

SALL2-Mediated Suppression of WNT Signaling Through Transcriptional Control of AXIN2 in Colorectal Cancer Cells

Aracelly Quiroz ^{1,2,3}, Emilia Escalona ⁴, Carlos Farkas ⁵, Diego Benítez-Riquelme ^{1,2}, Paulina Sepúlveda ^{1,2}, Mario Palma ^{1,2,†}, Paula Medina ^{1,2}, Carolina Delgado ³, Matías I. Hepp ⁵, Franz Villarroel-Espindola ⁶, Ariel F. Castro ^{1,2,*} and Roxana Pincheira ^{1,2,*}

- ¹ Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Biológicas, Universidad de Concepción, 4070409 Concepción, Chile; aracellyquiroz@udec.cl (A.Q.); diegobenitez@udec.cl (D.B.-R.); psepulv.ps@gmail.com (P.S.); mpalma@hsph.harvard.edu (M.P.); pmedina2018@udec.cl (P.M.)
- ² Laboratorio de Transducción de Señales y Cáncer, Facultad de Ciencias Biológicas, Universidad de Concepción, Concepción, Chile
- ³ Departamento de Especialidades, Facultad de Medicina, Universidad de Concepción, Concepción, Chile; carolinadelgado@udec.cl
- ⁴ MARLab, Instituto de Ciencias Biomédicas, Facultad de Ciencias de la Salud, Universidad Autónoma de Chile, Talca, Chile; emilia.escalona@uautonoma.cl
- ⁵ Laboratorio de Investigación en Ciencias Biomédicas, Departamento de Ciencias Básicas y Morfología, Facultad de Medicina, Universidad Católica de la Santísima Concepción, Concepción, Chile; cfarkas@ucsc.cl (C.F.); mhepp@ucsc.cl (M.I.H.)
- ⁶ Translational Medicine Laboratory, Fundación Arturo López Pérez Cancer Center, Santiago, Chile; franz.villarroel@falp.org
- * Correspondence: arcastro@udec.cl (A.F.C.); ropincheira@udec.cl (R.P.); Tel.: +56-412203794 (A.F.C.); +56-412203815 (R.P.)
- † Current address: Department of Molecular Metabolism, Harvard T.H. Chan School of Public Health, Boston, MA 02115, USA.

Aracelly Quiroz: <https://orcid.org/0009-0000-5221-4762>

Emilia Escalona: <https://orcid.org/0009-0005-1196-1282>

Carlos Farkas: <https://orcid.org/0000-0002-6245-2622>

Diego Benítez: <https://orcid.org/0000-0001-5470-5792>

Mario Palma: <https://orcid.org/0009-0007-9456-2096>

Matías Hepp: <https://orcid.org/0000-0003-0993-8794>

Franz Villarroel: <https://orcid.org/0000-0003-0080-2444>

Ariel Castro: <https://orcid.org/0000-0001-5583-4879>

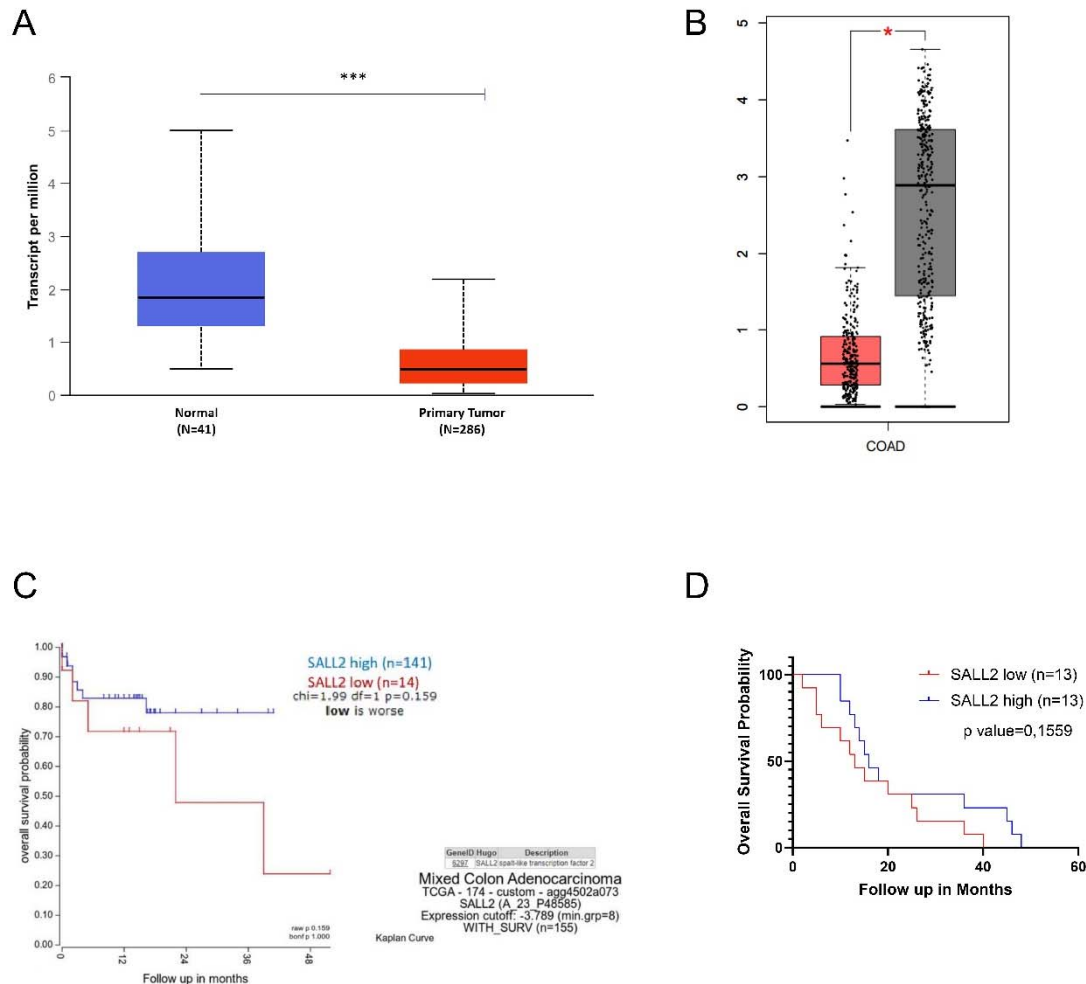
Roxana Pincheira: <https://orcid.org/0000-0003-4277-5245>

Corresponding author: Roxana Pincheira, Departamento de Bioquímica y Biología Molecular, Facultad Cs. Biológicas. Universidad de Concepción. Chile. Phone: 56-412203815; E-mail: ropincheira@udec.cl. Ariel Castro. Departamento de Bioquímica y Biología Molecular, Facultad Cs. Biológicas. Universidad de Concepción. Chile. Phone: 56-412203794; E-mail: arcastro@udec.cl.

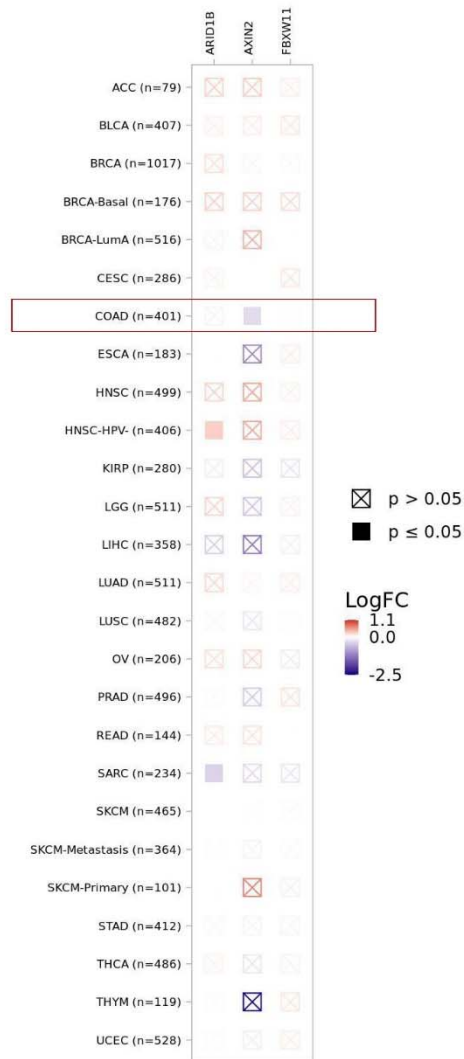
Running Title: The SALL2/AXIN2 axis-dependent regulation of the Wnt pathway

Keywords: SALL2, AXIN2, Wnt- β catenin, CHIR99021, XAV393, CRC

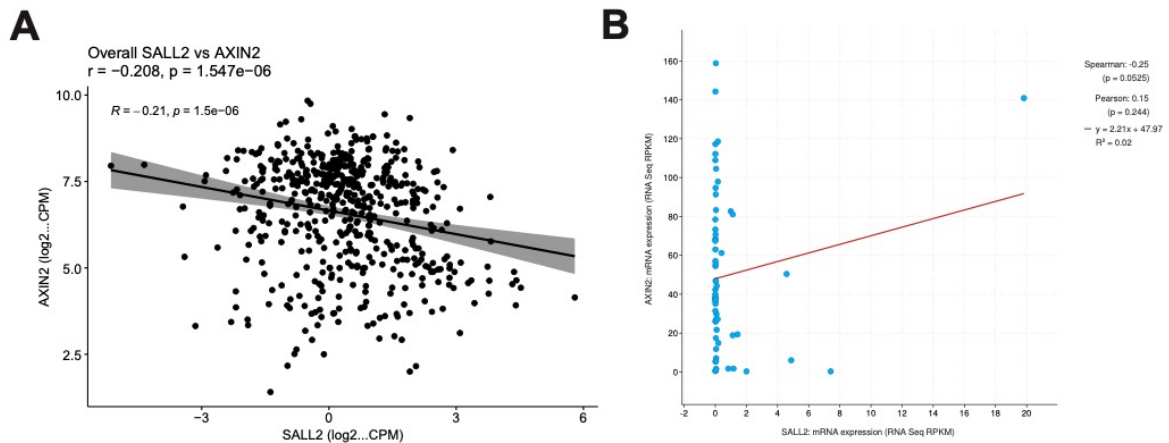
Supplementary figures:



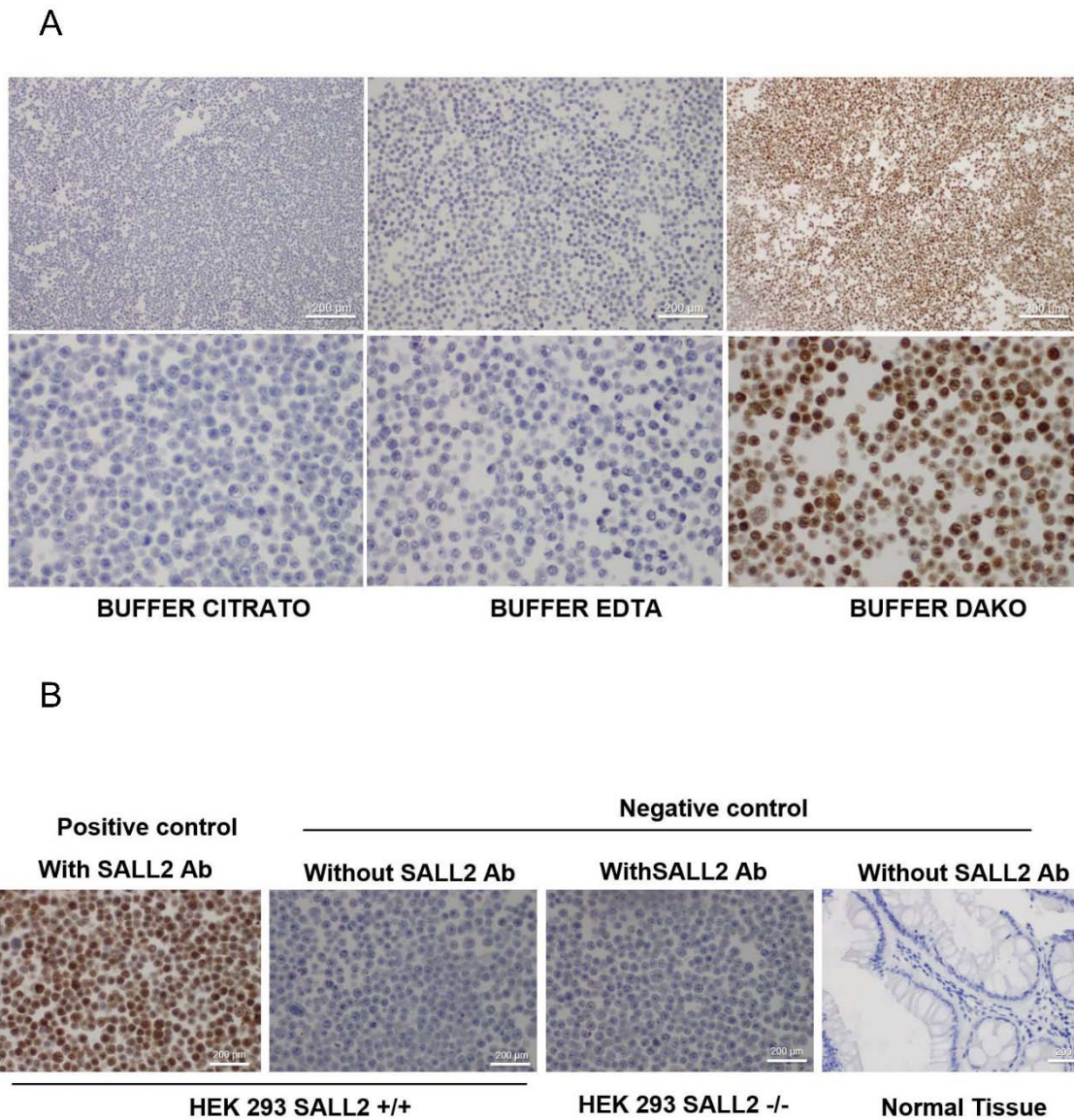
Supplementary Figure S1: *SALL2* expression is downregulated in CRC and related to overall survival **A)** *SALL2* mRNA expression levels in TCGA colon tumors (n = 286, blue bar) and matching normal tissue (n = 41, red bar) from UALCAN database ($p=7.5E-05$) **B)** The *SALL2* mRNA expression levels in TCGA normal (n=349) and GTEx cancer (n=275) data from GEPIA database ($p<0.05$). In red cancer tissue and grey normal tissue. **C)** Kaplan–Meier analysis of Overall survival (OS) curves for patients with high *SALL2* (n = 141) or low *SALL2* (n = 14) from TCGA Mixed colon adenocarcinoma cohort using R2 Genomics: dataset (<https://hgserver1.amc.nl/cgi-bin/r2/main.cgi>), *** $P < 0.001$, * $P < 0.05$. **D)** Kaplan–Meier analysis of OS curves for patients with high *SALL2* (n = 13) or low *SALL2* (n = 13) who died from cancer in our local study.



Supplementary Figure S2: *AXIN2* mRNA is downregulated in Colon Adenocarcinoma (COAD) tissues with *SALL2* genetic alterations. The functional heatmap table generated by the 'Gene Module' in the TIMER 2.0 platform shows the association between *SALL2*-mutated expression and Wnt pathway-related genes *ARID1B*, *AXIN2*, and *FBXW11* across TCGA cancer types. The red indicates a statistically significant positive association, and the blue indicates a statistically significant negative association. Gray denotes a non-significant result.



Supplementary Figure S3: Correlation between *SALL2* and *AXIN2* genes across bulk RNA sequencing datasets. **A)** Primary colorectal tumors (TCGA COAD). Scatter plot of log₂ CPM-normalized RNA seq counts for *SALL2* (x axis) and *AXIN2* (y axis) across all evaluable TCGA COAD samples (STAR Counts workflow). A best-fit linear regression (black line $\pm$ 95 % CI, grey band) illustrates a modest but significant inverse association (Pearson $r = -0.21$, $P = 1.5 \times 10^{-6}$; Spearman $\rho = -0.21$). **B).** Correlation of *SALL2* and *AXIN2* mRNA expression (RNA seq RPKM) in 60 colorectal cancer cell lines, visualized with the cBioPortal web server (dataset: Cancer Cell Line Encyclopedia – Broad 2019). Although Pearson’s correlation is weak and not significant ($r = 0.15$, $P = 0.244$), Spearman correlation shows a trend towards a negative relationship ($\rho = -0.25$, $P = 0.053$).



Supplementary Figure S4: Standardization of IHC staining and antibody specificity. **A)** SALL2 +/- HEK293 cells were processed based on the AgarCyto cell block preparation for IHC analysis. The efficacy of 3 different antigen retrieval buffers was tested (Citrate, EDTA, and DAKO), and the DAKO buffer was the most effective. Representative SALL2 IHC are shown (1:50 dilution). **B)** Representative SALL2 IHC images in SALL2 +/- HEK293 cells (1:50, positive control) and negative controls: SALL2 +/- HEK 293 cells without SALL2 antibody, SALL2 ^{-/-} HEK293 cells with the SALL2 antibody (1:50 dilution), and normal colon tissue without the SALL2 antibody.

SUPPLEMENTARY TABLES:

Supplementary Table S1: Clinical and Demographic Patient Information.

Diagnosis	n	Women	Men	Age at diagnosis
Normal	42	20/42 (47,6%)	22/42 (52,4%)	70
Adenoma	40	19/40 (47,5%)	21/40 (52,5%)	63,5
CRC	48	27/48 (56%)	21/48 (43,7%)	69,6
Total	130	66	64	67,7

Supplementary Table S2: CRC Patients Clinical characteristics

Clinical characteristics		SALL2 Negative		SALL2 Positive		p-value
		N	%	N	%	
Sex	Female	18	66,67	9	33,33	0,2357
	Male	16	76,19	5	23,81	
Age	> 60	26	83,87	5	16,13	0,0036
	< 60	8	47,06	9	52,94	
Tumor Size	< 5 cm	8	57,14	6	42,86	0,0903
	> 5 cm	26	76,47	8	23,53	
Tumor Localization	Colon	32	72,72%	12	27,27%	0,5695
	Rectum	2	50%	2	50%	
T	T1-T2	4	50,00	4	50,00	0,0778
	T3-T4	30	75,00	10	25,00	
N	N0	18	69,23	8	30,77	0,9503
	N1	6	75,00	2	25,00	
	N2	10	71,43	4	28,57	
Invasion	Mucosa	2	50,00	2	50,00	0,5649
	Submucosa	1	33,33	2	66,67	
	Serosa	31	75,61	10	24,39	
Microsatelital inestability	Positive	33	73,33	12	26,67	0,0700
	Negative	1	33,33	2	66,67	
β -catenin on the migratory front	Positive	24	80,00	6	20,00	0,0356
	Negative	10	55,56	8	44,44	
Linfovascular Permeation	Positive	19	76,00	6	24,00	0,2058
	Negative	15	65,22	8	34,78	
Ki67	Negative	22	75,86	7	24,13	0,2654
	Positive	12	63,15	7	36,84	

Supplementary Table S3: Primary antibodies.

Antibody	Clone	Source	Method	Dilution	Source
SALL2	HPA004162	SIGMA	WB	1:500	Rabbit
			IHQ	1:50	
ARID1B	(KMN1): sc- 32762	Santa Cruz	WB	1:350	Mouse
ARID1A	(C-7): sc- 373784	Santa Cruz	WB	1:350	Mouse
FBXW7	(A-4): sc- 518093	Santa Cruz	WB	1:350	Mouse
FBXW11	(C-6): sc- 390629	Santa Cruz	WB	1:350	Mouse
LEF1	(B-6): sc-374522.	Santa Cruz	WB	1:500	Mouse
DAAM2	(E-1): sc-515129	Santa Cruz	WB	1:500	Mouse
WNT7b	(H-8): sc-365459	Santa Cruz	WB	1:350	Mouse
WNT3a	(3A6): sc-136163	Santa Cruz	WB	1:350	Mouse
AXIN2	(C-6): sc-25302.	Santa Cruz	WB	1:500	Mouse
		Santa Cruz	IF	1:25	
β-catenin	(e-5): sc-7963	Santa Cruz	WB	1:100	Mouse
β-Actin	(C4): sc-47778	Santa Cruz	WB	1:20000	Mouse
GAPDH	(e-5): sc-7963	Santa Cruz	WB	1:20000	Mouse

HDAC 1	(C-19): sc-6298	Santa Cruz	WB	1:500	Goat
Flag	Anti-Flag M2, clone M2	SIGMA	WB	1:5000	Mouse
Cleaved PARP	(194C1439): sc-56196	Santa Cruz	WB	1:500	Mouse
Cleaved caspase 3	(31A1067): sc-56053	Santa Cruz	WB	1:500	Mouse

Supplementary Table 4: Primer sequences for qRT-PCR and ChIP

Partidores	Forward primer (5'-3')	Reverse primer (5'-3')
<i>AXIN2</i>	CCCATGCCTGTCTCTTCCAA	ATCCACGCATTTCTCCCTCT
<i>ACTIN</i>	GATGAGATTGGCATGGCTTT	CACCTTCACCGTTCCAGTTT
<i>PPIB</i>	AACGCAGGCAAAGACACCAA CG	TCTGTCTTGGTGCTCTCCACCT
<i>AXIN2</i> (-1420/- 1410/-1405/-1400)	CTGTTGCATTCCAGAGCCG	CCTTAAAGGGACAGCGCC
<i>AXIN2</i> (-784)	CGCCTTTGAAGTGCACAGTT	TTATGCAAAAGATCCGAGCGG
<i>AXIN2</i> (-112/-108)	TTATGGATGTGAGTCGGCGT	GAGGGGAAAAGGGGAGGG
<i>URR (ChIP)</i>	TGAAGCGGCTCTCAGTAACC	AGCTACCTGGGAACGTGAAA