Supporting Information

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SI Materials and Methods

Materials. PE-CD24, biotin-mouse lineage panel, biotin-CD31, and SAv-APC were purchased from BD Biosciences. FITC-CD29 and Pacific Blue-CD29 were from BioLegend. Biotin-CD140a was purchased from eBioscience. PTEN (A2B1) was from Santa Cruz Biotechnology. Anti-γ-H2AX and rabbit polyclonal anti-53BP1 were from Millipore and Novus Biologicals, respectively. Akt, p-Akt^{Ser473}, p-Akt^{Thr308}, Survivin (71G4B7E), β-catenin, mTOR, and p-mTOR^{Ser2448} were from Cell Signaling Technology. PTEN (Mm00477210_m1) and reagents for qPCR were all from Applied Biosystems. Texas Red- and Alexa 488-conjugated secondary antibodies for immunofluorescence were from Molecular Probes.

Immunohistochemistry. Cytospun cells were fixed with 4% paraformaldehyde (PFA) and washed with PBS three times before blocking with the blocking buffer (5% BSA/0.5% Tween-20 in 1× PBS) with the M.O.M blocking reagent (Vector Laboratories). Dilutions of 1:250 and 1:100 were used for primary antibodies against γ-H2AX and 53BP1, respectively, incubating at 4 °C overnight. Anti-mouse and anti-rabbit secondary antibodies were used for H2AX (1:500) and 53BP1 (1:500), 1 h at room temperature.

qPCR. After isolation of RNAs from the individual subpopulation, 1 μg of RNA per sample was treated with DNase I as recommended by the manufacturer, followed by cDNA synthesis using MutiScribe reverse transcriptase (Applied Biosystems) on a 2720 Thermal Cycler (Applied Biosystems). cDNA (4 ng) was used in qPCR containing Taqman Gene Expression Master Mix (Applied Biosystems), probe, and primers for PTEN (18 μM for primer and 5 μM for probe). qPCR was performed using the StepOnePlus real-time PCR system and software (Applied Biosystems) with the following cycles: 50 °C for 2 min, 95 °C for 10 min, and 95 °C for 15 s followed by 60 °C for 1 min for 40 cycles. qPCR data were normalized with 18S rRNA.

Intracellular Reactive Oxygen Species Analysis Among Various Subpopulations. Cells labeled with CD29 and CD24 as described in *Flow Cytometry* in *Materials and Methods* were then stained with MitoSOX Red (Invitrogen, 5 μM, incubated at 37°C for 10 min) and DCF-DA (Sigma, 10 μM, incubated at 37 °C for 30 min) according to manufacturers' protocols, followed by flow cytometry analysis.

MTS Assay (Cell Proliferation Assay). Cells were plated in flat-bottom 96-well plates at a density of 5×10^3 cells per well supplemented with mammosphere medium plus 5% FBS immediately after FACS sorting. After 3 days, CellTiter 96 AQueous One Solution Reagent (Promega) was added to each well according to the manufacturer's instructions. After 2 h in culture the cell viability was determined by measuring the absorbance at 450 nm, using a Multiskan Plus Plate Reader (Thermo Electron Corporation).

Preparation of Single Normal Mammary Epithelial Cells. Mammary epithelial cells (MECs) were isolated as described (1). Briefly, the no. 4 inguinal mammary glands (lymph nodes removed) from 8- to 10-week-old virgin female BALB/c mice were minced into small pieces. The digestion buffer [DMEM/F12 supplemented with 100 μg/mL gentimycin, antibiotic–antimycotic, and collagenase type III (225 units/mL, Worthington)] was used to digest the gland for 1.5 h at 37 °C on a rotary shaker shaking at 125 rpm. Samples were pipetted every 30 min and centrifuged at 600 × *g* for 10 min to pellet the organoids. Samples were washed four times in washing buffer at 425 × *g* for 2 s and washed once with PBS before trypsinization in 0.5 g/L trypsin per 0.2 g/L EDTA in saline for 10 min. HBSS$^+$ [HBSS (Invitrogen) containing 2% FBS and 10 mM Hepes buffer (Invitrogen)] was added to dilute out trypsin. Cells were filtered through 40-μm filters and centrifuged at 671 × *g* for 5 min before labeling with antibodies.

Protein Extraction, SDS/PAGE, and Western Blot. Freshly FACS-isolated TICs and MS enriched for TICs, as well as FACS-isolated non-TICs and the total tumor cells cultured on plastic, which are depleted in TICs, were centrifuged at 200 × *g* for 10 min at 4 °C. Approximately 3 packed cell volumes of RIPA [50 mM NaCl, 150 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate], supplemented with the phosphatase inhibitor PhosSTOP (Roche), were added to the cell pellets. Mixtures were homogenized and incubated on ice for 15 min. Protein supernatant was collected by spinning the lysis at 16,000 × *g* for 10 min at 4 °C. A total of 3 μL of the solution was saved for protein quantification using Bradford Assay according to manufacturer's protocol.

1. Zhang M, et al. (2008) Identification of tumor-initiating cells in a p53-null mouse model of breast cancer. *Cancer Res* 68:4674–4682.

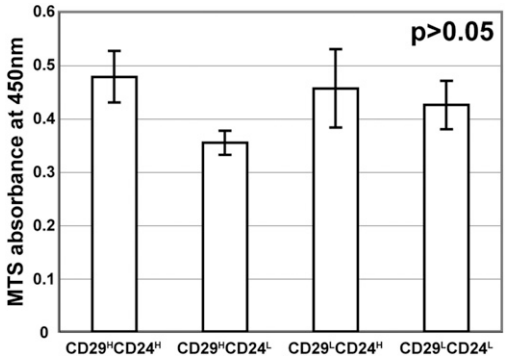

Fig. S1. Proliferation rate is similar among various sorted subpopulations. A total of 5×10^3 cells/per well from each subpopulation after FACS sorting were plated into flat-bottom 96-well plates. After 3 days of culturing, cell proliferation was measured using an MTS assay. The results represent the mean ± SD of two experiments performed in triplicate.

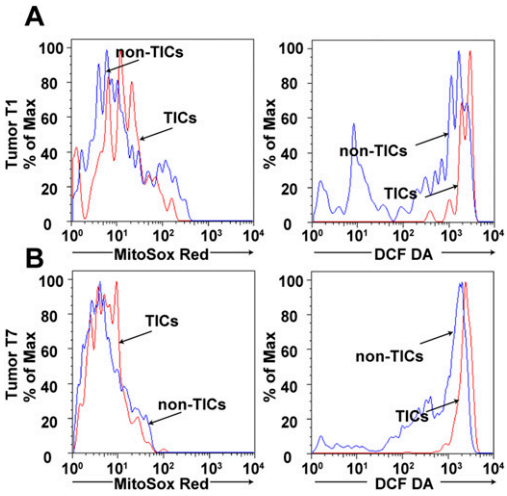

Fig. S2. Levels of the ROS were same among the TICs vs. the non-TICs. Dissociated cells from tumors T1 (*A*) and T7 (*B*) were stained with the identified TIC markers, CD29, CD24, and MitoSOX (5 μM) or DCF-DA (10 μM), followed by FACS analysis to measure their intracellular ROS levels.

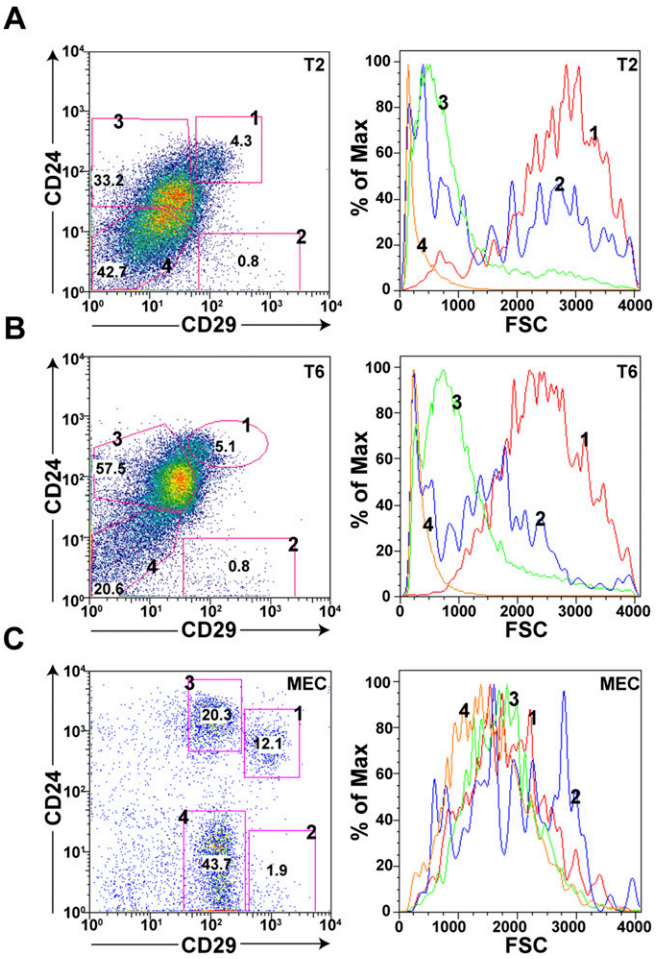

Fig. S3. TICs are larger in size as compared with the other subpopulations. Collagenase-digested tumors T2 (*A*) and T6 (*B*) were FACS sorted on the basis of CD29 and CD24 expression. Forward scatter (FSC), whose measurement is related to cell size, was analyzed using FlowJo (version 8.8.6; Tree Star) among various subpopulations and compared with that of the MECs (*C*).

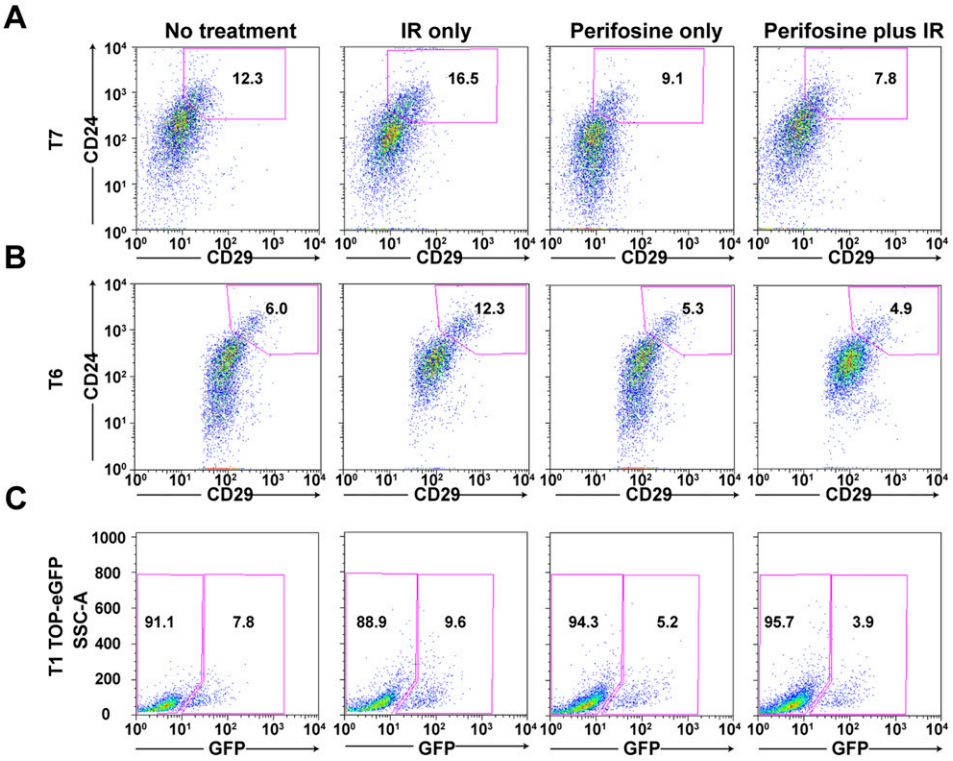

Fig. S4. Akt inhibitor perifosine sensitized radiation-resistant mammary tumorspheres enriched for TICs in vivo. Tumors T7 (*A*), T6 (*B*), and TOP-eGFP transduced T1 (*C*) treated with IR, perifosine, and perifosine followed by IR treatment were digested and FACS analyzed on the basis of CD29 and CD24 expression (in tumors T6 and T7) and GFP expression (in TOP-eGFP transduced T1). Percentages of TICs (*A* and *B*) and GFP-positive cells (*C*) were labeled accordingly.

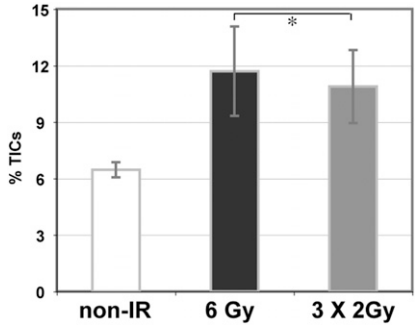

Fig. S5. IR treatment (6 Gy vs. 3 × 2 Gy). Mice with tumors (T1) were IR treated with a single dose of 6 Gy or 3 doses of 2 Gy every 16 h. Forty-eight hours after treatment, tumors were excised and dissociated as described, followed by antibody labeling and FACS analysis. The percentage of TICs from IR-treated (6 Gy and 3 × 2 Gy) as well as from non-IR treated tumors was determined by FACS analysis. Five tumors were included in each group. No significant differences were observed between the 6-Gy and 3 × 2 Gy treatment groups (*, $P > 0.05$).