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Supplemental Information

s-SHIP Promoter Expression Identifies Mouse Mammary Cancer Stem Cells

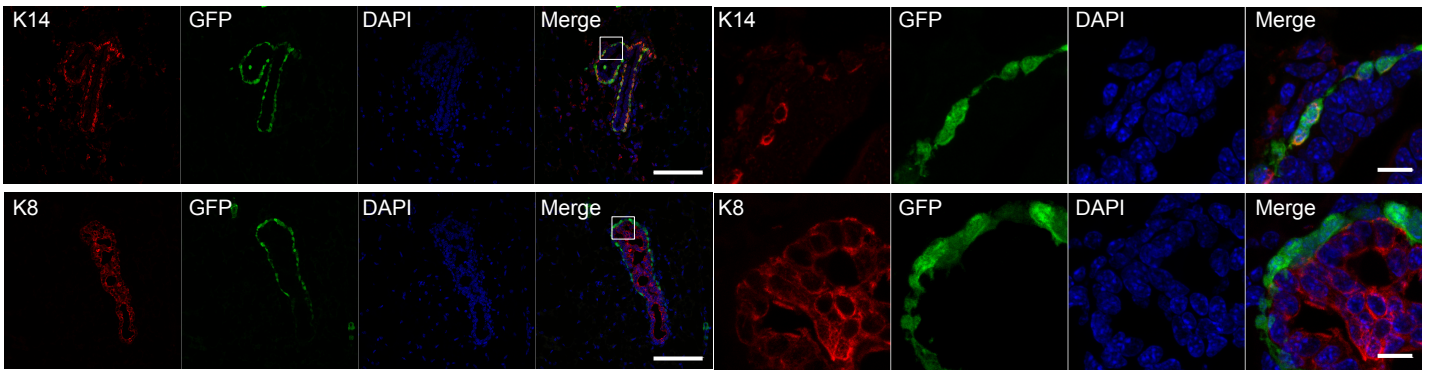
Lu Tian, Marie-José Truong, Chann Lagadec, Eric Adriaenssens, Emmanuel Bouchaert, Hélène Bauderlique-Le Roy, Martin Figeac, Xuefen Le Bourhis, and Roland P. Bourette

Figure S1

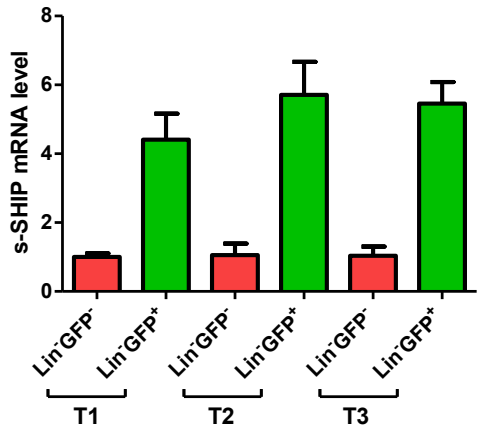
A

N° Animal	Type	Pattern	Infiltration	Stroma	Necrosis	Mitotic index	Peritumoral emboli
688	Carcinome	solid papillary with tubular carcinoma	2	1	3	150	2
796	Carcinome	solid papillary with tubular carcinoma	2	1	2	40	0
877	Carcinome	solid papillary with tubular carcinoma	3	1	1	104	3
921	Carcinome	solid papillary with tubular carcinoma	1	2	2	161	0
687	Carcinome	solid papillary with tubular carcinoma	3	2	2	86	1
766	Carcinome	tubulopapillary features	1	1	3	20	0
951	Carcinome	tubulopapillary features	2	1	2	77	2
978	Carcinome	tubulopapillary features	2	1	2	45	3
765	Carcinome	solide epithelial	1	1	1	81	3
808	Carcinome	solide epithelial	3	2	1	138	2
673	Carcinome	mixture of epithelial and spindle morphology	1	1	3	158	1
885	Carcinome	mixture of epithelial and spindle morphology	1	2	1	77	1
959	Carcinome	mixture of epithelial and spindle morphology	2	3	0	116	1
977	Carcinome	mixture of epithelial and spindle morphology	1	3	3	38	3

B



C



D

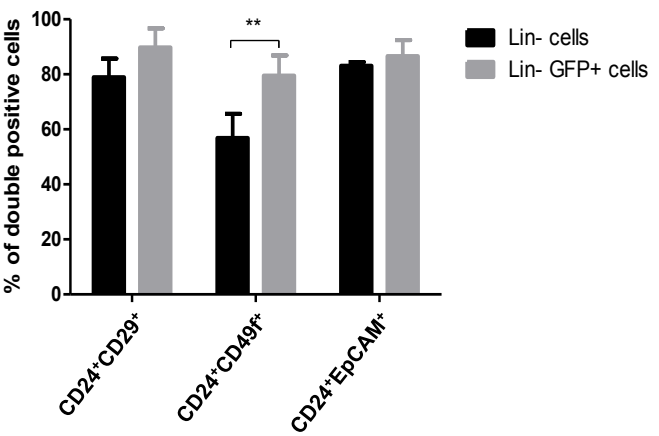
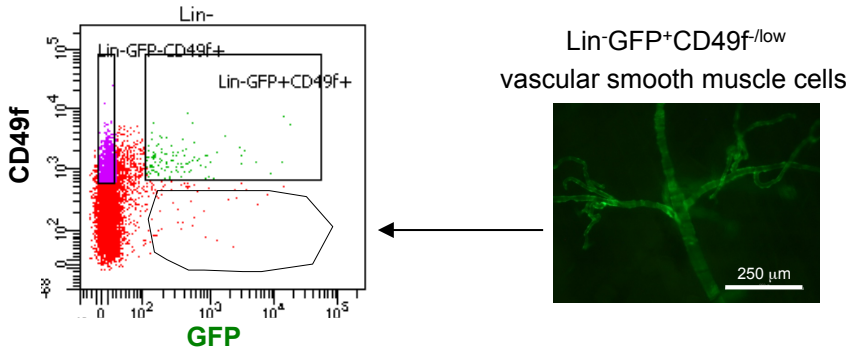


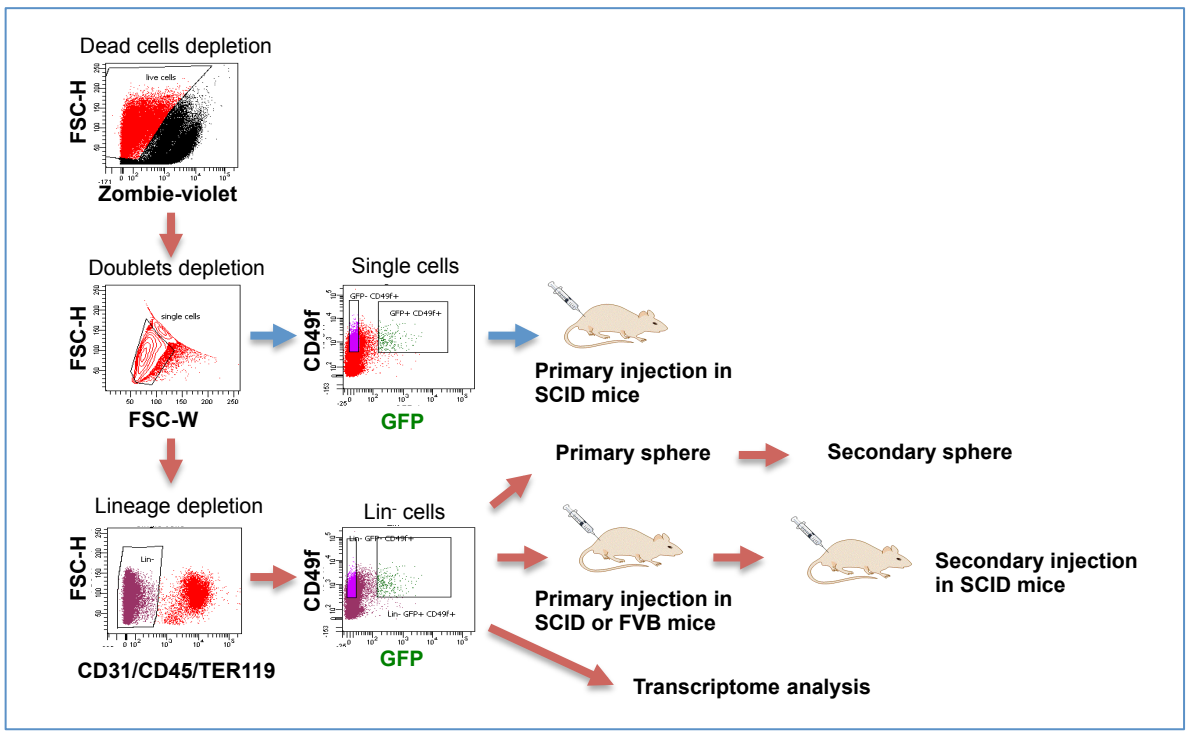
Figure S1. Analysis of mammary tumors from s-SHIP-GFP/C3(1)-Tag bi-transgenic mice. Related to Figure 1.
(**A**) Anatomopathological analysis of fourteen tumors isolated from 4 to 6-month-old bi-transgenic (biTg) mice.
(**B**) Representative photographs (n=3) of immunofluorescent staining for cytokeratin 14 (K14, upper panels), cytokeratin 8 (K8, lower panels) (red) and with DAPI nuclear stain (blue) of 7-week-old mammary gland of biTg mice containing GFP^+ cells (green) (scale bar = 100µm left panels and 10 µm right panels). (**C**) Expression level of endogenous s-SHIP mRNA was assessed from Lin^-GFP^- and Lin^-GFP^+ cell populations isolated from three different tumors (T1 to T3) using RT-qPCR.
(**D**) Flow cytometry analysis of CD24, CD29, CD49f and EpCAM cell surface marker expression on Lin^- cells and Lin^-GFP^+ cells. Data represent mean values ± SEM of three different tumors. *p* values were determined by Student's t-test $^{**}p < 0.01$.

Figure S2

A



B



C

Primary injection in SCID mice without lineage depletion

	5000 cells	1000 cells	500 cells	100 cells	Tumor initiating cell frequency (95% CI)
CD49f$^+$ GFP$^-$	5/6	2/5	2/5	1/14	**1/2001** (1/4184 - 1/957)
CD49f$^+$ GFP$^+$	6/6	5/5	4/5	10/14	**1/129** (1/249 - 1/67)

P=2.25^{e-09}

D

Primary injection in FVB mice

	1000 cells	100 cells	Tumor initiating cell frequency (95% CI)
Lin$^-$ CD49f$^+$ GFP$^-$	1/11	1/14	**1/5918** (1/24165 - 1/1449)
Lin$^-$ CD49f$^+$ GFP$^+$	3/11	6/14	**1/1154** (1/2400 - 1/554)

P=0.0166

Figure S2. Experimental design for cell sorting and analysis of tumorigenic potential of s-SHIP/GFP$^+$ cells. Related to Figures 2, 3 and 4.

(**A**) Flow cytometry analysis showed a minor population of Lin$^-$ GFP$^+$ CD49$^{low/-}$ (left panel) corresponding to vascular smooth muscle cells (right panel) as previously described (Bai and Rohrschneider, 2010). Scale bar: 250 µm. (**B**) Cell sorting strategy and experimental design. FACS plot shows gates drawn for cell sorting of either CD49f$^+$GFP$^+$ and CD49$^+$GFP$^-$ cells, or Lin$^-$CD49f$^+$GFP$^+$ and Lin$^-$CD49f$^+$GFP$^-$ cells. (**C-E**) The collected cell populations were injected into recipient female mice in a limiting dilution manner. Mice were monitored until tumors were observed or up to 7 months if no tumors were detected. Tumor-initiating cell frequencies were generated by ELDA: extreme limiting dilution analysis. (**C**) Primary injection in immunodeficient SCID mice. (**D**) Primary injection in immunocompetent FVB mice.

Figure S3

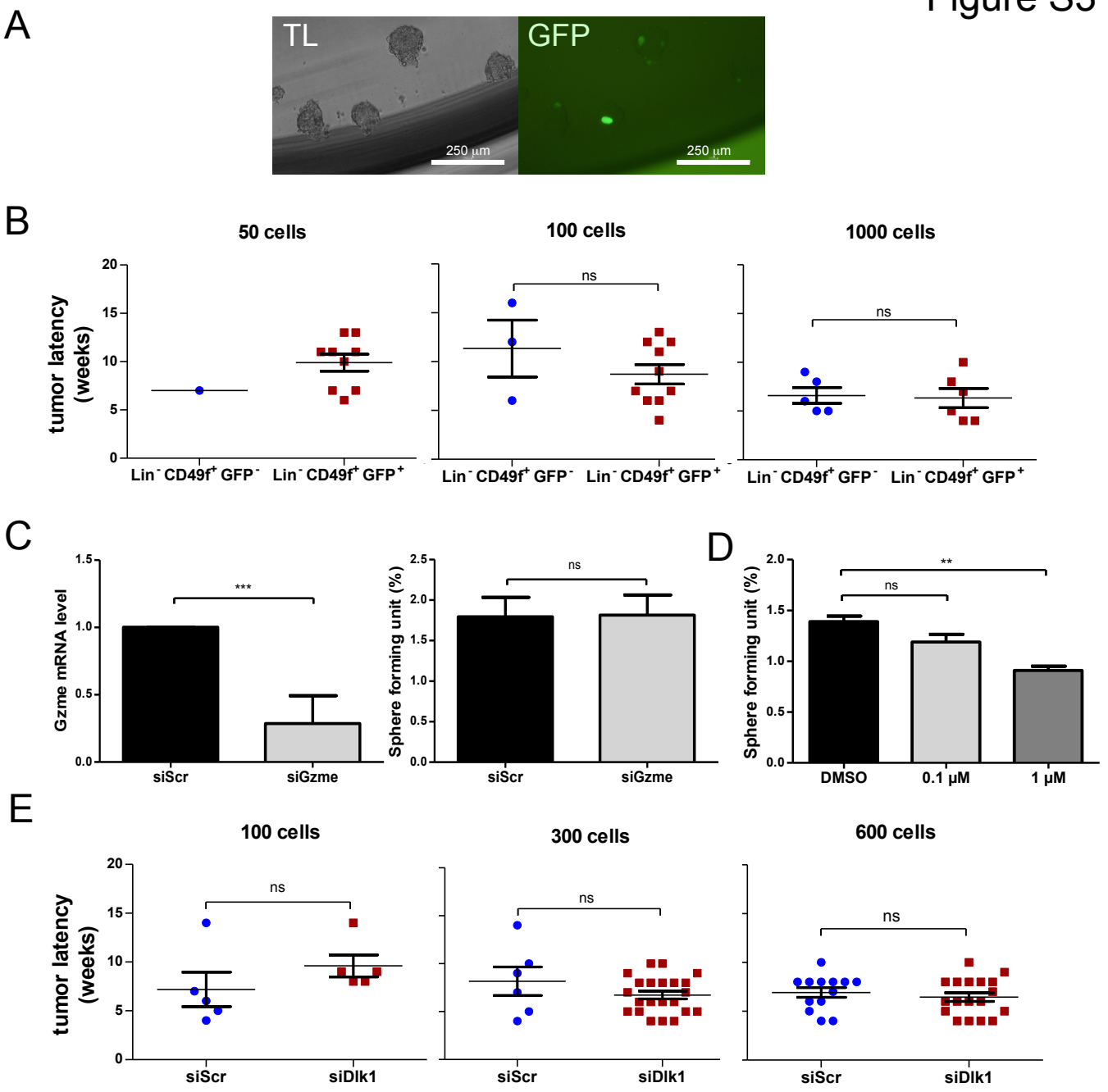


Figure S3. (A) Long-term maintenance of sphere-forming potential of s-SHIP/GFP$^+$ tumor cells (related to Figure 2), (B) tumor latency of primary injection of Lin$^-$CD49f$^+$GFP$^+$ cells in SCID mice (related to Figure 3A), (C) Inhibition of *Gzme* expression did not modify the sphere-forming potential of Lin$^-$CD49f$^+$GFP$^+$ tumor cells (related to Figure 4), (D) Notch signaling inhibition decreased sphere-forming potential of Lin$^-$CD49f$^+$GFP$^+$ cells (related to Figure 4), (E) Tumor latency of siScr- (control) and si*Dlk1*- treated Lin$^-$CD49f$^+$GFP$^-$ cells injected in SCID mice (related to Figure 4F).

(A) Spheres derived from GFP$^+$ cells can be maintained through at least four passages and GFP$^+$ cells persisted during sphere passaging (n=3 independent experiments). Representative photographs of spheres from the 4th passage was shown. Scale bar: 250 µm. **(B)** Tumor latency of primary injection in SCID mice; mice were monitored twice a week and tumor graft latency was measured as the time between implantation and the development of a palpable tumor. (**C**) si*Gzme* or control siScramble (siScr) were introduced into Lin$^-$CD49f$^+$GFP$^+$ cells isolated from bi-Tg mammary tumors. 48h after transfection, the level of *Gzme* mRNA was determined by RT-qPCR (left panel). siScr or si*Gzme* transfected Lin$^-$CD49f$^+$GFP$^+$ cells were plated under spheres conditions and spheres were numbered after 7 to 10 days in culture (right panel). Data represent mean values ± SEM of six independent experiments. (**D**) Lin$^-$CD49f$^+$GFP$^+$ cells were plated under spheres conditions in the presence of Compound E (0.1 µM or 1 µM) or carrier (DMSO). Data represent mean values ± SEM of four independent experiments. **(E)** Tumor latency of si*Dlk1*- or siScr-Lin$^-$CD49f$^+$GFP$^-$ cells injected in SCID mice; mice were monitored twice a week and tumor graft latency was measured as the time between implantation and the development of a palpable tumor. (**B-E**) *p* values were determined by Student's test ***$p<0.001$ **$p<0.01$ ns= not significant.

Supplemental Experimental Procedures

Mouse genotyping

FVB-Tg(C3-1-Tag)cJeg/JegJ mice were from Charles River Laboratories (L'Arbresle, France). FVB/N transgenic 11.5kb-GFP mice have been described previously (Rohrschneider et al, 2005, Brocqueville et al, 2010). FVB/N wild-type mice were from Janvier Labs (France) and CB17 SCID mice were from Institut Pasteur de Lille (France). Animals were housed and bred in accordance with institution guidelines for humane animal treatment. For genotyping of bi-transgenic mice, tail clips were digested in lysis buffer (KAPA Biosystems). Samples were diluted 1:10 and employed for PCR reactions to amplify T antigen cDNA using the primers TA1: 5'-GACCTGTGGCTGAGTTTGCTCA-3' and TA2: 5'-GCTTTATTTGTAACCATTATAAG-3'. Products of the amplification were analyzed by agarose gel electrophoresis.

Histology

Mammary tumors were fixed in 4% formaldehyde (ThermoFisher) at 4°C overnight, dehydrated in ethanol, and cleared in toluene. Dehydrated tumors were embedded in paraffin and cut into 4 µm sections. Tissue sections were dewaxed, rehydrated and stained with Hematoxylin and Eosin (Sigma). After staining, tissue sections were dehydrated and mounted with Eukitt® Quick-hardening mounting medium (Sigma). Images were acquired using bright field microscopy (Nikon).

Isolation of mammary tumor cells

Tumors were harvested from 4 to 6-month-old female bi-transgenic mice and minced using razor blades and digested using Mouse Tumor Dissociation Kit (Miltenyi Biotec) according to the manufacturer's instructions. After digestion, cells were filtered through 40µm cell strainers and washed once with washing buffer (DMEM medium) (Gibco). Then, cells were suspended in Red Blood Cell Lysis Solution (Miltenyi Biotec) in order to eliminate red blood cells. Cells were washed with PBS buffer (no Tris buffer and protein free), and were suspended in diluted Zombie Violet® solution (1/100, Zombie Violet™ Fixable Viability Kit, BioLegend) and incubated at room temperature, in the dark, for 15 minutes. Cells were washed once with complete medium (DMEM medium supplemented with 10% of bovine fetal serum (FBS) and 1X ZellShield ® (Minerva Biolabs). Then, cells were resuspended in complete medium before labeling with antibodies.

Flow cytometry, antibodies, and cell sorting

Single cells were preincubated with rat anti-mouse CD16/CD32 antibody (1:50; eBioscience #14-0161-85) for 10 min at 4°C, stained with antibodies for 30 min at 4°C with agitation, washed and resuspended in complete medium before analysis. Antibodies used were as follows: APC rat anti-human/mouse CD49f (1:100; eBioscience #17-0495-82), PE-Cy7 rat anti-mouse CD24 (1:100; Biolegend #101822), APC Armenian hamster anti-mouse CD29 (1:100; eBioscience #17-0291-82), Alexa Fluor 647 rat anti-mouse CD326 (EpCAM) (1:100; BioLegend #118212); and lineage markers PE rat anti-mouse CD31 (1:100; eBioscience #12-0311-83), PE rat anti-mouse CD45 (1:100; eBioscience # 12-0451-83), PE rat anti-mouse TER-119 (1:100; eBioscience #12-5921-83). The stained specimens were then analyzed or sorted using FACSAria flow cytometer (BD Biosciences). For data analysis, the horizontal and vertical lines of the gates were set using isotype and unstained controls. To define the GFP gate, tumor cells that do not express GFP were obtained from mammary tumors of 5-month-old FVB C3(1)Tag mice. Selection criteria that included side scatter and forward scatter profiles, depletion of zombie violet-positive cells and depletion of Lin-positive cells were used. Cells with appropriate GFP and CD49f status were then collected. After sorting, cells were washed with PBS and counted with trypan blue to exclude dead cells and determine cell numbers.

Tumor injection

SCID and FVB/N female mice (6–8 weeks of age) were sedated via inhalation of isoflurane (Baxter SAS, France). Sorted cells, transfected or not with siRNA, were suspended in 50µl of PBS, which was then mixed with 50µl of Growth Factor Reduced Matrigel (354230; BD Biosciences). The cell mixture was then injected subcutaneously in the region next to the nipple of the third thoracic gland of the mice using a 29G x 12.7 mm needle. Mice were observed weekly for 1–7 months for tumor formation. All tumorigenic cell frequencies were calculated using ELDA: Extreme Limiting Dilution Analysis (Hu and Smyth, 2009).

Mammosphere-forming assay

Single cell suspensions were plated at 25,000 cells/ml in serum-free DMEM/F12 medium, supplemented with 2% B27 (Invitrogen), 20 ng/ml FGF (Peprotech), 20 ng/ml EGF (Peprotech), 4 µg/ml heparin (Sigma), 5 µg/ml

insulin (Sigma) and 0.5μg/ml hydrocortisone (Sigma) for 7-14 days on 96-well ultra-low attachment plate (Corning). Cells were fed with fresh medium every three days and passaged using Trypsin/EDTA (Lonza). E-compound (Stem Cell Technologies) was dissolved in DMSO. Numbers and sizes of the mammospheres were determined under phase contrast microscopy. Only spheres that were larger than 50 μm were numbered. Sphere-forming potential was evaluated as follow: sphere-forming unit (SFU) = spheres/input cells x 100. Fluorescence microscopy was used to detect GFP-positive cells. Pictures were taken on a Zeiss Axio-Observer Z1 microscope.

RNA isolation and quantitative RT–PCR

Total RNA was extracted using the RNeasy Micro Kit (Qiagen). Reverse transcription (RT) was performed using QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. Diluted cDNAs were transferred to 96-well PCR optical plates (Axygen). KAPA SYBR FAST qPCR kit (Kapa Biosystems) was used. qRT-PCR was performed using the Agilent Mx3000P detection system (Agilent Technologies). Relative mRNA levels were determined following normalization to the housekeeping genes: Actin, HPRT, Rpl38, and analysis of the comparative threshold cycle (2-ΔΔCt) method. Primer sequences were as follows: s-SHIP forward, 5'-GTTCCCACTAGTTGTTGAACTTTACCTT-3'; s-SHIP reverse, 5'-CAACGTCCACTTTGAGATGCAT-3'; GFP forward, 5'- AAGGCTACGTCCAGGAGCGCA -3'; GFP reverse, 5'- TGCCGTCCTCGATGTTGTGGC -3'; Actin forward, 5'- GATCTGGCACCACACCTTCT -3'; Actin reverse, 5'- GGGGTGTTGAAGGTCTCAAA -3'; HPRT forward, 5'- GAGAGCGTTGGGCTTACCTC -3'; HPRT reverse, 5'- ATCGCTAATCACGACGCTGG -3'; RPL38 forward, 5'- GGTTCTCATCGCTGTGCGG – 3'; RPL38 reverse, 5'- TGACAGACTTGGCATCCTTCC – 3'. Dlk1 forward 5'-GAAATAGACGTTCGGGCTTG-3'; Dlk1 reverse 5'-AGGGAGAACCATTGATCACG-3' ; Gzme forward 5'-CCACAACATCAAGGCTAAGG-3' Gzme reverse 5'- GCATGATGTCACTGAAGAAG –3'

Immunofluorescence and antibodies

Tumors were fixed in 4% paraformaldehyde overnight and put into 30% sucrose overnight, embedded in O.C.T., frozen on dry ice, and cut into 5-μm sections. Tissue sections were permeabilized with 0.5% Triton X-100, blocked with 5% FBS, and then incubated sequentially with primary antibodies overnight at 4°C, and with secondary antibodies for 45 min at room temperature. Slides were washed three times with PBS after each antibody incubation, and sections were mounted in Prolong Gold (Invitrogen). Primary antibodies used for this study were rabbit anti-cytokeratin 14 (1:500; Covance #PRB-155P), rat anti-cytokeratin 8 (1:100; Merk #MABT329, clone TROMA-1), Fluorochrome-conjugated secondary antibodies were Alexa Fluor 594 F(ab')2 fragment of goat anti-rabbit IgG (1:1000; Molecular Probes #A-11072) and Alexa Fluor 594 F(ab')2 fragment of goat anti-rat IgG (1:1000; Molecular Probes #A-11007). Sections were counterstained with DAPI (1 μg/mL; Sigma) to visualize cell nuclei. Pictures were taken on a LSM 710 confocal microscope (objectives Plan apochromat, Carl Zeiss, Germany).

Microarray analysis

Total RNA was prepared using the RNeasy micro kit (Qiagen) according to manufacturer's instructions, including the additional step of DNase treatment. Total RNA yield and quality were further assessed on a NanoDrop ND-1000 and an Agilent 2100 bioanalyzer (Agilent Technologies, Massy, France). One color whole Mouse (074809 slides) 60-mer oligonucleotides 8x60k microarrays (Agilent Technologies) were used to analyze gene expression. cRNA labeling, hybridization, and detection were carried out according to supplier's instructions (Agilent Technologies). For each microarray, Cyanine 3-labeled cRNA was synthesized with the low input QuickAmp labeling kit from 20ng of total RNA. RNA Spike-In was added to all tubes and used as positive controls of labeling and amplification steps. The labeled cRNA was purified and 600 ng of each cRNA were then hybridized and washed following manufacturer's instructions. Microarrays were scanned and data extracted using Agilent Feature Extraction Software© (FE version 10.7.3.1). Statistical comparisons and filtering were achieved with the Genespring® software version GX14.5 (Agilent Technologies). After a 75 percentile normalization of raw data, non-expressed probes in all conditions were removed, followed by a paired t-test with a p-value cut-off at 0.01 and a fold change cut off at +/-2. Further investigations were carried out using Ingenuity Pathway Analysis© Software (Ingenuity® Systems, www.ingenuity.com, Redwood City, CA, USA).

siRNA transfection

For *Dlk1* or *Gzme* knockdown analysis, dissociated and FACS-sorted $Lin^-CD49f^+GFP^-$ or $Lin^-CD49f^+GFP^+$ cells from the bi-transgenic tumors (10^5 cells/ well) were suspended into 24-well ultra-low attachment plates (Corning), and were transfected with ON-TARGET plus Non-targeting Pool as empty control and ON-TARGET plus *Dlk1* siRNA or ON-TARGET plus *Gzme* siRNA (Dharmacon-SMART pool) respectively, by using Lipofectamine RNAiMAX (Life Technology) for 45 min in a final volume of 500 μL serum-free mammosphere medium. The transfected cells were then collected and washed once with mammosphere medium in order to

remove transfection reagent and siRNA. Cells were counted and suspended in mammosphere medium (500 or 2000 cells/well) into 96-well ultra-low attachment plates. The sphere-forming potential was analyzed 7-10 days after culture. Downregulation of *Dlk1* or *Gzme* mRNA was verified by RT-qPCR 2 days and 8 days after transfection.