

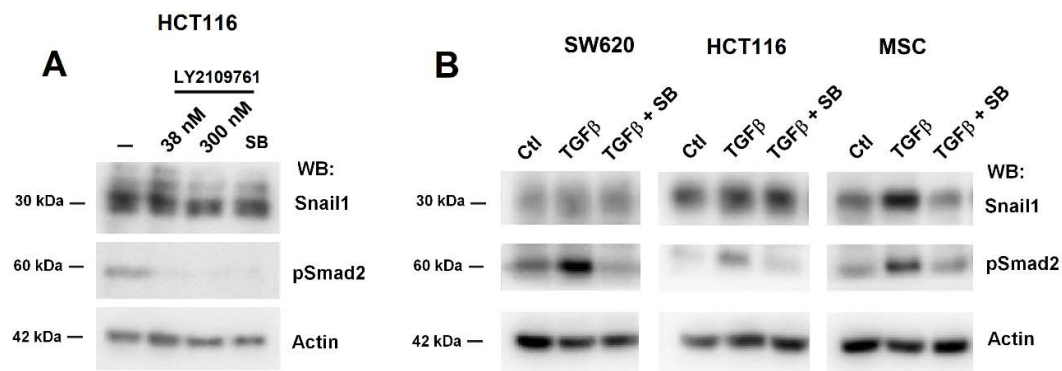
Appendix for

Non-canonical Wnt controls colon tumor cell growth, chemoresistance and tumor-dependent fibroblast activation.

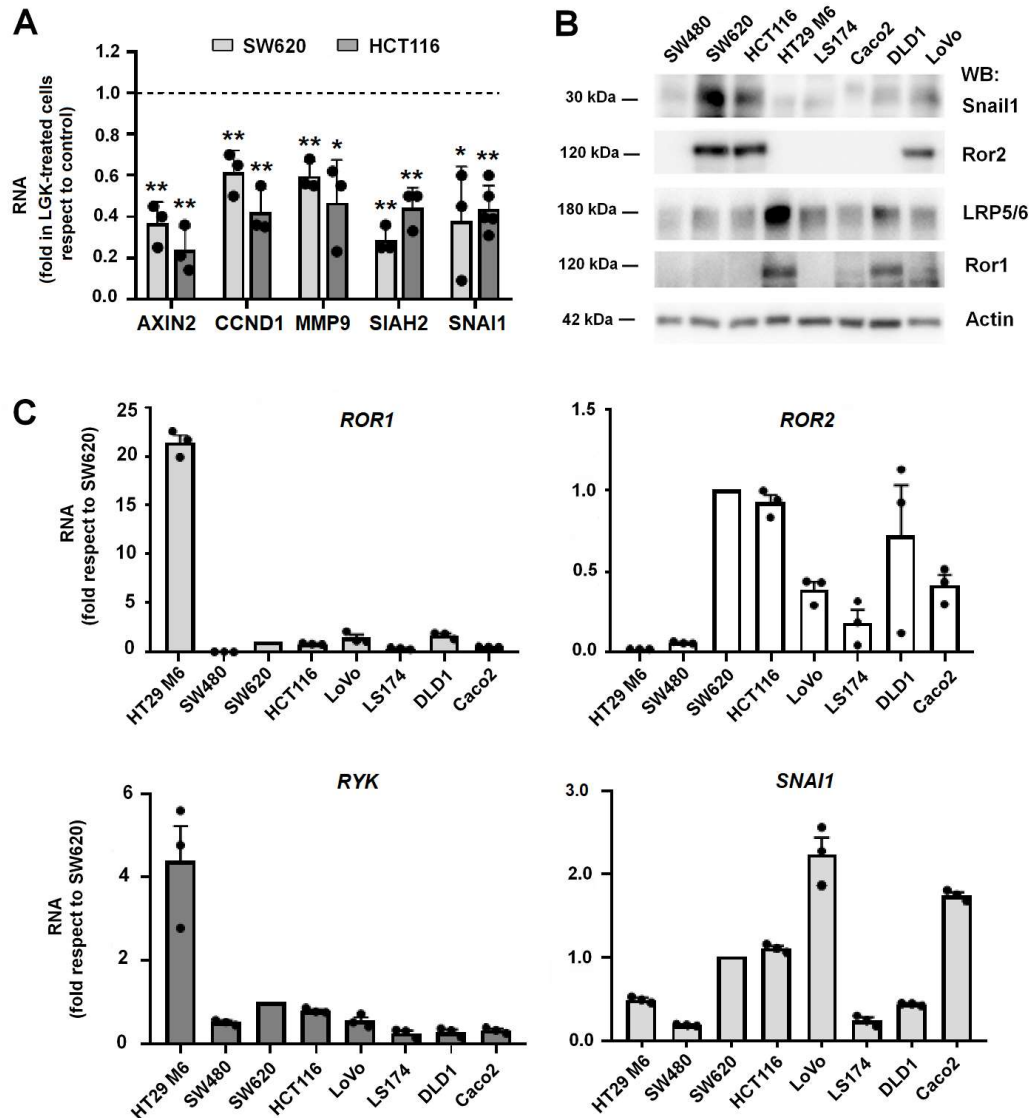
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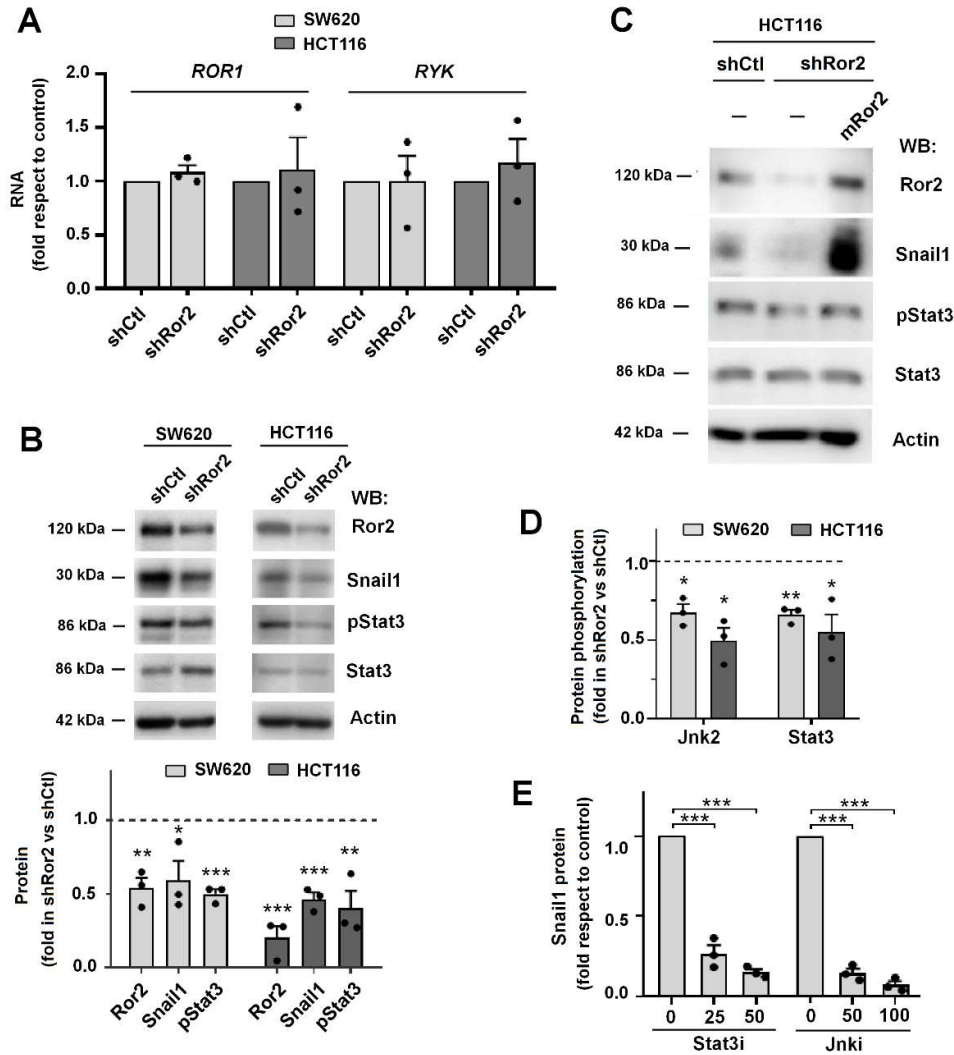


Appendix Figure S1. TGF β does not control Snail1 expression in colon cancer cells. **A**, HCT116 cells were treated for 16 h with 38 or 300 nM of LY2109761, concentrations that inhibit TGF β receptor I or I and II. Cells were lysed and proteins analyzed by WB. **B**, SW620, HCT116 or MSC cells were treated with TGF β (5 ng/ml) and TGF β receptor inhibitor SB505124 (SB, 5 μ M) when specified. Total protein extracts were prepared after 1 h of treatment and proteins were analyzed by WB. Source data are available online for this figure.



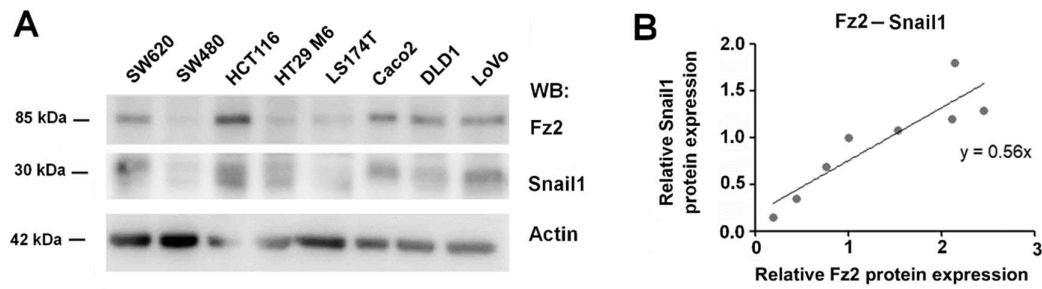
Appendix Figure S2. Snail1 and Ror2 expression correlates in colon cancer cells.

A, RNA was isolated from HCT116 and SW620 cells treated with LGK (10 μ M) for 16 h. Expression of canonical Wnt (*AXIN2* and *CCND1*) or non-canonical Wnt target genes (*MMP9* and *SIAH2*) and *SNAI1* was assessed by qRT-PCR. **B**, Snail1, Ror2, LRP5/6 and Ror1 protein levels were analyzed by Western blot in total extracts from the indicated cells. **C**, Expression of *ROR1*, *ROR2*, *RYK* and *SNAI1* was determined by qRT-PCR in different cell lines and values represented versus those obtained in SW620 cells. In A and C, the mean $\pm$ SD of the results of three biological replicates performed in triplicate is shown. *, $p < 0.05$; **, $p < 0.01$. Source data are available online for this figure.

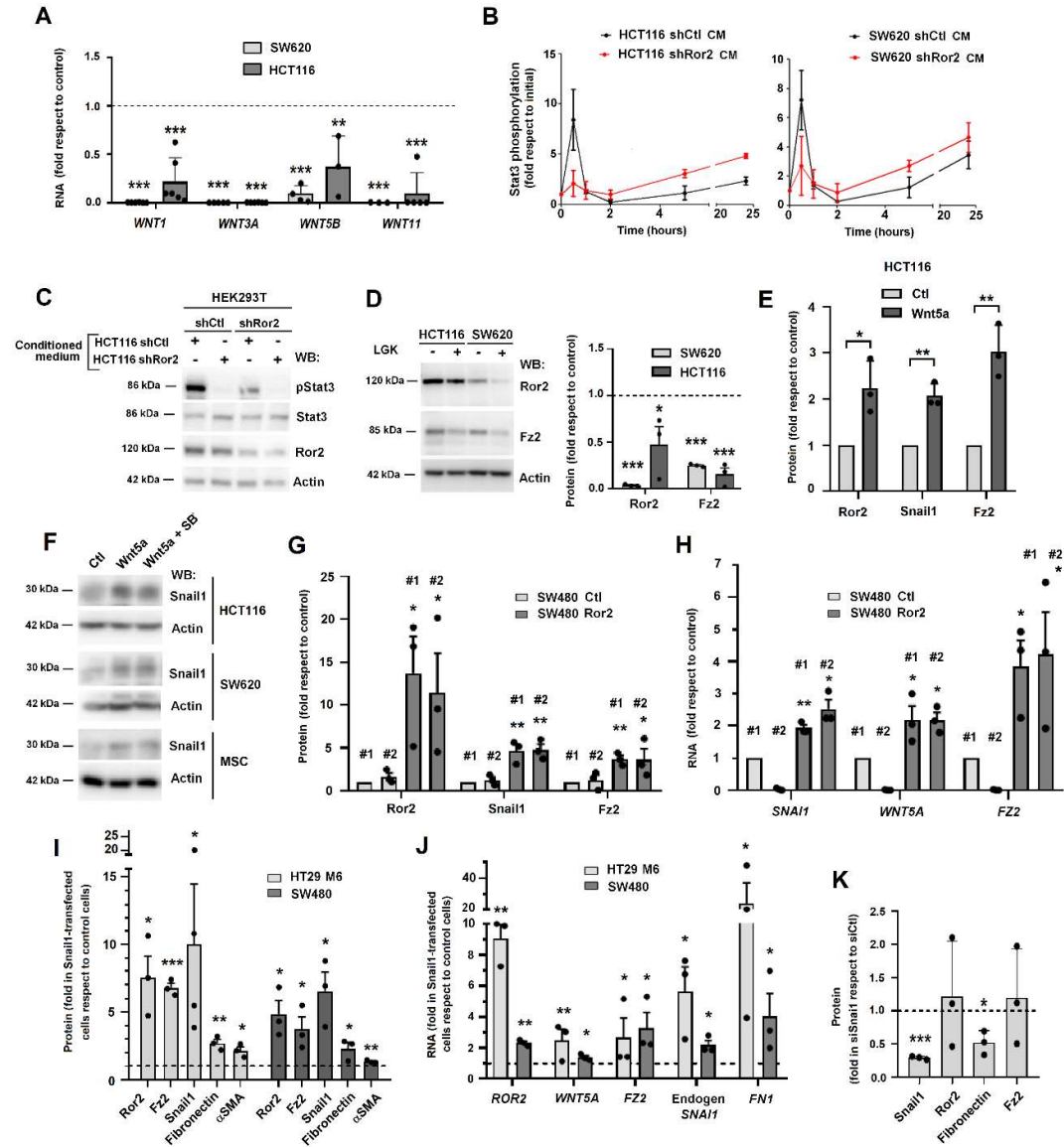


Appendix Figure S3. Ror2 depletion decreases Snail1 levels. **A**, RNA was isolated from HCT116 and SW620 stably transfected with shCtl or shRor2 (#1). Expression of ROR1 and RYK was assessed by qRT-PCR. The figure shows the mean $\pm$ SD of the results of three independent experiments. **B**, **C**, SW620 and HCT116 cells were transfected with shCtl or shRor2 (#2) and a murine Ror2 cDNA when indicated. Total cell extracts were prepared and analyzed by WB. Signals from three biological replicates from B were densitometered and represented in the lower panel. Values correspond to fold variation in shRor2 versus shCtl cells. **D**, **E**, Quantification of the WBs shown in Figs 1D and 1F, respectively (mean $\pm$ SD of three biological replicates). The dashed line in B

(lower) and D corresponds to the protein levels in shCtl cells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Source data are available online for this figure.



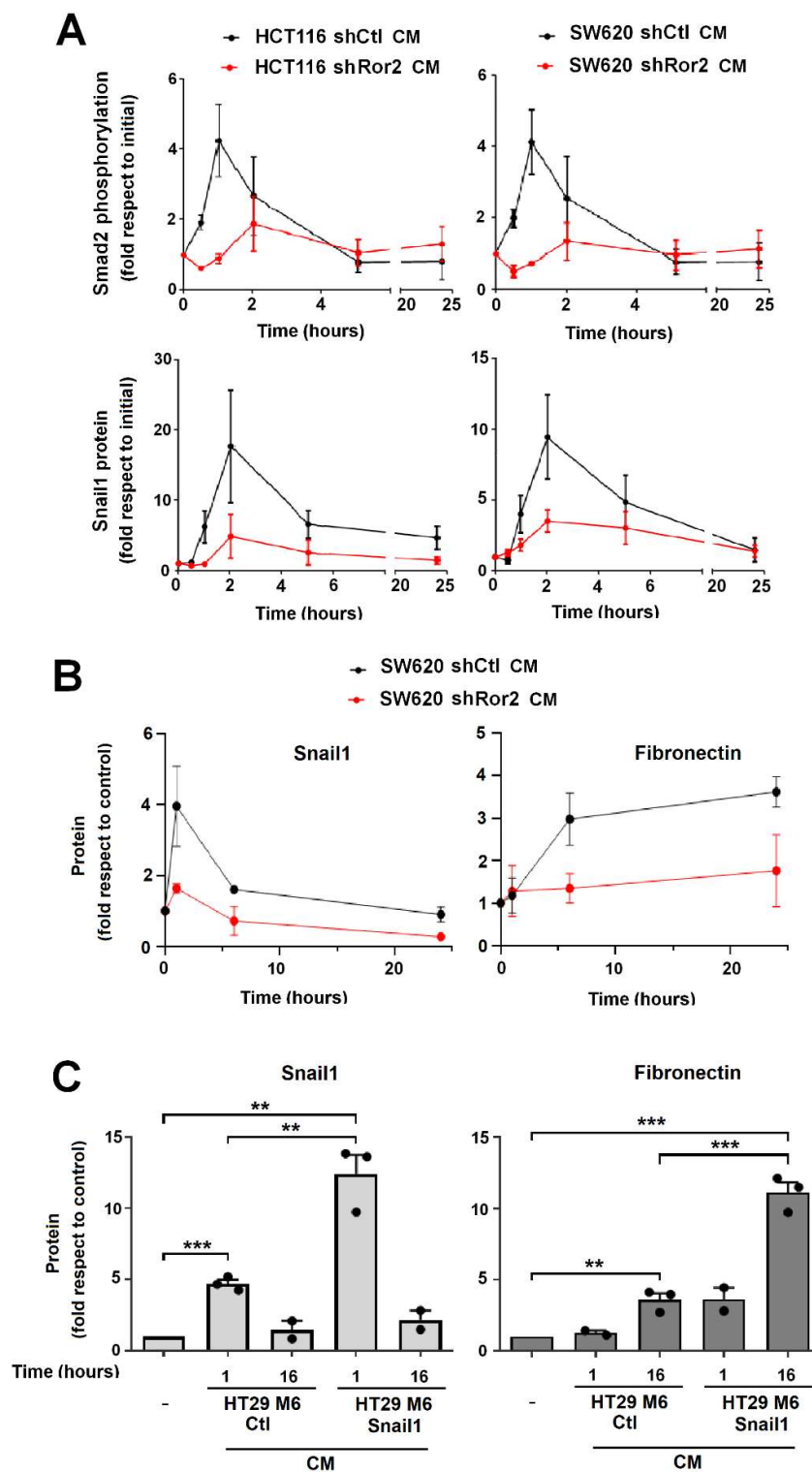
Appendix Figure S4. Snail1 and Fz2 expression correlates in colon tumor cells. A, Fz2 and Snail1 expression was analyzed by WB in total extracts from colon cancer cell lines. **B,** Signals from determinations performed in panel A were densitometered and represented. Values from SW620 cells were taken as reference. Source data are available online for this figure.



Appendix Figure S5. Wnt5a, Ror2 and Snail1 create a self-stimulatory signaling pathway. **A**, RNA was isolated from the indicated cells and analyzed by qRT-PCR.

Values are represented with reference to shCtI cells (dashed line). The figure shows the mean $\pm$ SD of three to five biological replicates. **B**, Quantification of WBs shown in Fig 2C (mean $\pm$ SD of two biological replicates). **C**, HEK293 cells, either transfected with shCtI or shRor2, were treated with conditioned media from control or Ror2-depleted HCT116 cells for 16 h and analyzed by WB. **D**, Extracts from HCT116 and SW620 cells treated with LGK (10 μ M) for 16 h were prepared and analyzed by WB. A quantification

of three experiments is shown at right. **E**, Quantification of WBs shown in Fig 2D (mean $\pm$ SD of three different experiments). **F**, HCT116, SW620 and MSC cells were treated with Wnt5a in combination with SB (5 μ M) for 16 h. Snail1 levels were analyzed by WB. **H** and **J**, RNA was isolated from the indicated cells and analyzed by qRT-PCR. SW480 Ctl (#1) was used as reference in G. Values represented in J correspond to fold variation in Snail1-transfected cells versus control cells. The figures show the mean $\pm$ SD of three biological replicates. **G**, **I** and **K**, Quantification of the results presented in Figs 2F, 2G and 2H, respectively. Values represented in I and J correspond to fold variation in Snail1-transfected cells versus control cells; in K, to fold variation in siSnail1 versus siCtl cells. The figures show the mean $\pm$ SD of the results of three biological replicates. The dashed line corresponds to the protein or RNA levels in control cells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Source data are available online for this figure.

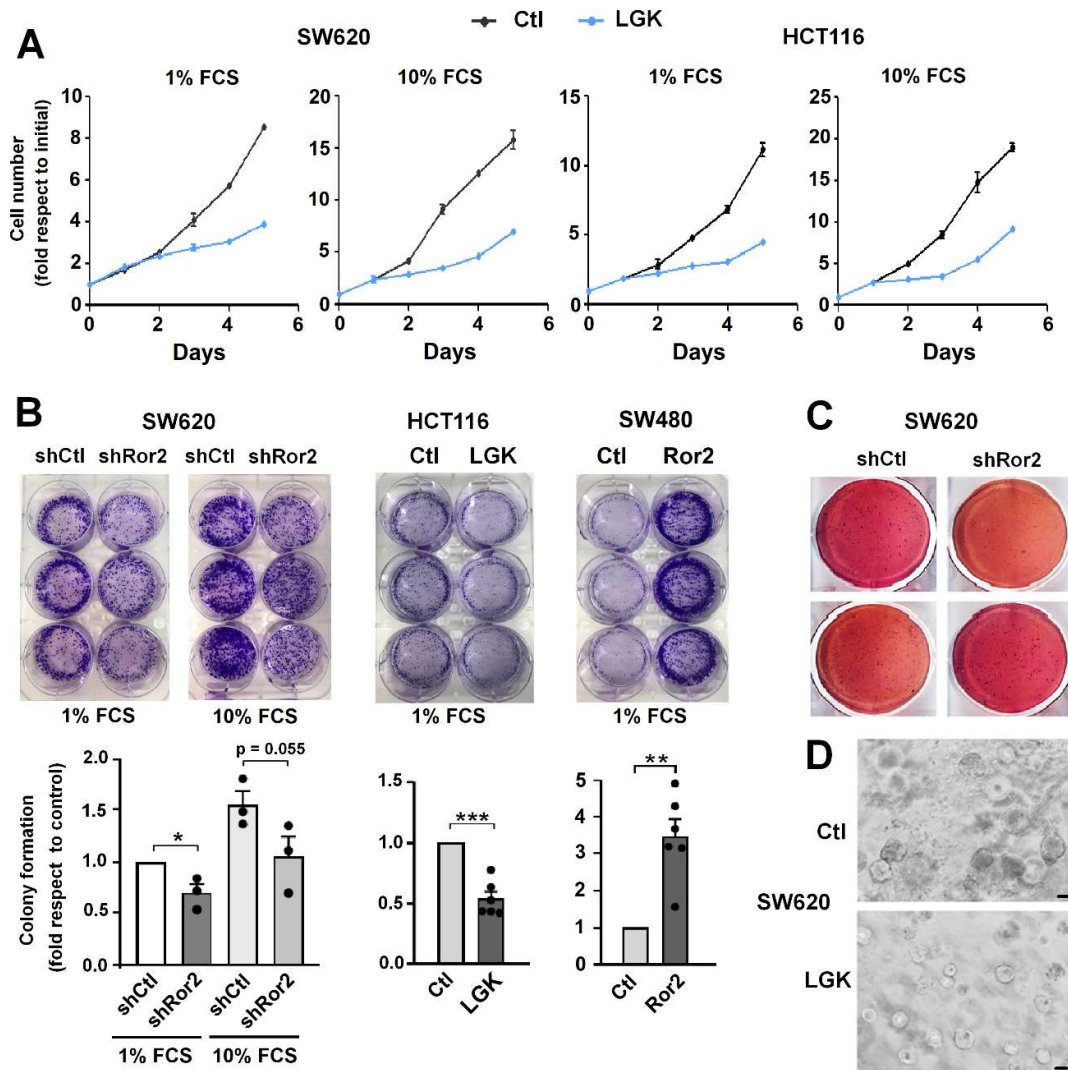


Appendix Figure S6. Wnt5a and Ror2 increase TGF β production and fibroblast

activation. **A**, Signals from three independent experiments carried out in Fig EV2C

were densitometered and represented (mean $\pm$ SD). **B**, **C**, Quantifications of the WB

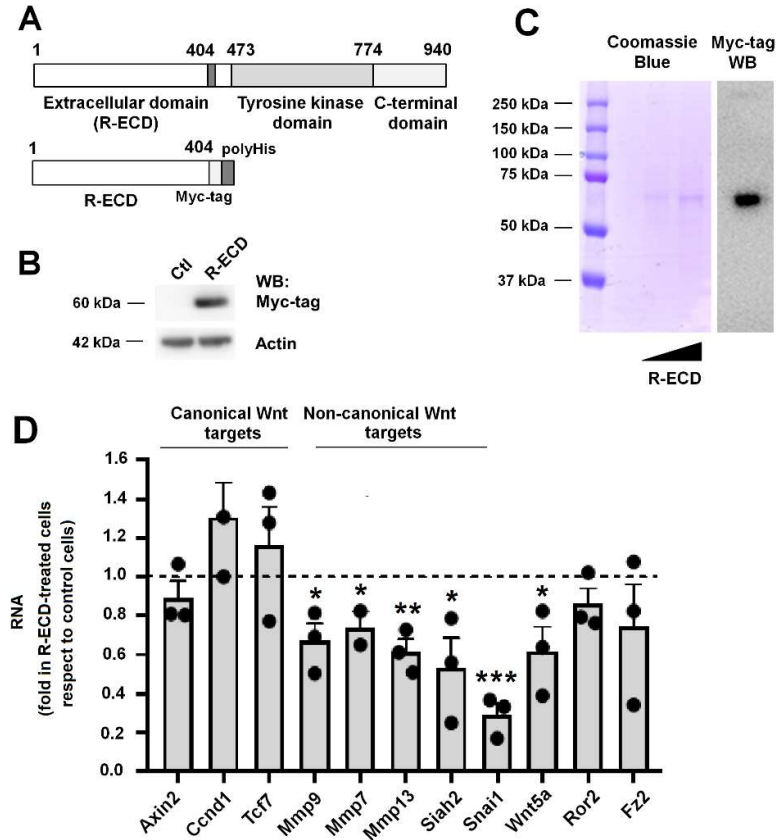
shown in Figures 3C and 3F, respectively. The mean $\pm$ SD of two (B) or three (C) independent experiments is shown. **, $p < 0.01$; ***, $p < 0.001$. Source data are available online for this figure.



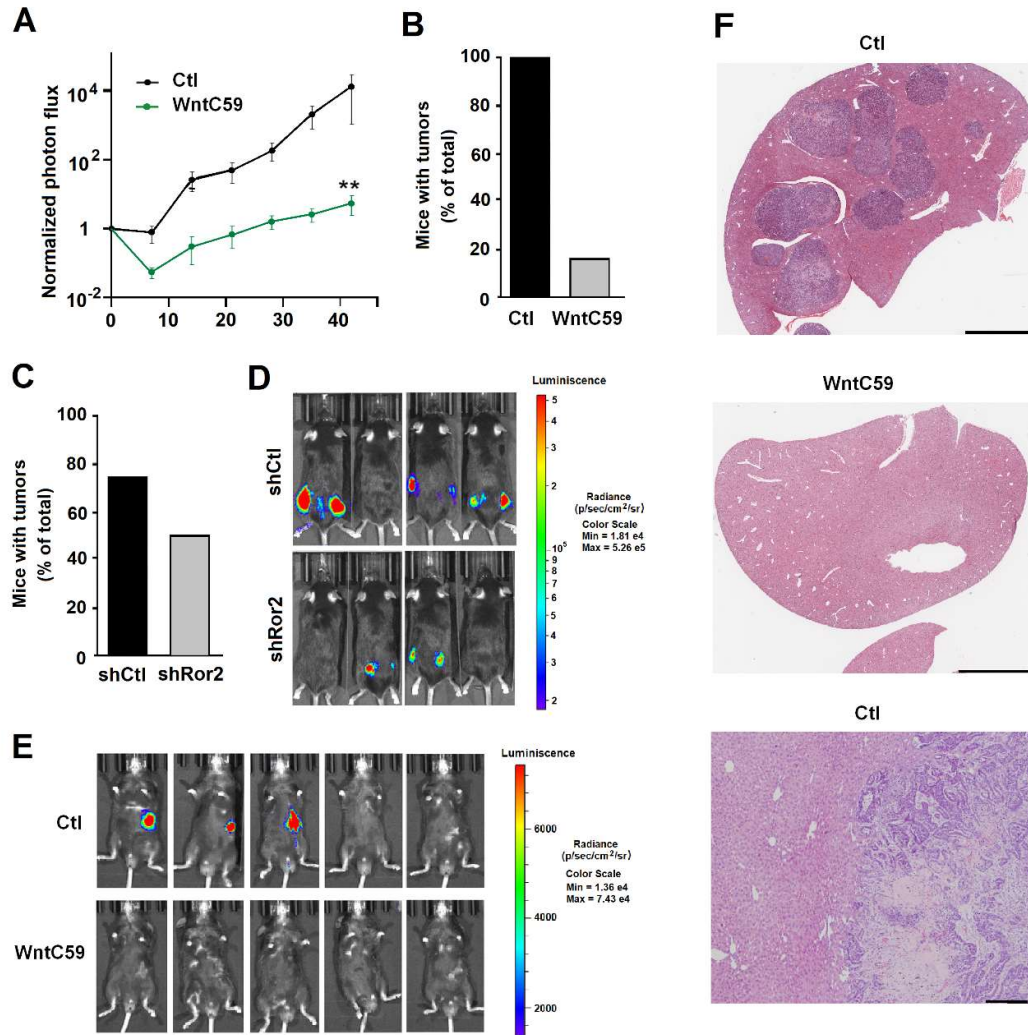
Appendix Figure S7. Inhibition of non-canonical Wnt decreases colony formation.

A, Cell number was determined by the MTT assay for HCT116 cells treated with LGK (10 μ M, every three days) and grown with the indicated FCS concentration. **B**, Clonogenic assay was carried out with Ctl or Ror2-depleted SW620 cells, HCT116 treated with LGK or SW480 over-expressing Ror2. Cells were grown at the indicated concentrations of FCS for 10 days. Colonies were stained with crystal violet and counted using ImageJ software. Results were presented as the mean $\pm$ SD of triplicate samples from at least three biological replicates. The micrograph shows the results of a representative experiment. **C**, Representative micrographs of the effect of Ror2 depletion

on SW620 soft-agar colony formation (21 days). **D**, Representative micrographs of tumor organoids formed by SW620 cells cultured in the presence of LGK. The bar corresponds to 25 μm . *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Source data are available online for this figure.

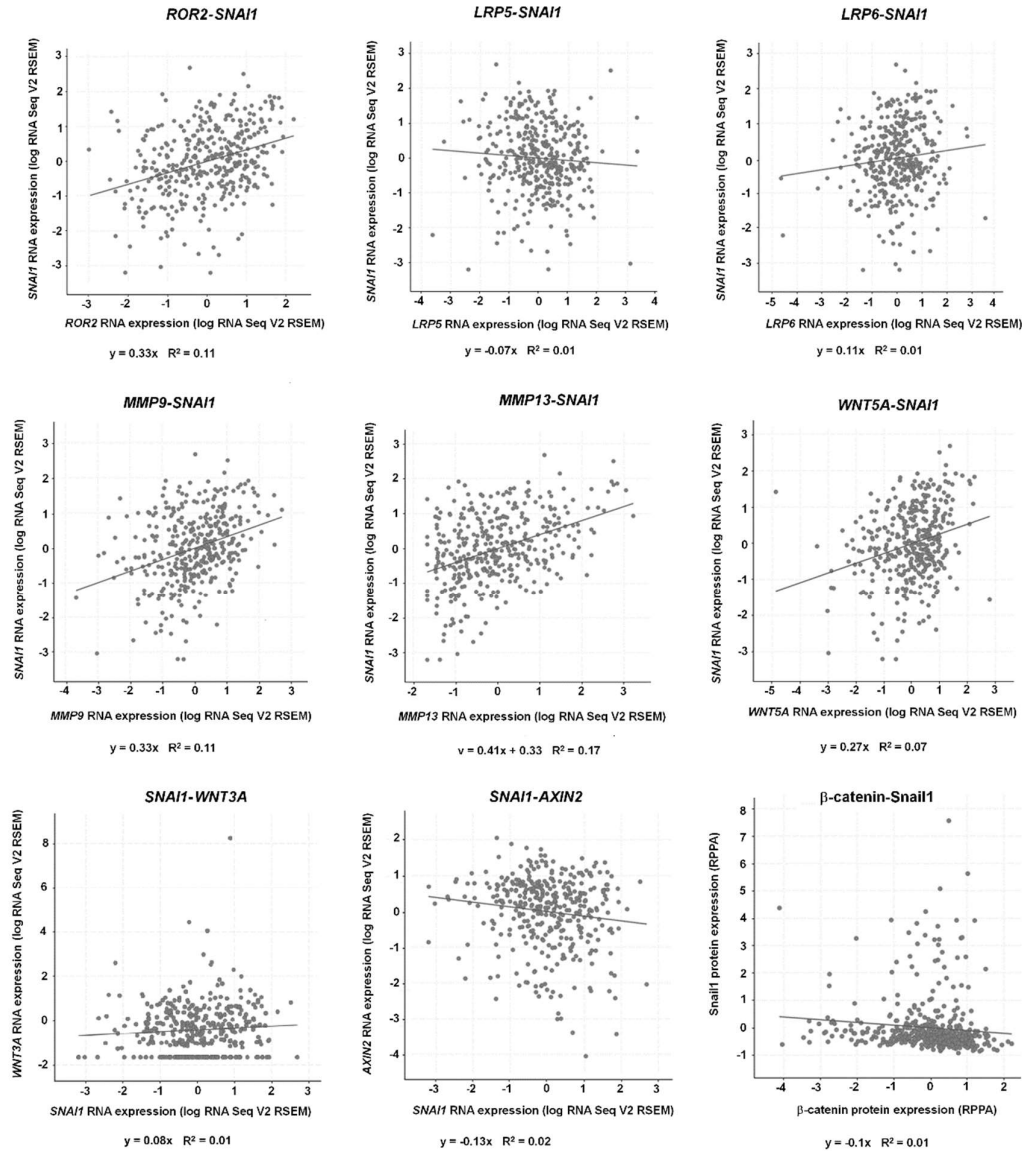


Appendix Figure S8. Expression of a dominant negative Ror2 mutant inhibits non-canonical Wnt. **A**, Diagram of full length Ror2 and Ror2 extracellular domain (R-ECD). **B**, **C**, HEK293 cells were transiently transfected with a plasmid encoding the R-ECD. The panels show WB from total cell extracts (**B**) and a Coomassie Blue staining and a WB of Nickel-agarose-purified R-ECD (**C**). **D**, RNA was isolated from MTO cells treated with 100 ng/ml R-ECD for 16 h. Expression of canonical and non-canonical Wnt target genes was assessed by qRT-PCR and represented. The dashed line corresponds to the RNA levels in control cells. The figure shows the mean $\pm$ SD of three biological replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Source data are available online for this figure.

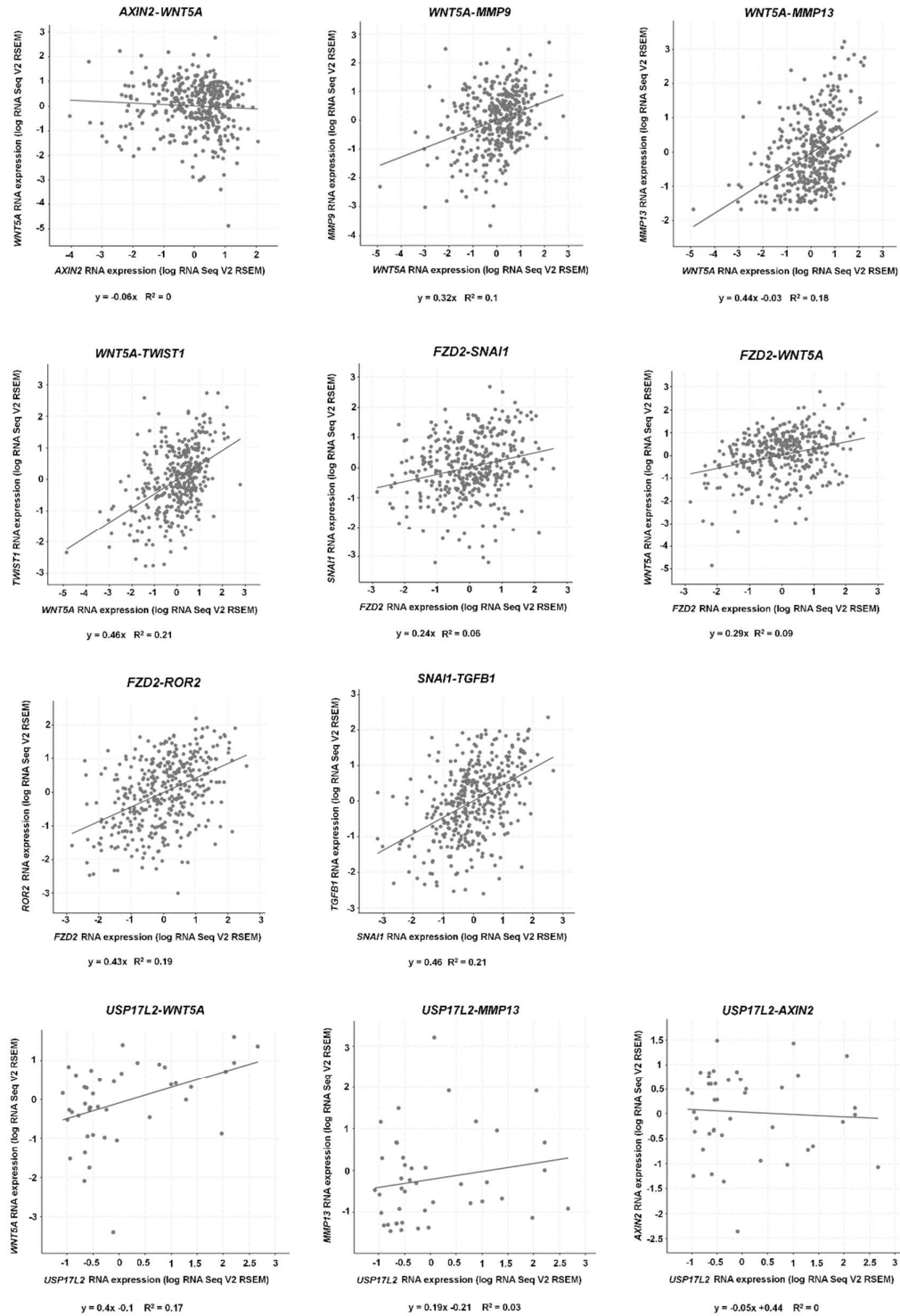


Appendix Figure S9. Inhibition of non-canonical Wnt decreases the tumorigenic potential of primary colon tumor cells. **A, B**, C57BL6J mice were injected subcutaneously with MTO and treated with WntC59 or vehicle since day 1. The kinetics of *in vivo* luminescence (**A**) (mean $\pm$ SD of the values in five mice) and percentage of mice with tumors (**B**) are shown. **C, D**, C57BL6 mice were injected subcutaneously in both flanks with MTO cells either shCtl or shRor2. The percentage of tumor formed are shown in **C**; representative IVIS images of each condition after 38 days, in **D**. **E**, C57BL6 mice were injected in the spleen with MTO cells and treated since day 1 with WntC59 or vehicle. Representative IVIS images of each condition after 26 days are shown. **F**, Micrographs of H/E stained livers from control animals with metastases or from WntC59-

treated mice. A magnification of a metastasis is shown at the lower panel. Scale bars correspond to 4 mm (upper and middle) or 100 μ M (lower). **, $p < 0.01$. Source data are available online for this figure.



Appendix Figure S10. Expression of *SNAI1* associates to *WNT5A* and *Wnt5A*-target genes and not to *WNT3A* in colon tumors. The correlation between the RNA expression (log RNA seq V2 RSEM) of the indicated genes is represented. Data was obtained from the TCGA Colorectal Adenocarcinoma Firehose Legacy database and analyzed in the cBioPortal.



Appendix Figure S11. Expression of *WNT5A* and Wnt5A-target genes correlates with that of *FZ2*, *TWIST1* and *USP17L2* (Dub3) in colon tumors. The correlation between the RNA expression (log RNA seq V2 RSEM) of the indicated genes is

represented. Data was obtained from the TCGA Colorectal Adenocarcinoma Firehose Legacy database and analyzed in the cBioPortal.

Appendix Table S1. Primers used for RT-PCR analysis

Gene	Forward	Reverse
Human		
<i>ASCL2</i>	5'-CATAACTTGAGCTGCTGGAGG	5'-TGCTCGTGTCCAGATCTTGG
<i>AXIN2</i>	5'-TGTCTTAAAGGTCTTGAGGGTTGAC	5'-CAACAGATCATCCCATCCAACA
<i>BTRC</i>	5'-CACTCACAGCTTTCCAGACAT	5'-TGCTGCAAGAGAAGGCACTC
<i>CDH1</i>	5'-CCTGGGCAGAGTGAATTTTG	5'-GGCGTAGACCAAGAAATGGA
<i>CCND1</i>	5'-CTCAGACTTGCGCGTCACAG	5'-CAGAACACGGCTCACGCTTA
<i>FBXL5</i>	5'-CTTACCCAGACTGACATTTTCAGATTC	5'-GAAGACTCTGGCAGCAACCAA
<i>FBXL14</i>	5'-AACGGATCCACCATGGAGACCCACA TCTCATGC	5'-CAAGATATCCCCTTCTGGAGCTTCCC
<i>FZD2</i>	5'-GTGCCGCTCTATCTGTGAGC	5'-CTCCGCTCCTCGGAGTGGTT
<i>HPRT</i>	5'-GGCCAGACTTTGTTGGATTG	5'-TGCGCTCATCTTAGGCTTTGT
<i>KRT20</i>	5'-ACACGGTGAAGTATGGGAGC	5'-GCGTTGGTTTCGTACCACTG
<i>LGR5</i>	5'-TGGAGGAGTTACGTCTTGCG	5'-AGAGCTTCTGTGGGTACGTG
<i>MMP9</i>	5'-TTCAGGGAGACGCCATTTC	5'-TGGGTGTAGAGTCTCTCGCT
<i>MMP13</i>	5'-AAGGACCCTGGAGCACTCAT	5'-CCTGGACCATAGAGAGACTGGA
<i>ROR1</i>	5'-AGTGCCTACCTCATCATGGAAC	5'-GAGCCATAGATGGTGGACCG
<i>ROR2</i>	5'-CTCGAGTAAAAGGGACACCAGAGTG GC	5'-AAGCTTCCCTTATATTCCTCAGGCTTC AA
<i>RYK</i>	5'-AGCTCCAACCACTTCTACGC	5'-AACCACCTTCTACGCGTGTGT
<i>SIAH2</i>	5'-TTTGCCATCGTCCTGCTCAT	5'-GGCTGTGTGCGAAAACAAG
<i>SMOC2</i>	5'-ACAGTCAGGTCCAGTGTACAC	5'-TTCATTTACGGAACCCGGGC
<i>SNAI1</i>	5'-GTGCCTCGACCACTATGCC	5'-GCTGCTGGAAGGTAAACTCTGG
<i>TGFB1</i>	5'-AAGTGGAGCAGCACGTGGAG	5'-CAGCCGGTTGCTCAGGTATC
<i>TGFB2</i>	5'-TACTACGCCAAGGAGGTTTACAAA	5'-TTGTTTACAGGCACTCTGGCTTT
<i>TGFB3</i>	5'-CTGGCCCTTTACAACAGC	5'-CACTGAGGACACATTGAAGC
<i>TWIST1</i>	5'-CAGCTACGCCTTCTCGGTCT	5'-CTCGGACAAGCTGAGCAAGA
<i>USP17L2</i>	5'-GGTCTTTGTCTCCAGAGGGC	5'-TGATGCTACAGGCAGTGACC
<i>USP27X</i>	5'-CGTCCCTGGCCCATCTTATC	5'-CAGGGCTTAAGGGTCTTGGG
<i>USP37</i>	5'-TCCTGCCTGTAGCCAGAAG	5'-GGCTTTGTTTCGCTCCACTG
<i>WNT1</i>	5'- CAAGATCGTCAACCGAGGCT	5'-TCACACGTGCAGGATTTCAT
<i>WNT3A</i>	5'- CTTTGCAGTGACACGCTCAT	5'-CGAACTCCCGAGACACCATC
<i>WNT5A</i>	5'-GCTCCCCGACAGCCTGGTCT	5'-AGGACATGGCCGCTGGGTGT

<i>WNT5B</i>	5'-GGATGGCTGTGAGCTCATGT	5'-CACGATCTCCGTGCACTTCT
<i>WNT11</i>	5'-GCCAAGTTTTCCGATGCTC	5'-GACACCCCATGGCACTTACA
<i>ZEB1</i>	5'-CGGCGCAATAACGTTACAAA	5'-TTTCACTGCTCCTCCCTGGT
Mouse		
<i>Ascl2</i>	5'-CGAGGATTTTTCGAGGACGC	5'-GTGGACGTTTGACCTTCAC
<i>Axin2</i>	5'-CCAAGTGTCTCTACCTCATTTTCCG	5'-GGTTTGTGGGTCTCTTCATAGC
<i>Cdh1</i>	5'-GCGCTCCTGCTCCTGCTCCTGCTG	5'-CAGCCAGCCTGTCTCCCTCTC
<i>Ccnd1</i>	5'-CCTGGGCAGAGTGAATTTTG	5'-GGCGTAGACCAAGAAATGGA
<i>Fn1</i>	5'-AGCAAGCCTGAGCCTGAAGAG	5'-GCGATTTGCAATGGTACAGCT
<i>Fz2</i>	5'-AATAAGAAGCTTATCGCTG	5'-TTAATACTCGAGGACGGTA
<i>Krt20</i>	5'-GCAGTGGTACGAAACCAACG	5'-CTGCAGCCAGCTTAGCATTG
<i>Lgr5</i>	5'-CATCAGGTACCGGAGC	5'-TAATGTGCGAGGCACCATTC
<i>Mmp7</i>	5'-TGTGGGGCAAAGAGATCCCC	5'-CCTCGCGCAAAGCCAATC
<i>Mmp9</i>	5'-GGCGTCGTGATCCCCACTTAC	5'-CCGTGTAGAGTCTCTCACTAGGGCA
<i>Mmp13</i>	5'-GAACACTCAAATGGTCCCAAACG	5'-GTTGTAGCCTTTGGAAGTCTTGTC
<i>Ror2</i>	5'-GCTAGCACAGGCTGCAAGCTG	5'-AGCTAGCGCCCGAGCATGGCT
<i>Siah2</i>	5'-TTTGCCATCGTCCTGCTCAT	5'-GGCTGTGTCGAAAACAAG
<i>Smoc2</i>	5'-TCAGCCCCAAGGAGATGAAG	5'-TGGTCTTGTTCTGCCGACTC
<i>Snai1</i>	5'-GCGCCCGTCGTCCTTCTCGTC	5'-CTTCCGCGACTGGGGTCTCT
<i>Tcf7</i>	5'-CGCGGGATAACTACGGAAGA	5'-TAGCAAGCAGTTCTGTGGGG
<i>Wnt5A</i>	5'-AGGAGTTCGTGGACGCTAGA	5'-ATACTGTCCTACGGCCTGCTT