# Figure S1

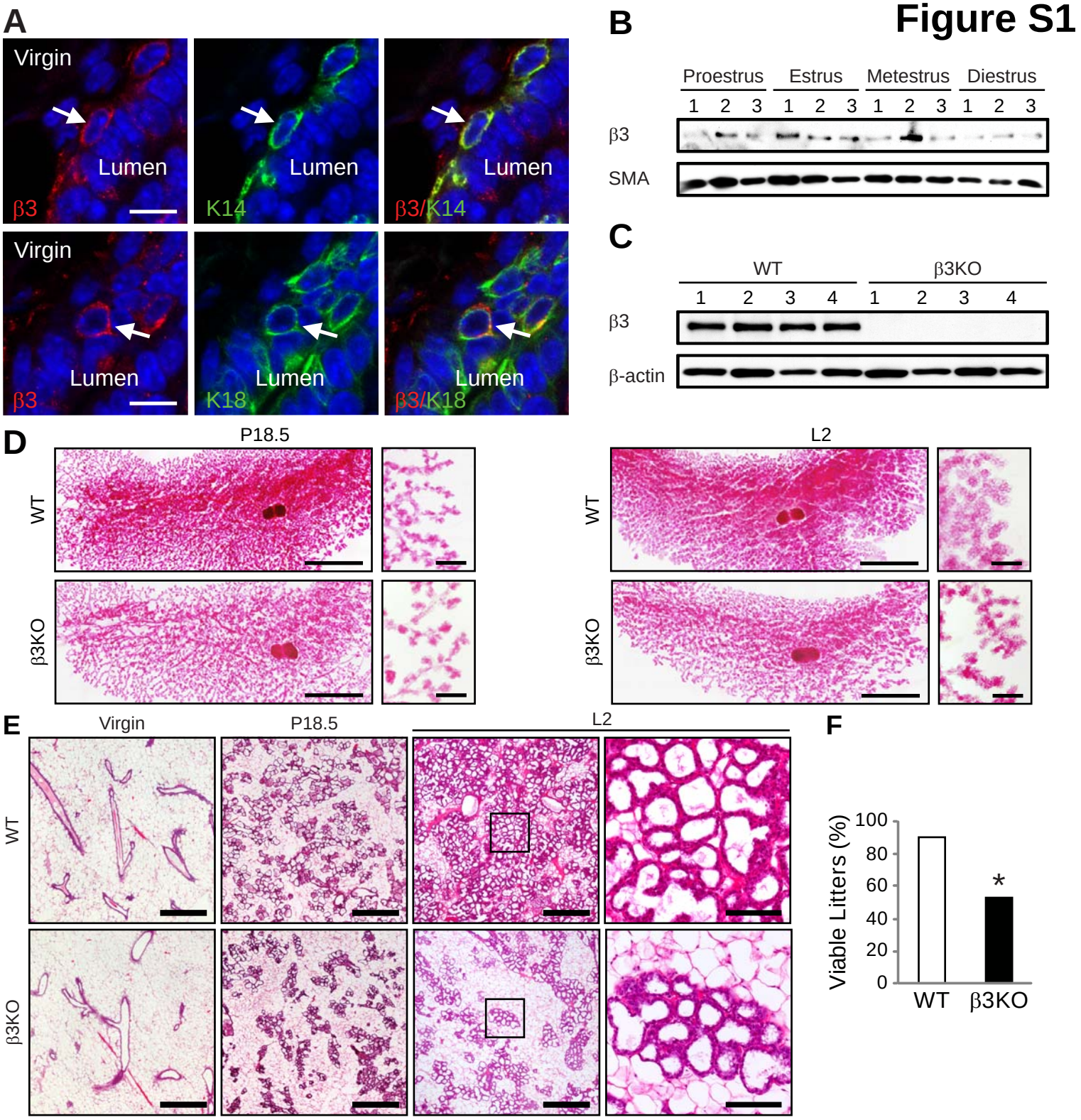



**Figure S1, Related to Figure 1. β3 is required for mammary gland development during pregnancy**

(A) Representative images of immunofluorescent staining for β3 and the basal marker cytokeratin 14 (K14) (top panels) or the luminal marker cytokeratin 18 (K18) (lower panels) in the virgin murine mammary gland. Scale bars, 10 μm.

(B) Western analysis for β3 and SMA (loading control) in whole mammary gland lysates from mice at different stages of the estrus cycle. Estrus stage was determined by cytological analysis of vaginal smears.

(C) Immunoblot for β3 and β-actin (loading control) in lysates from WT and β3KO P12.5 mammary glands.

(D) Mammary gland whole-mounts from pregnant day 18.5 (P18.5) and lactating day 2 (L2) WT and β3KO mice. Scale bars, 5 mm (low magnification), and 500 μm (high magnification).

(E) H&E-stained sections from paraffin-embedded mammary glands. Scale bars, 500 μm (low power) and 100 μm (high power). (D,E) Virgin; WT, n=8, β3KO, n=7, P18.5; WT, n=13, β3KO, n=10, L2; WT, n=24, β3KO, n=15.

(F) Histogram showing the percentage of viable litters raised to weaning by WT versus β3KO dams. WT, n=72, β3KO, n=53. Statistics were evaluated by Fisher's exact test. *$P$<0.05.

# Figure S2

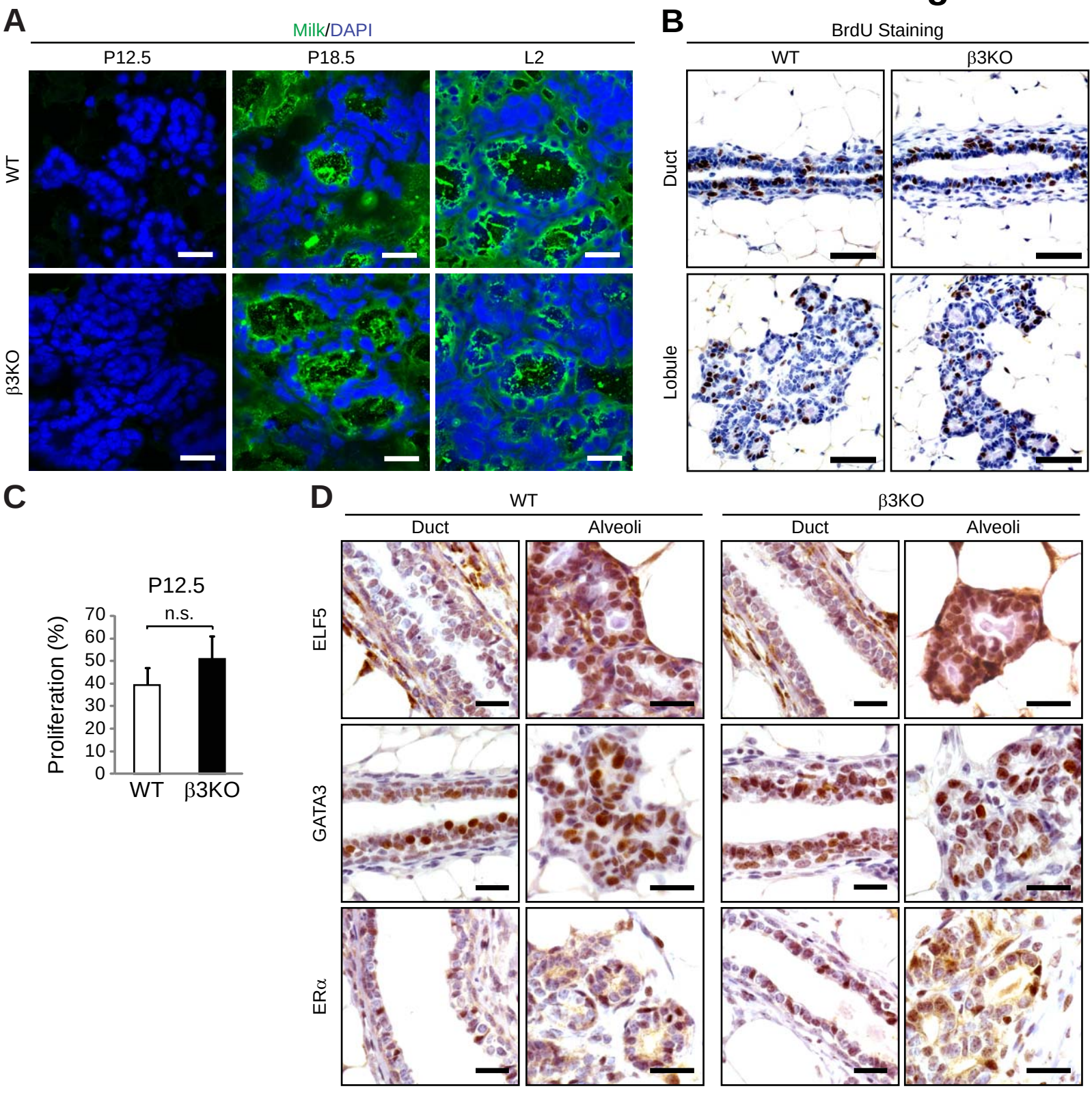



**Figure S2, Related to Figure 1. β3 is dispensible for secretory activation, proliferation and luminal marker expression**

(A) Representative immunofluorescent images showing staining for milk protein in WT and β3KO mammary glands at various stages. For each stage, WT, n=3, β3KO, n=3. Scale bars, 25 μm.

(B) Representative images showing 5-Bromodeoxyuridine (BrdU) incorporation (brown stain) in P12.5 mammary glands from two different WT and β3KO mice. WT, n=9, β3KO, n=6. Scale bars, 50 μm.

(C) Histogram depicting the amount of proliferation in WT and β3KO P12.5 mammary glands expressed as the percentage of Ki-67+ nuclei per field. Data represent the mean ± s.e.m. from at least 5 fields per gland and statistical analysis performed by unpaired two-sided Student's T-test. WT, n=3, β3KO, n=3. n.s. = not significant ($P$>0.05).

(D) Immunohistochemistry for ELF5, GATA3 and ERα in WT and β3KO P12.5 mammary glands. Shown are representative examples comparing staining in ducts and alveoli. WT, n=5, β3KO, n=5. Scale bars, 25 μm.

# Figure S3

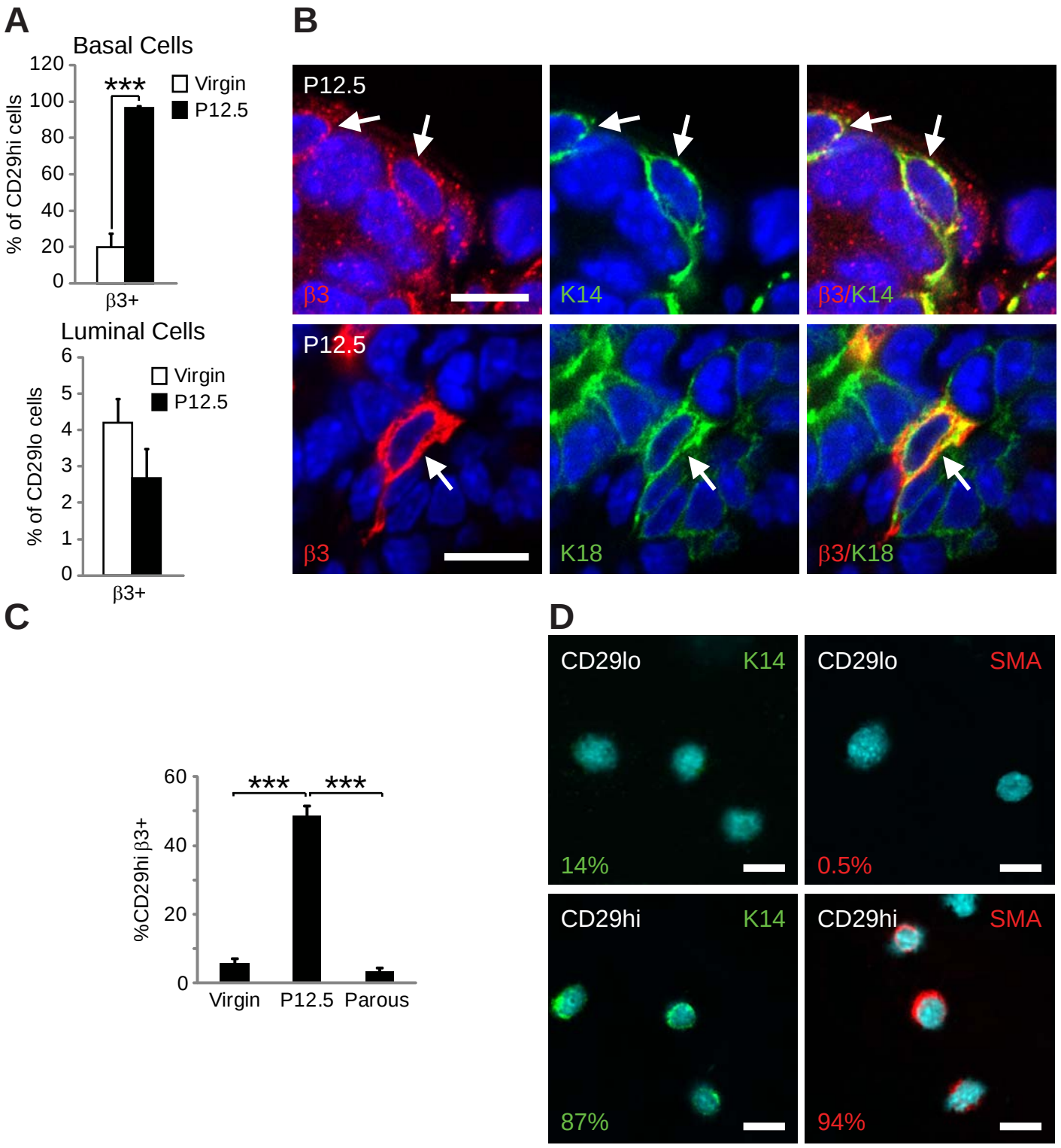



**Figure S3, Related to Figure 2. β3 is expressed in the MaSC-enriched pool during pregnancy**

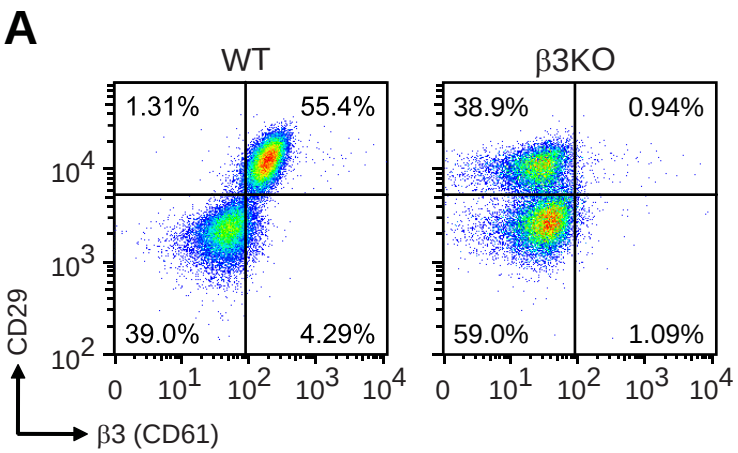
(A) Histograms showing the percent of Lin$^-$CD24$^+$ CD29$^{hi}$ basal cells (top) or CD29$^{lo}$ luminal cells (bottom) that are β3$^+$ in virgin and P12.5 mammary glands. Virgin, n=8, P12.5, n=13. *P*=0.0000093 for virgin vs P12.5 basal cells. Data shown are mean ± s.e.m. and were analyzed by unpaired two-sided Student's T-tests. ****P*<0.001.

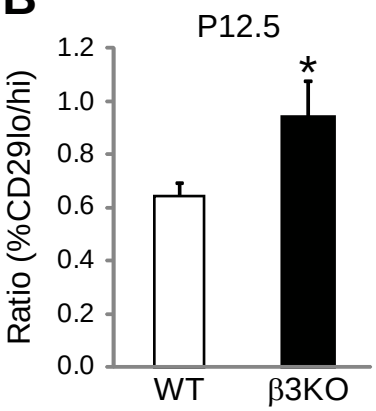
(B) Representative images of β3 immunostaining with basal (K14) and luminal (K18) markers in WT P12.5 mammary glands. Arrows mark β3$^+$ cells. Scale bars, 10 μm.

(C) Histogram showing the percentage of β3$^+$ CD29$^{hi}$ cells present in the live cell Lin$^-$CD24$^+$ fraction from virgin, P12.5 and single parous (8 weeks involuted) mammary glands as assessed by flow cytometry. Virgin, n=8, P12.5, n=12, Parous, n=7. Data represent the mean ± s.e.m. and statistical analysis was performed by Student's T-tests. ****P*<0.0001.

(D) Immunofluorescent staining for the basal markers K14 and SMA in cytospun CD29$^{lo}$ and CD29$^{hi}$ sorted cells. Scale bars, 20 μm.

# Figure S4

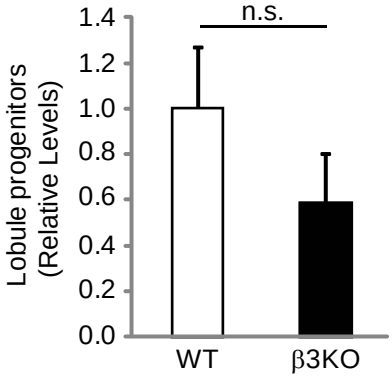



C

**Table 1 | Effect of β3 deletion on mammary repopulation**

| Number of CD29hi cells injected per mammary fat pad | Number of outgrowths per number of injected fat pads | |
|---|---|---|
| | WT | β3KO |
| 10,000 | 6/12 | 3/12 |
| 5000 | 3/4 | 3/4 |
| 2500 | 3/6 | 1/6 |
| 1000 | 1/20 | 0/18 |
| 100 | 1/16 | 1/18 |
| Repopulating Frequency (95% Confidence Interval) | 1/9186 (1/15,987 - 1/5278) | 1/18,663 (1/38,031 - 1/9159) |
| *P*-value | 0.106 | |



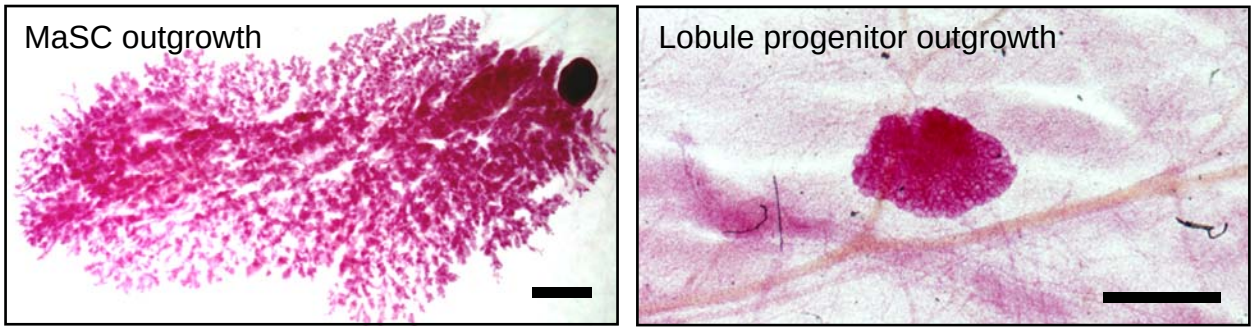




**Figure S4, Related to Figure 3. β3 is required for MaSC expansion**

(A) Representative FACS density plots from WT and β3KO P12.5 mammary glands showing the live, Lin$^-$CD24$^+$ cells expressed according to their CD29 and β3 status.

(B) Histogram depicting the ratio of CD29$^{lo}$ to CD29$^{hi}$ cells in P12.5 mammary glands from WT and β3KO mice. P12.5; WT, n=14, β3KO, n=13.

(C) Table showing the frequency of MaSC outgrowths from P12.5 WT and β3KO CD29$^{hi}$ cells.

(D) Histogram showing the relative levels of lobule progenitor cells in the CD29$^{hi}$ pool from WT and β3KO P12.5 donor mice. n=4 independent experiments. (B,D) Data represent the mean ± s.e.m. and statistical analysis was performed by Student's T-tests. *$P$<0.05. n.s. = not significant ($P$>0.05).

(E) Representative images of MaSC and lobule progenitor outgrowths from P12.5 CD29$^{hi}$ cells harvested at lactating day 2. Scale bars, 2 mm (left panel) and 1 mm (right panel).

# Figure S5

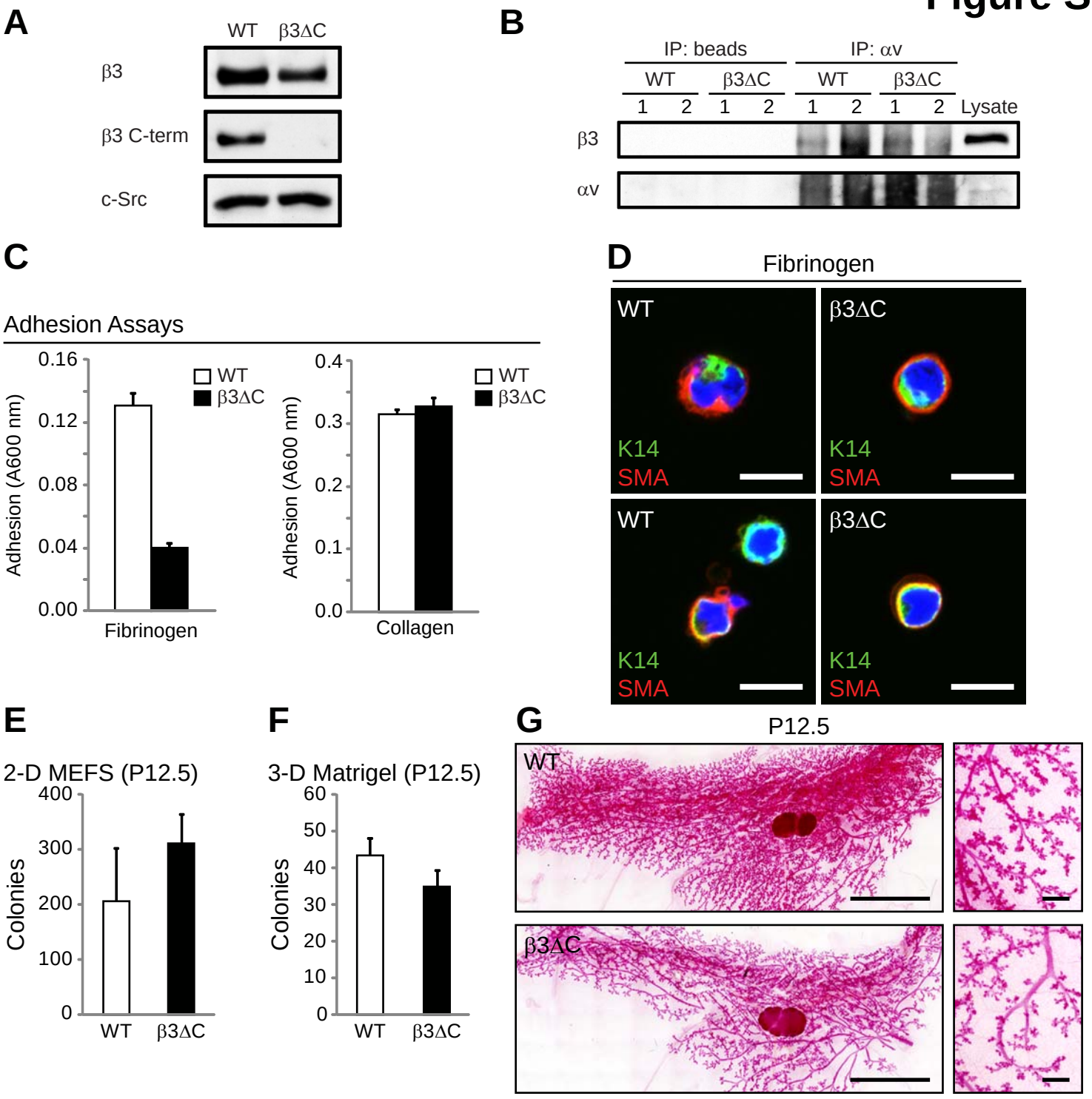



**Figure S5, Related to Figure 4. A β3 signaling deficient mutant (β3ΔC) attenuates MaSC clonogenicity and mammary gland morphogenesis during pregnancy, similar to β3 deletion**

(A) Immunoblot showing β3 expression in whole mammary gland lysates from P12.5 WT and β3ΔC knock-in mice.

(B) Detection of an αvβ3 heterodimer by immunoprecipitation (IP) of αv or beads alone (control) in whole mammary gland lysates from 2 WT and 2 β3ΔC P12.5 mice followed by immunoblotting for β3 and αv. Lysate from a WT P12.5 mammary gland is shown as a control for molecular size.

(C) Histograms displaying the results of adhesion assays comparing P12.5 WT and β3ΔC mammary cells on 20 μg/mL fibrinogen or 10 μg/mL type I collagen. Specific adhesion was assessed relative to wells coated with BSA alone.

(D) Immunofluorescent images of WT or β3ΔC P12.5 mammary cells specifically adhered to 20 μg/mL fibrinogen and stained for K14 and αSMA. Nuclei are stained blue in all panels. Scale bars, 10 μm.

(E) Total colony counts from P12.5 WT and β3ΔC colonies grown on irradiated MEF's. WT, n=5, β3ΔC, n=4.

(F) Colony numbers from P12.5 WT and β3ΔC Matrigel experiments. WT, n=4, β3ΔC, n=3. (C,E,F) Bars represent the mean ± s.e.m. and statistics were analyzed by Student's T-test (E,F).

(G) Mammary gland whole-mounts from P12.5 WT and β3ΔC knock-in mice. WT, n=12, β3ΔC, n=20. Scale bars, 5 mm (low magnification), and 500 μm (high magnification).

# Figure S6

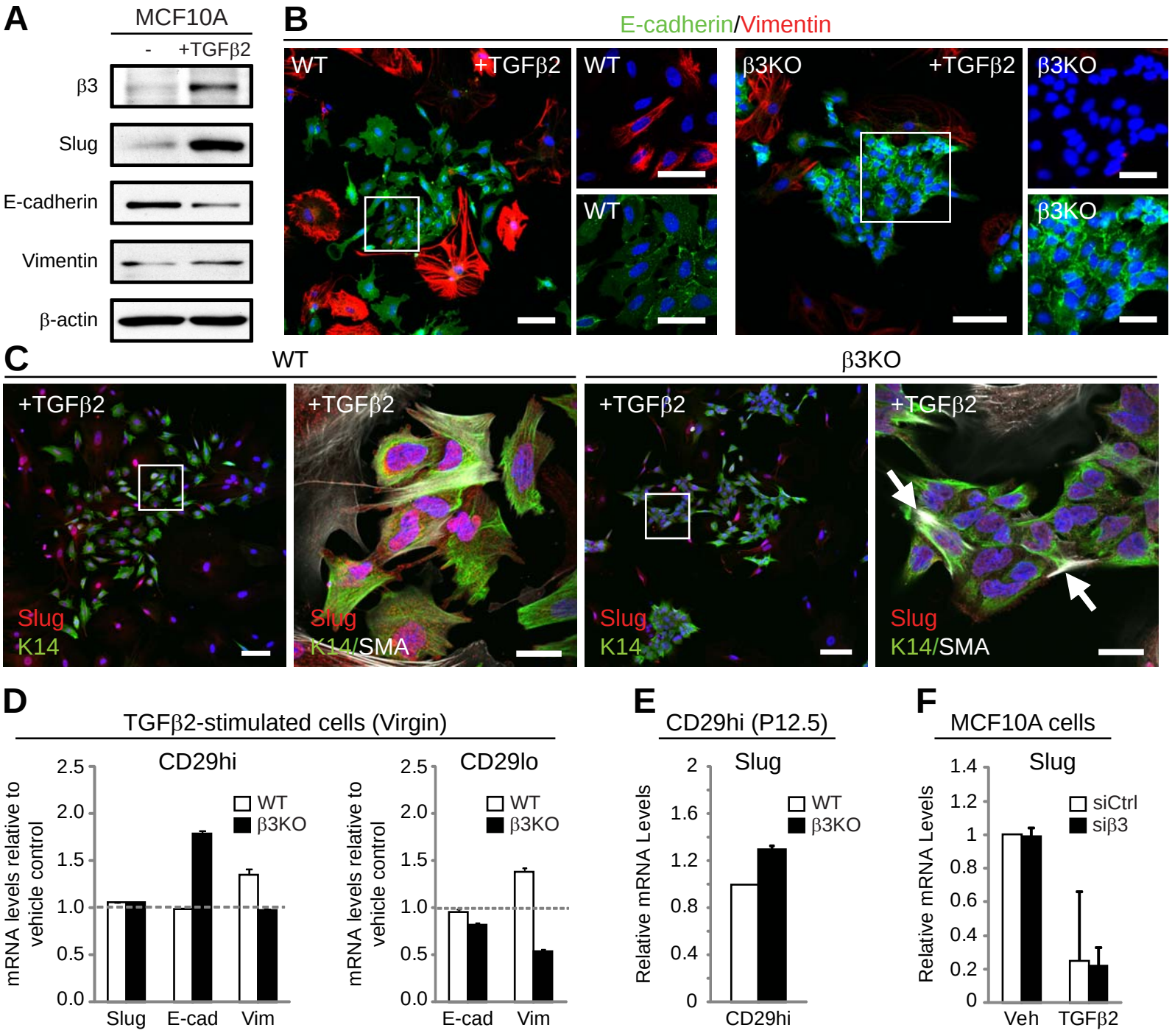






**Figure S6, Related to Figure 6. αvβ3 is required for expression of Slug protein, but not mRNA**

(A) Western analysis of MCF10A lysates from cells stimulated with vehicle or TGFβ2 and probed with the indicated antibodies. β-actin is shown as a loading control.

(B,C) Representative examples of 2-D colonies from pooled virgin WT and β3KO mammary cells stimulated with TGFβ2 and stained with the indicated antibodies. (B) Immunofluorescent staining for E-cadherin and Vimentin. Scale bars, 100 μm (left panels), 50 μm (right panels). (C) Slug expression in single colonies (left panels) with areas in boxes shown at high-power (right panels). Arrows indicate K14+SMA+ cells. Scale bars, 100 μm (left panels), and 25 μm (right panels). (B,C) Nuclei are stained blue in all panels.

(D-F) qPCR results displaying the relative amount ($2^{-\Delta\Delta CT}$) of the specified mRNAs. (D) $CD29^{hi}$ and $CD29^{lo}$ sorted cells from virgin WT and β3KO mammary glands were stimulated with TGFβ2 and compared to vehicle control for mRNA expression of Slug, E-cadherin (E-cad) and Vimentin (Vim) after 24 hr. Slug is undetectable in $CD29^{lo}$ cells. (E) $CD29^{hi}$ sorted cells from WT and β3KO P12.5 mammary glands were assessed for expression of Slug mRNA. (F) MCF10A cells transfected with control or β3 siRNA and stimulated with TGFβ2 or vehicle control for 24 hr were assayed for Slug mRNA expression. (D-F) For each assay, samples were run in triplicate and 18S rRNA (D,E) or β-actin (F) was used as a loading control. Bars represent the mean fold change ± s.e.m.

# Figure S7

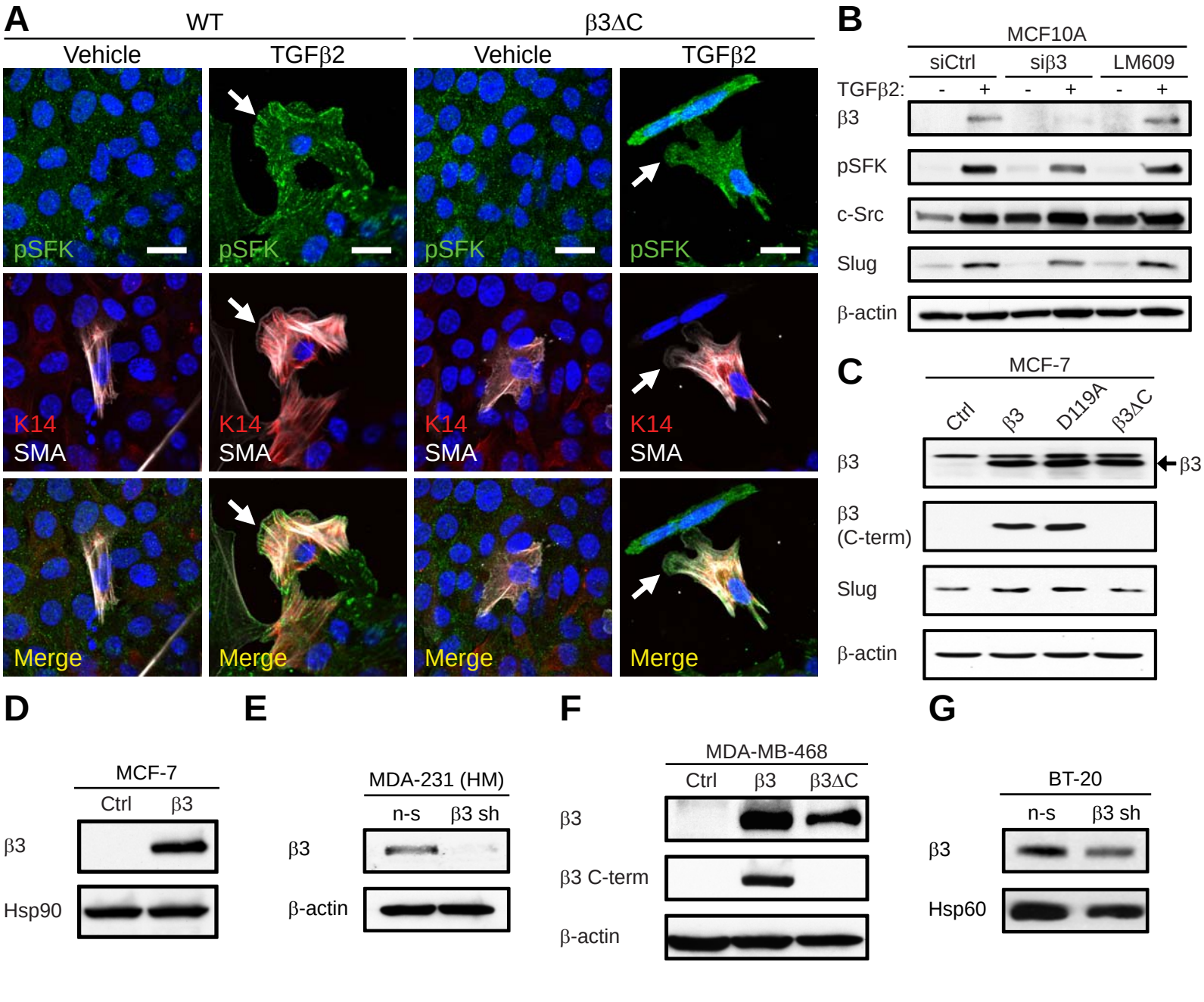



**Figure S7, Related to Figures 6 & 7. β3 is associated with Src activation and Slug expression in non-transformed and breast cancer cell lines**

(A) Representative immunofluorescent images showing pY416 Src family kinase (SFK) expression in $K14^{+}SMA^{+}$ virgin WT and β3ΔC mammary cells stimulated with TGFβ2 (arrows). Nuclei are stained blue in all panels. Scale bars 20 μm.

(B) Immunoblots from MCF10A cells transfected with control or β3 siRNA or treated with an αvβ3 function-blocking antibody (LM609) and stimulated with TGFβ2 or a vehicle control.

(C) Western analysis of MCF-7 cells transiently transfected with vector alone (Ctrl), full-length β3, β3 D119A (ligand binding deficient) or β3ΔC (signaling deficient) cDNAs. (B,C) β-actin is shown as a loading control.

(D-G) Western blots for β3 and the indicated loading controls in tumor cell lysates. (D,F) MCF-7 (D) and MDA-MB-468 cells (F) stably expressing vector alone (Ctrl), full-length β3, or β3ΔC mutant cDNAs (F). (E,G) MDA-MB-231 (HM) (E) or BT-20 (G) cells expressing a non-silencing control shRNA (n-s) or β3 shRNA (β3 sh).

## Supplemental Experimental Procedures

### Mice

β3KO mice on a mixed 129/BL6 background were purchased from Jackson Labs. For mammary outgrowth experiments, β3KO mice were backcrossed onto an FVB/N background (Harlan). β3ΔC knock-in mice on a C57BL6 background were generated as described previously (Ablooglu et al., 2009). Tumor experiments were performed using NOD/SCID/IL2 receptor gamma chain knock-out mice (NSG) purchased from Jackson Labs. All research was conducted under protocols approved by the UCSD animal subjects committee and is in accordance with the guidelines set forth in the NIH Guide for the Care and Use of Laboratory Animals.

### Cell lines

A highly metastatic variant of the MDA-MB-231 cell line, MDA-MB-231 (HM) (Munoz et al., 2006) was a gift from Dr. Robert Kerbel. MDA-MB-468 and MCF-7 cells were gifts from Dr. Steve Gonias. MDA-MB-231, MDA-MB-468 and MCF-7 cells were cultured in complete DMEM medium (DMEM supplemented with 10% fetal bovine serum (FBS) + 1:100 L-glutamine, sodium pyruvate, non-essential amino acids, and antibiotic/antimycotic). BT-20 cells were a gift from Dr. Jing Yang and cultured in αMEM with 10% FBS + 1:100 L-glutamine and antibiotic/antimycotic. MCF10A cells (ATCC) and HMECs (Lonza) were purchased and cultured in MEGM (Lonza) according to manufacturer's directions.

**Histological analysis, immunohistochemistry and immunofluorescence**

For immunohistochemical staining of formalin-fixed paraffin-embedded tissues, antigen retrieval was performed in citrate buffer at pH 6.0 and 95°C for 20 min. Sections were blocked in 5% goat serum, incubated overnight at 4°C in primary antibody (CD61, Abcam; Elf-5, Santa Cruz; GATA3, Cell Signaling Technology; ERα, Abcam) followed by biotin-conjugated anti-rabbit IgG and an avidin–biotin peroxidase detection system with 3,3′-diaminobenzidine substrate (Vector) then counterstained with hematoxylin. Whole-mount mouse mammary glands were fixed in Carnoy's solution and stained with carmine. Samples were imaged on a Nikon Ti/E inverted microscope using Nikon Elements software. For quantitation of duct/alveoli density, 3-4 4x images were randomly sampled from H&E-stained paraffin sections from each mouse and analyzed with Metamorph software. For frozen sectioning of mouse mammary glands, glands were first fixed in acetone at -20°C overnight, washed in 30% sucrose at 4°C overnight then frozen in OCT and 7 micron sections cut. Alternatively, cells in culture were fixed briefly in 2% paraformaldehyde/PBS for 10 min, and then permeabilized in 0.1% Triton/PBS for 5 min prior to staining. For immunofluorescence, frozen sections or fixed cells were blocked with 5% normal goat serum (sections), or 3% BSA/2.5% normal goat serum (cells) in PBS incubated in primary antibody (CD61, Abcam; Slug, Vimentin and pY416 SFK, Cell Signaling Technology; K18-FITC, Novus Biologicals; K14-FITC, Abcam; E-cadherin, BD Biosciences; αSMA-Cy3, Sigma; milk protein (RAM/TM), Nordic Immunology) overnight at 4°C followed by secondary (Alexafluors 488, 568, 647, Invitrogen) at room temperature for 1 h. Samples were imaged on a Nikon Eclipse C1 confocal microscope using EZ-C1 3.50 imaging software (Nikon). BrdU analysis was

performed using the BrdU *In-Situ* Detection Kit (BD Biosciences) according to manufacturers' instructions for formalin fixed paraffin embedded sections. Briefly, 1 mg of BrdU was delivered by intraperitoneal injection into day 12.5 pregnant mice and mammary glands harvested 3 h post-injection.

**Lysates and immunoblotting**

Whole mammary gland lysates were prepared by pulverizing glands flash frozen in liquid nitrogen with a mortar and pestle and then lysing the tissue with RIPA lysis buffer (100 mM Tris pH 7.5, 150 mM sodium chloride, 0.1% deoxycholate, 0.1% SDS, 50 mM NaF, Protease inhibitor cocktail (Roche), 2 mM PMSF, 2mM sodium orthovanadate). The lysate was further processed with a hand-held tissue homogenizer and cleared. Whole cell lysates were prepared from cell lines with the same lysis buffer combined with scraping. The following antibodies were used for immunoblotting: β3, Slug, Vimentin, SP1, pY416 SFK and c-Src (Cell Signaling Technology), β3 C-term (Abcam), E-cadherin (BD Biosciences), αSMA and β-actin (Sigma). Standard Western blotting procedures were performed.

**Mammary cell preparation**

The fourth (abdominal) and third (thoracic) mammary fat pads were finely minced using scalpels and dissociated in Ham's F12/Dulbecco's modified Eagle's medium 1:1 (v:v), supplemented with 10 mM Hepes, 2% bovine serum albumin fraction V, 5 μg $ml^{-1}$ insulin, 0.5 μg $ml^{-1}$ hydrocortisone, 10 ng $ml^{-1}$ cholera toxin, 300 units $ml^{-1}$ collagenase and 100 units $ml^{-1}$ hyaluronidase at 37°C for 8 h. The epithelial-cell-rich pellet was

collected by centrifuging the cell suspension at 80x*g* for 4 min followed by one wash with F12/DME/10 mM Hepes and pelleted. Cells were triturated for 5 min in 0.05% trypsin-0.025% EDTA solution with 100 µg $ml^{-1}$ DNase I (Worthington) to generate a single-cell suspension, Trypsin Neutralizing Solution (Lonza) was added and the cells collected by centrifugation then suspended in 5% bovine serum albumin fraction V and filtered through a 40 µm nylon cell strainer.

**Flow cytometry and mammary outgrowth assays**

Single cell suspensions were blocked in 0.5% BSA/PBS + 2 µg $ml^{-1}$ mouse IgG1 (BD) and stained with the following antibodies: CD24-PE (BD), CD29-Alexa647 (BioLegend), CD61-FITC (BD), CD31-biotin (BD), CD45-biotin (BD), Ter119-biotin (BD), Streptavidin-APC-Cy7 (BD). Cell sorting was performed using a FACS Diva or FACS Aria (BD). For outgrowth experiments, sorted cells were injected into the cleared abdominal fat pads of 3 week old syngeneic recipients. Estimated repopulating cell frequencies were calculated using the ELDA web-based tool (Hu and Smyth, 2009) (http://bioinf.wehi.edu.au/software/elda/).

**Immunoprecipitations**

The integrin αv subunit was pulled-down from 400 µg of whole mammary gland lysate from WT and β3ΔC P12.5 mammary glands. Briefly, lysates were pre-cleared with 20 µL of 50% protein A/G beads (Pierce) overnight while pre-incubating αv antibody (Santa Cruz) with beads (2 µg of antibody per 20 µL of beads slurry). Antibody-coated beads were then blocked with 5% BSA/PBS for 3 h at 4ºC prior to washing and adding 20 µL

of 50% bead slurry to pre-cleared lysates and incubating overnight at 4ºC. The beads were then washed 3x with lysis buffer prior to eluting proteins with 2x sample buffer and performing Western analysis.

**Real-time qPCR**

qPCR experiments were performed by collecting total RNA from whole mammary glands or cultured cells by Trizol extraction and reverse transcribing with the High Capacity cDNA Reverse Transcription kit (Life Technologies). Real-Time qPCR was performed using Quantitect SYBR Green PCR Mix (Qiagen) and run on a Light Cycler 480 (Roche). See the table below for all qPCR primer pairs. Relative mRNA levels were also examined using the Cells-to-CT kit (Life Technologies) according to manufacturer's instructions. Lysates were prepared from freshly sorted $CD29^{hi}$ and $CD29^{lo}$ cells (10,000) from virgin and P12.5 WT as well as P12.5 β3KO mammary glands. Alternatively, $CD29^{hi}$ and $CD29^{lo}$ cells were seeded into 96-well tissue culture treated dishes (coated with 2% Matrigel overnight) for 72 h prior to adding 5 ng $ml^{-1}$ TGFβ2 (Peprotech) or 0.1% BSA/PBS (vehicle) and preparing lysates 24 h later. Taqman Real-Time PCR assays for murine *itgαv*, *itgβ3, Snail2* (Slug), *E-cadherin, Vimentin,* and *18S rRNA* (control) were purchased from Life Technologies.

**qPCR Primers**

| Primer Name | Primer Sequence (5'-3') |
|---|---|
| **Mouse** | |
| Elf5-F | GATCTGTTCAGCAATGAAG |
| Elf5-R | GGTCTCTTCAGCATCATTG |
| GATA3-F | AGCCACATCTCTCCCTTCAG |
| GATA3-R | AGGGCTCTGCCTCTCTAACC |
| GAPDH-F | AGGTCGGTGTGAACGGATTTG |
| GAPDH-R | TGTAGACCATGTAGTTGAGGTCA |
| | |
| **Human** | |
| Itgb3-F | GCAATGGGACCTTTGAGTGT |
| Itgb3-R | GTGGCAGACACATTGACCAC |
| SP1-F | CTATAGCAAATGCCCCAGGT |
| SP1-R | TCCACCTGCTGTGTCATCAT |
| Snail2 (Slug)-F | ATATTCGGACCCACACATTACCT |
| Snail2 (Slug)-R | GCAAATGCTCTGTTGCAGTGA |
| β-actin-F | GGAGGAGCTGGAAGCAGCC |
| β-actin-R | GCTGTGCTACGTCGCCCTG |

**Adhesion assays**

48-well non-TC plates were coated overnight with 10 μg ml-1 type I collagen or 20 μg ml-1 fibrinogen and blocked with 5% BSA/PBS overnight prior to seeding with 200,000 digested cells from P12.5 WT or β3ΔC mammary glands in 200 μL of serum-free DMEM/0.1% BSA. Cells were allowed to adhere for 1 h prior to washing 1x with PBS and staining with 0.1% crystal violet/20% methanol/PBS for 1 h. Wells were then washed twice with water and air-dried prior to eluting with 200 μL methanol and reading the A600 nm. Specific adhesion was measured by subtracting away the average absorbance from wells coated with BSA alone. Alternatively, the assay was performed in an 8-well chamberslide (Lab-Tek) and cells were stained with K14 and αSMA for immunofluorescence as previously described.

**Cell transfection and lentiviral transduction**

Transient transfection of MCF-7 cells with β3 cDNA's in the pcDNA3.1 expression plasmid was performed with Lipofectamine 2000 (Invitrogen) as per manufacturer's instructions for 6-well dishes. Alternatively, MCF-7 cells were treated with 500 μg $ml^{-1}$ G418 for two weeks and 3 resistant clones selected and pooled for use in experiments. siRNA transfection of MCF10A cells was achieved using HiPerFect transfection reagent (Qiagen) as per manufacturer's instructions for a 10 cm dish. AllStars negative control, β3 and SP1 FlexiTube siRNAs were purchased from Qiagen. Stable knock-down of β3 was achieved by transducing MDA-MB-231 (HM) and BT-20 cells with lentivirus expressing a human-specific β3 shRNA/pGIPZ or a non-silencing shRNA/pGIPZ (Open Biosystems) and pooling three puromycin-resistant clones. MDA-MB-468 cells were transduced with lentivirus generated with the FG12 plasmid alone (empty vector) or FG12 containing β3 or β3ΔC cDNA.

**Luciferase reporter assays**

The pEZX-LvPG04 luciferase reporter plasmid alone or containing the β3 promoter were purchased from GeneCopoeia. These plasmids co-express secreted alkaline phosphatase (SEAP) as a control. Reporter assays were performed by transient transfection of MCF10A cells with either vector alone or the β3 promoter luciferase reporter using Lipofectamine 2000 (Invitrogen) as per manufacturer's instructions and stimulating cells 24 h later with 5 ng $ml^{-1}$ TGFβ2 (Peprotech) or vehicle control. Cell culture medium was sampled and assessed for luciferase activity relative to SEAP 48 hours post-stimulation using Secrete-Pair dual luminescence kit (GeneCopoeia). Both

luciferase and SEAP levels were measured in 96-well plates on a TECAN plate reader running Magellan software.

**Soft agar/tumorsphere assays**

Suspended cells in 0.3% agar/complete DMEM medium were cultured on top of a bottom layer of 1% agar in 48-well dishes. Additional media was added and cells cultured for 10–14 days prior to fixing and staining colonies with 0.1% crystal violet/20% methanol/PBS and counting colonies consisting of at least 5 cells from whole wells.

## Supplemental References

Ablooglu, A.J., Kang, J., Petrich, B.G., Ginsberg, M.H., and Shattil, S.J. (2009). Antithrombotic effects of targeting alphaIIbbeta3 signaling in platelets. Blood *113*, 3585-3592.

Munoz, R., Man, S., Shaked, Y., Lee, C.R., Wong, J., Francia, G., and Kerbel, R.S. (2006). Highly efficacious nontoxic preclinical treatment for advanced metastatic breast cancer using combination oral UFT-cyclophosphamide metronomic chemotherapy. Cancer Res *66*, 3386-3391.