Supporting Information

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SI Materials and Methods

Generation of Conditional c-Src–KO Mice. As the *src* locus spans more than 60 kbp, precluding the conditional removal of the whole gene, one loxP site was inserted upstream of exon 4, which contains the translational start site, and the second loxP site was inserted upstream of exon 7, according to the National Center for Biotechnology Information entry NM_009271. This would, in the presence of Cre recombinase, efficiently remove exons 4 to 6, which encode the first 121 aa of c-Src. To achieve this, a *src* genomic DNA fragment was isolated from a 129 genomic lambda phage library (Stratagene) by using a dilution PCR protocol with primers located 5′ to exon 4. A 13-kb fragment was cloned, reduced to a 6-kb fragment containing exons 4 to 7, and inserted into pBluscript 3.1. The positive selection cassette, containing a neomycin–IRES–GFP construct flanked by two FRT sites and one loxP site, was inserted in the HindIII site located 5′ of exon 7. The second loxP site was inserted in the AvrII site 5′ of exon 4. R1 embryonic stem cells [gift from Daniel Dufort (McGill University Health Center, McGill University, Montreal, Quebec, Canada)] were electroporated, selected with neomycin, and clonally amplified, and genomic DNA was extracted to identify properly targeted embryonic stem clones by using PCR and Southern blotting. Mice were generated by aggregation and chimeric mice screened for presence of the loxP-flanked allele by PCR. The positive selection cassette was removed by crossing chimeric mice with Actin-Flp–expressing mice.

PCR Strategy for Genotyping and Confirmation of src Gene Excision. To ensure proper gene targeting in vivo, PCR was performed on DNA extracted from tails and mammary glands of mice bearing conditional *src* alleles and expressing MMTV-Cre. The primer pair For5/Rev1 flanks the 3′ FRT-LoxP site. The primer pair For2/Rev1 binds to sequences in the original targeting vector, whereas primer Rev3 is located outside the targeting vector. Both Rev1 and Rev3 can be used with primer For2 to confirm the Cre-mediated recombination event. Genotyping was performed by PCR using the primers For2/Rev2, which flank the 5′ loxP site.

Preparation of Mammary Gland Protein Lysates. Excised mammary glands were finely chopped and dissociated in DMEM containing 2.4 mg/mL collagenase B (Roche), 2.4 mg/mL Dispase II (Roche), and antibiotics for 2 h at 37 °C. The resulting cell suspensions were washed three times by centrifugation and the pellets were resuspended in DMEM containing antibiotics. Five further centrifugal washes were performed by using brief pulses at 350 × *g* to remove stromal contamination. The pellets, which were enriched for mammary epithelial cells, were then lysed and subjected to SDS/PAGE and immunoblotting as described for cultured cells (*Materials and Methods*).

Immunostaining of Tissue Sections. Tissue sections were deparaffinized in three changes of xylenes, rehydrated, and heated in a pressure cooker in 10 mM citrate buffer (pH 6) for 15 min for antigen retrieval. For IHC, endogenous peroxidase activity was inactivated by incubation in 3% hydrogen peroxide/methanol for 20 min. Sections were then blocked in Power Block (Biogenex), incubated with primary antibody overnight at 4 °C, washed three times in PBS solution, and incubated with biotinylated (Vector Elite) or Alexa Fluor 488 and 555 (Invitrogen)-conjugated secondary antibodies for IHC or IF, respectively. After three further washes in PBS solution, IHC staining was visualized using the Vectastain ABC kit (Vector Laboratories) according to the manufacturer's instructions, and sections were then counterstained with hematoxylin, dehydrated, and mounted with Clearmount (Invitrogen). For IF, sections were counterstained with DAPI (Sigma), mounted with Immunomount (Thermo Scientific), and analyzed using a Zeiss LSM 510 confocal microscope. Primary antibodies were Cre recombinase (Covance), cleaved caspase-3 (Cell Signaling), Ki67 (NovoCastra), c-Src (Abgent), Active Src (Invitrogen), c-Yes (R&D Systems), Fyn (Millipore), PyVmT [gift of S. Dilworth (Department of Surgery and Cancer, Imperial College London, London, United Kingdom)], cytokeratin 8 (Fitzgerald), cytokeratin 14 (Covance), E-cadherin (BD Biosciences), and β-casein (Santa Cruz).

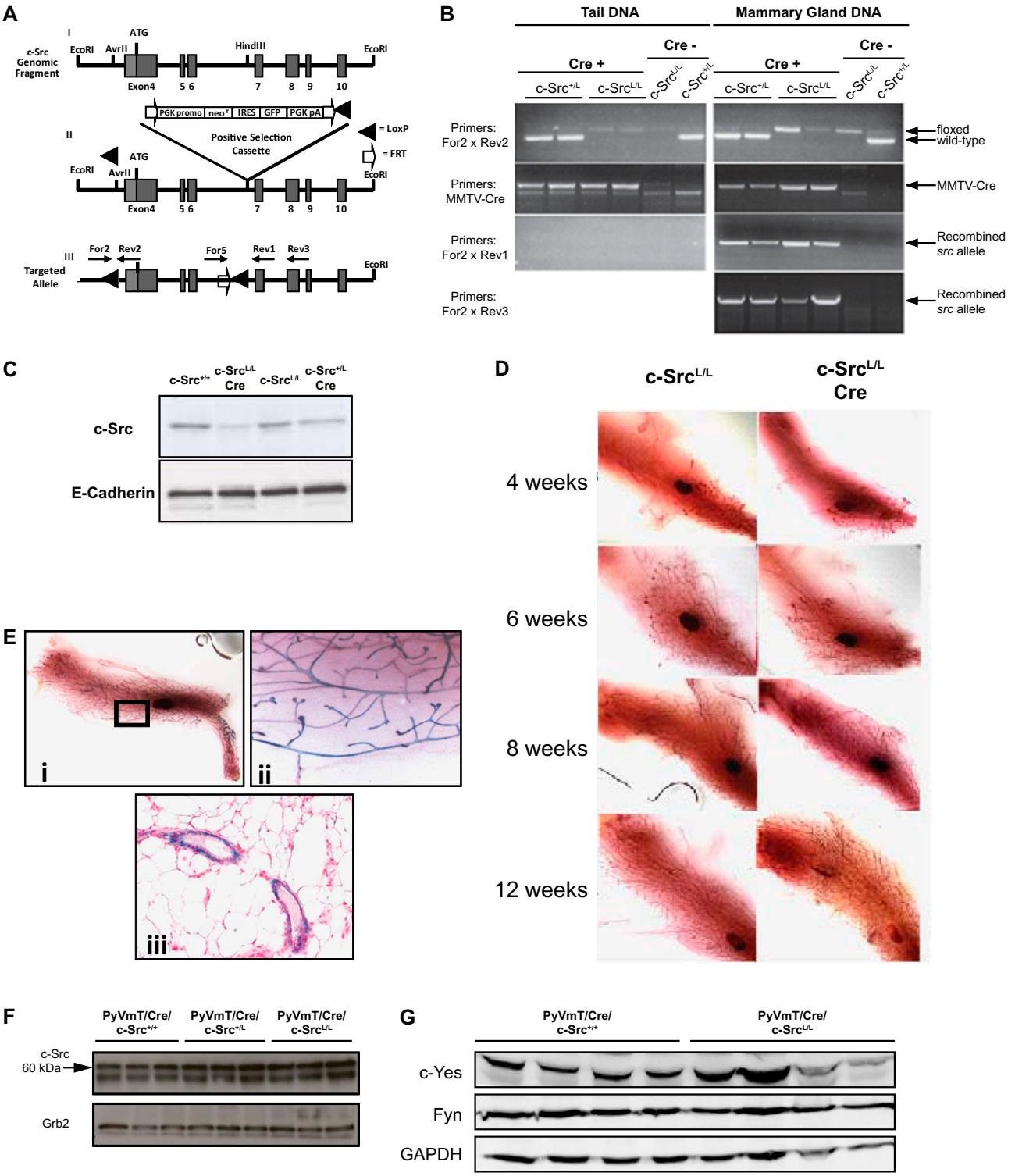


Fig. S1. (*A*) Schematic diagram illustrating the gene targeting strategy used to generate the conditional c-Src–KO mouse. The binding positions of PCR primers used for genotyping and to detect Cre-mediated recombination are indicated. (*B*) DNA was isolated from the tail and mammary gland of PyVmT/Cre/c-Src$^{L/L}$, PyVmT/c-Src$^{L/L}$, PyVmT/Cre/c-Src$^{+/L}$, and PyVmT/c-Src$^{+/L}$ mice. PCR was performed to confirm the genotype of each mouse using primers For2/Rev2 to identify targeted and WT *src* alleles. Specific PCR primers were also used to confirm the presence or absence of MMTV-Cre. To detect the recombination of the targeted alleles by Cre recombinase, PCR primers For2/Rev1 or For2/Rev3 were used. (*C*) Mammary gland protein lysates were prepared from c-Src$^{+/+}$, c-Src$^{L/L}$, c-Src$^{L/L}$/Cre, and c-Src$^{+/L}$/Cre mice and immunoblotted for c-Src to confirm deletion at the protein level, and for E-cadherin to verify that a similar proportion of the protein in each lysate was from epithelial cells. (*D*) Mammary gland whole mounts of WT and c-Src–deleted mice at 4, 6, 8, and 12 wk of age. (*E*) (*i*) Mammary glands from GTRosa26/Cre/c-Src$^{L/L}$ mice were stained in situ to detect β-gal activity and whole-mounted. (*ii*) Higher magnification of the boxed region in *i* shows β-gal–positive (blue) cells in the mammary epithelium. (*iii*) Hematoxylin-stained cross section of a paraffin-embedded, β-gal–stained GTRosa26/Cre/c-Src$^{L/L}$ mammary gland. (*F*) c-Src expression in PyVmT/Cre/c-Src$^{+/+}$, -c-Src$^{+/L}$, and -c-Src$^{L/L}$ end-stage mammary adenocarcinomas was determined by immunoblotting. The 60-kDa c-Src band is indicated by an arrow. Grb2 was used as a loading control. (*G*) Fyn and c-Yes expression in PyVmT/Cre/c-Src$^{+/+}$ and -c-Src$^{L/L}$ end-stage mammary adenocarcinomas was determined by immunoblotting. GAPDH was used as a loading control.

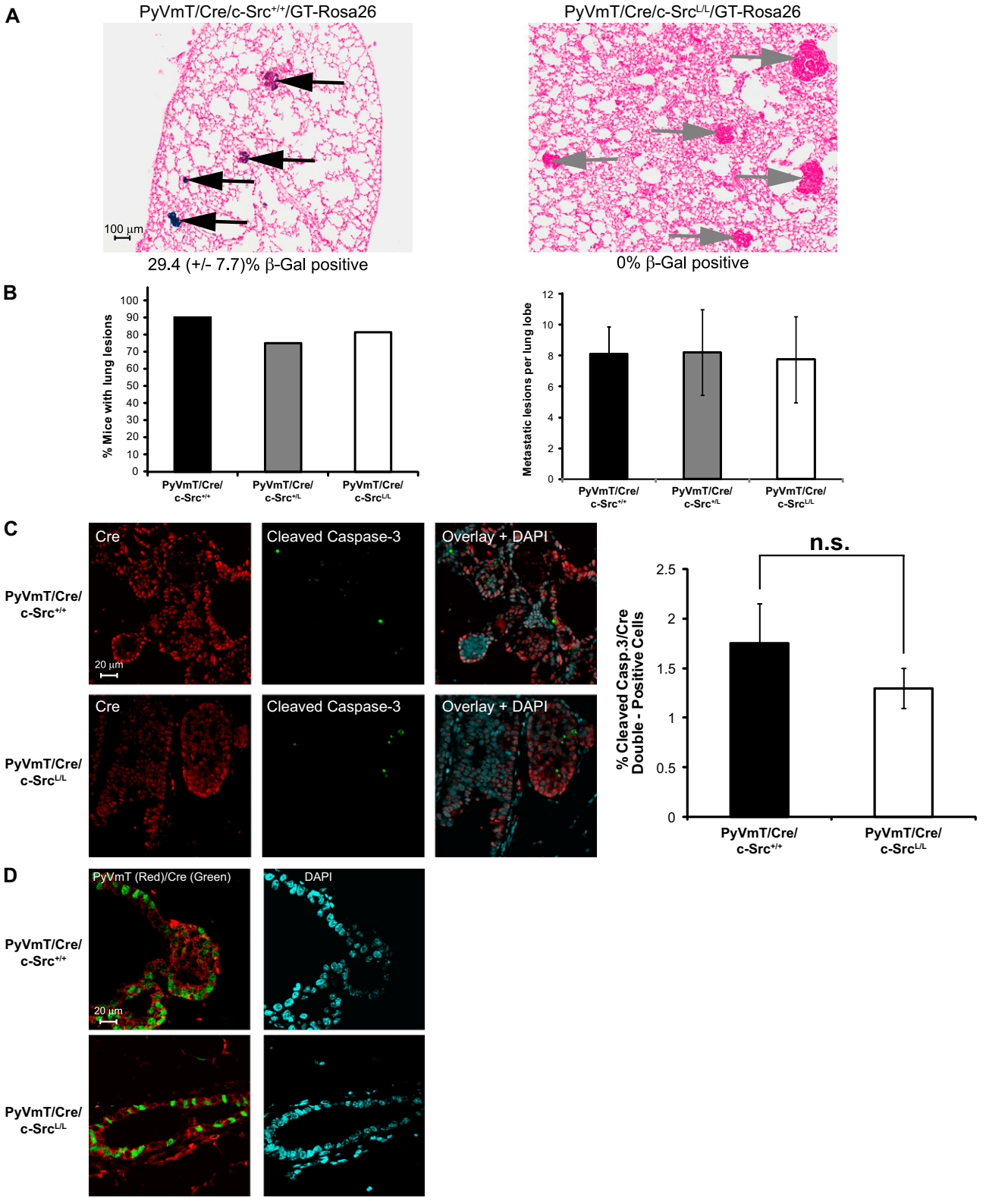


Fig. S2. (*A*) Lungs from PyVmT/Cre/c-Src$^{+/+}$ and PyVmT/Cre/c-Src$^{L/L}$ mice bearing the GTRosa26 Cre reporter gene were stained in situ for β-gal activity and paraffin-embedded sections were examined for lung metastases. Black arrows (*Left*) indicate metastatic lung lesions with positive β-gal staining. Gray arrows (*Right*) indicate lung lesions lacking positive β-gal staining. (Scale bars: 100 μm.) The percentage of β-gal–positive lung metastases in WT (n = 215 lesions, eight mice) and c-Src–conditional (n = 251 metastases, eight mice) strains is indicated under each image. (*B*) Bar graphs indicate the percentage of mice of each genotype with lung metastases (*Left*) and the average number of lesions per lung lobe (*Right*). Error bars represent SEM. No statistically significant differences were observed (unpaired Student t test). (*C*) *Left*: Representative images of IF staining of mammary gland sections from 8-wk-old PyVmT/Cre/c-Src$^{+/+}$ (*Upper*, n = 5) and PyVmT/Cre/c-Src$^{L/L}$ (*Lower*, n = 10) mice to detect expression of Cre and cleaved caspase 3, a marker of apoptosis. (Scale bar: 20 μm.) *Right*: Quantification of Cre/cleaved caspase-3 double-positive cells (n.s., not significant at $P > 0.05$, unpaired Student t test). (*D*) Expression of PyVmT and Cre was examined by IF staining of mammary gland sections from 8-wk-old PyVmT/Cre/c-Src$^{+/+}$ and PyVmT/Cre/c-Src$^{L/L}$ mice. Images are representative of mammary glands from three mice per genotype. (Scale bar: 20 μm.)

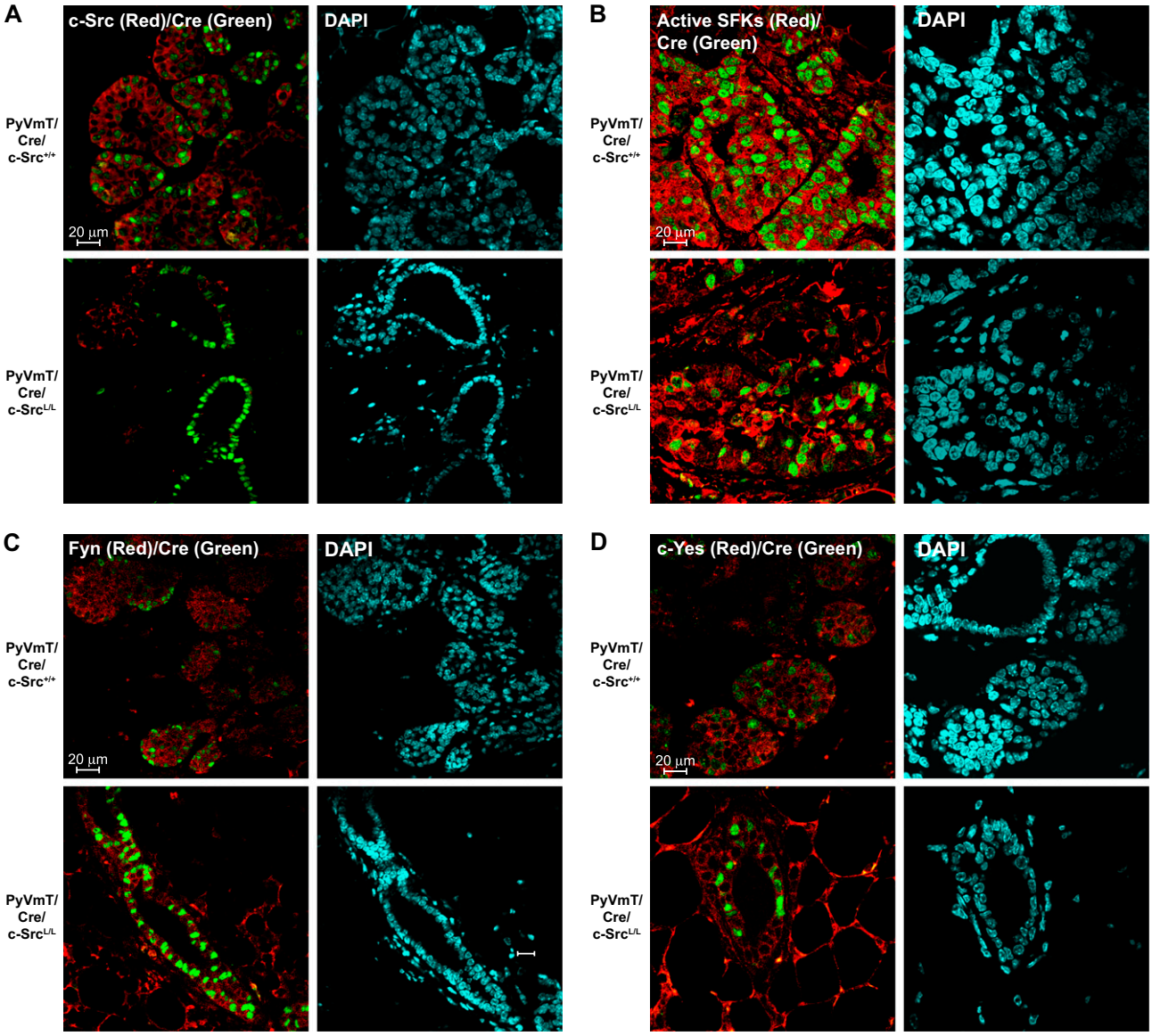


Fig. S3. (*A–D*) Representative images of IF staining of mammary gland sections from 8-wk-old PyVmT/Cre/c-Src$^{+/+}$ and PyVmT/Cre/c-Src$^{L/L}$ mice to detect expression of SFKs and Cre recombinase as indicated. Images are representative of mammary glands from at least three mice. (Scale bar: 20 μm.)

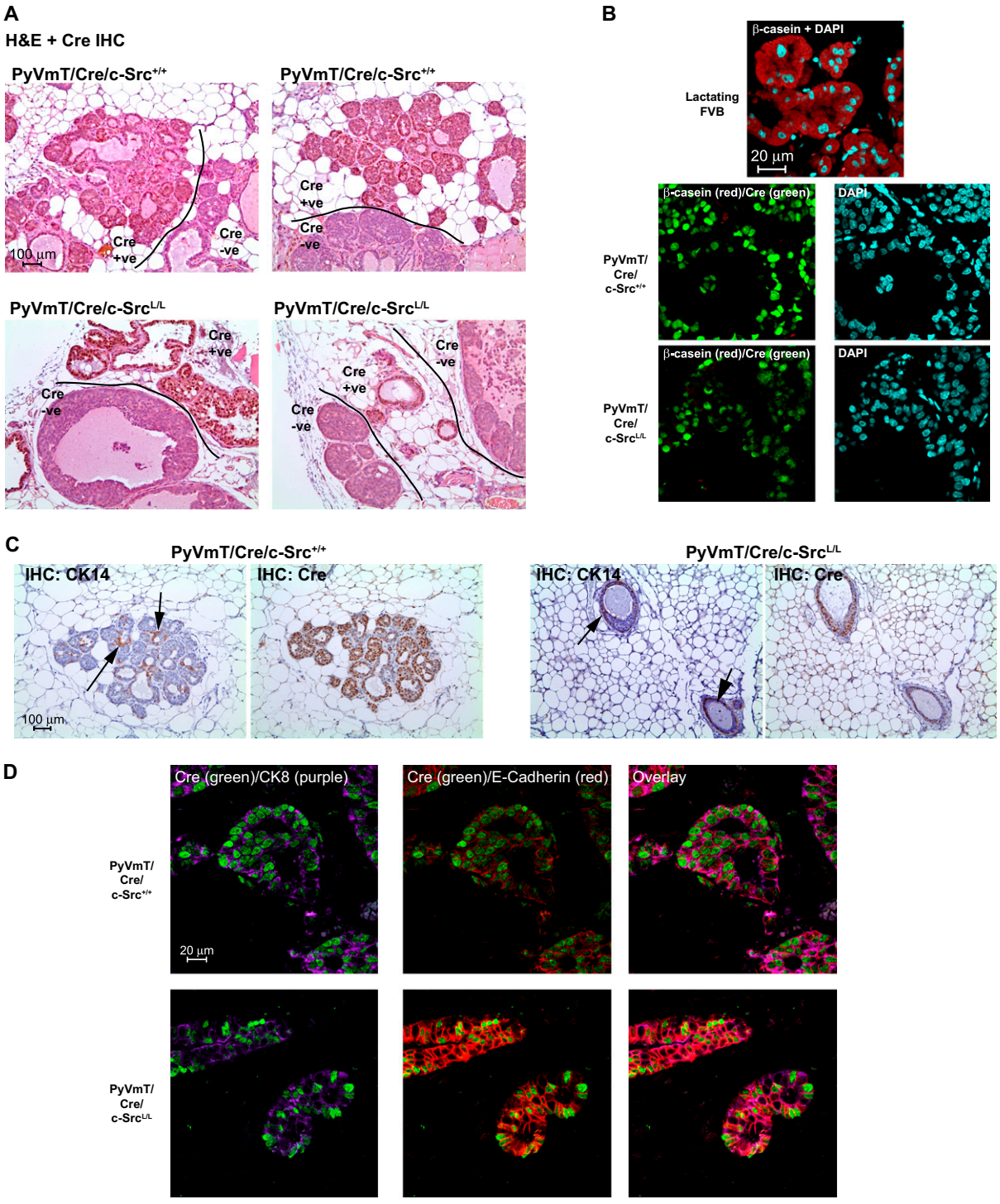


Fig. S4. (*A*) Mammary gland sections from 8-wk-old PyVmT/Cre/c-Src$^{+/+}$ and PyVmT/Cre/c-Src$^{L/L}$ mice were stained with H&E. Cre recombinase was detected simultaneously by immunohistochemistry so the histology of lesions could be correlated with Cre expression and hence c-Src status. Black lines divide regions containing lesions with positive Cre staining (Cre positive, brown nuclear stain) from regions containing Cre-negative lesions. (Scale bar: 100 μm.) (*B*) Mammary gland sections from 8-wk-old PyVmT/Cre/c-Src$^{+/+}$ and PyVmT/Cre/c-Src$^{L/L}$ mice were stained with anti–β-casein and Cre antibodies by IF. A mammary gland from a lactating WT FVB mouse was used as a positive control for β-casein staining. (Scale bar: 20 μm.) (*C*) Adjacent serial mammary gland sections from 8-wk-old PyVmT/Cre/c-Src$^{+/+}$ and PyVmT/Cre/c-Src$^{L/L}$ mice were stained with antibodies to detect expression of cytokeratin 14 (CK14) and Cre by IHC. The same regions were identified in the adjacent sections to compare CK14 staining in cells with and without Cre expression. Black arrows point to examples of regions containing CK14-positive cells. (Scale bar: 100 μm.) (*D*) Mammary gland sections from 8-wk-old PyVmT/Cre/c-Src$^{+/+}$ and PyVmT/Cre/c-Src$^{L/L}$ mice were stained with antibodies to detect Cre, cytokeratin 8 (CK8), and E-cadherin by IF. (Scale bar: 20 μm.)

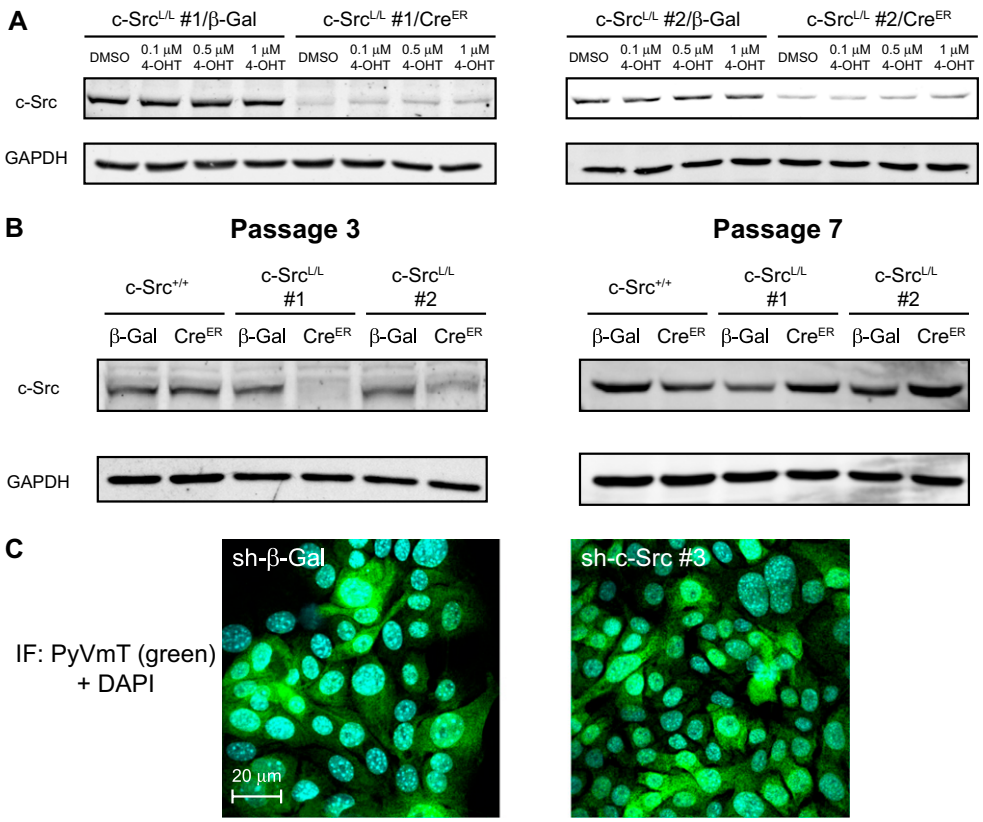


Fig. S5. (*A*) Sets of c-Src$^{L/L}$/β-gal and -/Cre^{ER} cell lines derived from two independent tumors were treated with the indicated doses of 4-hydroxytamoxifen (4-OHT) for 48 h and cultured for a further 72 h. Cell lysates were immunoblotted with the indicated antibodies. (*B*) Cell lines stably expressing Cre^{ER} or β-gal were lysed after the indicated number of passages in culture, and lysates were immunoblotted with the indicated antibodies. (*C*) Representative images of PyVmT IF staining in control and c-Src–silenced PyVmT cells grown on glass coverslips. (Scale bar: 20 μm.)

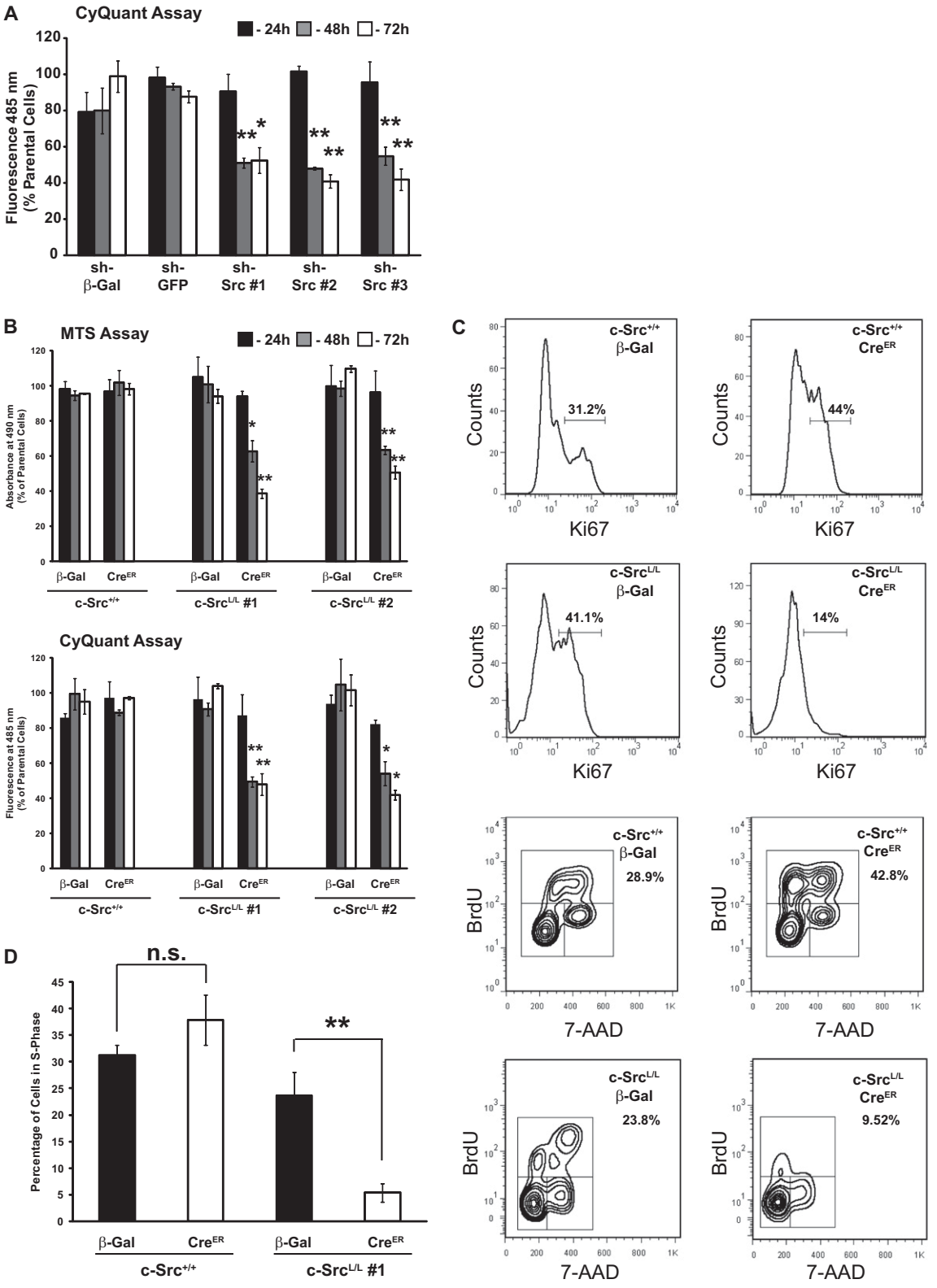


Fig. S6. (*A*) PyVmT cell lines stably expressing control or c-Src shRNAs were analyzed by using the CyQuant proliferation assay ($n = 3$). Data are normalized to values obtained from parental cells at each time point. Error bars represent SEM; $*P < 0.05$ and $**P < 0.01$, unpaired Student *t* test comparing normalized values of each sh-Src cell line to the sh-β-gal control. (*B*) PyVmT cell lines stably expressing Cre^{ER} or β-gal were analyzed by using MTS and CyQuant proliferation assays ($n = 3$). Data are normalized to values obtained from parental cells at each time point. Error bars represent SEM; $*P < 0.05$ and $**P < 0.01$, unpaired Student *t* test comparing normalized values for Cre^{ER} and β-gal. (*C*) *Upper:* Ki67 expression in PyVmT cell lines stably expressing Cre^{ER} or β-gal was determined by flow cytometry. Data are representative of two independent experiments. The percentage of Ki67-positive cells is indicated on each histogram. *Lower*: BrdU incorporation in PyVmT cell lines stably expressing Cre^{ER} or β-gal was determined by flow cytometry. BrdU staining (*y* axis) is plotted against DNA content by using the DNA-binding dye 7-AAD (*x* axis). The percentage of BrdU-positive cells is indicated on each plot. (*D*) Bar graph of quantification of BrdU incorporation reveals the average proportion of cells in S-phase ($n = 3$). Error bars represent SEM; $**P < 0.01$, n.s., not significant at $P > 0.05$, unpaired Student *t* test.

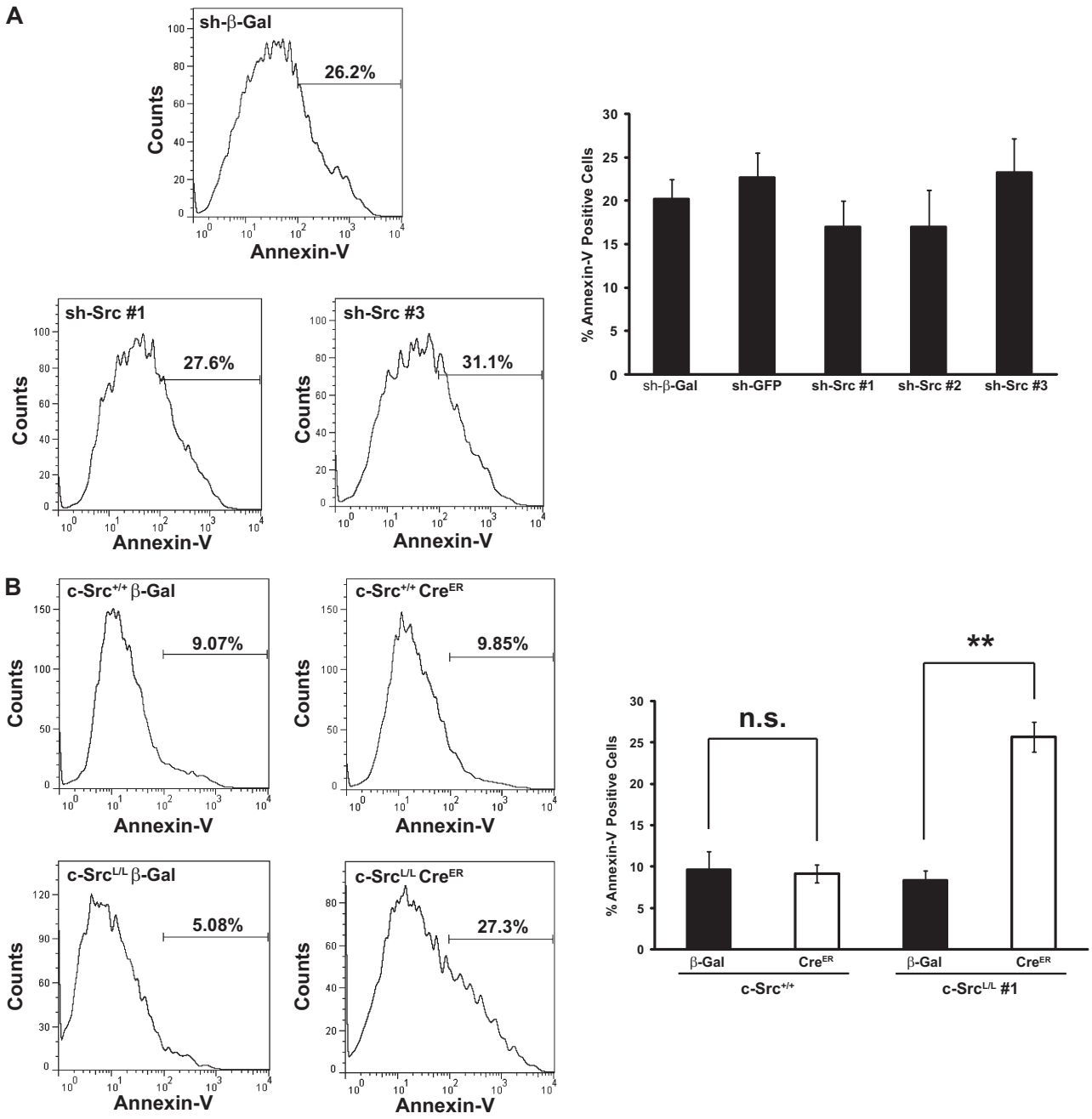
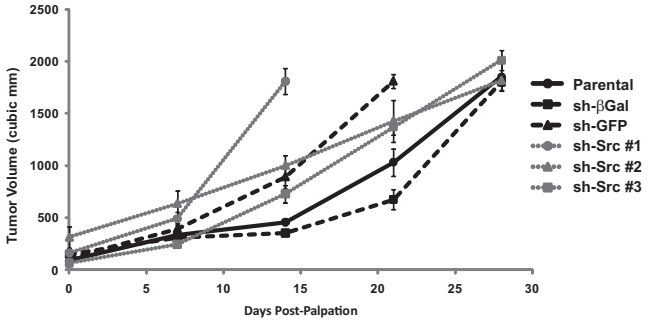


Fig. S7. (*A*) Annexin-V staining in cell lines stably expressing shRNAs targeting c-Src or negative control shRNAs was determined using flow cytometry. *Left*: Example histograms showing annexin-V staining in each cell line. The percentage of annexin-V–positive cells is indicated on each histogram. *Right*: Bar graph of quantification of the annexin-V–positive population for each cell line ($n = 4$). Error bars represent SEM. There was no statistically significant difference between any c-Src shRNA cell line and the sh-β-gal or sh-GFP control (unpaired Student *t* test). (*B*) Annexin-V expression in cell lines stably expressing Cre^{ER} or β-gal was determined using flow cytometry. *Left*: Example histograms showing Annexin-V staining in each cell line. The percentage of annexin-V–positive cells is indicated on each histogram. *Right*: Bar graph of quantification of the annexin-V–positive population for each cell line ($n = 4$). Error bars represent SEM; $^{**}P < 0.01$, n.s., not significant at $P > 0.05$, unpaired Student *t* test.

A

Cell Line	Mean Tumor Onset (Days)	S.E.M.
Parental	25.9	1.4
sh-β-Gal	28.2	1.3
sh-GFP	25.2	0.7
sh-Src #1	39**	1.8
sh-Src #2	37.6**	3.5
sh-Src #3	32.2**	1.7

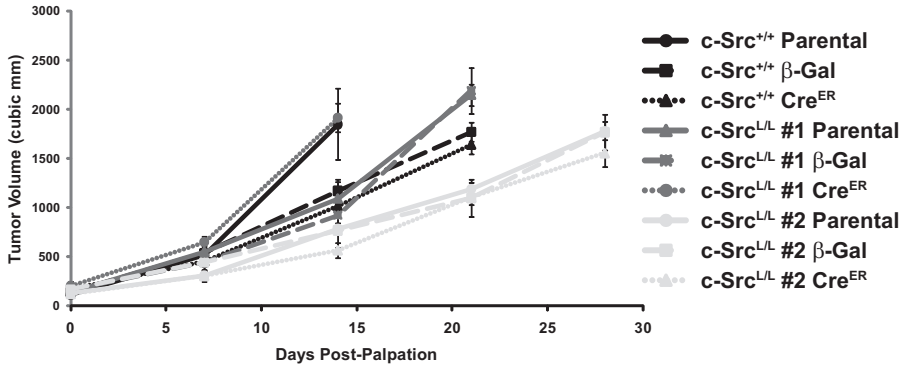


B

Cell Line	Mean Tumor Onset (Days)	S.E.M.
c-Src$^{+/+}$ Parental	24	0
c-Src$^{+/+}$ β-Gal	25.4	1.4
c-Src$^{+/+}$ Cre^{ER}	25.4	1.4

Cell Line	Mean Tumor Onset (Days)	S.E.M.
c-Src$^{L/L}$ #1 Parental	27	0
c-Src$^{L/L}$ #1 β-Gal	28.4	1.4
c-Src$^{L/L}$ #1 Cre^{ER}	65.5*	10.7

Cell Line	Mean Tumor Onset (Days)	S.E.M.
c-Src$^{L/L}$ #2 Parental	32.6	1.4
c-Src$^{L/L}$ #2 β-Gal	35.8	1.8
c-Src$^{L/L}$ #2 Cre^{ER}	57.2**	1.2



C

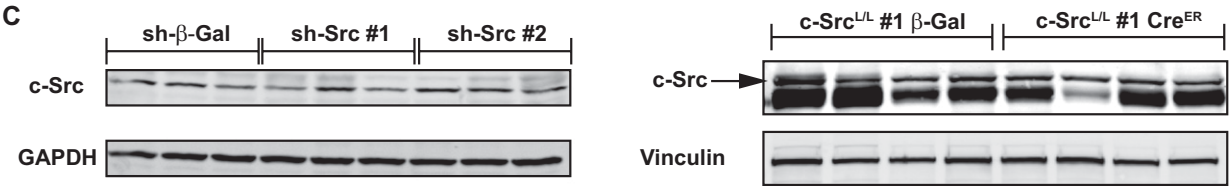


Fig. S8. (*A*) *Left*: Chart of elapsed time between injection and the onset of palpable mammary tumors for PyVmT parental cells and cell lines with stable expression of c-Src shRNAs or negative control shRNAs (n = 10). **P < 0.01, unpaired Student t test versus parental, sh-β-gal, and sh-GFP. *Right*: Tumor volume was determined by using caliper measurements and plotted against time after palpation. (*B*) *Upper*: Charts show elapsed time between injection and onset of palpable mammary tumors for parental cells and cell lines stably expressing β-gal or Cre^{ER} (n = 5). *P < 0.05 and **P < 0.01, unpaired Student t test versus β-gal control. *Lower*: Tumor volume was determined by using caliper measurements and plotted against time since palpation. (*C*) Tumor lysates from immunocompromised mice with mammary fat pad injection of c-Src–deficient cell lines and controls were immunoblotted with the indicated antibodies. Arrow (*Right*) indicates the 60-kDa c-Src band. GAPDH and vinculin were used as loading controls.

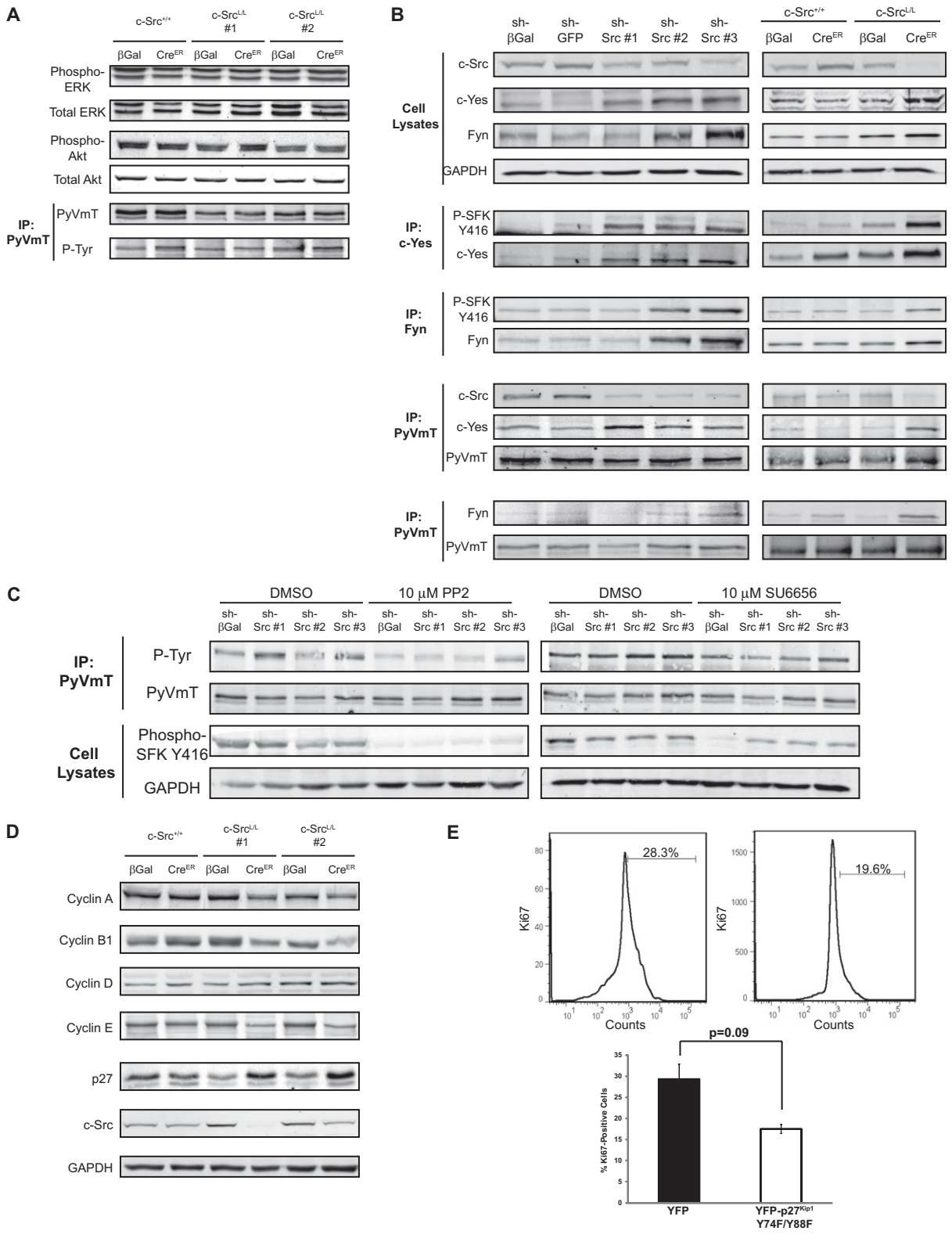


Fig. S9. (*A*) PyVmT cell lines stably expressing Cre^{ER} or β-gal were lysed, and immunoblotting was performed by using the indicated antibodies. In the bottom two rows, PyVmT was immunoprecipitated from cell lysates and immunoblotting was performed with the indicated antibodies. (*B*) Stable c-Src–deficient and control cell lines were lysed and immunoblotted with the indicated antibodies. (*C*) Stable c-Src–silenced and sh-β-gal control cell lines were treated with PP2 or SU6656 (both at 10 μM) or DMSO (vehicle control) for 24 h. Cells were lysed and PyVmT was immunoprecipitated. Immunoprecipitates were immunoblotted with anti-PyVmT and -phosphotyrosine antibodies. Cell lysates were immunoblotted with anti–phospho-SFK Y416 and -GAPDH antibodies. (*D*) PyVmT cell lines stably expressing Cre^{ER} or β-gal were lysed and immunoblotted for the indicated proteins. (*E*) PyVmT cells expressing YFP (*Upper Left*) or a YFP-tagged p27^{Kip1} Y74F/Y88F mutant (*Upper Right*) were analyzed for Ki67 expression by flow cytometry. Bar chart shows the average of three independent experiments.