DOI: 10.1038/ncb2725

nature cell biology

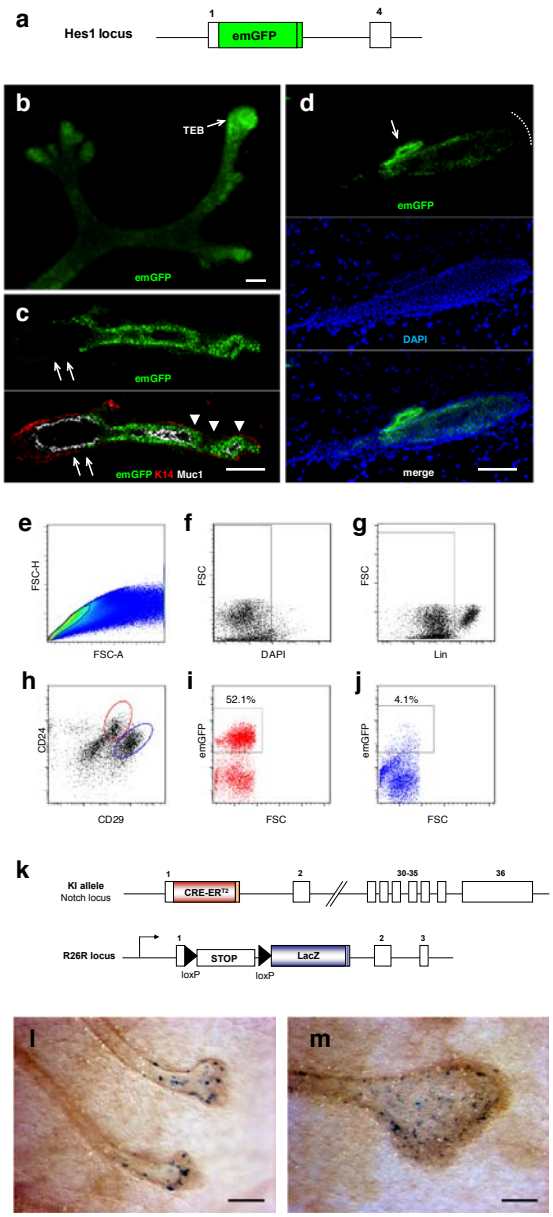

Figure S1 Notch pathway activity in pubertal mammary glands. **a,** Schematic presentation of the targeted allele in the Hes1-emGFP^{SAT} knock-in reporter line. The emGFP cassette with a polyadenylation signal (pA) is placed in frame with the start codon in exon1. White boxes represent exons. **b-d**, Representative immunofluorescence images of mammary glands from 6 week old Hes1-emGFP^{SAT} females (n=3 mice). The emGFP reporter construct is expressed in cells with active Notch signaling. **b**, Whole-mount preparation showing emGFP expression in a typical actively growing mammary ductal tree. **c,** Thin cross-section of a typical actively growing mammary duct co-labelled with the anti-Muc1 (white) and anti-K14 (red) antibodies. Arrows mark proximal, more mature portion and arrowheads mark the distal portion of the duct. **d,** Thick cross-section of a typical end bud that has reached the edge of the mammary fat pad (white dotted line) and a budding lateral branch (arrow). Cell nuclei co-stained with DAPI (blue). Scale bars: 100 μm. **e-j**, Representative FACS analysis of pubertal Hes1emGFP glands. Notch signaling is active in approximately half (52.1%) of all viable luminal cells (CD24+CD29^{low} population) and in a small fraction (4.1%) of the CD24+CD29^{high} population. (h) Expression of CD24 and CD29 in viable Lin$^-$ population. Red gate, CD24+CD29^{low}; blue gate,CD24+CD29^{high} population. (i,j) Analysis of emGFP expression in red gate(i) and blue gate (j). Values are expressed as mean of two independent experiments (also see source data in table S1). **k**, Schematic presentation summarizing the genetic strategy used to induce LacZ expression in *Notch2* expressing cells and their progeny. The N2-CreERT2^{SAT} knock-in line has the Cre-ERT2 cassette inserted into the first exon of *Notch2*. One allele of the targeted *Notch2* locus expresses the tamoxifen inducible Cre-ER recombinase under the control of the *Notch2* promoter, while the other allele remains functional. N2-CreERT2^{SAT} mice are crossed to R26R^{LacZ} reporter strain and bi-genic progeny treated with 4-OHT in order to induce LacZ expression. White boxes represent exons. **l,m** Distribution of LacZ$^+$ cells in medium-size and large bulbous TEBs in early puberty. x-gal labeled mammary gland whole mount preparations from N2-CreERT2^{SAT} /R26R^{LacZ} females induced with 4-OHT at early puberty (4 weeks of age) and sacrificed after 24h. **l**, A typical medium size TEB. **m**, A typical large, bulbous TEB. Scale bar: 100 μm.

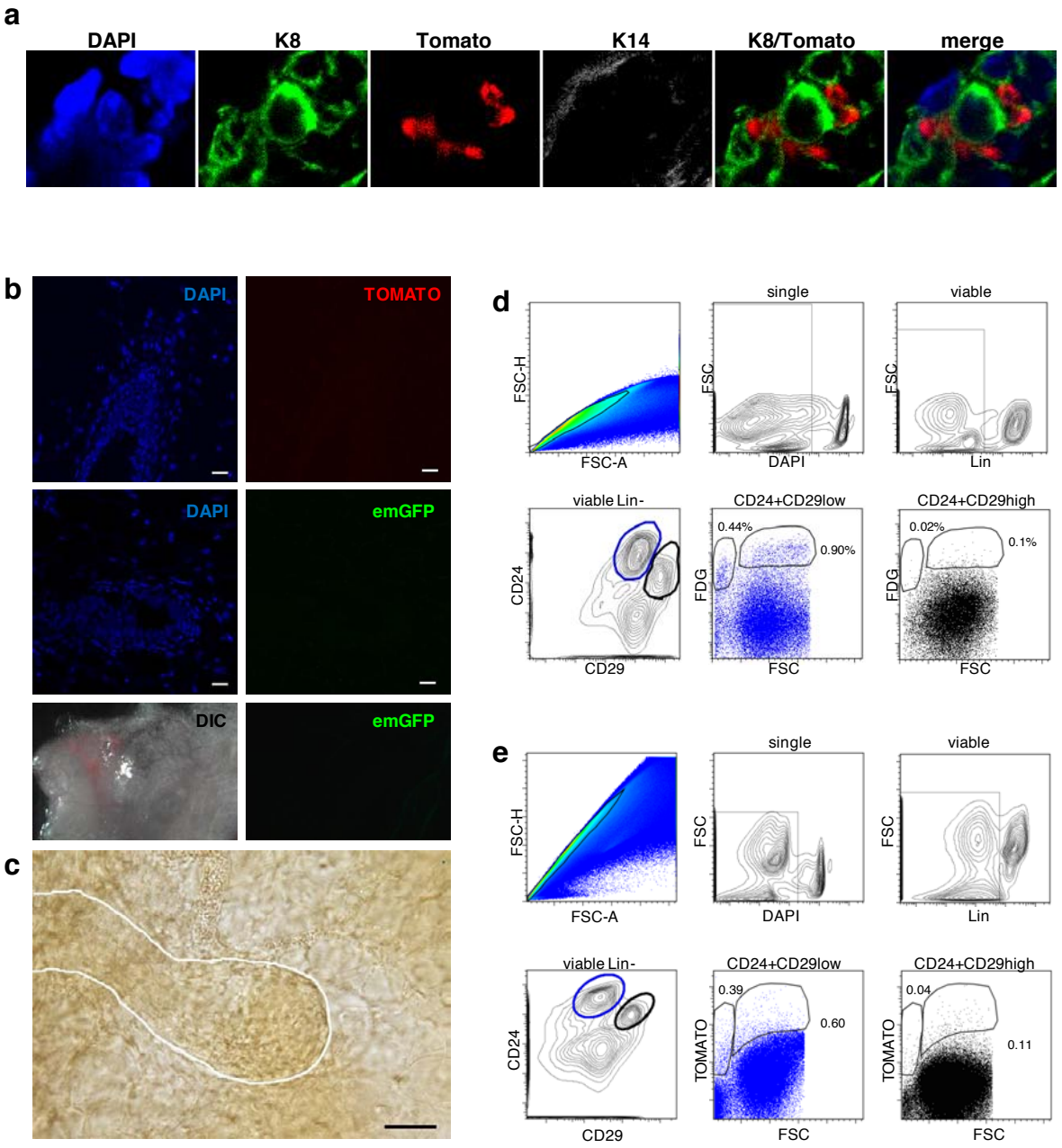

Figure S2 Control immunofluorescent images and FACS analyses. **a,** Immunofluorescent images for individual markers shown in Figure 1h. **b,** Immunofluorescent images of mammary gland sections and a mammary wholemount from control N2-CreERT2^{SAT}/R26R^{TOM} pubertal females untreated with 4-OHT. Nuclei co- stained with DAPI (upper two panels). **c**, High magnification image of x-gal labeled mammary wholemount from control N2- CreERT2^{SAT} /R26R^{LacZ} pubertal female (untreated with 4-OHT). Scale bars: 20 μm (a,b); 200 μm (d). **d,e,** FACS analysis of Notch2^{+} lineages, using two different reporter systems (FDG versus Tomato). N2-CreERT2^{SAT} /R26R^{LacZ} (d) and N2- CreERT2^{SAT} /R26R^{TOM} (e) pubertal females. PE conjugated antimouse CD24 in (d) was substituted with PE-CY5 conjugated antimouse CD24 in (e). Mammary glands pooled from 3 mice for each experiment.

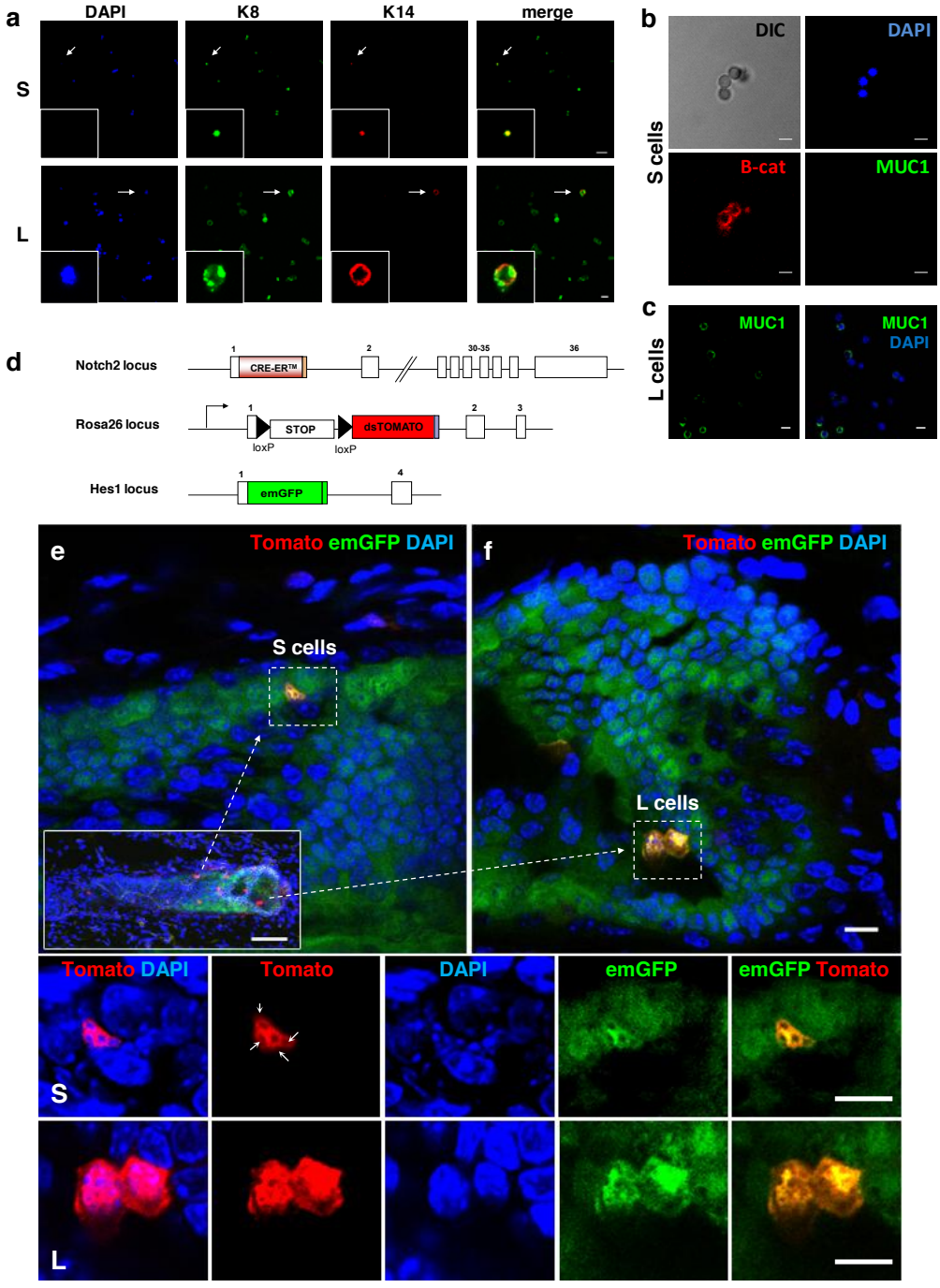

Figure S3 *In vitro* and *in vivo* marker analysis of S and L cells. **a-c**, Representative confocal images of freshly sorted S and L populations from pubertal N2-CreERT2^{SAT} /R26R^{LacZ} females (gated in **Figure 2h)**. Mammary glands pooled from 3 mice. Co-labeled with DAPI and lineage specific antibodies. **a**, Anti-K8 (green), anti-K14 (red) antibody. Insets are close-ups of cells marked with arrow. **b,c** Anti-Muc1 (green) and anti-β-catenin (red) antibody. Representative z-stack images (0.1 um resolution, also see Supplementary movie). 100% of S and L cells are positive for the luminal marker K8 (n=400 and 800 cells, respectively) very rare subset is dual-positive K8^{+}K14^{+} (2.7% S and 3% L cells) (also see **Figure 2p,q**). Scale bars: 10 μm (a,c); 2.8 μm (b). **d**, Schematic presentation summarizing the genetic strategy used to analyze Notch pathway activity in *Notch2* expressing cells and their progeny. Trigenic N2-CreERT2^{SAT} /R26R^{Tom}/ Hes1emGFP mice are treated with 4-OHT in order to induce Tomato expression. White boxes represent exons. **e,f** Immunofluorescence images of pubertal mammary duct (inset in e) containing a group of four small Tomato^{+} cells (S cells) flanking a single large Tomato^{-} luminal cell (e) and a pair of large Tomato^{+} cells (L cells) (f). Tomato (red), emGFP (green). Nuclei co-labeled with DAPI. Mammary gland thick frozen section (20um) from trigenic N2-CreERT2^{SAT} /R26R^{Tom} /Hes1emGFP female induced at 6 weeks of age and sacrificed at 8 weeks of age. Lower panels are close-ups of areas marked with white dashed squares in (e) and (f), respectively. Arrows point to individual S cells. Scale bars: 100 μm (inset in e); 10 μm (e,f, close-ups).

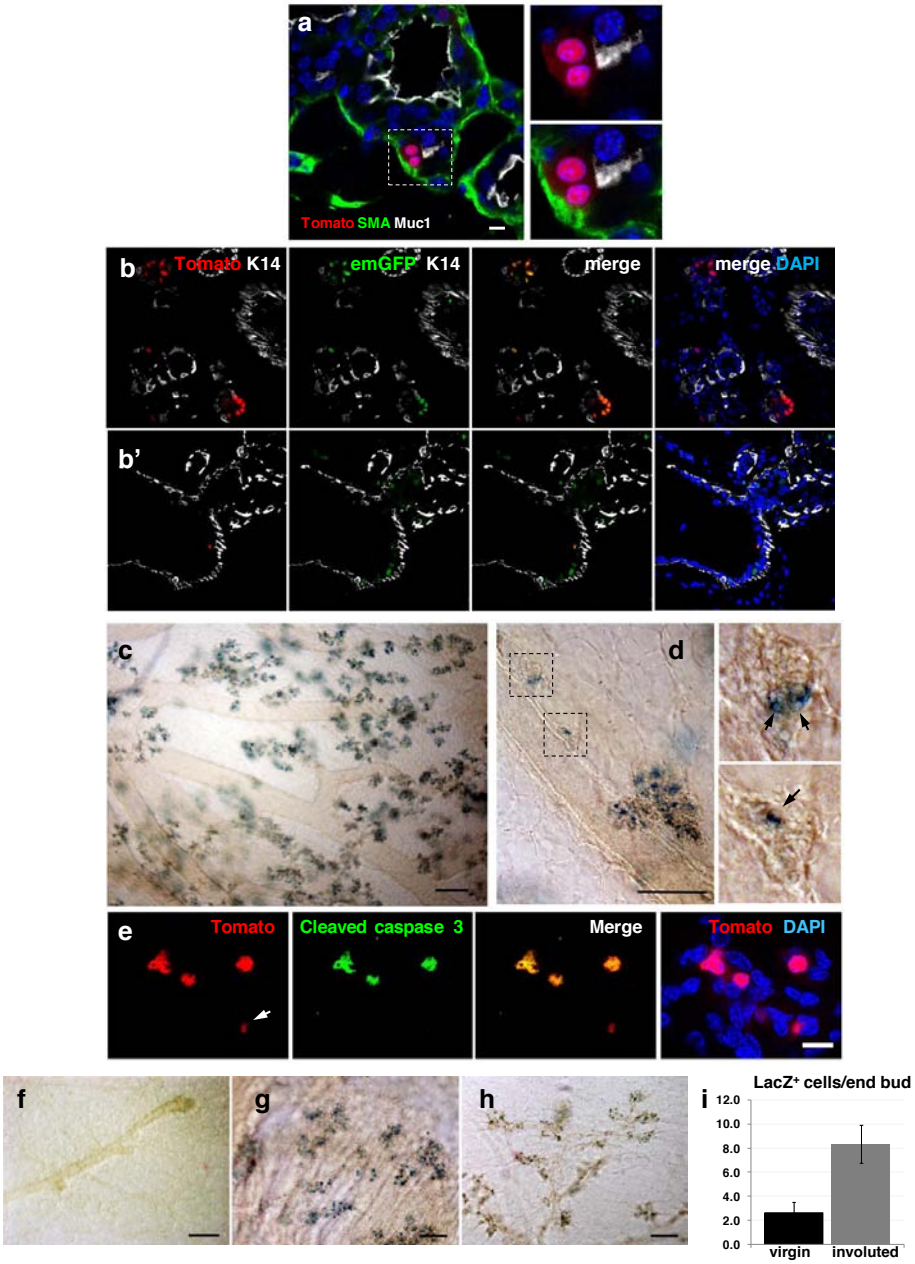

Figure S4 Marker analysis and the fate of L-alveolar cells in lactation and involution. **a,** Immunofluorescence image of a typical alveolus containing a pair of large alveolar Tomato$^+$ cells (L-alveolar). Mammary gland frozen section co-labelled with anti-Muc1 (white) and anti-SMA (green) antibodies. Nuclei co-labelled with DAPI. Tomato (red). N2-CreERT2^{SAT} /R26R^{Tom} female induced with 4-OHT at 7 weeks of age and sacrificed during lactation. Small panels are close-up of the area marked with the white square in the main panel. Scale bar: 10 µm. **b,b'** Notch signaling pathway activity in L-alveolar cells. Immunofluorescence images of alveoli (b) and a large duct (b') in the lactating mammary gland from a tri-genic N2-CreERT2^{SAT} /R26R^{Tom}/ Hes1emGFP reporter female induced with 4-OHT at 7 weeks of age and sacrificed at lactation. L-alveolar and L-ductal cells (Tomato$^+$ cells, red) express the Notch signalling reporter emGFP (green). (b) Area of the gland in close proximity to the nipple. Two alveoli contain multiple L-alveolar cells arranged in arrays. Frozen sections co-labelled with anti-K14 antibody (white). Nuclei co-labelled with DAPI. Scale bar: 20 µm. **c-e,** The fate of L-alveolar cells in involution. **c,d** x-gal labelled whole-mount preparations of a typical involuting mammary gland at low (c) and high (d) magnification. Insets in (d) are enlarged images of the areas in the main panel marked with squares depicting collapsing alveoli with a pair (upper inset, arrows) and a single (lower inset, arrow) L-alveolar cell. N2-CreERT2^{SAT} /R26R^{LacZ} female induced with 4-OHT at 7 weeks of age and sacrificed during involution (4 days after forced weaning). Scale bars: 200 µm (c); 100 µm (d). **e**, Immunofluorescence image of a representative collapsed alveolar cluster containing several apoptotic L-alveolar cells. A single L-alveolar cell is not undergoing apoptosis (arrow). Mammary gland frozen section co-labelled with anti- cleaved caspase 3 antibody (green). Nuclei co-labelled with DAPI. Tomato (red). N2-CreERT2^{SAT} /R26R^{Tom} female induced with 4-OHT at 7 weeks of age and sacrificed during involution (4 days after forced weaning). Scale bar: 10 µm. **f-h**, The fate of L-alveolar cells at different stages of involution. x-gal labelled mammary whole-mounts showing typical end buds in adult nulliparous female (f) and two females at different stages of involution (g,h). N2-CreERT2^{SAT} /R26R^{LacZ} females induced with 4-OHT at 7 weeks of age and sacrificed at 10 weeks of age (f), 4 days after forced weaning (g) and 8 days after forced weaning (h). Scale bars: 100 µm. **i**, Histogram depicting the number of large LacZ+ cells (L cells) in virgin (2.6±0.9) and involuted end buds 8 days post-weaning (8.4±1.6). Data is expressed as mean±SD, n=34 end buds per condition from 3 mice.

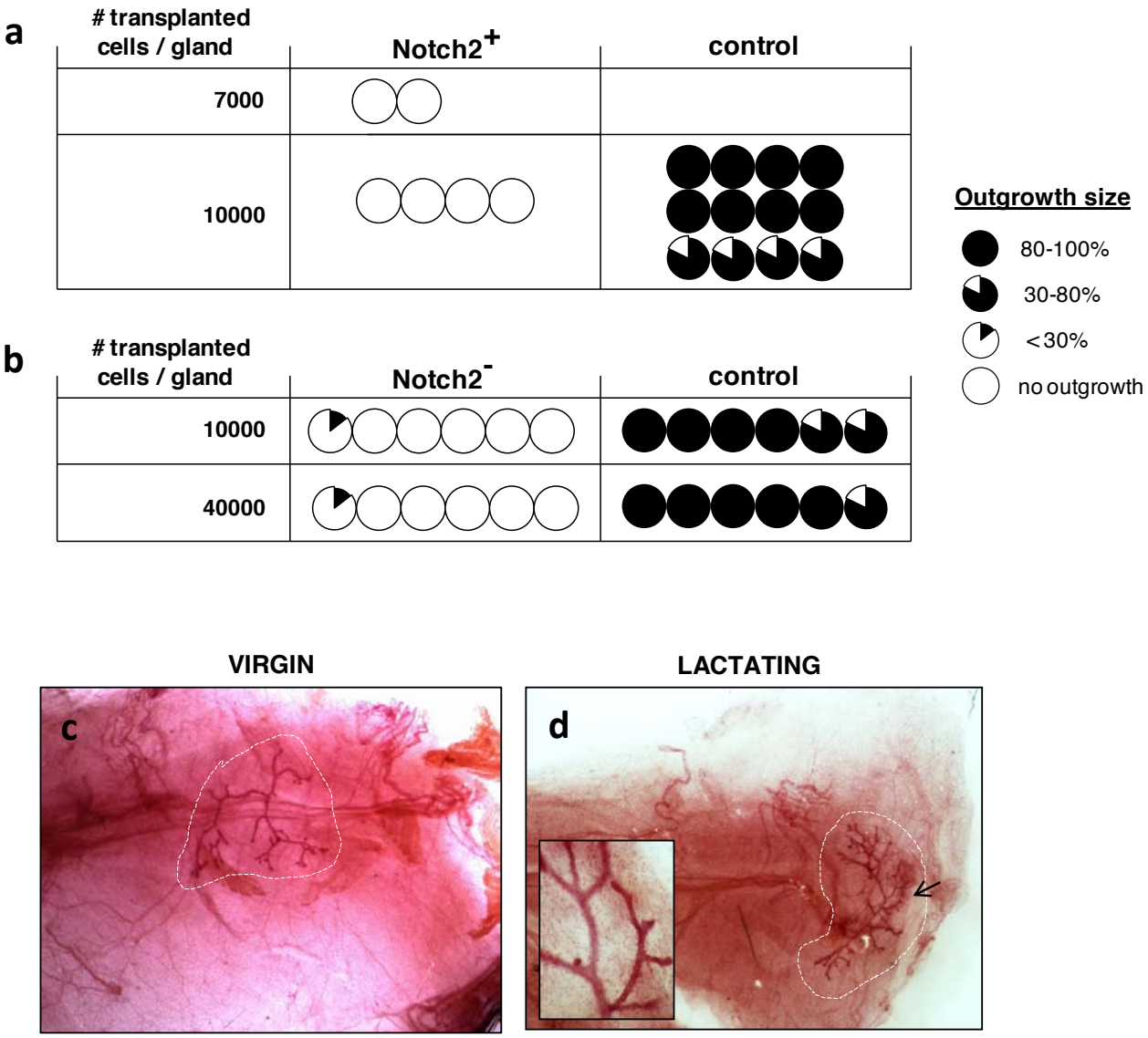

Figure S5 Reconstitution assays. **a,b**, Recipient virgin females sacrificed 9 weeks post-transplantation.Each circle represents one mammary gland; the blackened region represents the area filled with outgrowth. **c,d** Images of mammary wholemounts labeled in x-gal and co-stained with Carmine alum. **c,** Notch2- (FDG-) outgrowth in virgin recipient (40000 cells). **d**, Notch2- (FDG-) outgrowth in lactating recipient, one day post-partum (40000 cells). Insert is a close-up of the area labeled with an arrow. Outgrowths are circled with white dashed line.

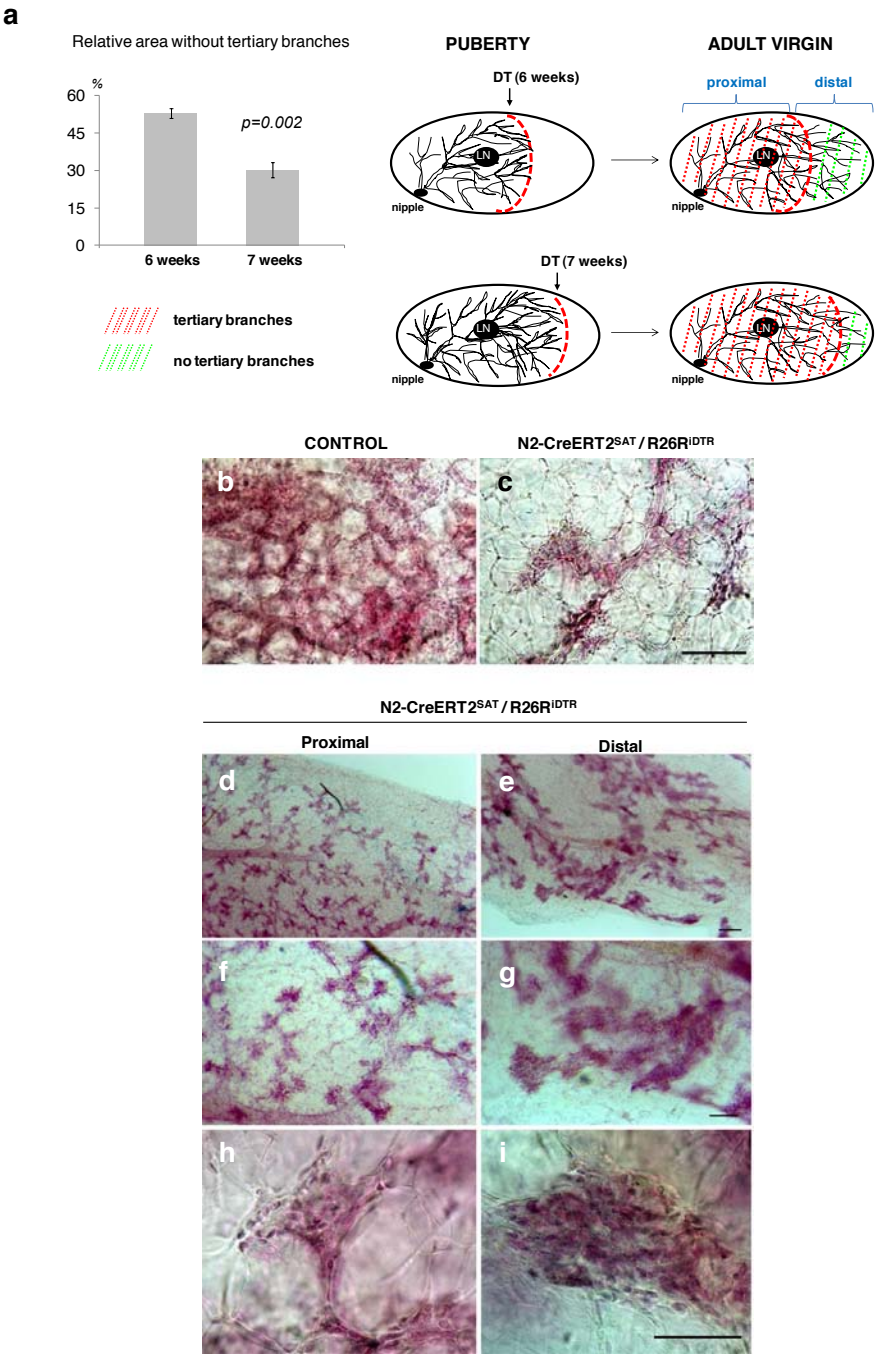

Figure S6 Ablation of pubertal *Notch2*$^{+}$ lineages impairs formation of tertiary branches and alveolar clusters. **a,** Histogram depicting the relative size of the areas without tertiary branching in inguinal glands from animals induced with DT at 6 weeks (53%±2%) and 7 weeks (30%±3%) of age. Data is expressed as mean±s.e.m.; p=0.002, paired t-test; n=4 inguinal glands per time point. The values are calculated relative to area of the gland between the lymph node and the distal end of the fat pad. Individual values are presented in the table S1. Schematic presentation of inguinal glands depicting correlation between the age of the animal at the time of DT administration and the relative size of the areas exhibiting proximal and distal tertiary branching phenotype. Puberty: the age of the animal and approximate position of the actively growing end buds (red dashed line) at the time of *Notch2*$^{+}$ cell ablation (i.e. DT administration). Adult virgin: Approximate position of the margin between the segments with and without tertiary branches (i.e proximal and distal relative to the nipple). Total 16 glands from 4 mice were analyzed (2 inguinal and 2 thoracic glands per mouse). Thoracic glands in DT treated animals exhibited the same proximal/distal phenotype as inguinal glands. **b-i,** Representative carmine alum stained whole-mount preparations of mammary glands from lactating females (1 day post partum, primiparous). Control (R26R^{iDTR}) (b) and N2-CreERT2^{SAT} /R26R ^{iDTR} females (c-i) induced with 4-OHT and DT at 6 weeks of age. (b,c) Alveolar clusters are absent in the gland with ablated *Notch2*$^{+}$ lineages (c). (d-i) Representative images of the proximal and distal segment of the same mammary gland. (f,g) Close-up views of (d,e). (h,i) High power magnification images of typical tertiary structures in proximal (h) and distal (i) segment. Scale bars: 100 µm (b,c,f,g); 200 µm (d,e); 50 µm (h,i).