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Supplemental Information

Lineage-Restricted Mammary Stem Cells Sustain the Development, Homeostasis, and Regeneration of the Estrogen Receptor Positive Lineage

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Supplemental Experimental Procedures

Mice. RosaYFP (Srinivas et al., 2001) mice were obtained from the Jackson Laboratory. TetOCre (Perl et al., 2002) mice were provided by A. Nagy. TetOH2B-GFP (Tumbar et al., 2004) mice were provided by E. Fuchs. Mice colonies were maintained in a certified animal facility in accordance with European guidelines. These experiments were approved by the local ethical committee (CEBEA).

Generation of ER-rtTA mice. The rtTA fragment from pTetON Advanced plasmid preceded by the β-globin intron and followed by a SV40 polyA signal was subcloned into pBluescript II SK+. The 4-kb sequence upstream the ATG codon of the murine Esr1 gene, obtained from the BAC clone RP24-222G13 (BACPAC Resources Center, Children's Hospital Oakland Research Institute) using the forward primer 5'-ATGTTGGCTTATGGTTTGAATGGG-3' and the reverse primer 5'-TGCAGAGACTCAGAAGCAAAGAGC-3', was cloned upstream of the β-globin intron. The resulting ER-rtTA fragment of 5.6 kb was released from the backbone by NotI digestion and was microinjected into fertilized oocytes to generate transgenic mice (in the transgenic facility of the Université catholique de Louvain, Brussels, Belgium). 4 transgenic founders were first identified by PCR, out of 34 mice born. Expression profiles of the ER-rtTA founders were screened with reporter TetOH2B-GFP mice. 1 founder expressed the GFP in cells expressing the endogenous ER, and was used throughout this study.

Targeting H2B-GFP expression. ER-rtTA/TetOH2B-GFP adult female mice were induced by oral administration of doxycycline food diet (1g/kg, BIO-SERV) during 5 days and analyzed at the end of the treatment.

Targeting YFP expression. 4 weeks old ER-rtTA/TetOCre/RosaYFP female mice were induced by oral administration of doxycycline food diet (1g/kg, BIO-SERV)

during 5 days, and analyzed at different time points after induction, as specified in figure legends. For saturation experiments, 4 weeks old ER-rtTA/TetOCre/RosaYFP female mice were induced by oral administration of doxycycline food diet (1g/kg, BIO-SERV) combined with doxycycline diluted in drinking water (2g/l, AG Scientific) and 3 intraperitoneal injections per week (200µl of 10mg/ml doxycycline diluted in PBS) during 28 days.

Histology and immunostaining. Dissected MGs were pre-fixed for 2h in 4% paraformaldehyde at room temperature. Tissues were washed three times with PBS for 5 min and incubated overnight in 30% sucrose in PBS at 4°C. Tissues were embedded in OCT compound (Tissue Tek) and kept at -80°C. 5 µm sections were cut using a HM560 Microm cryostat (Mikron Instrument).

Sections were incubated in blocking buffer (5% horse serum/ 1% bovine serum albumin/ 0,2% Triton in PBS) for 1h at room temperature. Primary antibodies were incubated overnight at 4°C. Sections were rinsed three times for 5 min in PBS and incubated with secondary antibodies in blocking buffer for 1 hour at room temperature. Nuclei were stained With Hoechst 33342 dye (Sigma) and slides were mounted in mounting medium (DAKO) supplemented with 2,5% Dabco (Sigma).

For figures 3D, 3E, 4B and 4G, thick sections of 100 µm were cut and staining was performed as for the 5 µm sections, except that the secondary antibodies were incubated overnight at 4°C instead of 1 hour at room temperature.

The following primary antibodies were used: Anti-ERα (Rabbit, 1/500, sc-542, Santa Cruz Biotechnology), Anti-K8 TROMA-1 (Rat, 1/1000, Developmental Studies Hybridoma Bank), Anti K14 (Chicken, 1/1000, PRB-155P-0100, Covance), Anti-GFP (Chicken 1/2000, ab13970, Abcam), Anti-GFP (Goat 1/1000, ab6673, Abcam), Anti-PR (Rabbit 1/200, MA5-14505, ThermoFisher Scientific).

The following secondary antibodies were used: Anti-Rat RRX-conjugated (1/400, Jackson), Anti-Rat Cy5-conjugated (1/400, Jackson), Anti-Rat AlexaFluor 488-conjugated (1/400, LifeTechnology), Anti-Rabbit RRX-conjugated (1/400, Jackson), Anti-Rabbit AlexaFluor 488-conjugated (1/400, LifeTechnology), Anti-Chicken Cy5-conjugated (1/400, Jackson), Anti-Chicken AlexaFluor 488-conjugated (1/400, LifeTechnology), Anti-Goat AlexaFluor 488-conjugated (1/400, LifeTechnology).

Microscope image acquisition. Unless otherwise stated, images were acquired at room temperature using a Zeiss LSM780 multiphoton confocal microscope fitted on an Axiovert M200 inverted microscope equipped with C-Apochromat (X40 = 1.2 numerical aperture) water immersion objectives (Carl Zeiss). Optical sections of minimum 1024X1024 pixels were collected sequentially for each fluorochrome. The data sets generated were merged and displayed with the ZEN software. Images from Images from figures 4 B, C, H, I were acquired with EC-Plan Neofluoar 10X/0.30 M27 objective. Image from Figure 2B was acquired on an Axio Observer Z1 Microscope using X40 Zeiss EC Plan-NEOFLUAR objectives, with an AxioCamMR3 camera and using the Axiovision software (Carl Zeiss).

Mammary cell preparation. MGs were dissected and the lymph nodes removed before processing. Samples were washed in HBSS and cut in pieces of 1 mm^3 with scissors. Samples were digested for 2h at 37°C under shaking in 300U/ML collagenase (Sigma)/ 300 µg/ml hyaluronidase (Sigma) in HBSS. EDTA at a final concentration of 5 mM was added for 10 min to the resultant organoid suspension, followed by 0.25% Trypsin/EGTA for 2 min. Samples were then filtrated through 40-µm mesh and rinsed in 2% FBS/PBS.

Cell labelling and flow cytometry. All steps of cell labelling were performed in PBS supplemented with 2% bovine serum. Two million cells per condition were incubated

in 500 µl primary antibody dilution for 30 min on ice, with shaking every 10 min. Primary antibodies were washed and cells incubated with secondary antibodies, with shaking every 10 min. Secondary antibodies were washed and cells were resuspended in 2.5 µg/ml DAPI (Invitrogen) before analysis.

Primary antibodies used were: APC-conjugated anti-CD45 (1/100, clone 30-F11, 17-0451, eBiosciences), APC-conjugated anti-CD31 (1/100, clone 390, 17-0311, eBiosciences), APC-conjugated anti-CD104a (1/100, clone APA5, 17-1401, eBiosciences), PECy7-conjugated anti-CD24 (1/100, clone M1/69, 560535, BD Biosciences), AlexaFluor700-conjugated anti-CD29 (1/100, clone HMβ1-1, 102218, Biolegend), PE-conjugated anti-CD49b (1/100, clone DX5, 553858, BD Biosciences), PerCP/Cy5.5-conjugated anti-Sca1 (1/100, clone D7, 108124, Biolegend), Biotin-conjugated anti-CD133 (1/100, clone 13A4, 13-1331, eBiosciences). Secondary antibody used was: APC-Cy7-conjugated streptavidin (1/400, 554063, BD Biosciences).

For BrdU staining, cells suspension from 4w and 10w old CD1 mice injected with 50mg/kg 5-Bromo-2-deoxyuridine (Sigma, B5002) 8h prior to analysis were stained as described above followed by BrdU staining using BD BrdU Flow kit (BD Biosciences 552598) using manufacturer’s instruction except that anti-BrdU FITC (1/50, BD Biosciences 347583) was used instead of the one provided in the kit.

Data analysis was performed on a FACS Fortessa using the FACS DiVa software (BD Biosciences).

Mammary fat pad transplantation and analysis. One 1mm^3 non digested MG fragment coated with matrigel or 100000 unsorted mammary cells resuspended in 10 µl 75% DMEM/ 25% matrigel were injected into the number 4 glands of 4w old NodScid female mice that had been cleared of endogenous epithelium. Recipient mice

were mated 4 weeks after the transplantation, and were killed 10 days later, at mid-pregnancy. Recipient glands were dissected, fixed and embedded in OCT for analysis by immunofluorescence. An outgrowth was defined as an epithelial structure comprising ducts and branchings.

Quantification of ER+ LCs

For quantification of proportion of ER+ cells within LCs in figure 1B, 3 mice per time point were analyzed and a total of 213, 349, 364, 1497 and 515 LCs were analyzed respectively for P7, 5w, 8w, pregn and invo.

Quantification of YFP+ in ER+ LCs

For quantification of proportion of YFP+ cells within ER+ LCs described in figure 2N, 4 mice were analyzed per time point and a total of respectively 2335 and 2485 ER+K8+ cells were analyzed.

Quantification of YFP+ in CD24+CD29low LCs

For quantification of YFP+ cells in CD24+CD29low population described in figure 2G and 2S, respectively 3, 4, 3 and 4 mice were analyzed for no, 3d, 4w and 10w time points and minimum 100000 CD24+CD29low cells were analyzed per sample.

For quantification of YFP+ cells in CD24+CD29low population described in figure 3J, respectively 3, 4, 4 and 4 mice were analyzed for 4w, pregn, invo and 2nd invo time points and minimum 100000 CD24+CD29low cells were analyzed per sample.

Quantification of BrdU+ in LCs

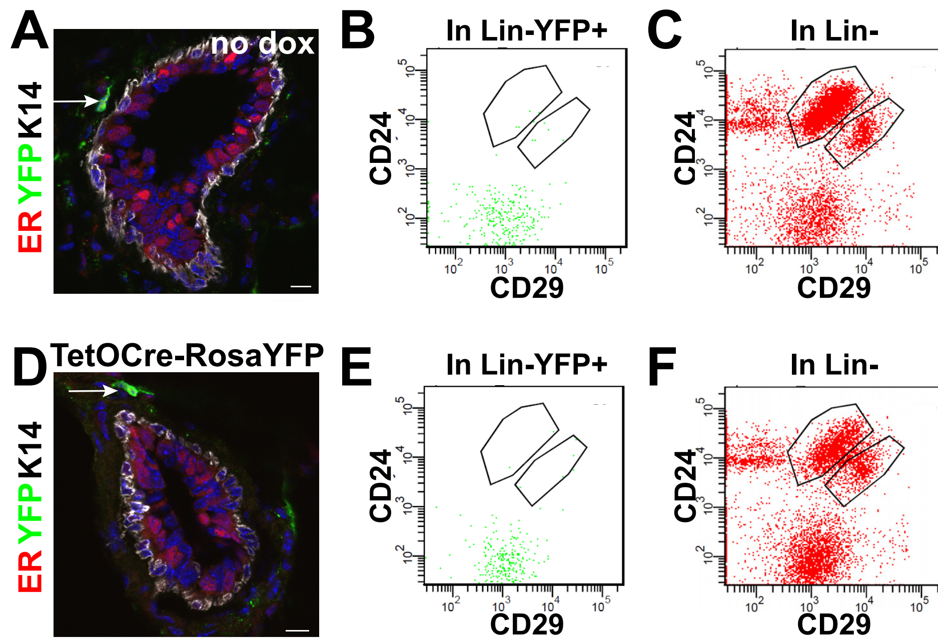
Quantification of BrdU incorporation shown in figure 1C was performed in respectively 5 and 4 mice for 4w and 10w time points and a minimum of 10000 LCs were analyzed per sample.

Quantification of absolute number of LCs

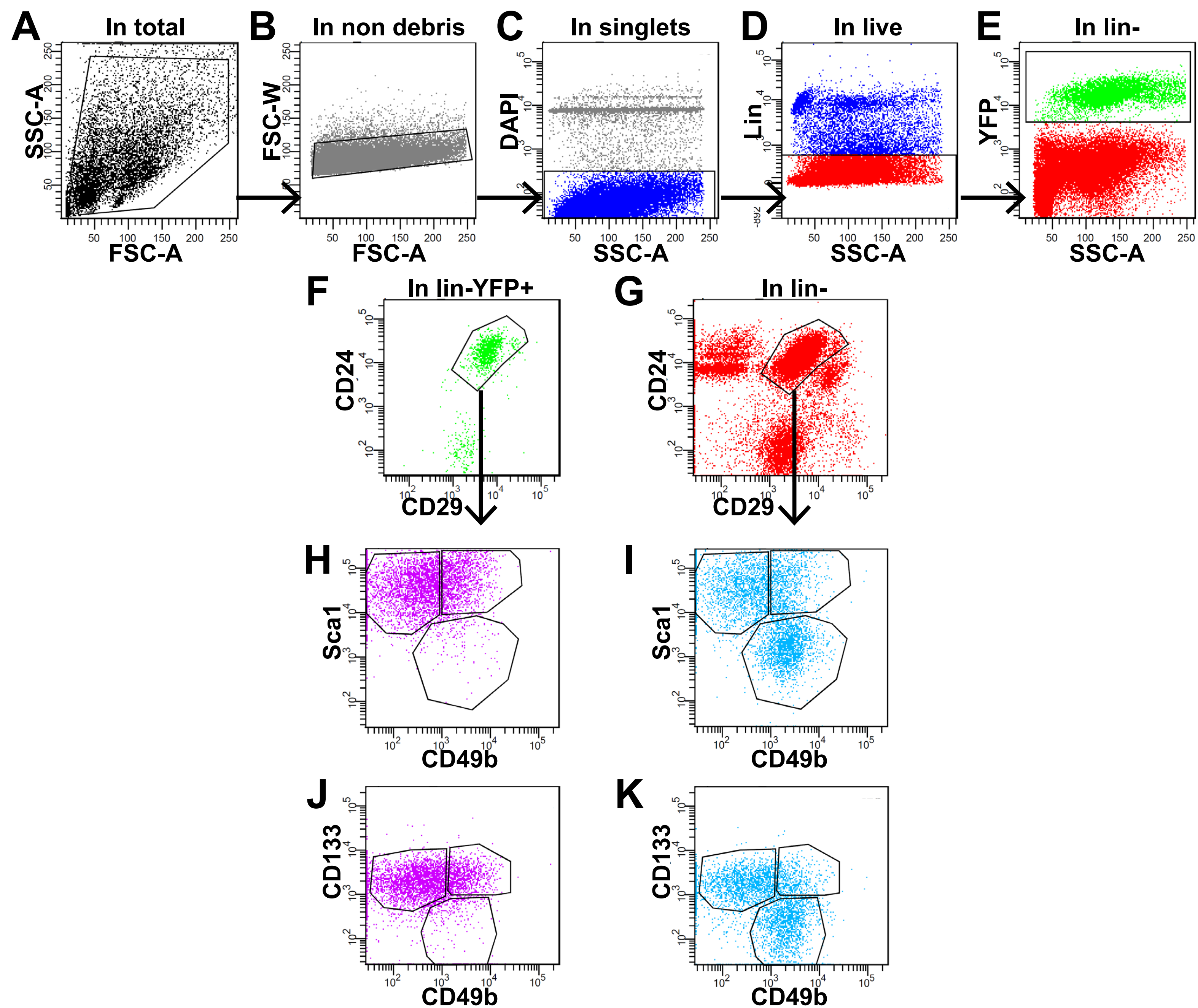
For assessing the absolute number of LCs, total number of cells in samples was counted after cell preparation using Neubauer Improved cell counter (Blau Brand). FACS staining was performed as described above, and fraction of the different populations compared to total cells was analyzed based on FACS analysis. Absolute number of LCs was calculated by multiplying the total number of cells counted by the fraction of the LCs compared to total cells on FACS analysis.

For quantification of number of CD24+CD29low cells at 4w and 10w described in Figure 2H, 2 inguinal glands (one #4 and one #5 glands) from female CD1 mice were processed per sample. 10 mice were analyzed per time point.

For quantification of number of CD24+CD29low and YFP+ CD24+CD29low cells in adult and pregnant mice, 3 thoracic glands (one #1, one #2 and one #3) from female 10w old virgin or 2w pregnant ERrtTA/TetOCRE/Rosa-YFP mice were processed. Respectively 6 and 4 mice were processed in adult and pregnancy.



Supplemental Figure S1. Leakiness of TetOCre transgene in mesenchyme cells of the MG. Related to Figure 2. A-C, 5 weeks old ER-rtTA/TetOCre/RosaYFP mice without Dox treatment showing labelling of mesenchymal cells in the MG. A, Immunostaining of ER (red), YFP (green), K14 (white) and nuclei (blue). Arrow points to a YFP+ cell in the mesenchyme. **B,** C. FACS analysis of CD24 and CD29 in YFP+ cells **(**B) and in Lin- cells **(**C) show that YFP+ CD24- are observed without Dox treatment. **D-**F, 5 weeks old TetOCre/RosaYFP mice without Dox treatment also showing labelling of mesenchymal cells in the MG, demonstrating that the leakiness is due to TetOCre and not ER-rtTA transgene. D, Immunostaining of ER (red), YFP (green), K14 (white) and nuclei (blue). Arrow points to a YFP+ cell in the mesenchyme. **E,** F. FACS analysis of CD24 and CD29 in YFP+ cells **(**E) and in Lin- cells **(**F) show that YFP+ CD24- are observed in TetOCre/RosaYFP mice



Supplemental Figure S2. FACS analysis of Sca1, CD133 and CD49b expression in LCs. Related to Figure 2. A-E, Unicellular suspension of mammary cells from ER-rtTA/TetOCre/RosaYFP mice (in this example, induced at puberty and analyzed 3d later) stained for Lin (CD31, CD45, CD140a), CD24, CD29, Sca1, CD133 and CD49b were gated as shown in **A** to eliminate debris, doublets were discarded with gate shown in **B**, the living cells were gated by DAPI dye exclusion as shown in **C**, the non-epithelial Lin positive cells were discarded in **D**, and the YFP+ cells were gated as shown in **E**. **F, G**, CD24 and CD29 expression was studied in YFP+ cells (**F**) or in Lin- cells (**G**) to define the CD24+CD29low luminal population. **H, I**, Sca1and CD49b expression was studied in luminal YFP+ cells (**H**) and in total luminal cells (**I**). **J, K**, CD133 and CD49b expression was studied in luminal YFP+ cells (**J**) and in total luminal cells (**K**).