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Supplemental Information

Dachshund Depletion Disrupts Mammary Gland Development and Diverts the Composition of the Mammary Gland Progenitor Pool

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Dachshund depletion disrupts mammary gland development and diverts the composition of the mammary progenitor pool

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Running Head: DACH1 expands murine mammary gland stem cells *in vivo*.

Keywords: DACH1, mammary gland, breast cancer, TGFβ, SARA, stem cells.

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Transgenic mice. All the animal studies were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University. The mice were housed and maintained at the animal facility in Thomas Jefferson University. The *Dach1*$^{fl/fl}$ mice (Chen et al., 2015; Pierce et al., 1993) (which remove the same section of the *Dach1* locus that is deleted in the knockout mice strain (Davis et al., 2001)), were intercrossed with the *ROSA26*$^{\text{Cre-ERT2}}$ mice (expressing CRE-ERT2 fusion protein under the control of the ubiquitous *ROSA2*6 promoter, The mice were kindly provided by Dr. Thomas Ludwig at Columbia University, New York, NY.). The Cre-ERT2 fusion protein binds with tamoxifen and is then transported to the nucleus to induce the deletion of floxed alleles (de Luca et al., 2005) and activate *ROSA26*$^{\text{mT-mG}}$, a cell membrane-targeted, two-color fluorescent Cre-reporter allele. Prior to Cre recombination, tdTomato (mT) red fluorescence will be expressed in almost all cells. In the presence of Cre recombinase, cells will express cell membrane-localized EGFP (mG) fluorescence instead of the red fluorescence. The Jackson Laboratory Stock No. 007576 was previously described (Muzumdar et al., 2007). *Dach1*$^{fl/fl}$ mice were mated with either *ROSA26*$^{CreERT2/CreERT2}$ or *ROSA26*$^{\text{mTmG/mTmG}}$ mice to generate *Dach1*$^{fl/wt}$*ROSA26*$^{CreERT2/CreERT2}$ (Cre mice) and *Dach1*$^{fl/wt}$*ROSA26*$^{mTmG/mTmG}$ (Cre reporter mice). *Dach1*$^{fl/wt}$*ROSA26*$^{CreERT2/+}$ mice were then mated with *Dach1*$^{fl/wt}$*ROSA26*$^{mTmG/+}$ mice to generate *Dach1*$^{fl/fl}$*ROSA26*$^{CreERT2/mTmG}$ mice and *Dach1*$^{wt/wt}$*ROSA26*$^{CreERT2/mTmG}$ littermates. Six-week-old female mice were administered tamoxifen by intra-peritoneal injection (2mg/25g body weight per day for 5 days) to induce the deletion of floxed alleles. The extent of deletion was assessed by PCR-based DNA analysis. Littermates of *Dach1*$^{wt/wt}$*ROSA26*$^{CreERT2/mTmG}$ were used as controls to decrease individual differences and avoid potential effects of tamoxifen on mammary gland development and gene expression (Shehata et al., 2014). Mammary squashes and analysis of mammary gland development were conducted as previously described (Rowlands et al., 2003)

Cell culture, reagents, mammosphere assays, expression vectors, DNA transfection, and statistical analyses. LA-7 cells were transiently transfected with an expression vector encoding DACH1 or a control vector as described (Wu et al., 2006) using the Lipofectamin^{TM} 2000 reagent (Cat. 11668-027, Invitrogen) according to the manufacturer's instructions. 48 hrs. after transfection, cells were detached using 0.25% trypsin and 0.5 mM EDTA for 5 min at 37°C in order to obtain a suspension of single cells which were then counted. The cells were then divided into three pools and used for either Real Time PCR analysis (RT-PCR), mammosphere formation or organoid formation assay. Thus, a portion of the cells were replated in 2D culture conditions for subsequent RNA

extraction and for quantitative Real Time PCR analysis, a portion of the cells were placed in non-adherent culture conditions for mammosphere formation and a portion of the cells were used in 3D collagen matrix for organoid formation assay.

For mammosphere assays, suspensions of single cells transfected with an expression vector encoding DACH1 or control vector were plated at a limiting dilution (500 cells/1.4 ml) in non-differentiating medium as described (Zucchi et al., 2007).

Tubule and organoid formation in 3D culture. For tubule/organoid formation, cells were suspended on ice in rat tail derived collagen prepared as previously described (Zucchi et al., 2007). Cells were embedded in collagen at the density of 1000 cells/mL. Aliquots of 500 μL of cell and collagen suspension were plated in triplicate into 48 well plates (Greiner, Twin-Helix). After incubation at 37°C for 1 hr., medium was added and subsequently replaced every 2.5 days. Organoid cultures were examined daily with light microscopy to assess tubule and organoid formation and photographed at days 7, 14 and 20. Evaluation of branching was assessed 3 days after seeding by examining 10 tubules for each condition in 3 independent experiments.

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Immunoprecipitation and Western Blot. Immunoprecipitation (IP) and Western blot assays were performed in cells as indicated. Antibodies used in Western blotting included anti-CD44 (sc-53068, Santa Cruz), goat polyclonal anti-β-actin (Cat #sc-1615, Santa Cruz), anti-β-casein (Cat #sc-30042, Santa Cruz), anti-Smad2/3 (BD Cat #610843), anti-phosphorylated Smad2/3 (Santa Cruz Biotechnology Cat # sc-11769-R), anti-phosphorylated Smad1/5/8 (Santa Cruz Biotechnology Cat #sc-12353-R), anti-SARA (Proteintech Cat # 14821-1-AP), anti-DACH1 (Proteintech Cat #10914-1-AP), or anti-FLAG M2 antibody (Sigma). Cells were lysed in immunoprecipitation (IP) buffer (10 mMTris-HCL at pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% IGEPAL CA-630, 10% glycerol, 0.2 mM sodium orthovanadate, 0.1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin). For each IP, 1 ml lysate (1 mg protein) and 2 μg anti-Smad2/3 (BD Cat #610843) or anti-SARA (Proteintech Cat #14821-1-AP) were incubated overnight at 4^{o}C. Immunoprecipitates were washed 5 times in IP buffer, and 30 μl of 2 x sample buffer was added to the bead pellet. The immunoprecipitates, as well as 50 μg proteins of the corresponding lysates, were separated by electrophoresis in 4–11% graded polyacrylamide gel, transferred to nitrocellulose filters, and immunoblotted with the indicated antibodies. Protein bands were detected using the enhanced chemiluminescence detection system (Amersham Biosciences). Laminin B1 antibody (Abcam

Cat #ab16048) was used as an internal control for nuclear protein abundance and vinculin (Sigma Cat #V9131) was used as a control for cytoplasmic fraction enrichment.

FACS analysis of stem cells. FACS analysis for mammary epithelial stem cells was conducted as outlined in prior publications (dos Santos et al., 2013; Jiao et al., 2016; Shackleton et al., 2006). Briefly, mammary glands were removed from 12 week old *Dach1*$^{fl/fl}$*;ROSA26*$^{CreERT2/mTmG}$ and *Dach1*$^{wt/wt}$*;ROSA26*$^{CreERT2/mTmG}$ mice. Both strains of mice were treated with tamoxifen at 6 weeks. The mammary gland was sectioned into 1 mm^3 pieces and subsequently digested with 300 U/ml collagenase and 100 U/ml hyaluronidase, 0.25% trypsin-2.1 mM EDTA, 5 mg/ml dispase and 0.1 mg/ml DNase and then treated with Hemolysis Buffer. The tissue debris was removed with a BD 40 μm cell strainer. The cells were blocked with normal IgG and rat anti-mouse CD16/CD32 antibody (2.4G2,BD Pharmingen) in 1:100 dilution for 30 min and then incubated with lineage marker (Allophycocyanin (APC) labeled anti-mouse CD31 (clone MEC 13.3), CD45 (clone 30-F11) and Ter119, all were from BD BioSciences Pharmingen) (1/50-1/100), phycoerythrin (PE)/Cy5-anti-mouse/rat CD29 (clone HMβ1-1, BioLegend) (1/200-1/400), and PE/Cy7-labeled rat anti-mouse CD24 (clone M1/69, BioLegend, San Diego) (1:200-1/400) for 1 h. All experiments were conducted at 4°C. Cell sorting was performed on a FACS LSRII cell sorter (BD Biosciences). Forward Scatter (FSC), Side Scatter (SSC), Green Fluorescent Protein (GFP), Tomato Fluorescent Protein, APC, PE/Cy5 and PE/Cy7 signals were recorded. The data were analyzed with FlowJo single cell analysis software (Tree Star, Inc., Ashland, OR).

Quantitative Real-time PCR. PCR analysis for luminal markers (Cdh1 (E-cadherin)), cytokeratin (CK18), alveolar marker (β-casein) and CD44 was conducted of LA-7 mRNA as previously described (Zucchi et al., 2007). Total RNA was isolated from murine mammary gland tissues and LA-7 cells, or LA-7 cells transfected with a DACH1 expressing vector or the control vector, using Trizol. RNA samples were treated with RQ1 DNase I (Promega Inc., Madison, WI) to remove contaminating DNA from RNA preparations followed by re-purification using the RNeasy Mini Kit (Qiagen, Valencia, CA). DNA-free RNA was subjected to reverse transcription reactions, performed using SuperScript™ III reverse transcriptase kit (Invitrogen, Carlsbad, CA). Following preparation of cDNA, SYBR Green based real-time PCR reactions were performed using Power SYBR Green Master Mix (Invitrogen, Carlsbad, CA) on an ABI Prism 7900HT system (Applied Biosystems Inc., Foster City, CA). Amplification of 18s rRNA was performed in every sample and the obtained Ct values for each sample were used for normalization. Primers for all

the genes/gene transcripts including 18s rRNA and HPRT (hypoxanthine phosphoribosyltransferase) are listed in Table 1, or for Cdh1, CK18, and CD44 as previously described (Zucchi et al., 2007).

SUPPLEMENTAL FIGURES

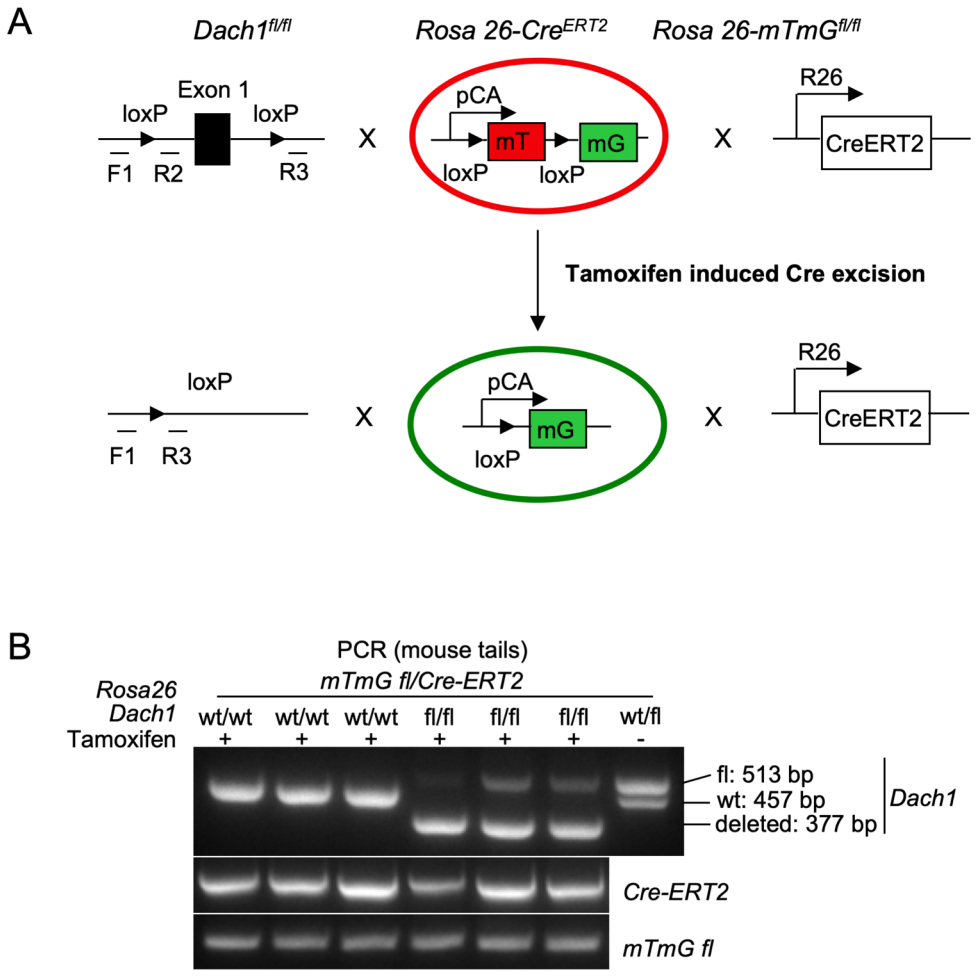

Figure S1. Multigenic mice analysis. (A). Schematic representation of the transgenic mice illustrating the Cre induced conversion from tomatoe red (mT) to green (mG) fluorescence and the consequent excision of the *Dach1*$^{fl/fl}$ allele. (B). Representative example of PCR-based genotyping for the multigenic mice with the specific PCR based products for the alleles as indicated.

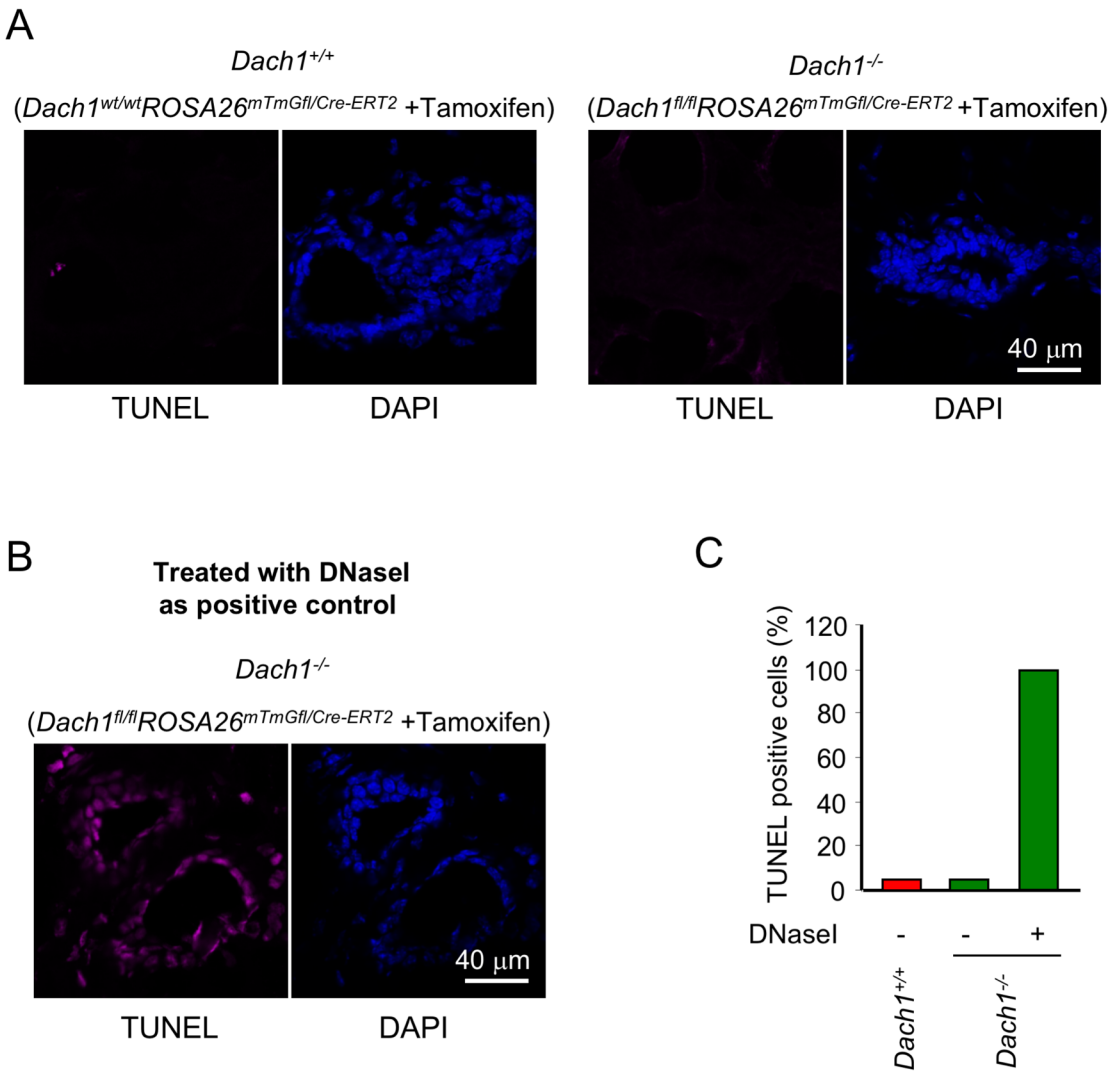

Figure S2. *Dach1* gene deletion does not affect mammary gland apoptosis. (A-C) Mammary gland apoptosis assessed by TUNEL staining for transgenic *Dach1* gene deletion mice with DNase 1 positive control, together showing no significant change in TUNEL staining in *Dach1* deletion mice mammary gland.

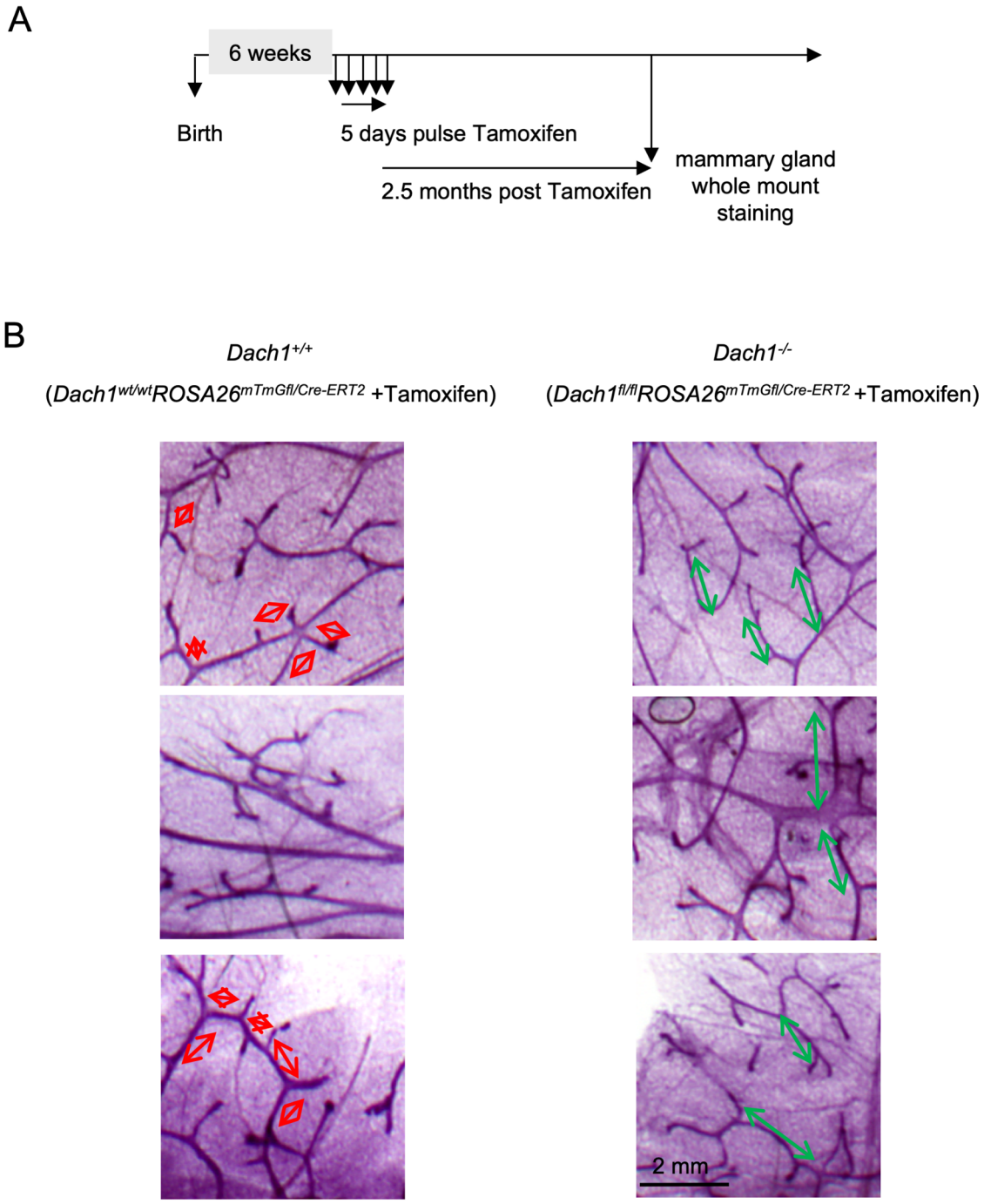

Figure S3. Transgenic mice mammary gland morphological analysis. (A) Schematic representation of the transgenic mice illustrating the timing of tamoxifen treatment to induce Cre expression, with subsequent washout. (B) Representative examples of mammary squashes from transgenic mice (*Dach1*$^{wt/wt}$*Rosa26*$^{mTmGfl/Cre-ERT2}$ and (C) *Dach1*$^{fl/fl}$*Rosa26*$^{mTmGfl/Cre-ERT2}$ as indicated.

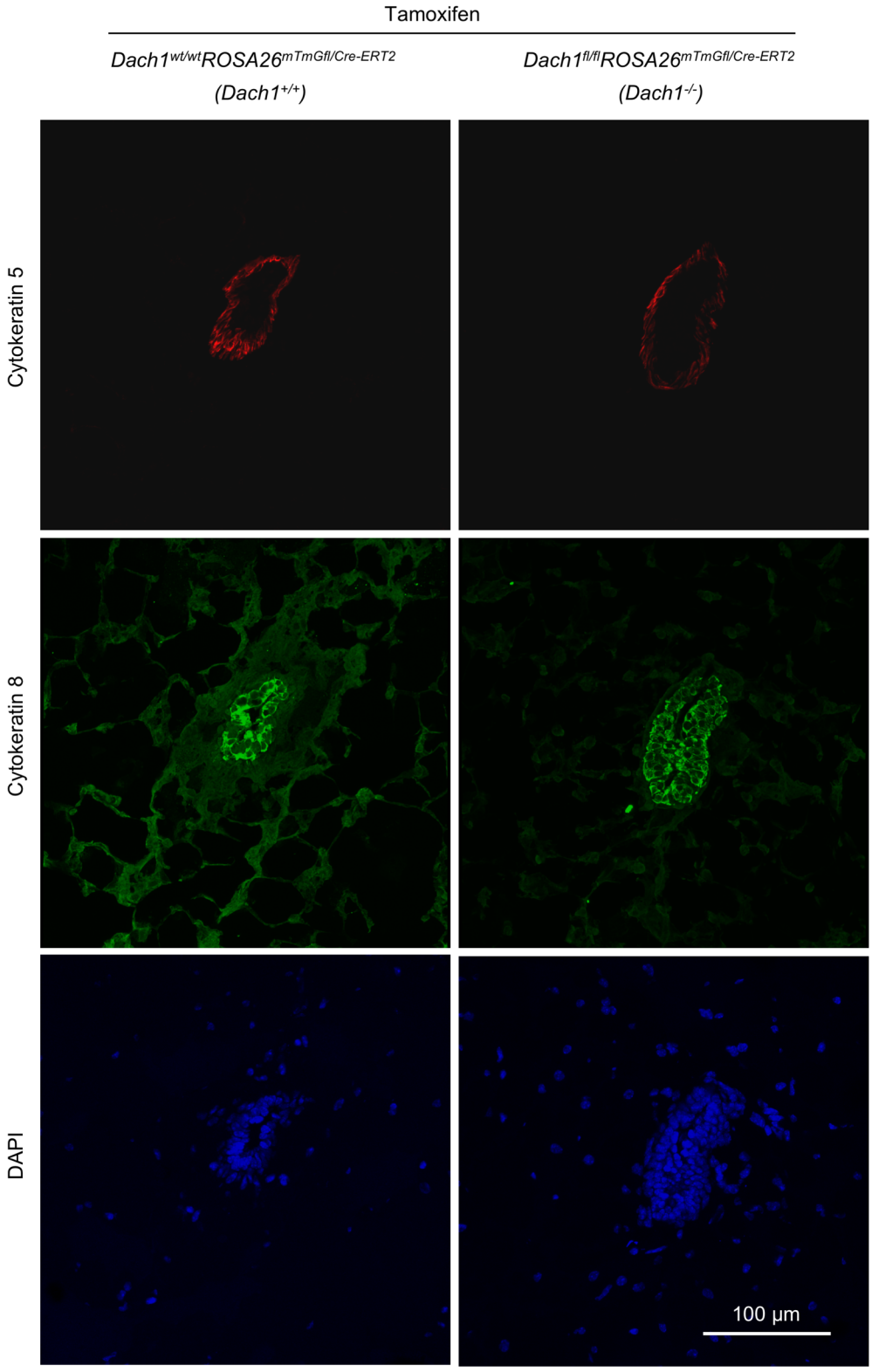

Figure S4. Double immunofluorescent staining of Cytokeratin 5 and 8 in *Dach1*$^{-/-}$ mouse mammary gland. Same images as figure 4A but with separated fluorescent channels.

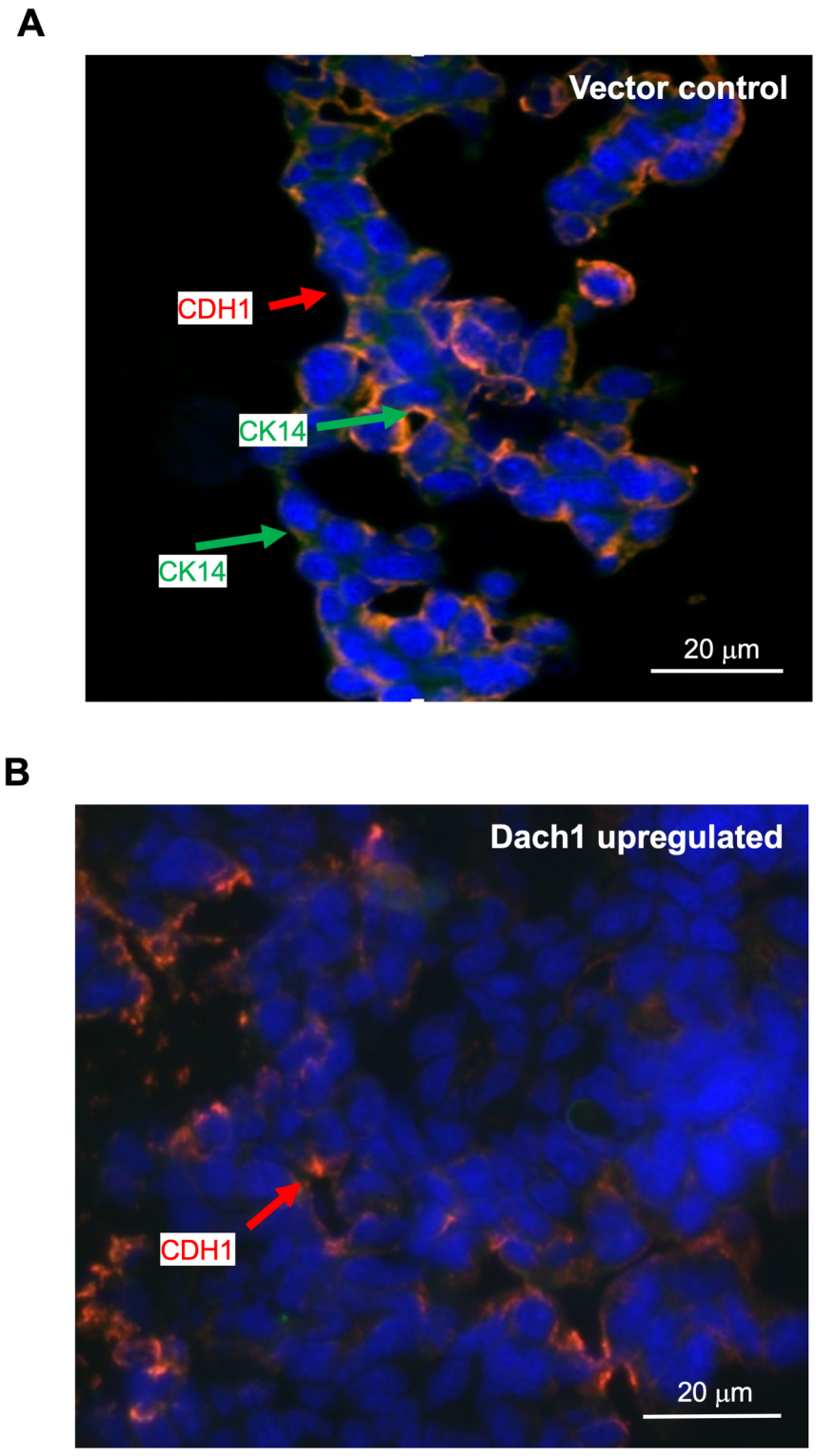

Figure S5. LA-7 generated 3D organoids and tubules stained for luminal and myo-epithelial markers. Same images as figure 6E but with higher resolution.

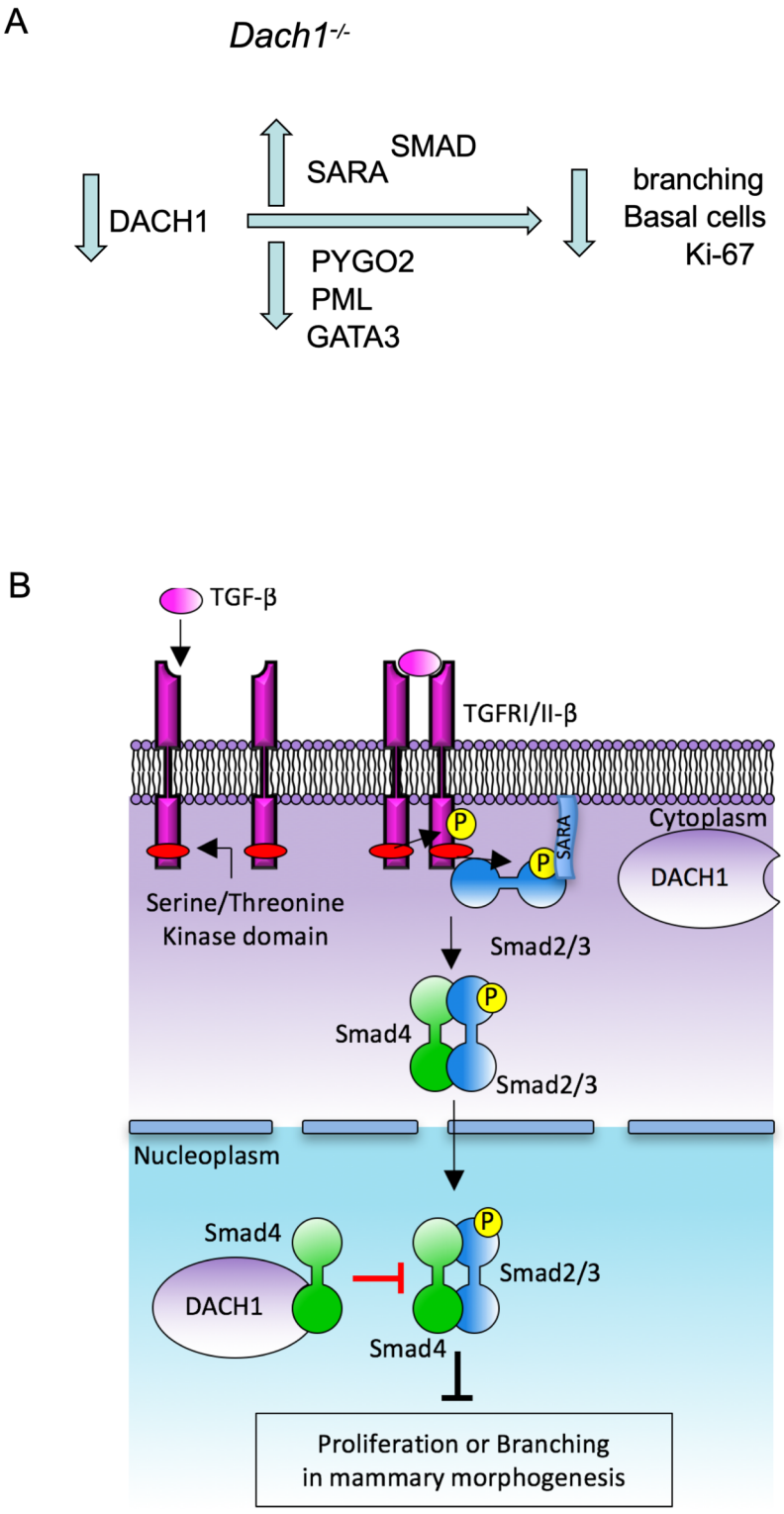

Figure S6. DACH1 restrains TGFβ signaling. (A,B). Schematic representation of a model in which association of DACH1 with SARA restrains TGFβ signaling.

Table 1. Primers for all the genes/ gene transcripts including housekeeping control genes/ gene transcripts.

Gene name	Species	Forward sequence	Reverse sequence	Note
Dach1	mouse	ACAAGTCAACCAGGAGGCTTAG	TTCACATGTAAGCAGTGTGGACT	genotyping PCR: *Dach1* floxed allele: 513bp; *Dach1* wild type allele: 457bp
Dach1	mouse	ACAAGTCAACCAGGAGGCTTAG	ACAACAATGTCCTGGGTGCTT	genotyping PCR: *Dach1* deleted allele: 377bp
Dach1	mouse	GGCTTCCTTTACGGTGGAGG	CAGCTTGGTGTAGACGGTGTG	real time PCR
Gata3	mouse	CTCGGCCATTCGTACATGGAA	GGATACCTCTGCACCGTAGC	real time PCR
Gata3	rat	GCAGGCGTTGCAAAGGTAGT	CTACCGGGTTCGGATGTAAGTC	real time PCR
Pml	mouse	CCAGAGGAACCCTCCGAAGA	GGCAGCGCAGAAACTGAAAT	real time PCR
Elf5	mouse	ATGTTGGACTCCGTAACCCAT	GCAGGGTAGTAGTCTTCATTGCT	real time PCR
Pygo2	mouse	AGCGAAGAAAGTCCAATACTCAG	GTTAGAAGCGACCAGATGATCC	real time PCR
Notch1	mouse	GATGGCCTCAATGGGTACAAG	TCGTTGTTGTTGATGTCACAGT	real time PCR
Notch1	rat	GTCAATGTTCGAGGACCAGATG	CTGATAGATGAAGTCGGAGATGACA	real time PCR
18s rRNA		GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	real time PCR
p21	rat	CAGACCAGCCTAACAGATTTCTATCA	GGCACTTCAGGGCTTTCTCTT	real time PCR
Cytokeratin14 (CK14)		CGCCTGGCGGCTGAT	TTGATGTCAGACTCCACATTTATGC	real time PCR
HPRT		TCCATTCCTATGACTGTAGATTTTAT CAG	AACTTTTATGTCCCCCGTTGACT	real time PCR

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