Parsing β-catenin's cell adhesion and Wnt signaling functions in malignant mammary tumor progression

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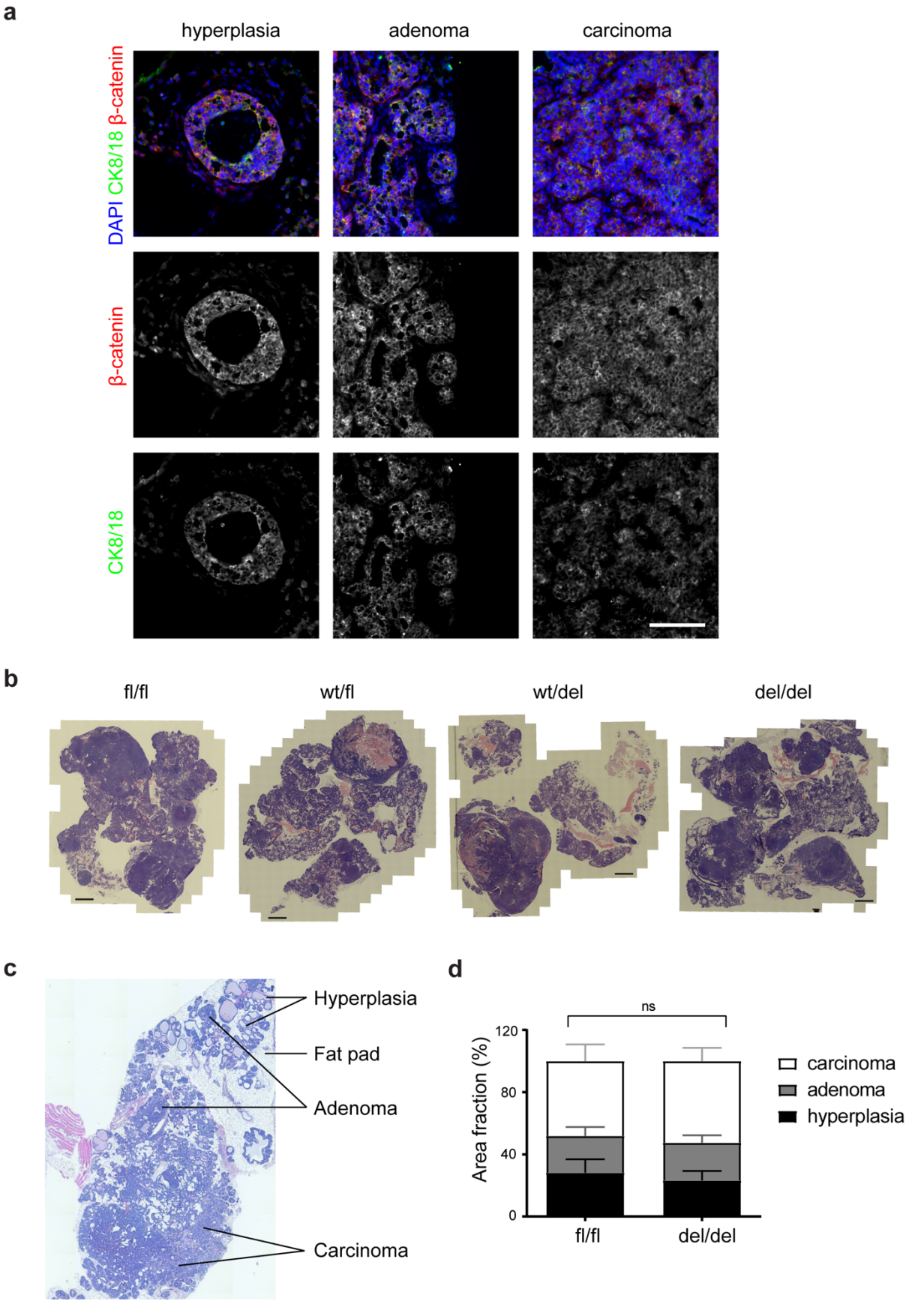
Supplementary Information

Supplementary Figures

Suppl Figure S1



Supplementary Figure S1. Mammary tumor cells escape Cre recombinase-mediated ablation of β-catenin.

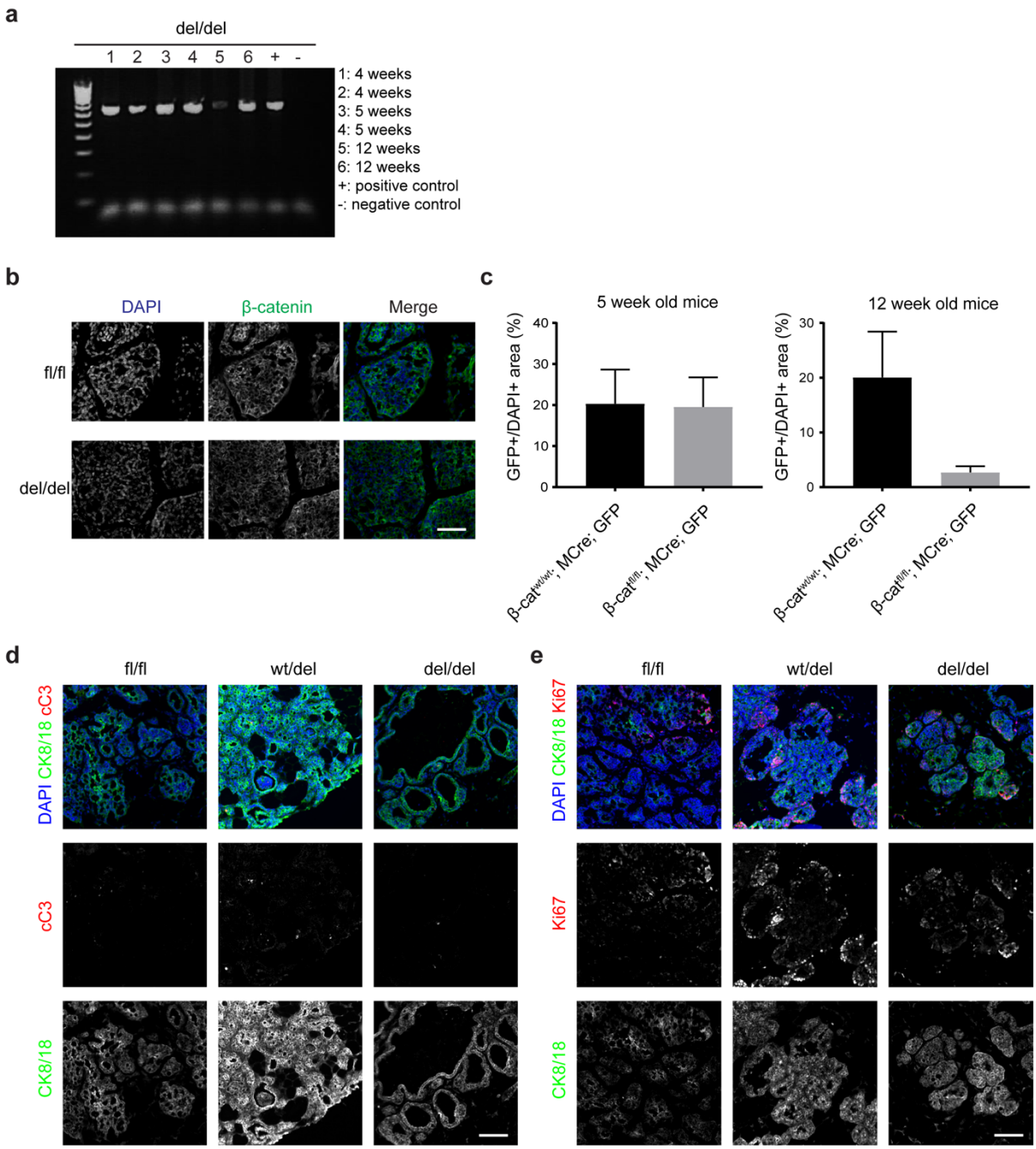
(a) Expression of β-catenin in the progressive stages of mammary tumor progression (hyperplasia, adenoma and carcinoma) in MMTV-PyMT transgenic mice. Representative histological sections of hyperplasia, adenoma and carcinoma were stained for CK8/18 to identify tumor cells for β-catenin and visualized by immunofluorescence microcopy. Scale bar, 100μm.

(b) No change in tumor progression in upon genetic ablation of β-catenin. Histological sections of tumors from β-catenin$^{fl/fl}$;MMTV-PyMT (fl/fl), β-catenin$^{fl/fl}$;MMTV-PyMT (wt/fl), β-catenin$^{wt/fl}$;MMTV-PyMT;MMTV-Cre (wt/del), and β-catenin$^{fl/fl}$;MMTV-PyMT;MMTV-Cre (del/del) mice at 12 weeks of age were stained with hematoxylin and eosin and visualized by wide field stitching microscopy. Scale bar, 2mm.

(c) The light microscopy image represents a hematoxylin and eosin-stained tumor section of the mammary gland of an MMTV-PyMT mouse showing different stages of breast cancer progression from hyperplasia to adenoma to carcinoma and the fat pad embedding the tumor, as indicated.

(d) Histopathological quantification of the different tumor stages in MMTV-PyMT;β-catenin$^{fl/fl}$ (fl/fl; n=7) and MMTV-PyMT;β-catenin$^{fl/fl}$;MMTV-Cre (del/del; n=11) mice. Data are displayed as mean ± SEM. Statistical analysis was performed using a Mann-Whitney U test. ns, not significant.

Suppl Figure S2



Supplementary Figure S2. β-catenin deficiency provokes cell death, and primary mammary tumors and metastasis retain β-catenin-expression.

(a) Genotyping of tumors of MMTV-PyMT;β-catenin$^{fl/fl}$;MMTV-Cre (del/del) mice, demonstrating an incomplete deletion of the β-catenin gene, in particular in 12 week-old mice. Positive control: after recombination of β-catenin$^{fl/fl}$ in a cell line; negative control: a β-catenin$^{fl/fl}$ cell line in the absence of Cre recombinase.

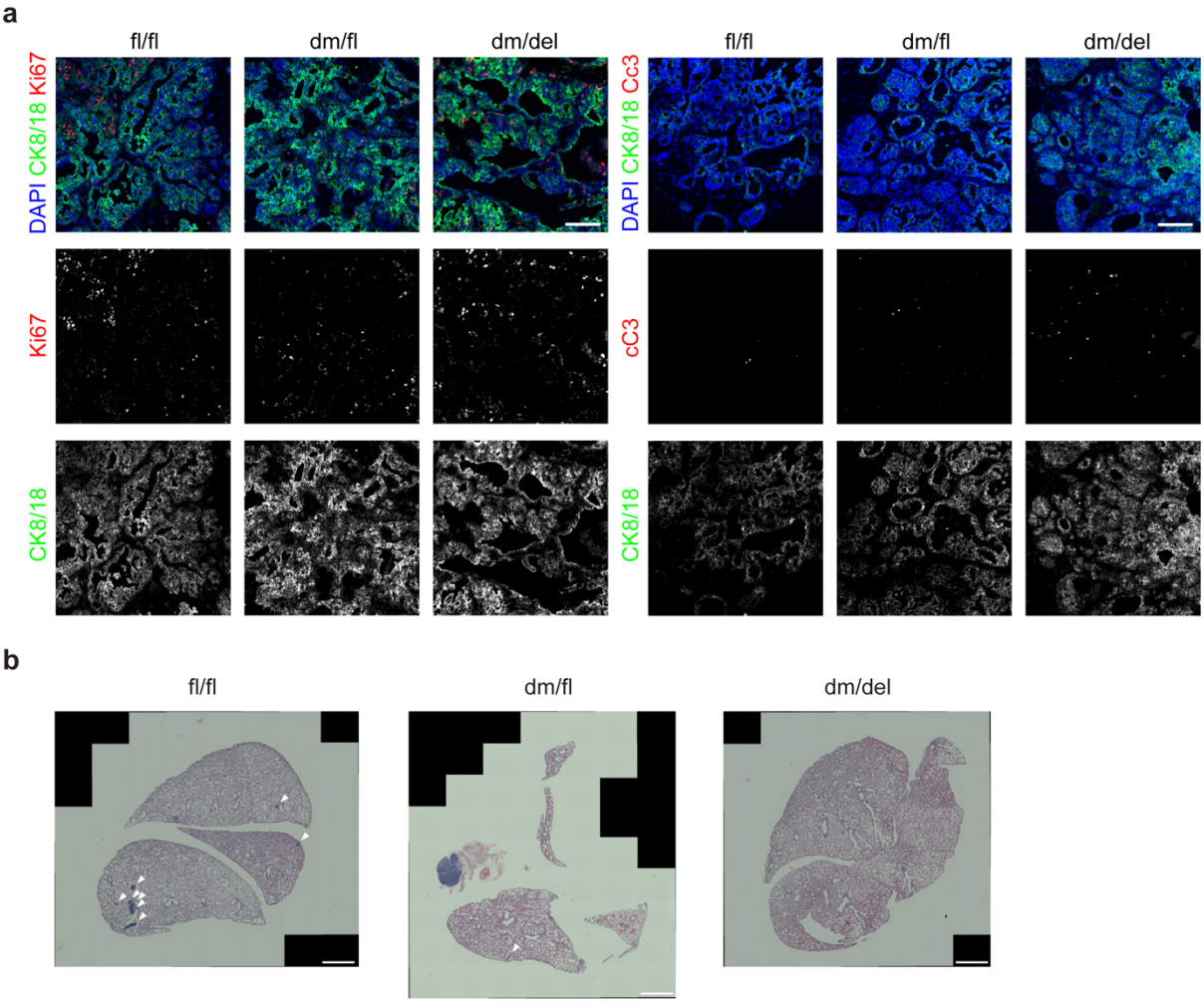
(b) Immunofluorescence (IF) staining of tumor sections of MMTV-PyMT;β-catenin$^{fl/fl}$ (fl/fl) and MMTV-PyMT;β-catenin$^{fl/fl}$;MMTV-Cre (del/del) mice for β-catenin, demonstrating that β-catenin is expressed by all tumor cells, including tumor cells of MMTV-PyMT;β-catenin$^{fl/fl}$;MMTV-Cre (del/del) mice. DAPI was used to visualize nuclei. Scale bar: 50μm.

(c) Quantification of the recombination efficiency of MMTV-Cre in the mammary gland using GFP reporter mice comparing MMTV-PyMT;β-catenin$^{wt/wt}$;MMTV-Cre;LSL-GFP and MMTV-PyMT;β-catenin$^{fl/fl}$;MMTV-Cre;LSL-GFP mice at 5 and 12 weeks of age. n=5. Data are displayed as mean ± SEM.

(d) Apoptotic cells were detected by immunofluorescence staining for cleaved Caspase-3 and CK8/18 for tumor cells in preneoplastic lesions of β-catenin$^{fl/fl}$;MMTV-PyMT (fl/fl; wildtype control), β-catenin$^{wt/fl}$;MMTV-PyMT;MMTV-Cre (wt/del; heterozygous knockout), and β-catenin$^{fl/fl}$;MMTV-PyMT;MMTV-Cre (del/del; homozygous knockout) mice at 5 weeks of age. DAPI was used to visualize nuclei. Dots in the graphs represent the individual imaging fields analyzed. Scale bar: 100μm. Quantification of cC3+ apoptotic cells is shown in Fig. 1b.

(e) Proliferating cells were detected by immunofluorescence staining for Ki67 and CK8/18 for tumor cells in preneoplastic lesions of β-catenin$^{fl/fl}$;MMTV-PyMT (fl/fl; wildtype control), β-catenin$^{wt/fl}$;MMTV-PyMT;MMTV-Cre (wt/del; heterozygous knockout), and β-catenin$^{fl/fl}$;MMTV-PyMT;MMTV-Cre (del/del; homozygous knockout) mice at 5 weeks of age. DAPI was used to visualize nuclei. Scale bar: 100μm. Quantification of Ki67+ proliferating cells is shown in Fig. 1c.

Supplemental Figure S3

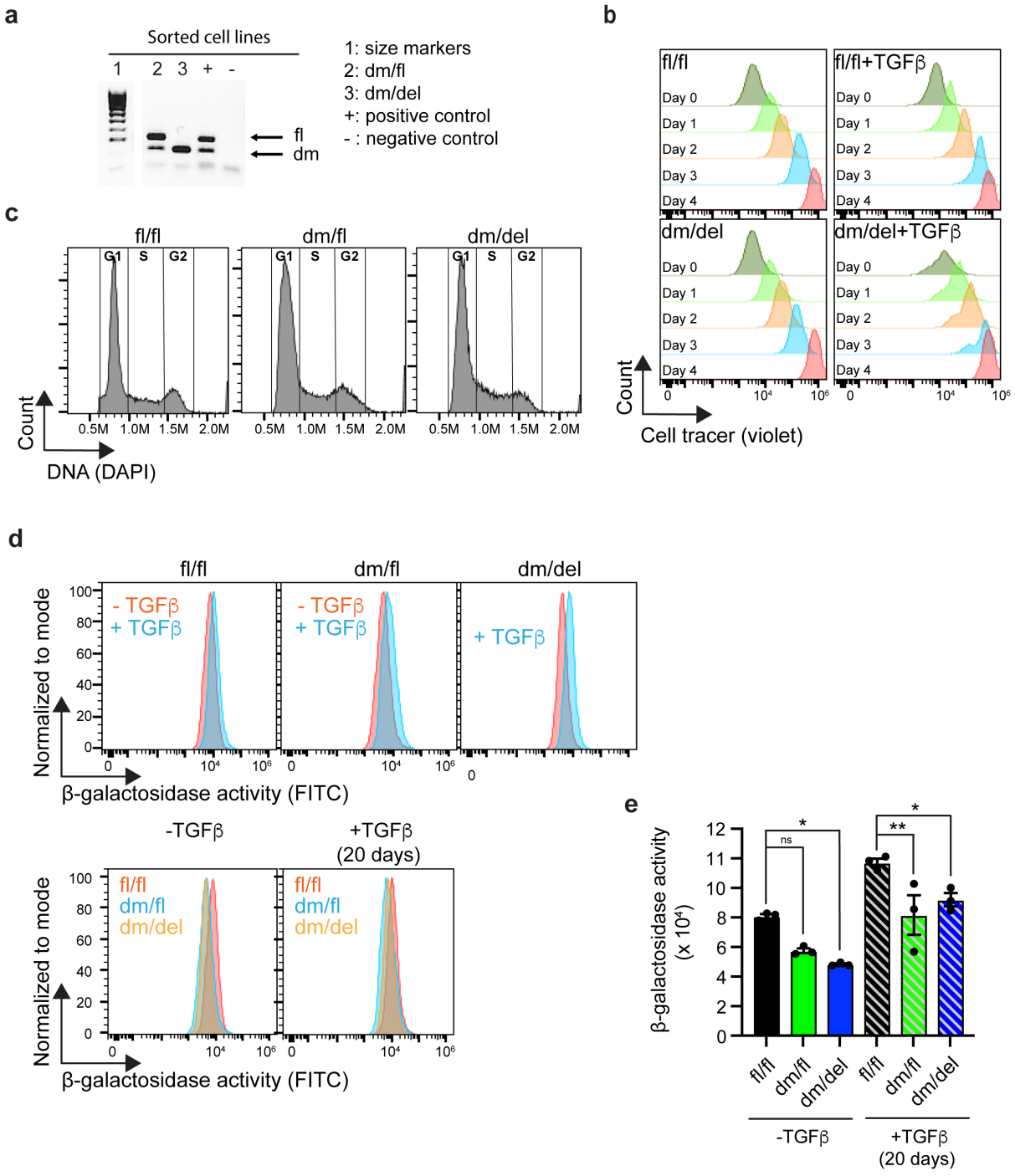


Supplementary Figure S3. Loss of β-catenin cofactor binding impairs tumor cell proliferation and reduces tumor cell apoptosis and lung metastasis formation.

(a) Immunofluorescence staining for Ki67 and CK8/18 to assess tumor cell proliferation (left panels) and for cleaved caspase 3 (cC3) and CK8/18 to assess tumor cell apoptosis (right panels) on tumor sections from β-catenin$^{fl/fl}$;MMTV-PyMT (fl/fl), β-catenin$^{dm/fl}$;MMTV-PyMT (dm/fl), and β-catenin$^{dm/fl}$;MMTV-PyMT;MMTV-Cre (dm/del) mice at 12 weeks of age. DAPI was used to visualize nuclei. Scale bar: 100µm.

(b) Reduced lung metastasis formation upon β-catenin transcriptional activity. Histological sections of lungs from β-catenin$^{fl/fl}$;MMTV-PyMT (fl/fl), β-catenin$^{dm/fl}$;MMTV-PyMT (dm/fl), and β-catenin$^{dm/fl}$;MMTV-PyMT;MMTV-Cre (dm/del) mice at 12 weeks of age were stained with hematoxylin and eosin and visualized by wide field stitching microscopy. White arrowheads indicate metastatic lesions. Scale bar, 2mm.

Supplemental Figure S4



Supplementary Figure S4. Effect of the loss of β-catenin's transcriptional activity on its subcellular localization and on tumor cell proliferation.

(a) Genotyping of cell lines established from tumors of the various genotype mice. Floxed alleles of β-catenin were removed by infection with Ad-Cre-IRES-GFP virus and flow cytometry sorting for GFP^+ cells. Primers RM41/RM42 amplify the *Ctnnb1* floxed allele (324bp) and wild-type or dm-mutant allele (221bp) (40).

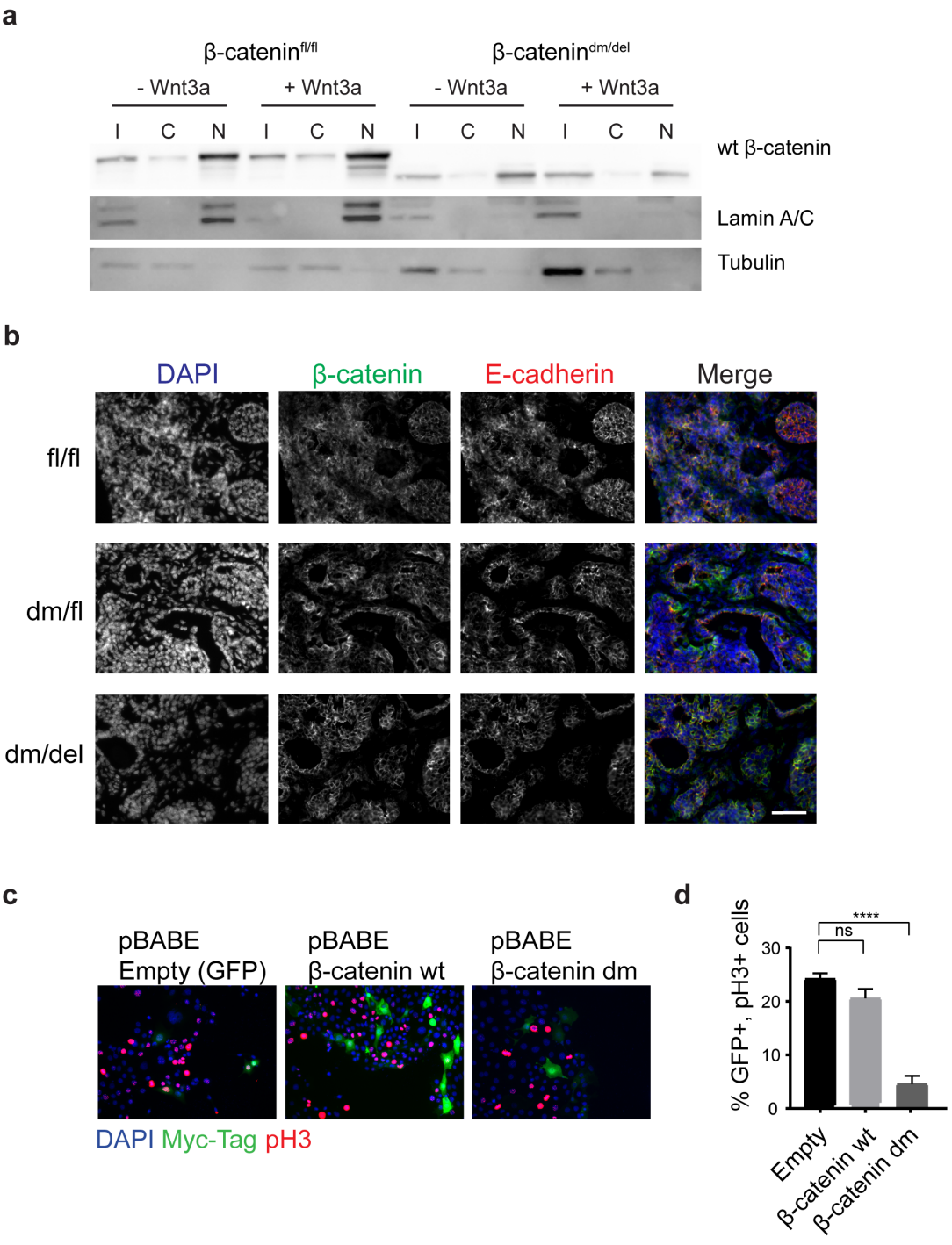
(b) Cell count analysis of fl/fl and dm/del cells by cell tracer staining and flow cytometry in the absence or presence of TGFβ over 4 days.

(c) Cell cycle analysis of fl/fl, dm/fl, and dm/del cells by DAPI staining and flow cytometry. Quantification of the cells in the various phases of cell cycle is shown in Figure 3b.

(d) fl/fl, dm/fl, and dm/del cells in the absence or in the presence of TGFβ for 20 days were stained for acidic β-galactosidase to mark cells in quiescence and analyzed by flow cytometry.

(e) Quantification of the flow cytometry analysis of the cell quiescence analysis described in (d). Data are displayed as mean ± SEM. Statistical analysis was performed using ordinary one-way ANOVA multiple comparison test. *, $p < 0.05$; **, $p < 0.01$; ns, not significant.

Supplemental Figure S5



Supplementary Figure S5. Effect of the loss of β-catenin's transcriptional activity on its subcellular localization and on tumor cell proliferation.

(a) Nuclear localization of β-catenin. fl/fl and dm/del cells were fractionated for nuclear and cytosolic protein content and the levels of β-catenin were determined by immunoblotting.

Lamin A/C was used as nuclear marker tubulin as cytosolic marker. I = total lysate input, C = cytosolic fraction, N = nuclear fraction. Note that due to its C-terminal deletion the dm mutant form of β-catenin exhibits a reduced molecular weight.

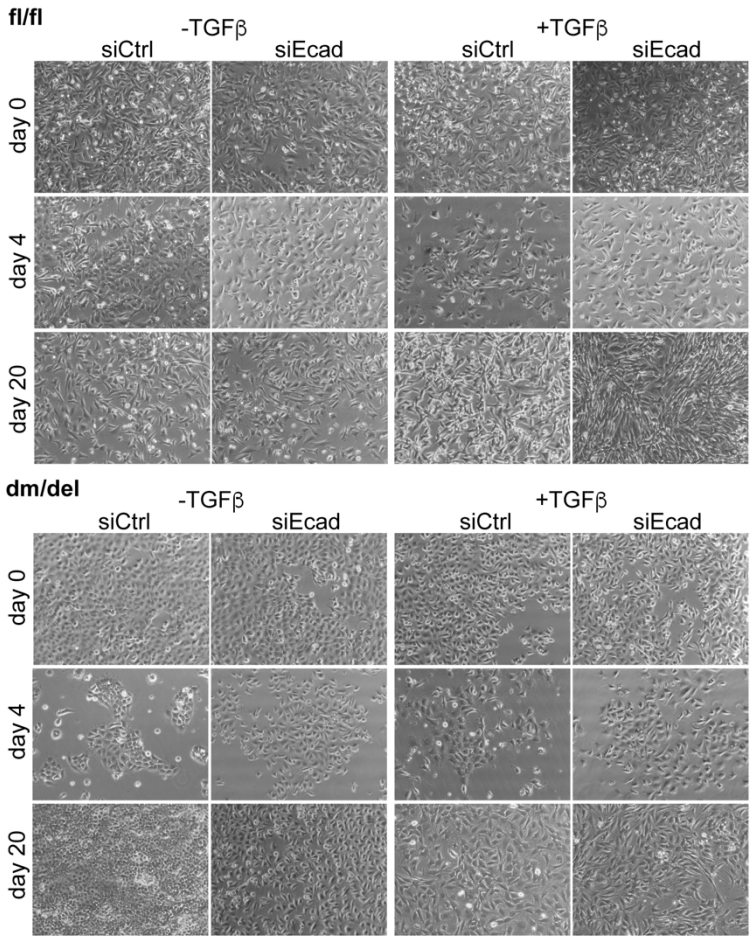
(b) Immunofluorescence staining for E-cadherin and β-catenin on tumor sections from twelve-week-old MMTV-PyMT transgenic mice expressing in their mammary tumor cells wild-type (fl/fl) β-catenin, one wild-type and one mutant allele of β-catenin (dm/fl) or only the mutant allele of β-catenin(dm/del). DAPI was used to visualize nuclei. Scale bar, 50μm.

(c) Transient transfection of Myc-tagged β-catenin^{wt} and β-catenin^{dm} or an empty vector expressing GFP in a β-catenin wild-type cell line (Py2T) and immunofluorescence visualization of the Myc-Tag or of GFP expression (green) and of pH3-positive proliferating cells (red). DAPI was used to visualize nuclei. Scale bar, 100μm.

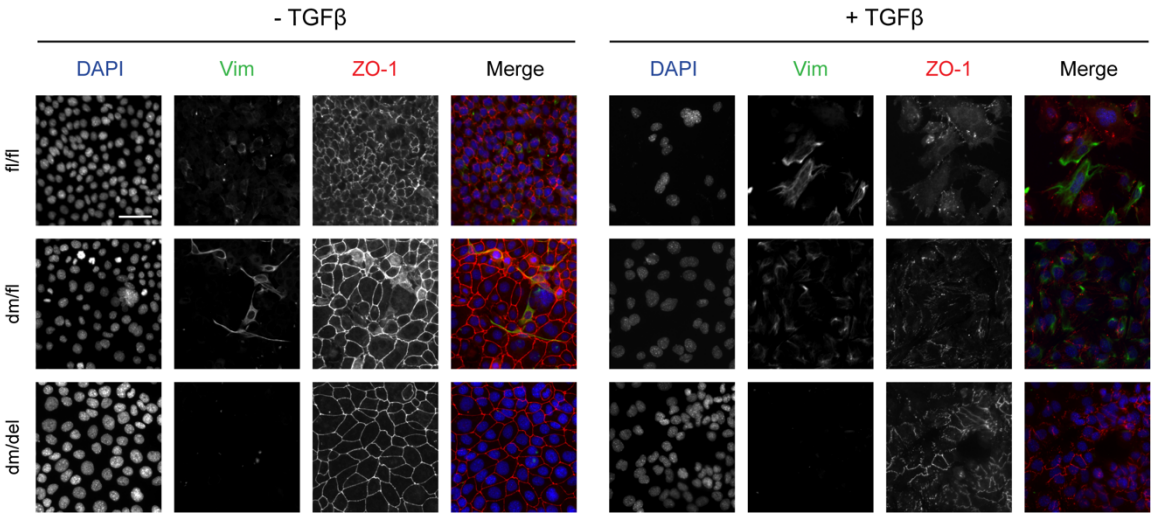
(d) The number of transfected and proliferating cells was quantified by Myc-Tag and pH3 staining, respectively, which revealed a reduced proliferation of cells transfected with the β-catenin mutant form compared to GFP-expressing cells or cells transfected to express wild-type β-catenin. Data are displayed as mean ± SEM. n=3. Statistical analysis was performed using ordinary one-way ANOVA multiple comparison test. ****, $p < 0.001$; ns, not significant.

Suppl Figure S6

a

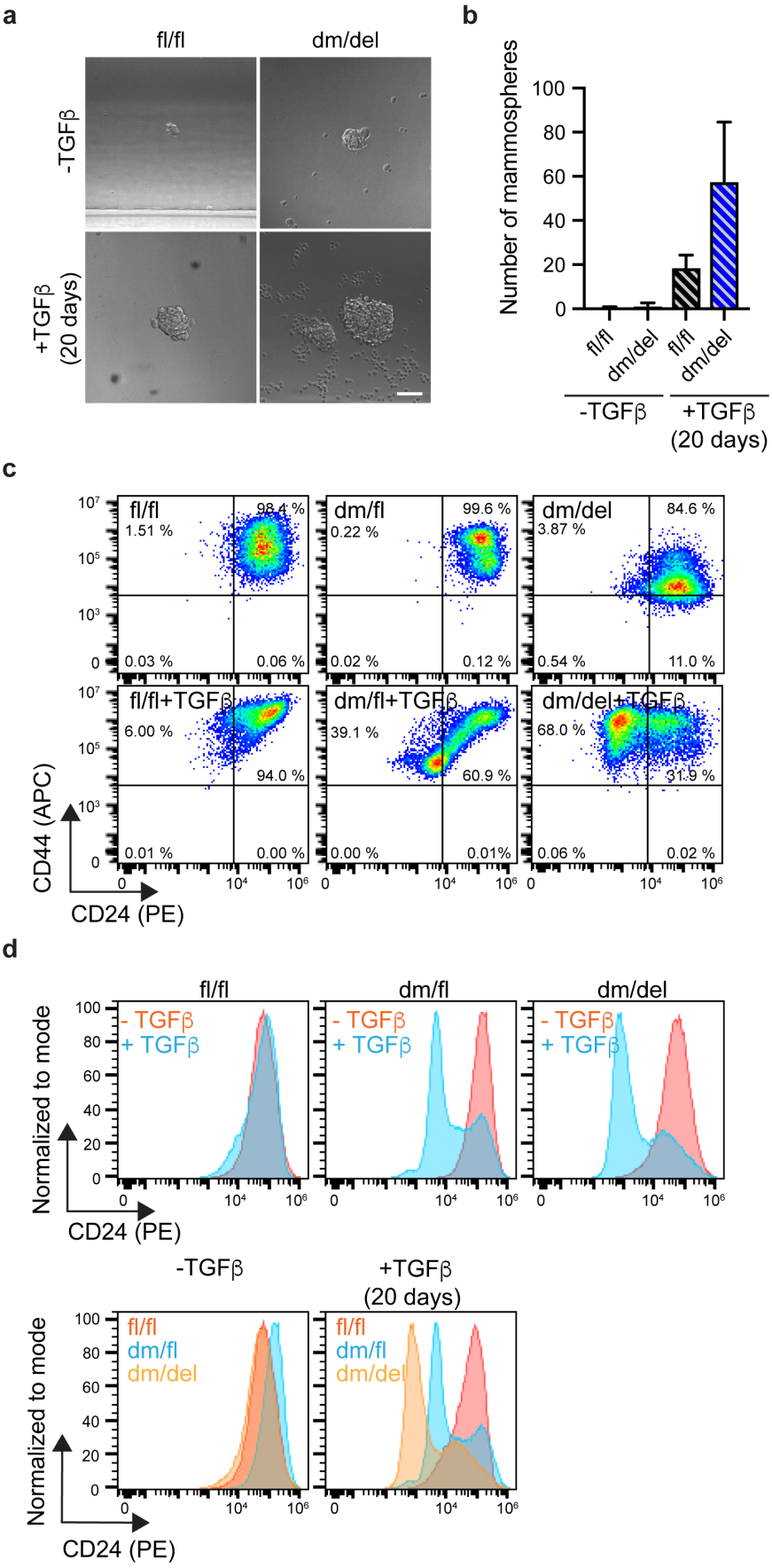


b



Supplementary Figure S6. The loss of β-catenin's transcriptional activity impairs EMT.
(a) Morphology of cell lines expressing wild-type or the dm mutant form of β-catenin in the absence of TGFβ or upon treatment with TGFβ for 4 or 20 days and or upon transfection with either Control siRNA (siCtrl) or siRNA against E-cadherin (siEcad). β-catenin wild-type-expressing cells (fl/fl) show a mesenchymal morphology upon TGFβ treatment or upon E-cadherin knockdown, while β-catenin dm mutant β-catenin-expressing cells (dm/del) do not elongate and remain in cell clusters.
(b) Immunofluorescence staining for vimentin (Vim; green) and Zona occludens 1 (ZO-1; red) on mammary tumor cells expressing only wild-type β-catenin (fl/fl), cells expressing one allele of β-catenin and one allele of dm mutant β-catenin (dm/fl), and cells expressing exclusively dm mutant β-catenin (dm/del). DAPI was used to visualize nuclei. Scale bar, 50μm.

Suppl Figure S7



Supplementary Figure S7. The loss of β-catenin's transcriptional activity impairs EMT yet increases stemness.

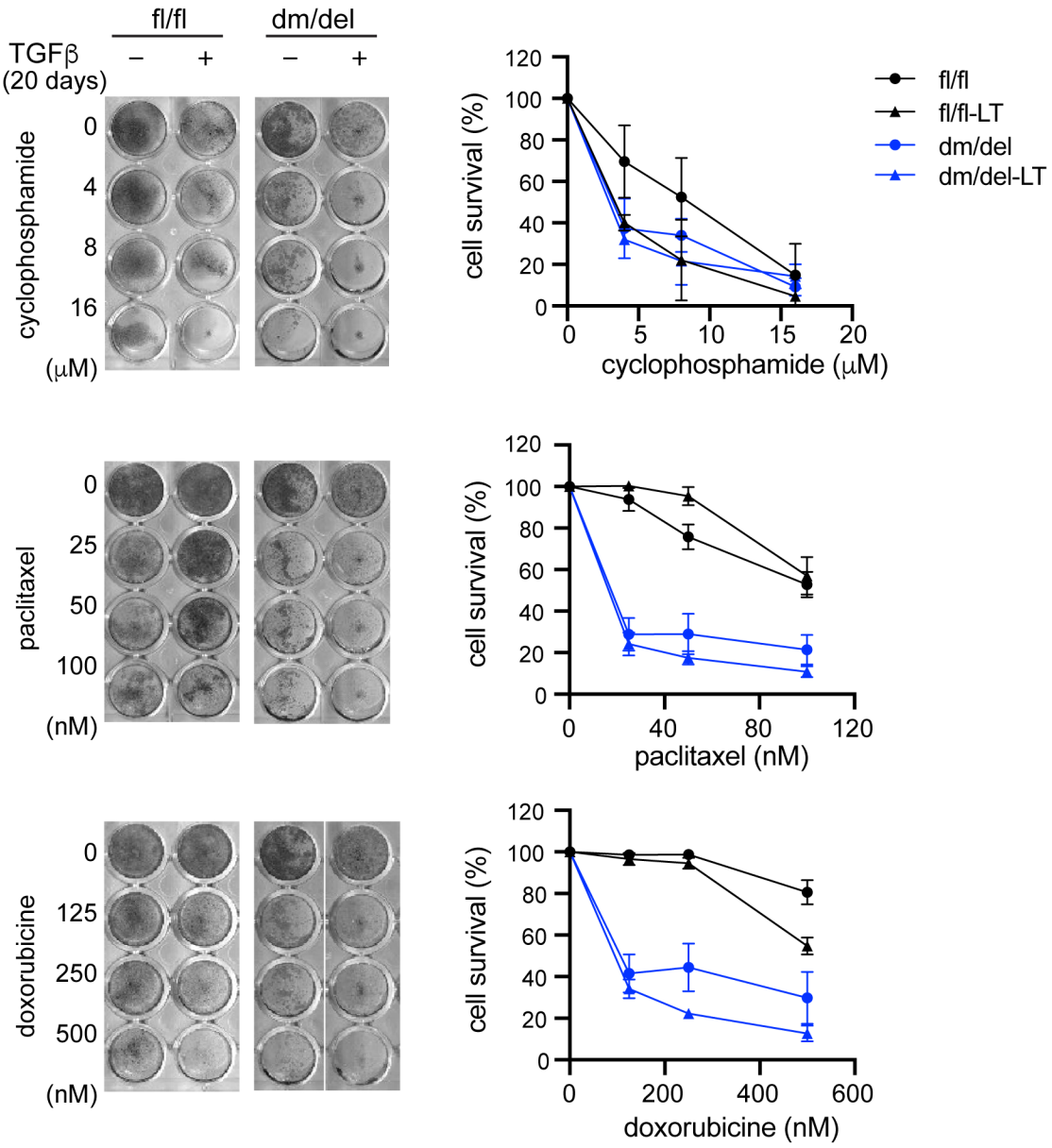
(a) Representative phase contrast microscopy pictures of mammospheres formed by fl/fl and dm/del cells. Scale bar, 100μm.

(b) Quantification of mammosphere formation of fl/fl and dm/del cells as shown in (b).

(c) fl/fl, dm/fl, and dm/del cells in the absence or presence of TGFβ for 20 days were stained with antibodies against the cancer stem cell markers CD44 and CD24 and analyzed by flow cytometry. The gating strategy and quantification is shown. Note that $CD44^{high}/CD24^{low}$ cells, considered as cancer stem cells in mouse, were increased in numbers in dm/del cells upon TGFβ treatment, while CD44 expression rather was reduced in the absence of TGFβ.

(d) Quantification of the CD24 expression in the cells analyzed in (c) to illustrate the reduction of CD24 expression by TGFβ treatment of dm/fl and dm/del cells as compared to fl/fl cells.

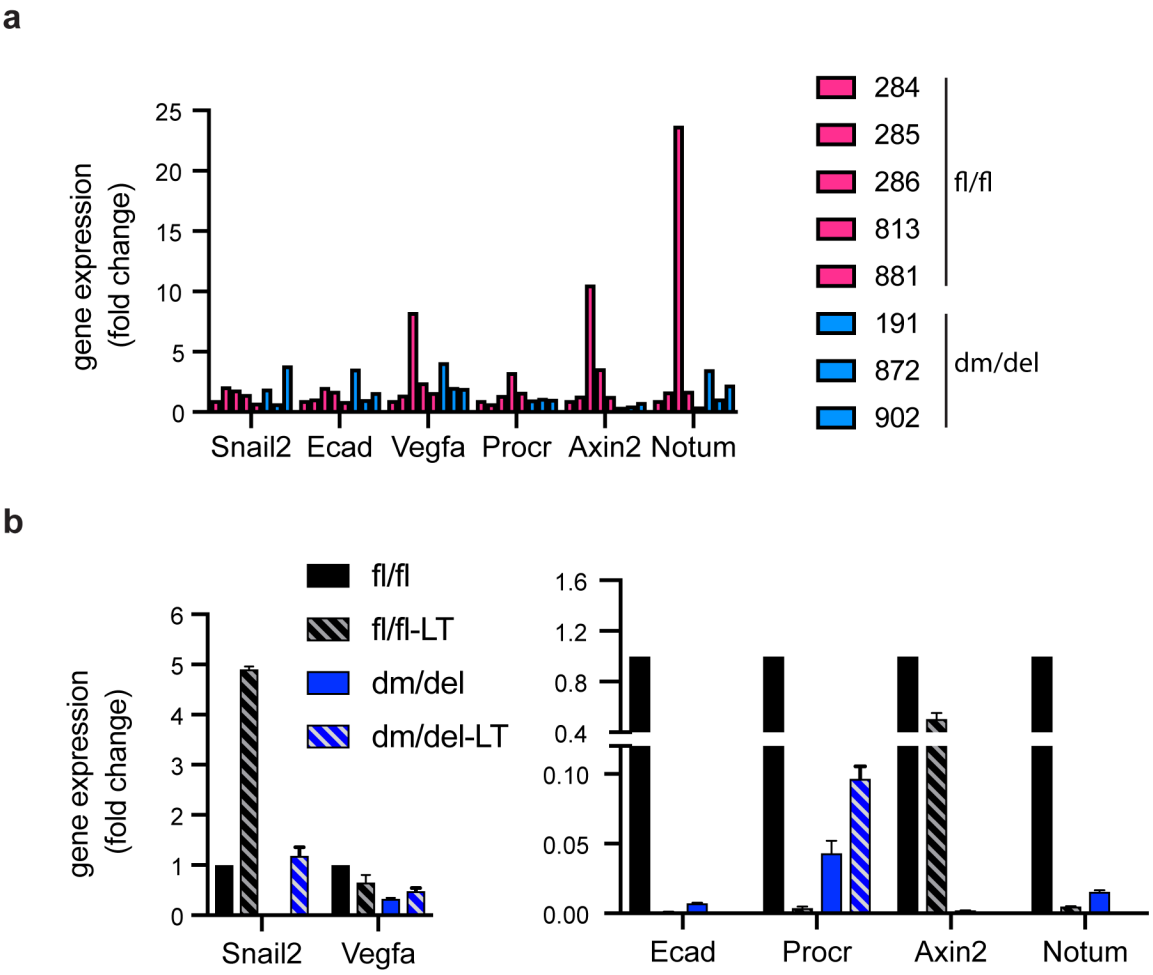
Suppl Figure S8



Supplementary Figure S8. The loss of β-catenin's transcriptional activity increases sensitivity to chemotherapy.

fl/fl and dm/del cells were treated with increasing doses of cyclophosphamide, paclitaxel or doxorubicin and without or with TGFβ for 20 days (LT). Representative pictures of colony formation are shown on the left and quantification of colony formation by crystal violet staining is shown on the right.

Suppl Figure S9



Supplementary Figure S9. Aberrant expression of Wnt and TGFβ target genes in β-catenin dm-mutant tumors and cultured cells.

(a,b) Quantitative RT-PCR analysis of the expression of classical Wnt target genes and EMT-related genes in primary tumors of fl/fl and dm/del mice (a) and in cell lines derived from these tumors (b).

Supplementary Methods

Histology

Tissues (mammary tumors and lungs) were fixed in 4 % paraformaldehyde overnight at 4°C followed by ethanol/xylene dehydration and subsequent embedding in paraffin. Paraffin-embedded samples were cut at 5µm thickness and subjected to Hematoxylin and Eosin (H&E)

staining. To quantify lung metastasis, 9 slides per lung separated each by 50µm were stained with H&E and the number and area of lung metastases were scored under the microscope at 10x magnification. Representative images of tumors and lung cross sections were acquired using a Zeiss Axio Imager Scanning Microscope with 10x objective. Tile scans were processed using Zen software (Zeiss).

Immunofluorescence microscopy

Tumors were fixed at 4°C in 4% paraformaldehyde for 2hrs followed by cryopreservation overnight in 20% sucrose/PBS prior to embedding in OCT-Compound freezing medium. Cryosections were cut at 7µm thickness and air-dried for 15min prior to rehydration in PBS. Tissue sections were permeabilized with 0.2% TritonX-100/PBS and blocked for 30min in 5% normal goat serum/PBS followed by incubation with the indicated primary antibodies at the appropriate dilution in blocking buffer overnight at 4°C. The next day, sections were incubated with fluorophore-coupled secondary antibodies (Alexa Fluor, Invitrogen) for 1hr at room temperature in the dark. The cell nuclei were counterstained with DAPI (D5942, Sigma-Aldrich). After staining, the coverslips were mounted on microscopy slides using DAKO Fluorescence Mounting Medium (S302380-2, Dako). Images were aquired using a Leica DMI 4000 fluorescence microscope with 20x and 40x objectives or using a
a Zeiss Axio Imager Scanning Microscope with 10x objective. Images were processed using Zen software and/or ImageJ (ImageJ, Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018)
For quantification of Ki67+ or Cc3+ and K8/18+ cancer cells in 12 weeks old mice each 12 imaging fields of 250000 μm^2 size were analyzed for each n=6 (Ki67) or n=5 (Cc3) mice. For 5 weeks old mice as many imaging fields as possibly for each n=3-4 mice were analyzed. Cancer cells were identified by cytokeratin 8/18 expression. For quantification of pH3+ cells without K8/18 staining cancer cells in 12 weeks old mice each 20 imaging fields of 250000 μm^2 size were analyzed for each n=5 mice.

Cell line derivation

Cells were isolated from breast tumors (mammary gland #2 and 3) of twelve-week-old MMTV-PyMT transgenic female mice (FVB/N background). Small tumor pieces were minced, and 10ml of pre-digestion buffer (10mM Hepes pH 7.4, 14mM NaCl, 0.67mM KCl, 1mM EDTA supplemented with 50g/ml gentamycin (Sigma, G1397) and 1X antibiotic-antimycotic (15240062, Thermo Fisher Scientific) were added and incubated for 30min at 37°C on a shaker.

After washing with PBS, the samples were digested using 6ml digestion buffer (10mM Hepes pH 7.4, 142mM NaCl, 0.67mM KCl, 0.67mM $CaCl_2$, 20mM Glucose supplemented with 1mg/ml Collagenase D (Roche, 11088858001), 50g/ml gentamycin (Sigma-Aldrich, G1397) and 1X antibiotic-antimycotic (15240062, Thermo Fisher Scientific) and incubated for 30min at 37°C on a shaker. Subsequently the samples were washed with PBS and then resuspended in growth medium and seeded in 10 cm plates. The medium was changed regularly and fibroblasts in culture were removed by differential trypsinization until only epithelial cells remained. The cell lines were further cultured in DMEM supplemented with glutamine, penicillin, streptomycin, and 10% FBS (F7524, Sigma Aldrich). To establish cell lines only expressing the mutant alleles, cells carrying one floxed allele and one mutant allele were seeded into 10cm plates and on the next day infected either with Adeno-CRE-IRES-GFP or with the Adeno-IRES-GFP virus as control using FuGENE® HD Transfection Reagent (E2311, Promega). Next day, the medium was changed, and after 3 days the cells were sorted for GFP-positive cells into 24 well plates using a BD FACSAria Flow Cytometer (BD Biosciences). Detachment of the cells was performed using trypsinization followed by two-times washing in 1x PBS and resuspension in 2% FBS, PBS and syringe filtering (40µm mesh filter) immediately before FACS sorting into a polystyrene round bottom tube (352054, FALCON) filled with DMEM medium. After centrifugation, supernatants were discarded, and cells were resuspended and seeded into 24-well plates.

Cell culture

Established cell lines from different tumor genotypes and Py2T cells (Waldmeier et al. 2012) were cultured in Dulbecco's modified Eagle's medium (DMEM) (D5671, Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (FBS 10% (F7524, Sigma-Aldrich), 2mM L-Glutamine solution (G7513, Sigma-Aldrich), 100 U penicillin and 0.1mg/ml streptomycin (P4333, Sigma-Aldrich). All cell lines were grown at 37°C, 5% CO_2, 95% humidity. To activate Wnt/β-catenin-mediated transcription, cells were treated with 100ng/ml recombinant murine Wnt3a for the indicated time periods. For EMT experiments, cells were treated with 2ng/ml recombinant human TGFβ1 every second day for the time periods indicated. To determine the effect of E-cadherin on EMT progression, the cells were transfected with Control siRNA (siCtrl: AGGUAGUGUAAUCGCCUUG) or siRNA against E-cadherin (siEcad: GGACGUCCAUGUGUGUGACUGUGAA).

To determine cell growth curves and doubling times, cells were seeded into 12 well plates in

duplicates. Next day the medium was changed, and the cells were cultured in a Cell IQ live cell imager (Model v.2) for 3 days. Cell IQ analysis software was used for quantification and analysis.

Adenovirus infection

Cells were plated onto 6cm dishes in duplicates and transfected the next day with Adeno-CRE-IRES-GFP or with Adeno-IRES-GFP virus as a control (1710 and 1761, Vector Biolabs) using Fugene HD transfection reagent (E2311, Promega). The following day, medium was changed and cell culture continued.

Transfection with expression constructs

A cDNA encoding the Myc-tagged dm mutant version of β-catenin were subcloned into the retroviral vector pBabe-puro. A cDNA encoding EGFP was subcloned from pEGFP-N3 (Clontech) into pBabe-puro and used as control. 4µg plasmid were used for transient transfection using Lipofectamine 3000 Reagent (L3000-015, Invitrogen) according to the manufacturer's instructions. Medium was changed the day after transfection.

Luciferase reporter assay

The firefly luciferase reporter constructs superTOPflash and superFOPflash (kindly provided by Konrad Basler, UZH Zürich) were used to quantify Wnt/β-catenin mediated transcriptional output. Cells were plated in duplicates in 24-well plates and transfected the following day with 500ng of the superTOPflash or superFOPflash *firefly* luciferase reporter plasmid and 10ng of a constitutive-active *Renilla* luciferase plasmid using Lipofectamine 3000 Reagent (Invitrogen) according to the manufacturer's instructions. Luciferase activities were measured using the Dual-Luciferase® Reporter Assay System (E1960, Promega). *Firefly* luciferase values were normalized to *Renilla* luciferase control values.

Transwell migration assay

50,000 untreated or long-term (>20 days) TGFβ-treated cells were suspended in 500µl of DMEM + 0.2% FBS and seeded into 24 transwell migration inserts (Corning, 353097) in duplicates. The bottom chambers were filled with 700µl of DMEM + 20% FBS to create a chemoattractant gradient. The cells were incubated in a tissue culture incubator at 37°C with 5% CO2. After 18 hours, inserts were fixed with 4% paraformaldehyde for 10 minutes. Cells

that had not crossed the membrane were removed with a cotton swab, and cells on the bottom of the membrane were stained with DAPI. Images of five fields per insert were taken with a fluorescence microscope (Leica DMI 4000) at 10 x magnification and DAPI stained nuclei were counted using an ImageJ software plugin developed in-house.

Mammosphere assay

Untreated or long-term (>20 days) TGFβ-treated cells derived from different tumor genotypes were suspended in serum-free DMEM-F12 medium containing 20ng/mL hEGF, 0.5µg/ml hydrocortisone, 5µg/ml insulin, 20ng/ml bFGF, 1U/mL heparin (Sigma Aldrich), B27 (Invitrogen) supplemented with 100U penicillin and 0.1mg/ml streptomycin (Sigma-Aldrich). A total of 12,000 cells/1.5 ml were then seeded in 6-well ultra-low attachment plates (Corning, CLS3471). 200µl medium was supplemented every third day and mammosphere were harvested 12 days after culture. Mammospheres were then fixed with 4% PFA for 1 h and washed with PBS followed by staining with DAPI (5µg/ml) for 10 min at room temperature and mounted with ibidi mounting medium (Ibidi, 50001). Numbers of mammospheres were counted under a microscope and images were taken with a spinning disk confocal microscope (Visitron Systems) at 20x magnification.

Treatment with chemotherapeutic agents

20,000 untreated or long-term (>20 days) TGFβ-treated cells were cultured in 24-well plate and treated with paclitaxel (0, 25, 50, 100 nM, T7191, Sigma-Aldrich), 4-Hydroperoxy cyclophosphamide (0, 4, 8,16 µM, sc-206885, Santa Cruz) or doxorubicin hydrochloride (0, 125, 250, 500 nM, D1515, Sigma-Aldrich) alone or coupled with TGFβ1 for 4 days. Cells were then fixed with 4% PFA for 10 min, washed with PBS and stained with crystal violet. After 10 min staining, the plates were washed with PBS for 3 to 4 times, air dried and scanned using an Epson scanner. Finally, cells were dissolved in 100% methanol, and crystal violet levels were quantified using a microplate reader at 595 nm wavelength.

Immunofluorescence of cultured cells

Cells were grown on glass coverslips (#1, 12mm round, Menzel–Glaser) and treated for 3 days with Wnt3a or 4 days with TGFβ. The cells were washed with PBS and then fixed with 4% paraformaldehyde/PBS for 15min at room temperature, followed by permeabilization with 0.2% TritonX-100/PBS for 5min. Subsequent blocking was performed with 3% BSA/0.0 %

Triton X-100/PBS for 1hr. Primary antibodies were then added in the appropriate dilution in 3% BSA/0.2% PBS-T for 2hrs at room temperature. Incubation with fluorophore-coupled secondary antibodies (Alexa Fluor, Invitrogen) was performed for 1hr at room temperature in the dark. Cell nuclei were counterstained with DAPI (D5942, Sigma-Aldrich). After staining, coverslips were mounted in Fluorescence Mounting Medium (S302380-2, Dako) on microscope slides and imaged using a fluorescence microscope (Leica DMI 4000) at 20x magnification.

Flow cytometry analysis

Untreated and long-term (>20 days) TGFβ-treated cells were trypsinized and washed with culture medium. To determine the breast cancer stem cell population, 10^6 cells were resuspended in 2% FBS in PBS containing 2mM EDTA (FACS buffer) followed by blocking with CD16/32 (Biolegend, 101302, 1:100) for 5 min at room temperature. Cells were washed with FACS buffer and stained with allophycocyanin (APC)-conjugated anti-CD44 (Biolegend, 103012) and PE-conjugated anti-CD24 (Biolegend, 101808) for 20 min in the dark on ice. Cells were then washed with FACS buffer and filtered through a 40µm mesh before loading on a Cytoflex LX 2-005 (Beckman Coulter).

For cell-cycle analysis, 10^6 cells were resuspended in 300µl of PBS. Ice-cold 100% ethanol was added dropwise to a final concentration of 70% (700µl) on a vortex, and the suspension was kept in -20°C for at least 30 min. After fixation, the cell suspension was washed with FACS buffer and incubated with DAPI (1µg/ml) for 10 min at room temperature. Cells were then washed with FACS buffer, filtered and analyzed on a Cytoflex LX 2-005.

For cell senescence analysis, β-galactosidase activity was measured using the CellEvent™ Senescence Green Flow Cytometry Assay Kit (ThermoFishers) according to the manufacturer's instruction. 10^6 cells were fixed with 2% PFA for 10 min at room temperature, washed with 1% BSA in PBS and incubated with Working Solution for at least 1 h at 37°C without CO2 in dark. After removing the Working Solution, the cells were washed with 1% BSA in PBS and resuspended with FACS buffer. Finally, β-galactosidase activity was analyzed on a Cytoflex LX 2-005 using a 488 nm laser and 530 nm/30 filter.

Cell proliferation was measured using the CellTrace™ Violet Cell Proliferation Kit (ThermoFishers) according to the manufacturer's instruction. 10^6 cells were resuspended in PBS and incubated with CellTrance Working Solution for 20 min at 37°C in dark. After incubation, 5 times the Working Solution volume of complete culture medium was added to the cells and incubated for 5 min at 37 °C. Cells were then washed with complete culture

medium and incubated for 10 min at 37 °C before analysis by flow cytometry, or seeded onto 6-well plates. Cells were analyzed on a Cytoflex LX 2-005 using a 405 nm laser and 450 nm filter every day from day 0 until day 4 after culture.
All data was processed and analysed with FlowJo^{TM} software (Becton, Dickinson and Company).

Immunoblotting

Cells were lysed in RIPA buffer (R0278, Sigma-Aldrich) supplemented with 1mM DTT, mM NaF, 2 mM sodium orthovanadate and 1X protease inhibitor cocktail (Sigma-Aldrich) for 30 min on ice followed by scraping into tubes and centrifugation for 10min at 10,000 rpm at 4°C. The supernatant was saved and the protein concentration was determined using a Bio-Rad Bradford assay according to the manufacturer's instructions. Equal amounts were prepared, diluted in loading buffer (10% glycerol, 2% SDS, 65mM Tris, 0.01mg/ml Bromphenolblue, 1% β-mercaptoethanol) and loaded onto a SDS polyacrylamide gel. Proteins were then transferred onto nitrocellulose membranes (10600002, Sigma-Aldrich) by wet transfer for 2hrs at constant current (0.33 A). Following blocking for 1hr in 5% milk prepared in TBS/0.05% Tween 20, membranes were incubated with primary antibodies overnight at 4°C. The next day, membranes were washed and incubated with HRP-conjugated secondary antibodies (Jackson Immunoresearch Laboratories and donkey anti goat IgG-HRP, sc-2020, Santa Cruz) for 1hr at room temperature and developed with Immobilon Western Chemiluminescent HRP Substrate (WBKLS0500, Millipore) and quantified using a Fusion Fx7 chemiluminescence reader.

Co-immunoprecipitation

β-catenin$^{fl/fl}$ or β-catenin$^{dm/del}$ cells were grown in 15cm plates, washed with cold PBS, scraped off in 300µl IP lysis buffer (20mM Tris pH 7.5, 10mM NaCl, 10% glycerol, 1% NP-40, 2mM EDTA, 1X PIC (protein inhibitor cocktail)) and collected in an Eppendorf tube. Incubation was performed at 4°C rotating for 20min. Subsequently samples were centrifuged at 4°C (10min, 16,000g), and supernatants were transferred to a new tube. Protein concentrations were measured using Bio-Rad Bradford solution according to the manufacturer's instructions. An input sample was taken and frozen. Per IP, 5µg antibody or IgG as a control were diluted in 200µl PBS/0.02% Tween-20 (PBS-T). Per IP 40µl DynaMagnetic Beads were prepared by washing 2 times with 500µl PBS-T. Beads were collected using a magnetic stand, antibody dilution was added and rotated for 15min at room temperature. The liquid was aspirated on the

magnetic stand and the lysate (1mg) was added and incubated overnight at 4 °C while rotating. The next day, beads were washed three times with 500µl IP wash buffer (15mM Tris-HCl pH 7.8, 100mM NaCl, 1X PIC) and then diluted in 40 µl 1X SDS-PAGE sample buffer and boiled for 5min. Empty beads were collected and the supernatant was loaded and analyzed by SDS-PAGE.

Cell fractionation assay

After 2 days Wnt3a treatment, cells were rinsed with PBS, and then scraped off with 1ml of PBS into an Eppendorf tube and pelleted by centrifugation. Cells were resuspended in 1ml RSB Buffer (10mM Hepes pH 6.2, 10mM NaCl, 1.5mM MgCl2, 1x PIC (P2714, Sigma-Aldrich)) and incubated for 30 min on ice. Samples were then homogenized using a syringe. 100µl were collected and frozen as input sample. The nuclei were collected by centrifugation for 2 min, 400 x g at 4ºC. The supernatant (cytoplasmic fraction) was collected. The nuclear fraction (pellet) was washed twice with 1ml RSB buffer and subsequently lysed adding EBC buffer (50mM Tris pH 7.5, 250mM NaCl, 1% Triton X-100, 1x PIC).

RNA Isolation and quantitative RT-PCR

Total RNA was isolated using TRI reagent (Sigma) according to the manufacturer's instruction. cDNA was synthesized using ImProm-II Reverse Transcriptase (Promega). qPCR was performed on a StepOnePlus machine (Applied Biosystems) using Power-Up SYBR green (Applied Biosystems). Target gene expression levels were normalized to Rpl19. Fold changes were calculated using the comparative Ct method (ΔΔCt). The following primers were used:

Rpl19	5'-CTCGTTGCCGGAAAAACA-3'
	5'-TCATCCAGGTCACCTTCTCA-3'
Snail2	5'-TGTGTCTGCAAGATCTGTGGC-3'
	5'-TCCCCAGTGTGAGTTCTAATGTG-3'
E-cadherin	5'-CGACCCTGCCTCTGAATCC-3'
	5'-TACACGCTGGGAAACATGAGC-3'
VEGF-A	5'- ACTGGACCCTGGCTTTACTG-3'
	5'-TCTGCTCTCCTTCTGTCGTG-3'
Procr	5'-CTCTCTGGGAAAACTCCTGACA-3'
	5'-CAGGGAGCAGCTAACAGTGA-3'
Axin2	5'-GGGGGAAAACACAGCTTACA-3'
	5'-TTGACTGGGTCGCTTCTCTT-3'

Notum	5’-CTGCGTGGTACACTCAAGGA-3‘
	5’-CCGTCCAATAGCTCCGTATG-3‘.