IH et al (2016) Wnt addiction of genetically defined cancers reversed by PORCN inhibition. *Oncogene* 35: 2197–2207
- Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M et al (2008) The epithelial–mesenchymal transition generates cells with properties of stem cells. *Cell* 133: 704–715
- Merlos-Suárez A, Barriga FM, Jung P, Iglesias M, Céspedes MAV, Rossell D, Sevillano M, Hernando-Momblona X, Da Silva-Diz V, Muñoz PN et al (2011) The intestinal stem cell signature identifies colorectal cancer stem cells and predicts disease relapse. *Cell Stem Cell* 8: 511–524
- Morel AP, Lièvre M, Thomas C, Hinkal G, Ansieau S, Puisieux A (2008) Generation of breast cancer stem cells through epithelial–mesenchymal transition. *PLoS One* 3: e2888
- Nusse R, Clevers H (2017) Wnt/ β -catenin signaling, disease and emerging therapeutic modalities. *Cell* 169: 985–999
- Pashirzad M, Sathyapalan T, Sahebkar A (2021) Clinical importance of Wnt5a in the pathogenesis of colorectal cancer. *J Oncol* 2021: 3136508
- Peinado H, Quintanilla M, Cano A (2003) Transforming growth factor beta-1 induces snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions. *J Biol Chem* 278: 21113–21123
- Ren D, Minami Y, Nishita M (2011) Critical roles of Wnt5a-Ror2 signaling in motility and invasiveness of carcinoma cells following snail-mediated epithelial–mesenchymal transition. *Genes Cells* 16: 304–315
- Rowan AJ, Lamlum H, Ilyas M, Wheeler J, Straub J, Papadopolou D, Bicknell D, Bodmer WF, Tomlinson IPM (2000) APC mutations in sporadic colorectal tumors: a mutational “hotspot” and interdependence of the “two hits”. *Proc Natl Acad Sci U S A* 97: 3352–3357
- Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B et al (2012) Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9: 676–682
- Solanas G, Porta-de-la-Riva M, Agustí C, Casagolda D, Sánchez-Aguilera F, Larriba MJ, Pons F, Peiró S, Escrivà MJ, Muñoz A et al (2008) E-cadherin controls beta-catenin and NF-kappaB transcriptional activity in mesenchymal gene expression. *J Cell Sci* 121: 2224–2234
- Sonego M, Pellarin I, Costa A, Vinciguerra GLR, Coan M, Kraut A, D'Andrea S, Dall'Acqua A, Castillo-Tong DC, Califano D et al (2019) USP1 links platinum resistance to cancer cell dissemination by regulating snail stability. *Sci Adv* 5: eaav3235
- Stanisavljevic J, Porta-de-la-Riva M, Batlle R, García de Herreros A, Baulida J (2011) The p65 subunit of NF- κ B and PARP1 assist Snail1 in activating fibronectin transcription. *J Cell Sci* 124: 4161–4171
- Stemmer V, deCraene B, Berx G, Behrens J (2008) Snail promotes Wnt target gene expression and interacts with beta-catenin. *Oncogene* 27: 5075–5080
- Sun M, Guo X, Qian X, Wang H, Yang C, Brinkman KL, Serrano-Gonzalez M, Joje RS, Zhou B, Engler DA et al (2012) Activation of the ATM–snail pathway promotes breast cancer metastasis. *J Mol Cell Biol* 4: 304–315
- Taketo MM, Edelmann W (2009) Mouse models of colon cancer. *Gastroenterology* 136: 780–798
- Tauriello DVF, Palomo-Ponce S, Stork D, Berenguer-Llergo A, Badia-Ramentol J, Iglesias M, Sevillano M, Ibiza S, Cañellas A, Hernando-Momblona X et al (2018) TGF β drives immune evasion in genetically reconstituted colon cancer metastasis. *Nature* 554: 538–543
- Van Amerongen R, Mikels A, Nusse R (2008) Alternative Wnt signaling is initiated by distinct receptors. *Sci Signal* 1: re9
- Villarreal A, Del Valle-Pérez B, Fuertes G, Curto J, Ontiveros N, García de Herreros A, Duñach M (2020) Src and Fyn define a new signaling cascade activated by canonical and non-canonical Wnt ligands and required for gene transcription and cell invasion. *Cell Mol Life Sci* 77: 919–935
- Viñas-Castells R, Frias A, Robles-Lanuza E, Zhang K, Longmore GD, García de Herreros A, Diaz VM (2014) Nuclear ubiquitination by FBXL5 modulates Snail1 DNA binding and stability. *Nucleic Acids Res* 42: 1079–1094
- Voloshanenko O, Schwartz U, Kranz D, Rauscher B, Linnebacher M, Augustin I, Boutros M (2018) β -Catenin-independent regulation of Wnt target genes by Ror2 and ATF2/ATF4 in colon cancer cells. *Sci Rep* 16: 3178
- Wu X, Tu X, Joeng KS, Hilton MJ, Williams DA, Long F (2008) Rac1 activation controls nuclear localization of beta-catenin during canonical Wnt signaling. *Cell* 133: 340–353
- Wu Y, Wang Y, Lin Y, Liu Y, Wang Y, Jia J, Singh P, Chi YI, Wang C, Dong C et al (2017) Dub3 inhibition suppresses breast cancer invasion and metastasis by promoting Snail1 degradation. *Nat Commun* 8: 14228
- Xiao Z, Chang L, Kim J, Zhang P, Hang Q, Yap S, Guo Y, Zhou Z, Zeng L, Hu X et al (2019) USP37 is a SNAI1 deubiquitinase. *Am J Cancer Res* 9: 2749–2759
- Yamanaka H, Moriguchi T, Masuyama N, Kusakabe M, Hanafusa H, Takada R, Takada S, Nishida E (2002) JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates. *EMBO Rep* 3: 69–75
- Yang J, Antin P, Berx G, Blanpain C, Brabletz T, Bronner M, Campbell K, Cano A, Casanova J, Christofori G et al (2020) Definitions and guidelines for research on epithelial–mesenchymal transition. *Nat Rev Mol Cell Biol* 21: 341–352
- Yook JI, Li XY, Ota I, Fearon ER, Weiss SJ (2005) Wnt-dependent regulation of the E-cadherin repressor snail. *J Biol Chem* 280: 11740–11748
- Zavadil J, Cermak L, Soto-Nieves N, Böttinger EP (2004) Integration of TGF- β /Smad and Jagged1/notch signalling in epithelial-to-mesenchymal transition. *EMBO J* 23: 1155–1165
- Zhang J, Tian XJ, Zhang H, Teng Y, Li R, Bai F, Elankumaran S, Xing J (2014) TGF- β -induced epithelial-to-mesenchymal transition proceeds through stepwise activation of multiple feedback loops. *Sci Signal* 7: ra91



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Expanded View Figures

Figure EV1. Ror2 modulates *SNAI1* transcription and *Snail1* protein stability.

- A Diagram of the two *SNAI1* promoter fragments indicating the position of the Stat3 and AP1 binding elements.
- B Promoter activity was analyzed in HCT116 and SW620 cells and represented with respect to the activity of the –869/+59 promoter for each cell line. Cells were treated with JNKi (50 μ M) and Stat3 inhibitor (S3I-201; 25 μ M) for 16 h.
- C A ChIP assay was performed as detailed in Methods in the indicated cells using c-Jun or Stat3 antibodies and an irrelevant IgG as control. Binding to *SNAI1* promoter was determined using primers specific for the distal (–659/+569; right panel) or proximal (–231/–155; left panel) parts of this promoter in order to assess Stat3 or c-Jun binding, respectively.
- D RNA was isolated from control and Ror2-depleted SW620 and HCT116 cells. Expression of *Snail1* ubiquitin ligases and *Snail1* deubiquitinases was assessed by qRT–PCR.

Data information: In (B–D), the results are presented as mean $\pm$ SD from three (B, C) or three to five (D) biological replicates performed in triplicate. ns, not significant; * $P < 0.05$; *** $P < 0.001$ (unpaired t -test). The dashed line in D corresponds to the RNA value in control cells.

Source data are available online for this figure.

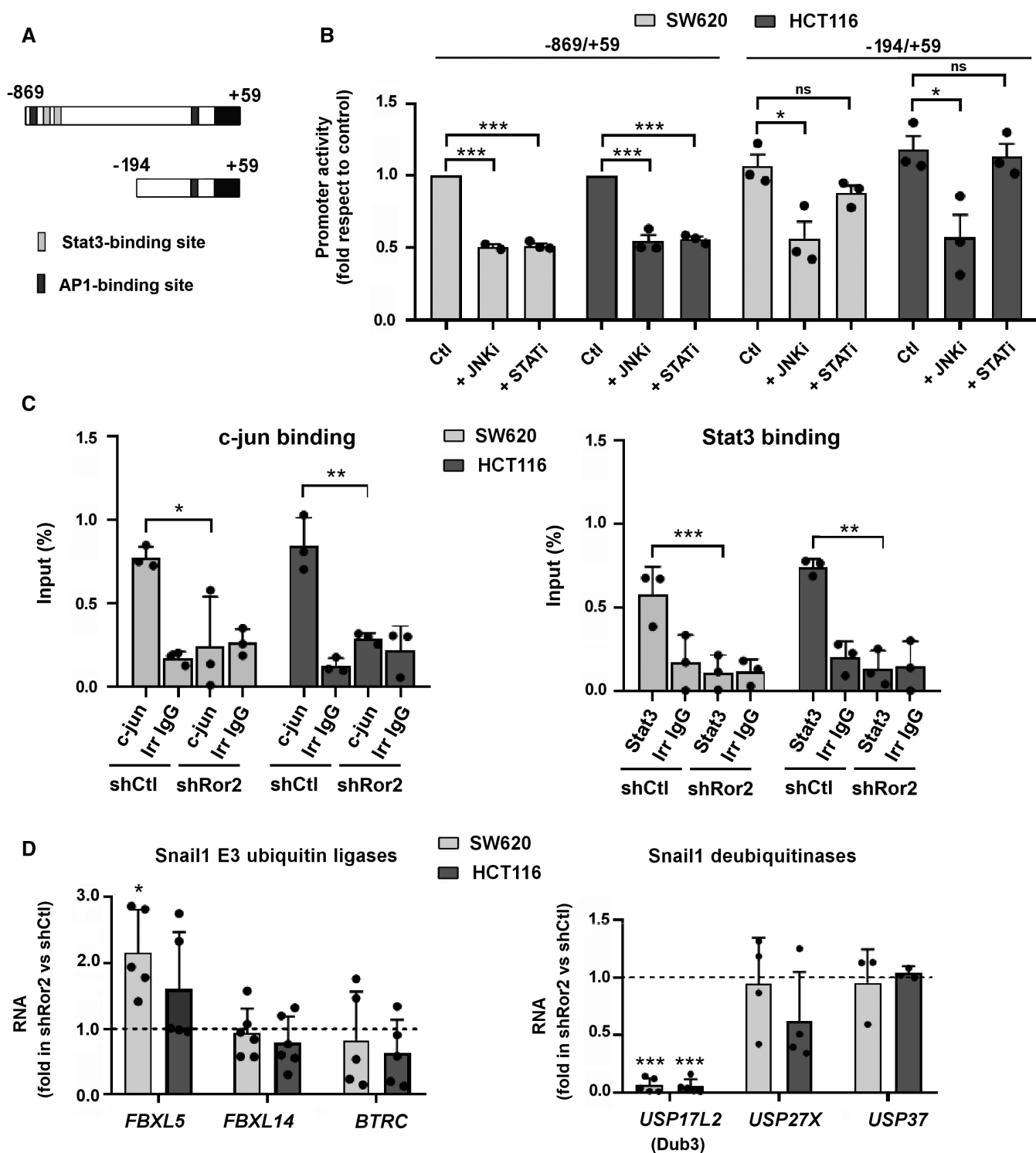


Figure EV1.

Figure EV2. Noncanonical Wnt stimulates TGF β production by tumor cells and fibroblast activation.

- A RNA was isolated from HCT116 and SW620 control or treated with LGK for 16 h. Expression of *TGFB2* and *TGFB3* was assessed by qRT-PCR. The figure shows the mean $\pm$ SD of three biological replicates performed in triplicate.
- B Conditioned medium from the indicated cells was obtained and used to stimulate HEK293T infected with TGF/SMAD reporter (mean $\pm$ SD of three biological replicates performed in triplicate).
- C–F Conditioned medium was also added to MSC cells previously transfected with shRor2 or shCtl when indicated. TGF β receptor inhibitor SB (5 μ M) was supplemented when mentioned. Cells were lysed and proteins analyzed by WB. Quantification of the experiments (mean $\pm$ SD of three biological replicates) is shown at the lower panel (for E) and in Appendix Fig S6A (for C).

Data information: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (unpaired t-test). The dashed line in (A) corresponds to the RNA value in control not-treated cells.

Source data are available online for this figure.

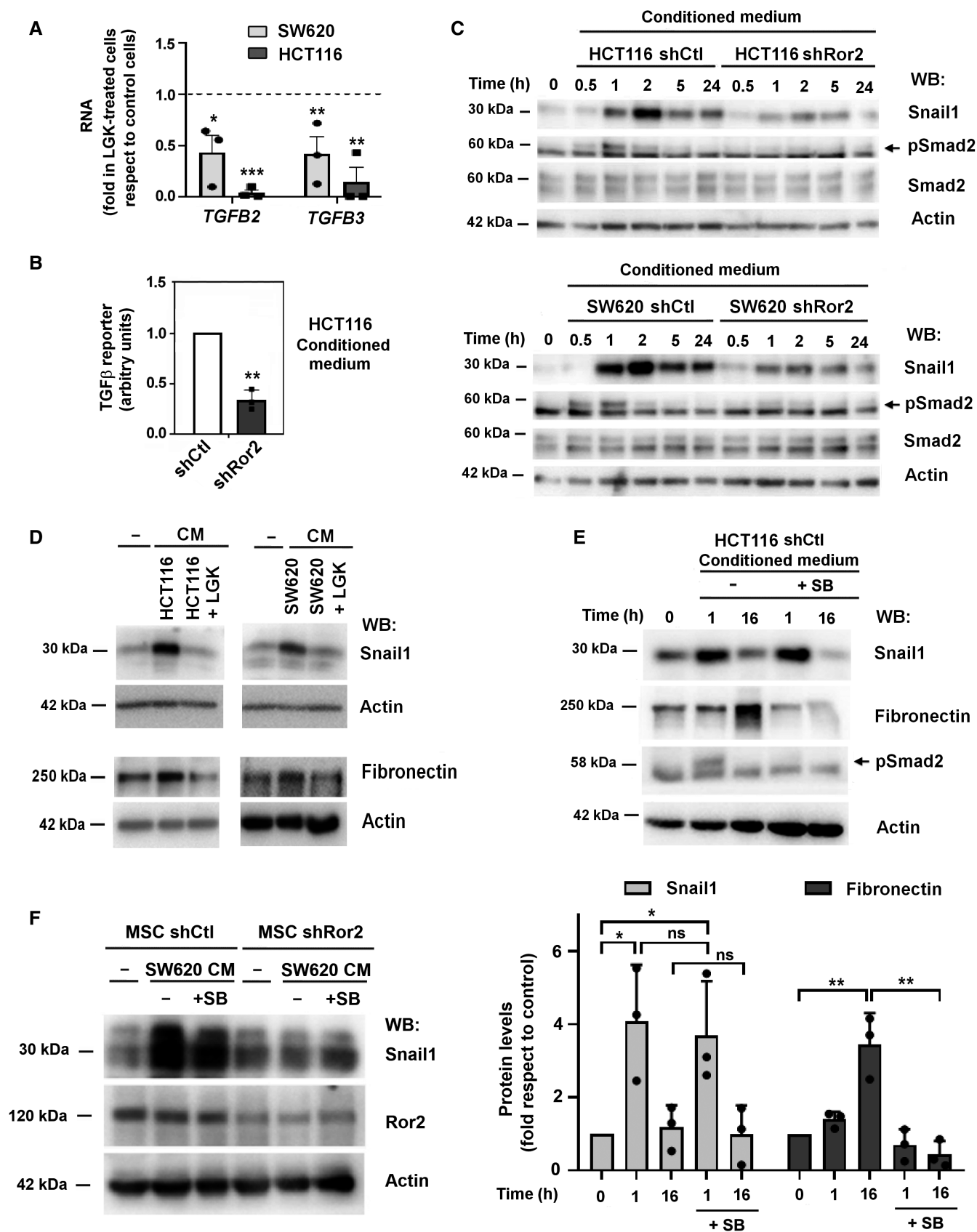


Figure EV2.

Figure EV3. Wnt5a/Ror2 modulates Snail1 increase promoted by chemotherapeutic drugs in colon tumor cells.

- A Viability of HCT116 cells was determined by MTT assay after treatment with the indicated concentrations of oxaliplatin and 5-Fluorouracil (5-FU) for 24 h. Results show the mean $\pm$ SD of two independent experiments performed in triplicate.
- B Snail1 protein quantification from HCT116 cells treated with the indicated drugs or Ror2-depleted. The mean of three or four biological replicates $\pm$ SD is shown.
- C, D HCT116 cells were treated with Wnt5a (100 nM or the indicated concentrations) and recombinant Wnt5a (50 ng/ml) combined with cisplatin (40 μ M) for 16 h.
- E RNA from cells treated with cisplatin for 16 h was analyzed by RT-PCR.
- F WB analysis of HT29 M6 stably overexpressing Snail1 and treated with cisplatin (40 μ M).
- G SW620 and HCT116 cell lines were treated with the indicated concentrations of oxaliplatin, doxorubicin (doxo) (5 nM) or 5-FU (100 μ M) for 16 h and analyzed by WB.
- H Cell lines were treated with cisplatin (40 μ M) and LGK974 for 16 h and analyzed by WB.
- I Snail1 protein quantification in MTO cells treated with the indicated drugs or infected with shCtl or shRor2. Mean $\pm$ SD of three (left) or two (right) biological replicates is shown.
- J Viability was measured by the CellTiter-Glo assay in MTO cells treated with R-ECD (0.2 μ g/ml) in combination with the indicated concentrations of cisplatin for 72 h. Results show the mean $\pm$ SD of three independent experiments performed in triplicate.
- K MTO cells were treated with R-ECD (0.2 μ g/ml) or the corresponding control, supplemented with cisplatin (100 μ M) when indicated and analyzed by WB.

Data information: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (unpaired t-test).

Source data are available online for this figure.

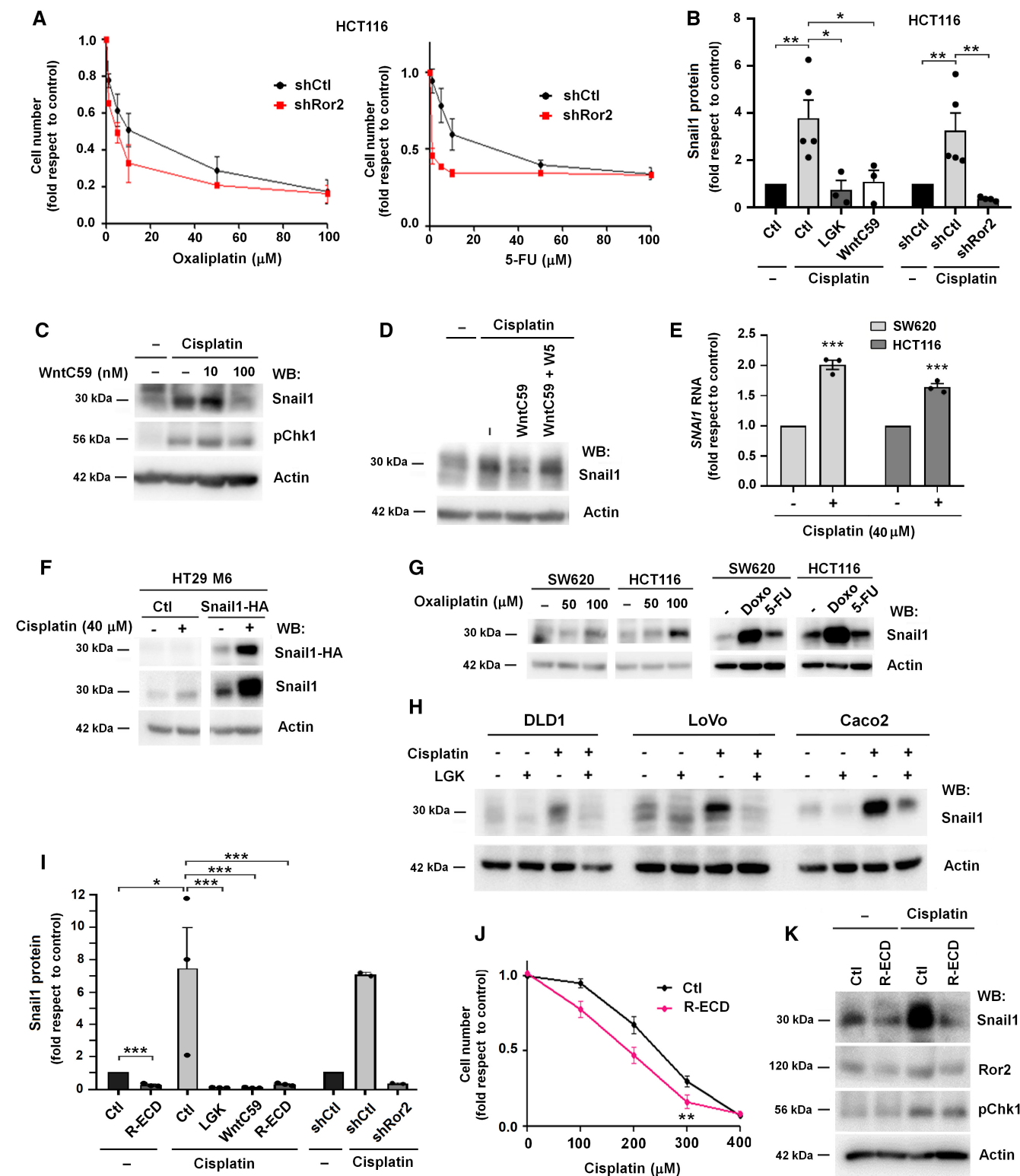


Figure EV3.

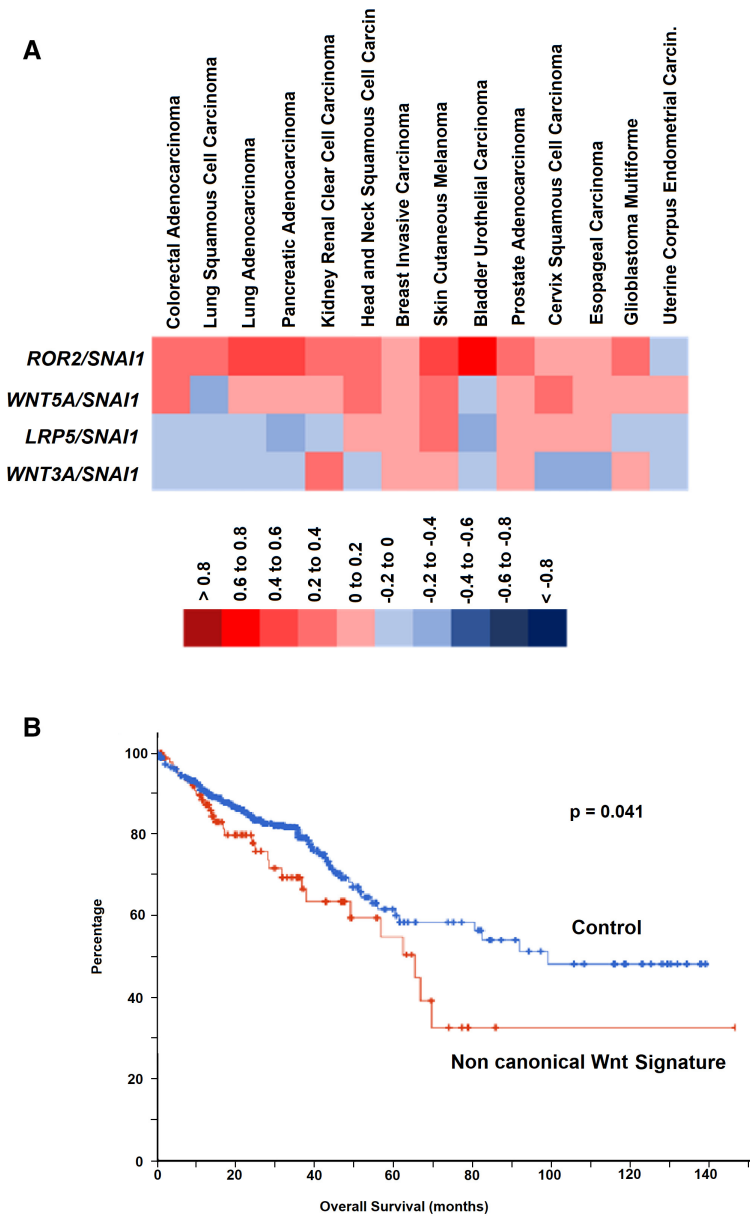


Figure EV4. Expression of *SNAI1* correlates with *WNT5A* and *ROR2* in other human tumors.

A Heat map of the association of *SNAI1* RNA and other genes in different neoplasms. Spearman coefficient was represented in a heatmap graph using GraphPad software. Data were obtained from the TCGA Firehose Legacy database and analyzed in the cBioPortal.

B Kaplan-Meier survival curves of patients with colon tumors presenting low (control) or high expression of the noncanonical Wnt signature (*ROR2*, *SNAI1*, *FZD2*, *WNT5A*, *MMP13*, *ZEB1*, *ACTA2*).

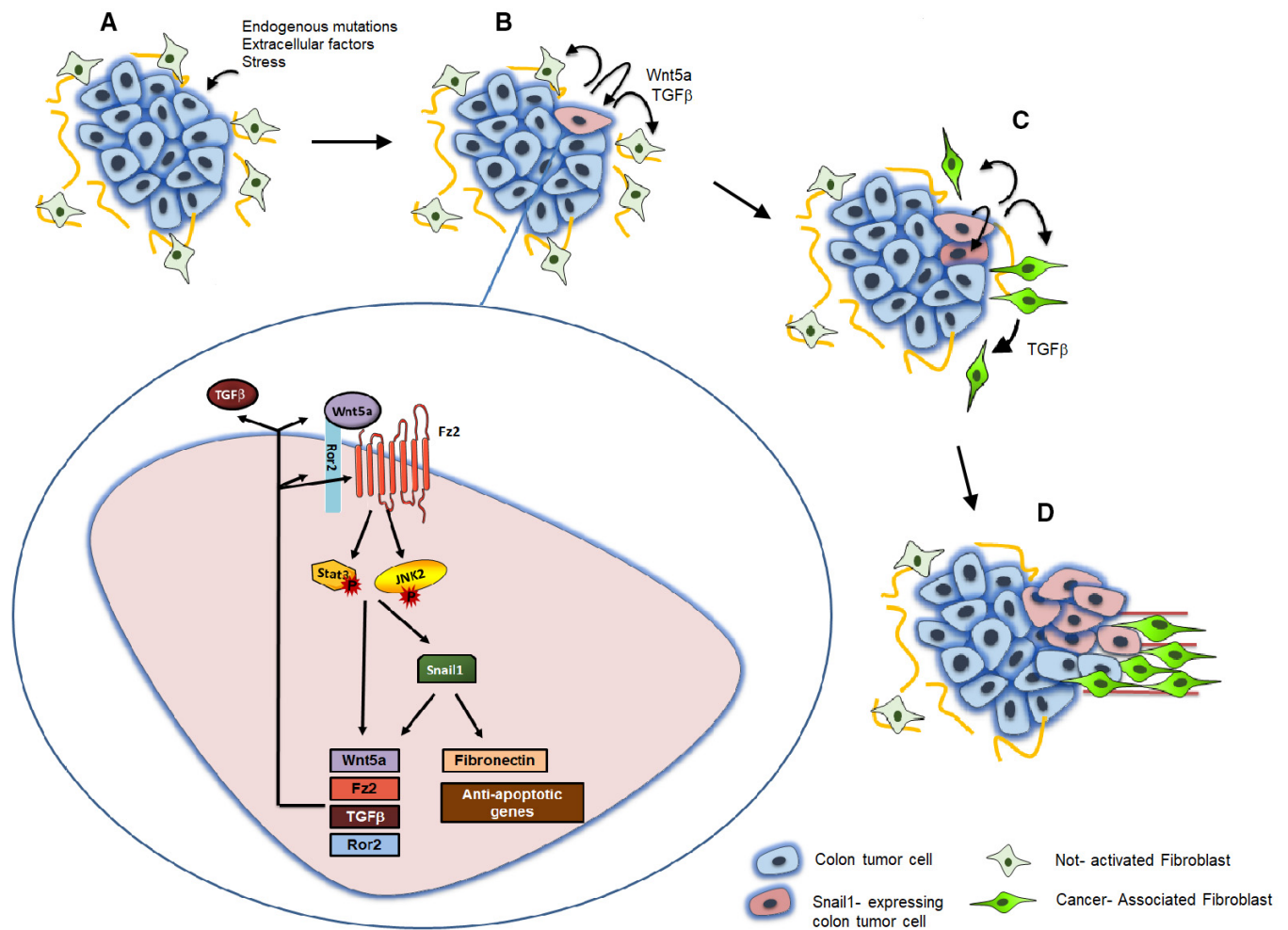


Figure EV5. Model for the control of Snail1 expression, EMT and fibroblast activation by noncanonical Wnt signaling.

As consequence of specific mutations, the sustained action of Wnt extracellular factors or microenvironmental stress conditions (a), some tumor cells (in pink) start to express Snail1 and undergo EMT (b). In these cells Snail1 not only increases the expression of mesenchymal markers and anti-apoptotic genes (see magnification), but also of Wnt5a, Ror2 and Fz2, creating a self-stimulation loop that amplifies and sustains the activity of the signaling pathway, and feeds the expression of Snail1 by activating Stat3 and JNK dependent pathways. Moreover, the expression of TGFβ, and other Wnt factors, is stimulated. TGFβ and Wnt5a activate fibroblasts placed in the microenvironment (c) and likely, other nearby tumor cells, and facilitate tumor invasion (d) and chemoresistance.