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Supplemental Information

Lineage Tracing of Mammary Epithelial Cells Using Cell-Type-Specific Cre-Expressing Adenoviruses

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Supplemental Figures

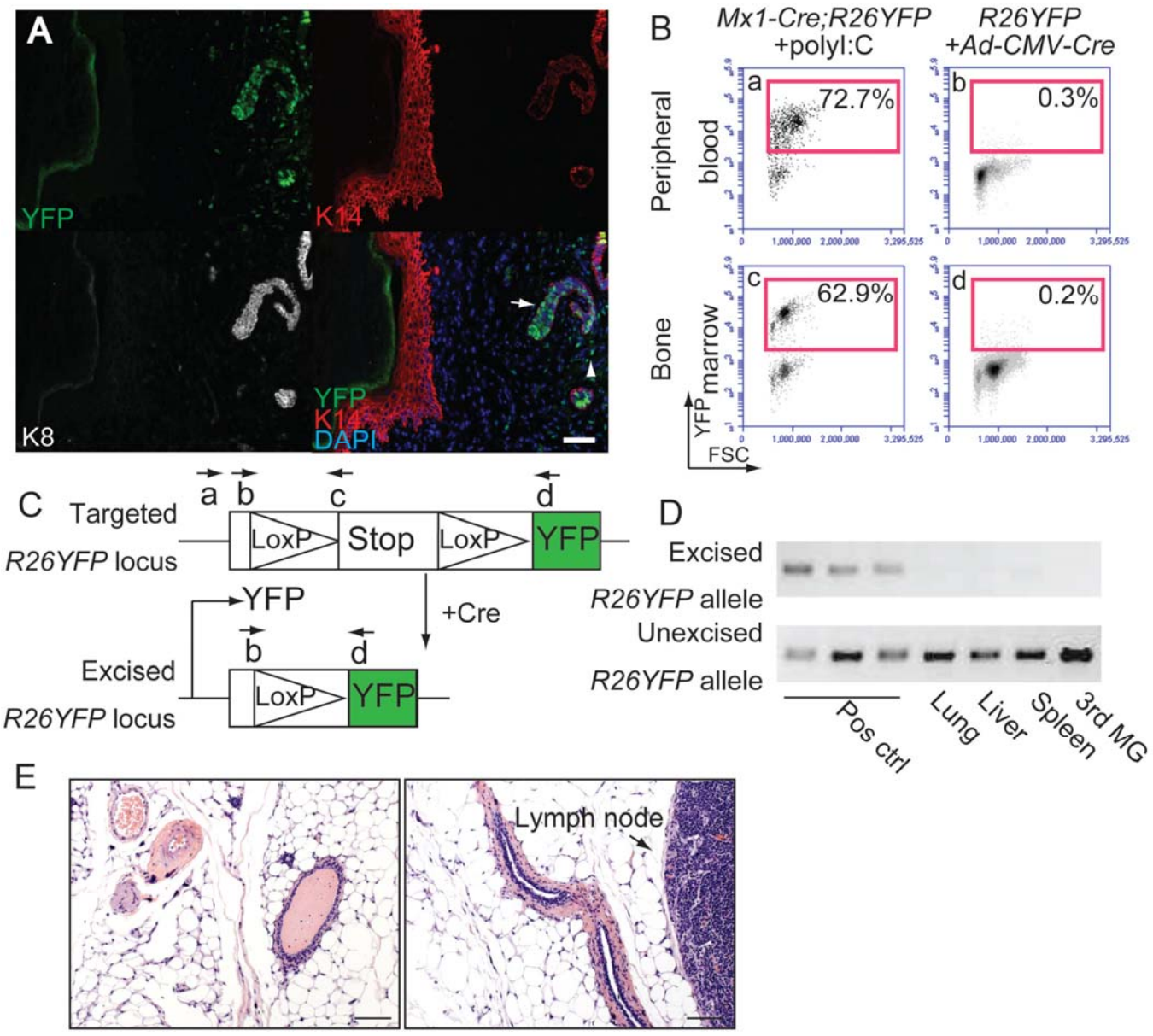

Figure S1-related to Figure 1. Characterization of *Ad-Cre* intraductal injection to mammary glands

(A) IF staining of skin surrounding the nipple area from a *R26YFP* female injected with *Ad-CMV-Cre*. YFP^+ cells can be detected in mammary ducts (arrow) and a few stromal cells (arrowhead) around the ducts. But skin cells (red $K14^+$ cells) were YFP^-. Scale bar = 50μm. **(B)** FACS (fluorescence-activated cell sorting) analysis of peripheral blood and bone marrow from *R26YFP* mice injected with *Ad-CMV-Cre*. Compared to *Mx1-Cre;R26YFP* control mice induced with poly(I:C), no obvious YFP^+ populations were detected in the peripheral blood and bone marrow of *R26YFP* mice injected with *Ad-CMV-Cre* (to their mammary glands). **(C)** Schematic diagram of primers designed to detect the unexcised *R26YFP* conditional knockin allele (primers a+c) and the excised *R26YFP* allele [i.e., YFP activated due to Cre-mediated excision of the floxed *Stopper* cassette (Stop), primers b+d]. **(D)** PCR for detection of Cre-mediated recombination at the *R26YFP* knockin allele using genomic DNA. Positive control DNA samples (Pos ctrl) were isolated from the 4^{th} mammary glands injected with *Ad-CMV-Cre*. Lung, liver, spleen and the 3^{rd} mammary gland (not injected with *Ad-CMV-Cre*) tissues were isolated from one of the *Ad-CMV-Cre* injected animals. **(E)** Hematoxylin & eosin (H&E) staining of mammary gland sections. No obvious inflammation was detected in mammary gland injected with adenovirus except the enlarged lymph node (arrow, 3 days after injection), which eventually reverted to normal size. Scale bar = 50μm.

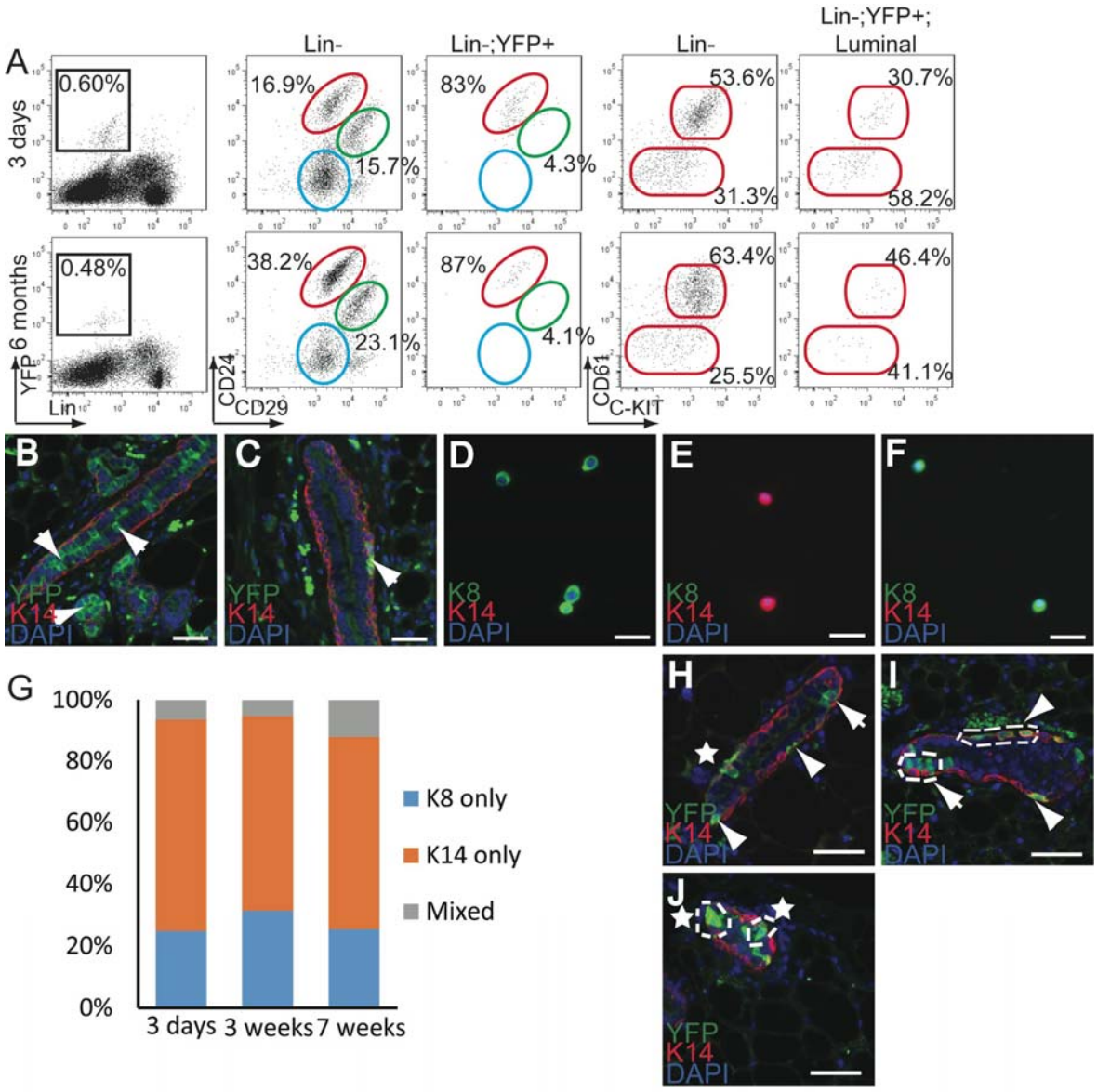

Figure S2-related to Figure 2. Lineage specificity of *Ad-K8-nlsCre* or *Ad-K14-nlsCre* induced genetic marking

(A) FACS analysis of *R26YFP*-only females injected with *Ad-K8-nlsCre* chased for 3 days or 6 months. YFP-marked MECs were restricted to the luminal gate (red circle, middle plot) and included both $CD61^+$ and $CD61^-$ luminal cells (right plot). **(B-C)** Mid-gestation mammary glands chased upon *Ad-K8-nlsCre* or *Ad-K14-nlsCre* injection at the nulliparous stage: **(B)** YFP^+ cells marked by *Ad-K8-nlsCre* at the nulliparous stage contributed to both ductal luminal (white arrows) and alveolar luminal (white arrowhead) cells. **(C)** YFP^+ ductal myoepithelial cells induced by *Ad-K14-nlsCre*. Scale bars = 50μm. **(D-F)** FACS sorted Lin^-YFP^+ cells from mammary glands injected with *Ad-K8-nlsCre* or *Ad-K14-nlsCre*: **(D)** Lin^-YFP^+ cells induced by *Ad-K8-nlsCre* are $K8^+K14^-$. **(E)** $K8^-K14^+$ cells (in the basal gate) induced by *Ad-K14-nlsCre*. **(F)** $K8^+K14^-$ cells (in the luminal gate) induced by *Ad-K14-nlsCre*. Scale bars = 50μm. **(G)** YFP-marked clones induced by *Ad-K14-nlsCre* subdivided based on K8 and K14 expression patterns were quantified as $K8^+K14^-$ (K8 only) clones, $K14^+K8^-$ (K14 only) clones, and Mixed clones with both $K8^+K14^-$ cells and $K14^+K8^-$ cells. Total clones at 3 days (125 clones), 3 weeks (192 clones) and 7 weeks (369 clones) after the initial *Ad-K14-nlsCre* injection were quantified. Quantification of multi-cell clones (at least 3 cells per clone, 31/192 and 134/369 such clones from 3-week and 7-week chase, respectively) is shown in Figure 2H (note at day 3, ~95% clones were 1- or 2-cell clones, therefore its multi-cell clone data was not plotted in Figure 2H**)**. **(H-J)** Representative pictures of clones induced by *Ad-K14-nlsCre*, including K14-only clones (white arrowheads), K8-only clones (white arrows) and Mixed clones (white stars) 3 weeks **(H)** and 7 weeks **(I-J)** after adenovirus injection. Multi-cell clones are indicated by dash lines. Scale bars = 50μm.

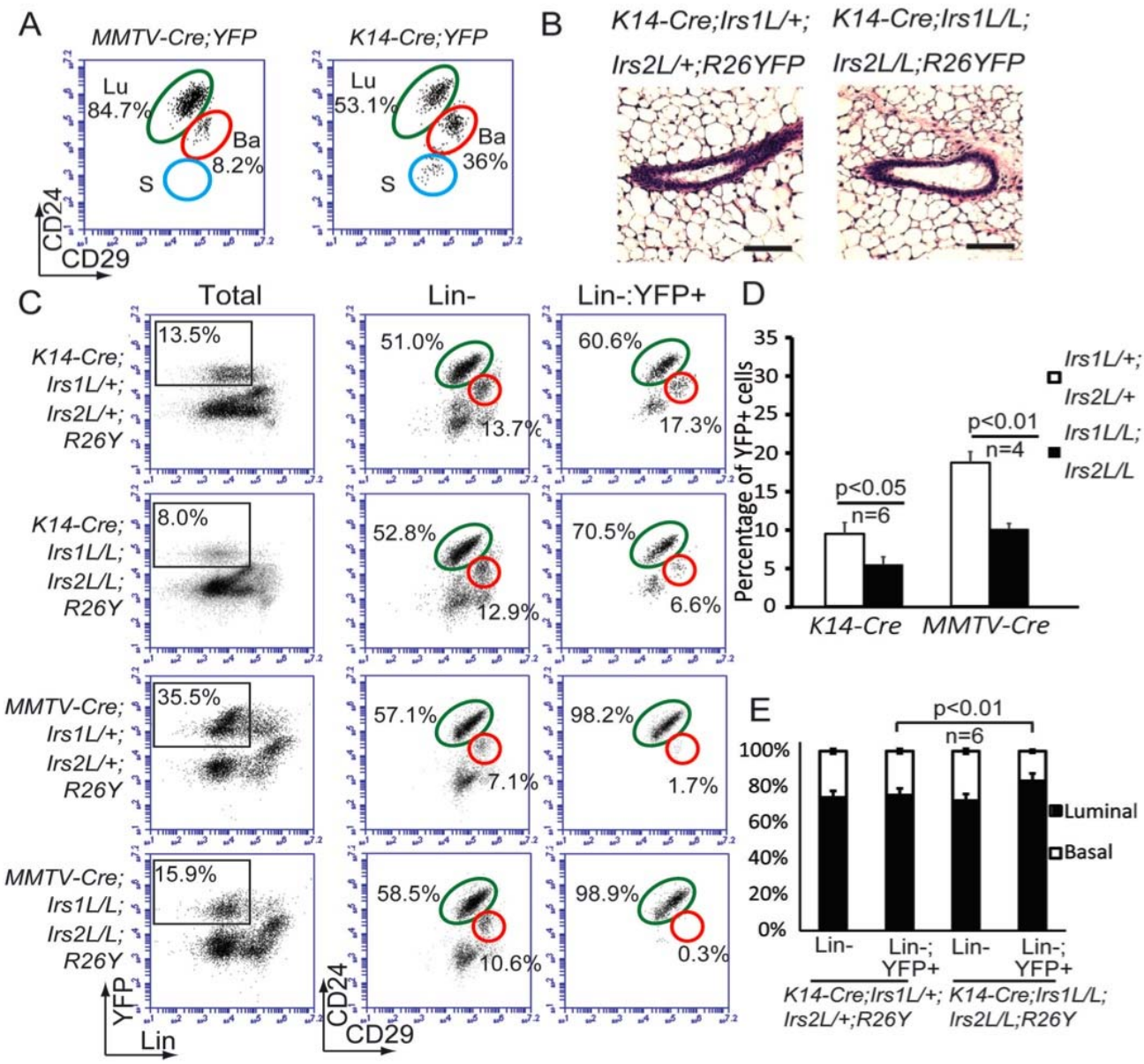

Figure S3-related to Figure 3. IRS1 and IRS2 are essential for maintaining the basal linage in adult mammary glands

(A) FACS analysis (gated for Lin^-YFP^+ cells) of lineage-marking in *R26YFP* females by conventional *K14-Cre* and *MMTV-Cre* transgenic mouse lines. *MMTV-Cre* mainly labels luminal cells (Lu, green circle) and a small number of basal cells (Ba, red circle), whereas *K14-Cre* labels both luminal and basal cells, as well as a small number of stromal cells (S, blue circle). **(B)** H&E staining of *K14-Cre;Irs1*$^{L/+}$*;Irs2*$^{L/+}$*;R26YFP* and *K14-Cre;Irs1*$^{L/L}$*;Irs2*$^{L/L}$*;R26YFP* female mammary glands showing normal morphology. Scale bars = 50μm. **(C)** *K14-Cre;Irs1*$^{L/L}$*;Irs2*$^{L/L}$*; R26YFP* and *MMTV-Cre;Irs1*$^{L/L}$*;Irs2*$^{L/L}$*;R26YFP* females had significant reduction in their YFP-marked (thus *Irs1/2*-null) MEC populations (left FACS plots); in addition, they also exhibited more profound decrease in their YFP-marked basal/myoepithelial population compared to that of the YFP^+ luminal cells (right FACS plots), compared to double heterozygous control females. **(D)** Quantification of decrease of Lin^-YFP^+ populations in *K14-Cre;Irs1*$^{L/L}$*;Irs2*$^{L/L}$*; R26YFP* and *MMTV-Cre;Irs1*$^{L/L}$*;Irs2*$^{L/L}$*;R26YFP* females compared to *K14-Cre;Irs1*$^{L/+}$*;Irs2*$^{L/+}$*; R26YFP* and *MMTV-Cre;Irs1*$^{L/+}$*;Irs2*$^{L/+}$*; R26YFP* double heterozygous control females. **(E)** Quantification of decrease of Lin^-YFP^+ basal populations in *K14-Cre;Irs1*$^{L/L}$*;Irs2*$^{L/L}$*; R26YFP* females compared to *K14-Cre;Irs1*$^{L/+}$*;Irs2*$^{L/+}$*; R26YFP* double heterozygous control females. *P* value is indicated.

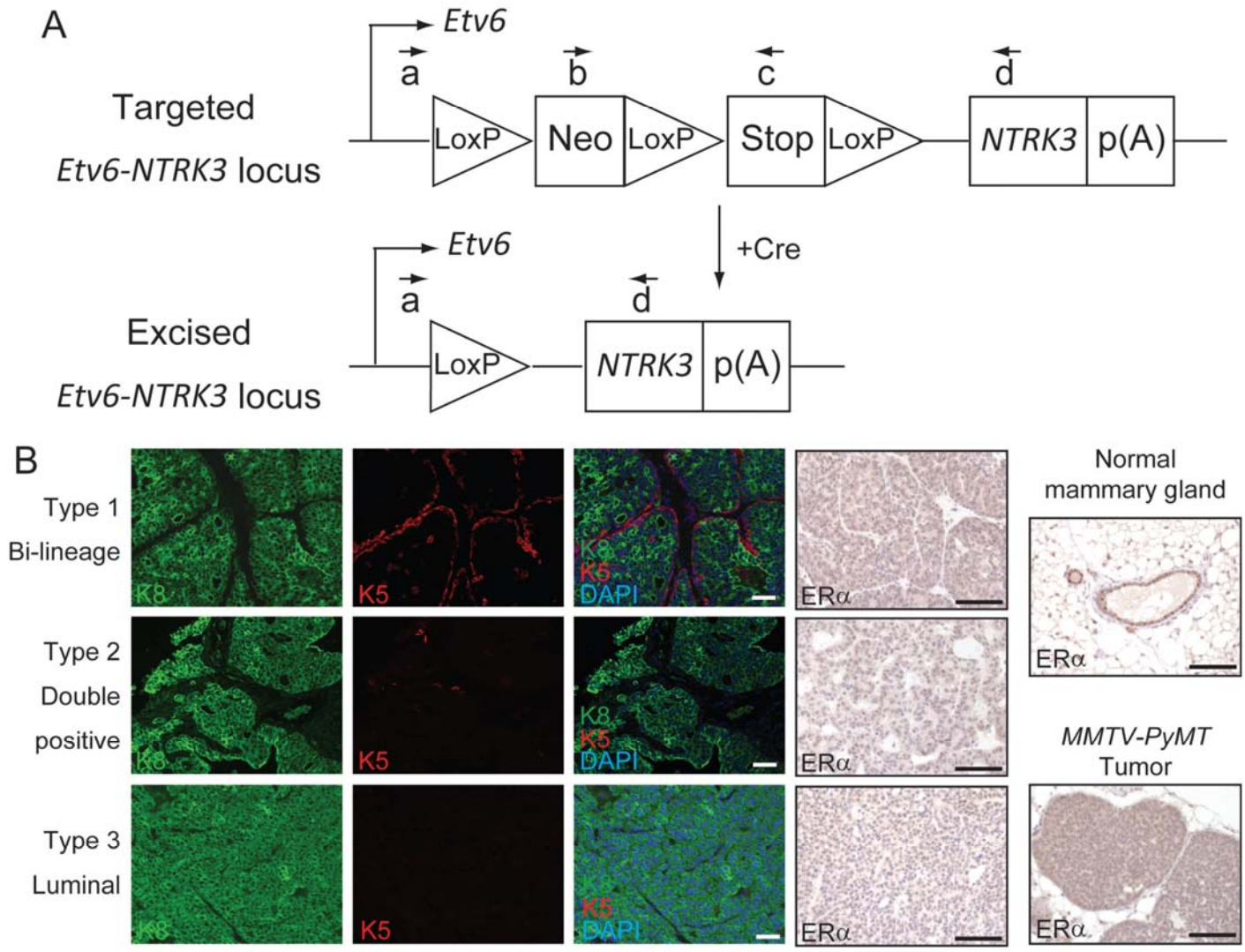

Figure S4-related to Figure 4. Characterization of EN mammary tumors induced by *Ad-K8-nlsCre*

(A) Schematic diagram of primers designed to detect the targeted *EN* conditional knockin allele (unexcised, primers b+c) and the excised *EN* allele (i.e., activated, primers a+d). **(B)** K5/K8 IF staining and ERα IHC staining of three types of mammary tumors developed in *EN/+* female mice injected with *Ad-K8-nlsCre*. ERα IHC staining of a normal mammary gland section (positive control) and a tumor section from an *MMTV-PyMT* female (negative control) were also shown. Note ERα staining intensities in EN mammary tumors were much lower than that of ER^+ cells in normal mammary gland (dark brown cells) and were largely comparable to that of *MMTV-PyMT* luminal tumor; some EN tumors (e.g., Type 1) contain tumor cells with slightly higher ERα staining intensity than those in *MMTV-PyMT* tumor. Scale bars = 50μm.

Supplemental Experimental Procedures

PCR and primers

Gene fragments, including nlsCre coding sequence, mouse *Keratin 8* promoter and human *Keratin 14* promoter, were amplified using Phusion High-Fidelity DNA polymerase (M0530L, New England Biolabs) and the following primers:

nlsCre forward: 5'-CCGACTCTAGCGGCCGCACCATGCCCAAGAAGAAGAGG-3'

nlsCre reverse: 5'-CGAAGCCGGCCGTCGACCTAATCGCCATCTTCC-3'

K8 forward: 5'-GGATGTGTTTAAACGGTGGATCACTTGCCCCCTCCGTTTG-3'

K8 reverse: 5'-AGCTGCTTCGCGGCCGCGGGACAGCGCCCAGCGAAGGCCC-3'

K14 forward: 5'-CTGTTCCGTTTAAACGGGCTCCGGAGCTTCTATTC-3'

K14 reverse: 5'-TGAGTGAAGGCGGCCGCCTCGGGTAAATTGGAAAGGG-3'

The *Stopper* excised and unexcised *R26YFP* (Figure S1C) and *Etv6-NTRK3 (EN)* (Figure S4A) conditional knockin alleles were assessed by PCR by using Taq 5X master mix (M0285s, New England Biolabs) and the following gene specific primers:

R26YFP allele (Figure S1C):

R26YFP-a: 5'-AAAGTCGCTCTGAGTTGTTAT-3'

R26YFP-b: 5'-GACGGTATCGTAGAGTCGAG-3'

R26YFP-c: 5'-GCGAAGAGTTTGTCCTCAACC-3'

R26YFP-d: 5'-CCGGACACGCTGAACTTGTG-3'

Etv6-NTRK3 (EN) allele (Figure S4A):

EN-a: 5'-AGCGTCTCAGTGTATATGGATATG-3'

EN-b: 5'-ACCGCATTAAAGCTTGGCTGGAC-3'

EN-c: 5'-TGGCAAGTGGTATTCCGTAAGAAC-3'

EN-d: 5'-CCGCACACTCCATAGAACTTGAC-3'

Mammary epithelial cell preparation, flow cytometry and sorting

Adenoviruses [diluted in injection medium (DMEM supplemented with 0.1% Bromophenol blue and 0.01M $CaCl_2$)] were introduced into mammary ducts via intraductal injection. The #4 MGs were dissected from injected animals at different time points after intraductal injection. After mechanical dissociation with a sharp scissors, the tissue were placed in digestion media for 1.5h at 37°C (Shackleton et al., 2006). The resultant organoid suspension was sequentially dissociated in 0.25% Trypsin-EDTA for 2min, 1mg/ml DNase for 2min and suspended in red blood cell lysis buffer for 2 min before passing through 40μm cell strainer. The resultant single MEC suspension was subjected to antibody labeling in 96-well plates. Fluorophore-conjugated antibodies were purchased from eBiosciences unless otherwise specified, including CD31-Biotin (clone 390), TER119-Biotin (clone Ter-119), CD45-Biotin (clone 30-F11), CD24-PE (clone M1/69), CD24-eFluor450 (clone M1/69), CD24-eFluor605NC (clone M1/69), CD29-APC (clone eBioHMb1-1), CD49f-APC (clone EbioGoH3), CD61-PE (clone 2C9.G3), C-KIT-PE-Cy7 (clone 2B8). Antibody incubation was performed at 4^oC for 10min. Flow cytometric analysis was performed on Accuri 6 and DXP11 flow cytometers. Cell Sorting was performed on BD Aria. Unstained cells were used to set up the negative gate and single color-stained cells were used to adjust compensation and/or set up the positive gate.

Immunostaining and clonal analysis

Tissue sections were deparaffinized and rehydrated using standard methods. Antigens were retrieved in 10mM sodium citrate buffer, pH6.0. For immunostaining, slides were blocked with 3% H_2O_2/Methanol to inhibit endogenous peroxidase. After 0.5% BSA/PBS blocking for 1 hour, slides was incubated for 1 hour at room temperature using one of the following antibody:

anti-Keratin 5 (Covance, PRB-160P, 1:1000)

anti-Keratin 14 (Covance, PRB-155P or SIG-3476, 1:1000)

anti-Keratin 8 (Covance, MMS-162P, 1:1000)

anti-GFP/YFP (Abcam, ab290, 1:1000)

The secondary antibodies used in IF staining were goat anti-mouse IgG conjugated with AF488 (A11029) or with AF647 (A31571), goat anti-rabbit IgG conjugated with AF488 (A11008), and goat anti-chicken IgG conjugated with AF594 (A11042) (all from Molecular Probes). For IHC staining of ERα, slides were incubated with ERα antibody (Santa Cruz Biotechnology, SC-542, 1:200) followed by detection using ImmPRESS Reagent kit (Vector, MP-7401).

For clonal analysis, after chased for a defined time period, *Ad-Cre*-injected MGs were fixed, embedded in paraffin and the whole MG was sectioned as sagittal sections. After staining for YFP, Keratin 8 (K8) and Keratin 14 (K14), single or clusters of YFP^+ cells that contact each other were defined as YFP-marked clones and their clone types were determined according to their Keratin-staining patterns (i.e., K8-only, or K14-only, or Mixed clones). Clones were further separated by the number of cells per clone and were scored. 3-4 MGs (biological replicates) for each time point were analyzed and their clone data were pooled together based on their clone types, and the frequency of each clone type was calculated as the percentage of the total number of clones, as described previously (Van Keymeulen et al., 2011).

Supplemental References

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Van Keymeulen, A., Rocha, A.S., Ousset, M., Beck, B., Bouvencourt, G., Rock, J., Sharma, N., Dekoninck, S., and Blanpain, C. (2011). Distinct stem cells contribute to mammary gland development and maintenance. Nature *479*, 189-193.