# Supporting Information

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### SI Materials and Methods

**Cells and Tissue Culture.** SUM cell lines were obtained from Dr. Stephen Ethier (Karmanos Institute, Detroit) and are commercially available (Asterand). MCF7, HCC1428, T47D, and BT-20 cell lines were purchased from ATCC. MCF7, HCC1428, T47D, and BT-20 cells were cultured in DMEM with 10% calf serum or in phenol-red–free DMEM, 2 mM L-glutamine, and 10% charcoal-dextran–stripped FBS for experiments with estrogen stimulation. SUM149PT and SUM159PT cells were cultured in Ham's F-12 medium with 5% calf serum, insulin (5 μg/mL), and hydrocortisone (1 μg/mL). All cell lines were grown at 37 °C in a 5% $CO_2$ incubator. Estrogen (17-β-estradiol) was dissolved in ethanol to a stock concentration of 1 μM; PD173074 (Sigma) was dissolved in DMSO to a stock concentration of 10 mM. All treatments, including those with conditioned media, lasted 6 d.

All human breast tissue procurement for these experiments was obtained in compliance with the laws and institutional guidelines, as approved by the institutional institutional review board committee from Tufts University School of Medicine. An $ER^+$, $Her2^-$ tumor was obtained from discarded material, and noncancerous breast tissue was obtained from patients undergoing elective reduction mammoplasty at Tufts Medical Center. Breast tissues were minced and enzymatically digested overnight with a mixture of collagenase and hyaluronidase as previously described (1, 2). Digested cells were plated briefly in serum (1–2 h) to deplete mammary fibroblasts from the organoid fraction of mammary fibroblasts. The organoids were dissociated to a single cell suspension by trypsinization and filtered with a 40-μm filter (BD Biosciences) to remove residual clustered cells. Immediately after dissociation, cells were assayed for mammospheres formation or were infected with lentivirus and then assayed. For the human-in-mouse tumors, mammary epithelial cells from three different patient samples were spin infected with lentivirus-encoding MyrP110α, kRasG12V, p53R175H, and CCND1 and implanted into humanized mouse mammary glands (1).

**Flow Cytometry.** Subconfluent cultures were trypsinized into single cell suspension, counted, washed with PBS, and stained with antibodies specific for the following human cell-surface markers: EpCAM (ESA)-FITC (clone VU-ID9, AbD Serotec), CD24-PE (clone ML5, BD Pharmingen); and CD44-APC (clone G44-26, BD Pharmingen). For each staining reaction, 100,000 cells were incubated with 4 μL of each antibody for 15 min at room temperature. Unbound antibody was washed off and cells were analyzed on a BD FACSCaliber no more than 1 h poststaining. Isotype controls included mouse $IgG_1$-FITC, mouse $IgG_{2a\kappa}$-PE, and mouse $IgG_{2b\kappa}$-APC (BD Pharmingen). When staining for ERα-FITC (clone SP1, Abcam) cells were stained sequentially with EpCAM (clone VU-ID9, Abcam), rat–anti-mouse PerCP (BD Pharmingen), and CD24-PE/CD44-APC (BD Pharmingen) before cells were fixed in 4% paraformaldehyde and 0.1% Saponin and incubated with ERα-FITC.

**Animals and Surgery.** All animal procedures were conducted in accordance with relevant national and international guidelines and according to the animal protocol approved by the Tufts University Institutional Animal Care and Use Committee. NOD/SCID mice were purchased from Jackson Labs. Female mice 5–7 wk of age were ovariectomized and allowed to recover for 4 wk before tumor cell injection. For tumor-seeding studies, the indicated numbers of MCF7 cells pretreated for 6 d with vehicle (EtOH), 1 nM 17-β-estradiol (E2), or 1 nM 17-β-estradiol and the FGFR inhibitor PD173074 (E2+ PD) were suspended in 1:1 (vol/vol) culture medium: Matrigel (BD Biosciences) mixture and injected into the fourth inguinal mammary gland. For SUM159 pretreatment experiments, intact 8-wk-old female NOD/SCID mice were injected into the fourth inguinal mammary gland ($n$ = 12 for each group) with 10,000 cells pretreated for 6 d with PD173074 or with FGF9.

**Tumorsphere Assays.** Viable dissociated single cells (~30,000/mL) were plated in 6-cm ultra–low-attachment plates (Corning) in the indicated media. Tumorspheres and mammospheres were allowed to form for 5 or 8 d, respectively, after which spheres were collected for analysis. Quantification of mammosphere and tumorsphere numbers was accomplished using a Multisizer 3 Coulter Counter (Beckman-Coulter) that provided number and size distributions with an overall sizing range of 40 μm to 336 μm. Tumorspheres and mammospheres were collected and pelleted at 800 rpm for 5 min and resuspended in 1 mL freshly filtered growth media, diluted in 20 mL 6:4 isoton II:glycerol diluent (Beckman-Coulter), and run in triplicate on the Multisizer 3.

**Conditioned Medium Experiments.** Subconfluent MCF7 cultures were treated with 1 nM 17-β-estradiol or EtOH for 6 d in phenol-red–free DMEM and 10% charcoal-dextran–stripped FBS (Invitrogen). Cultures were washed five times with PBS and incubated with fresh serum-free, phenol-red–free DMEM. Conditioned medium (CM) was harvested 72 h later, passed through a 0.2-μm filter, and frozen at −80 °C. For each experiment, at least three distinct batches of CM were combined and supplemented with 2 mM L-glutamine and 10% charcoal-dextran stripped FBS and fed to naive cells for a total of 6 d, with media changed every 2 d, after boiling for 5 min where specified.

**Cytokine Array and Quantification.** Serum-free CM was collected as described above. Human cytokine arrays (2000 series, RayBiotech) were exposed to conditioned medium from MCF7 cultures pretreated with either ethanol (vehicle) or estrogen and processed in accordance with the manufacturer's protocols. Exposed films of chemiluminescence signal obtained from dot blots were scanned, and the pixel intensity for each cytokine was quantified and normalized to IgG loading controls using ImageJ software (National Institutes of Health).

**Western Blot and Immunofluorescence.** For immunfluorescence (IF), cells were fixed in 4% paraformaldehyde and 0.1% saponin and permeabilized with 0.1% BSA and 0.25% Triton-X, both in PBS. Coverslips were mounted with Vectashield mounting medium plus DAPI (Vector Labs). Antibodies used for IF were ERα-FITC (clone SP1, Abcam), DUSP6 (clone 3G2, Novus), EpCAM (clone B29.1, Abcam), and Tbx3 (rabbit, Aviva). For Western blotting, 25 μg protein extract per sample denatured with heat and reducing agents, separated on a 4–12% acrylamide gel, and transferred to nitrocellulose. Antibodies used for Western blotting were Tbx3 (mouse, Abcam), FGFR3 (rabbit, Sigma), and β-actin (clone mAbcam 8226, Abcam).

**Isolation of RNA and Quantitative RT-PCR.** Cells were harvested by trypsinization, pelleted by centrifugation, and RNA isolation was performed using the RNAeasy kit (Qiagen) in accordance with the manufacturer's protocol. The RNA samples were then reverse transcribed using the iScript cDNA kit (Bio-Rad), and quantitative PCR was performed with Sybr green (Bio-Rad) on a Bio-Rad iCycler. Primers used are: GAPDH F-GAGTCAAC-

GGATTTGGTCGT R-GACAAGCTTCCCGTTCTCAG, Tbx3 F-TGGGGACCTCTGATGAGTCCT R-CCATGCTCCTCTTTGCTCTC, DUSP6 F-GCTATACGAGTCGTCGCACA R-CGTCCTTGAGCTTCTTGAGC, Wnt5a F-GGGAGGTTGGCTTGAACATA R-GAATGGCACGCAATTACCTT, ERα F-ATTTGAAGTGGGCAGAGAACAT R-CAATACCAACATCAGCCAGAAA, FGFR3 F-ACTGGGGAACAGTGGATGTC R-GGATGCCTGCATACACACTG, FGF9 F-TTTCTGGTGCCGTTTAGTCC R-GACTACCTGCTGGGCATCAA, Vimentin F-AGATGGCCCTTGACATTGAG R-GGTCATCGTGATGCTGAGAA, N-Cadherin F-ACAGTGGCCACCTACAAAGG R-CCGAGATGGGGTTGATAATG, E-Cadherin F-TGCCCAGAAAATGAAAAAGG R-GGATGACACAGCGTGAGAGA, Zeb-1 F-GATCAACCACCAATGGTTCC R-TTGCGCAAGACAAGTTCAAG.

**Lentiviral Constructs and Infection.** Bacterial glycerol stocks of MISSION shRNA were obtained (Sigma), and plasmid DNA was isolated by miniprep (Qiagen). Lentiviral expression construct for Tbx3 gene transduction was created using standard Gateway cloning techniques into the self-inactivating pLenti6.2/V5-DEST Gateway vector (Invitrogen). A WT human *Tbx3* cDNA clone (NM_016569.2–443) was generously provided by Josh LaBaer (Harvard Institute of Proteomics, Harvard Medical School. Boston, MA). The VSV-G-pseudotyped lentiviral vectors were generated by transient cotransfection of the above vectors with the VSV-G-expressing construct pCMV-VSV-G and the packaging construct pCMV DR8.2Dvpr (3), both generously provided by Inder Verma (Salk Institute), into 293T cells with the FuGENE 6 transfection reagent (Roche). Viral supernatant was collected and introduced to subconfluent SUM149, SUM159, MCF7, and HMEC cultures, or to patient-derived breast cancer cells. Lentiviral integration was selected with 1 μg/mL puromycin (for shRNAs), or with 10 μg/mL blasticidin (Tbx3) for 7 d.


1. Proia DA, Kuperwasser C (2006) Reconstruction of human mammary tissues in a mouse model. *Nat Protoc* 1:206–214.
2. Wu M, et al. (2009b) Dissecting genetic requirements of human breast tumorigenesis in a tissue transgenic model of human breast cancer in mice. *Proc Natl Acad Sci USA* 106: 7022–7027.
3. Miyoshi H, Blömer U, Takahashi M, Gage FH, Verma IM (1998) Development of a self-inactivating lentivirus vector. *J Virol* 72:8150–8157.


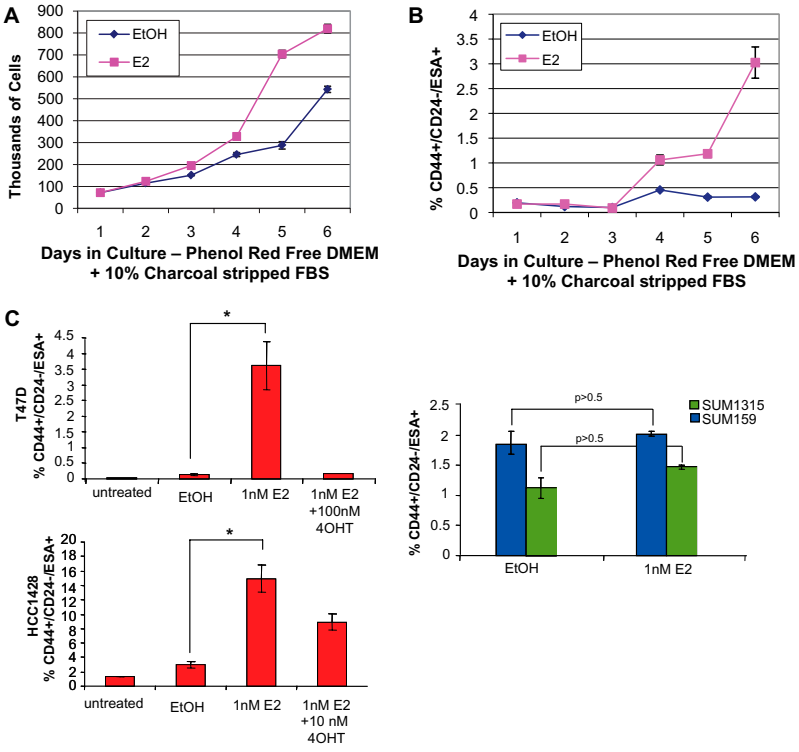


**Fig. S1.** (*A*) MCF7 cells were seeded in six-well plates at 100,000 cells per well. The next day, the cultures were switched to phenol-red–free DMEM with 10% charcoal-dextran FBS and either 1 nM estrogen or vehicle (EtOH). Each day, two wells per condition were trypsinized and counted. Average cell number per well per day is shown. (*B*) Cells described in *A* were assayed daily for percentage of $CD44^+/CD24^-/ESA^+$ cells by flow cytometry. (*C*) Average percentage of $CD44^+/CD24^-/ESA^+$ cells as measured by flow cytometry in the $ER\alpha^+$ cell lines T47D (*$P < 0.01$) and HCC1428 (*$P < 0.0001$), or in the $ER\alpha^-$ cell lines SUM159 and SUM1315, following 6-d treatment with either 1 nM 17-β-estradiol (E2) or vehicle (EtOH). Data are mean ± SEM; $n = 5$ biological replicates.

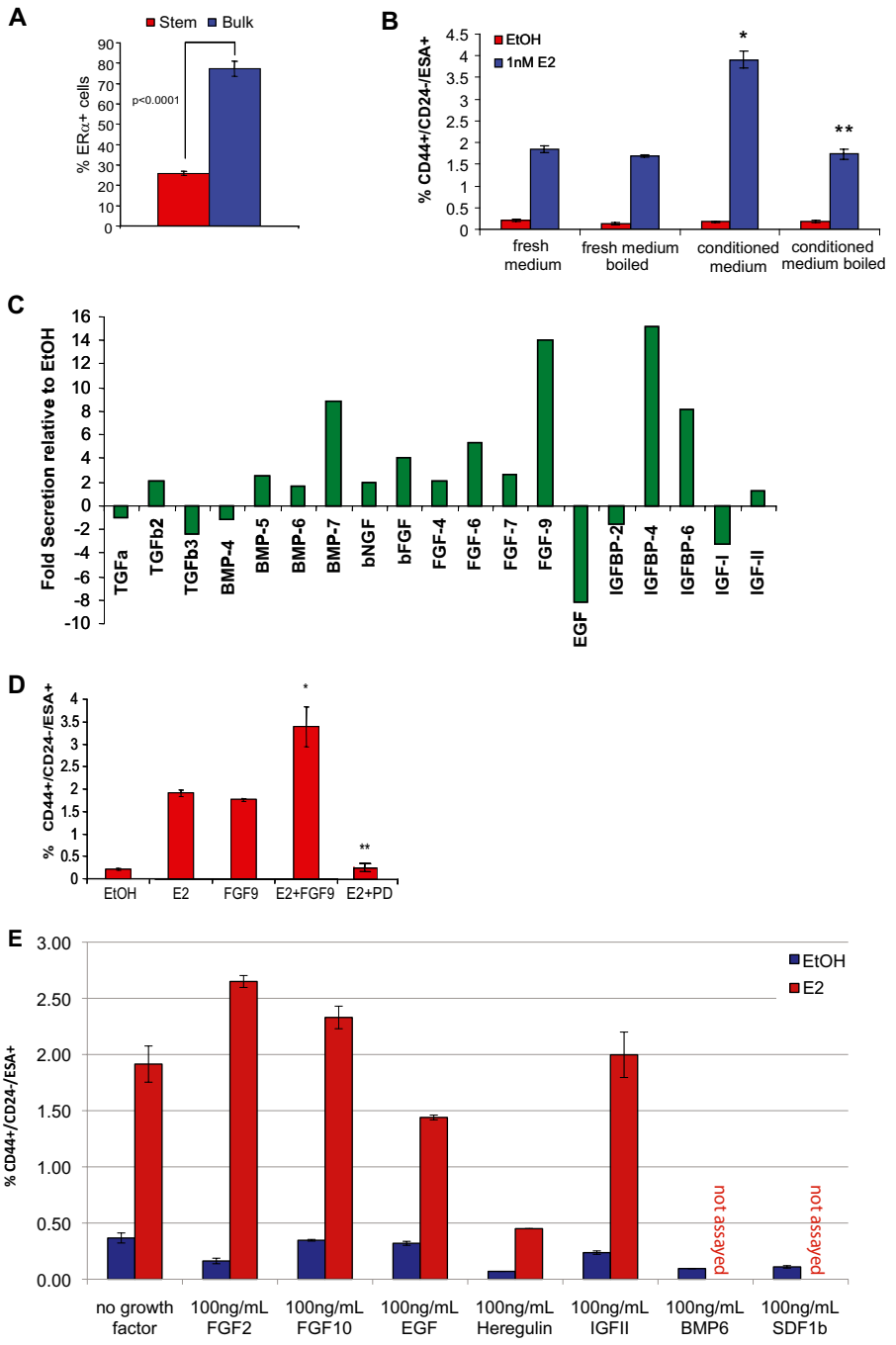


**Fig. S2.** (*A*) Quantification of ERα immunofluorescence staining on sorted and cytospun MCF7 cells. Graph represents percentage of ERα$^+$ cells for from seven fields per sort with an average of 56 nuclei per field ± SEM. (*B*) Average percentage of CD44$^+$/CD24$^-$/ESA$^+$ cells as assayed by flow cytometry in MCF7 cultures following 6-d treatment with fresh estrogen, vehicle (EtOH), or conditioned media from EtOH- or E2-pretreated MCF7 cells. *$P$ < 0.001 E2 CM vs. EtOH CM. Where indicated, unconditioned or conditioned media was boiled before feeding the cells. For unconditioned media containing fresh 1 nM 17-β-estradiol, the estrogen was added before boiling to show its relative heat stability. *$P$ < 0.04 E2 CM vs. E2 CM boiled. Data are mean ± SEM; $n$ = 4 biological replicates. (*C*) Cytokine array quantification of proteins secreted by MCF7 cells in response to estrogen (E2). All data are normalized to the respective IgG controls, and the fold increase in secretion is shown as the E2 pixel value divided by the EtOH pixel value for matched exposure lengths. (*D*) Average percentage of CD44$^+$/CD24$^-$/ESA$^+$ cells in MCF7 cultures treated with 1 nM 17-β-estradiol (E2), recombinant human FGF9 (100 ng/mL), or FGF9 and E2 in the absence of serum. *$P$ < 0.05 E2+FGF9 vs. E2; **$P$ < 0.001 E2 vs. E2+PD. Data are mean ± SEM; $n$ = 6 biological replicates. (*E*) Average percentage of CD44$^+$/CD24$^-$/ESA$^+$ cells in MCF7 cultures following 6-d treatment with EtOH vehicle, 1 nM 17-β-estradiol (E2), fibroblast growth factor 2 (FGF2), fibroblast growth factor 10 (FGF10), human epidermal growth factor (EGF), recombinant human heregulin (HRG), insulin-like growth factor 2 (IGFII), bone morphogenic protein 6 (BMP6), or stromal-derived factor 1-β (SDF1β). Data are mean ± SEM.

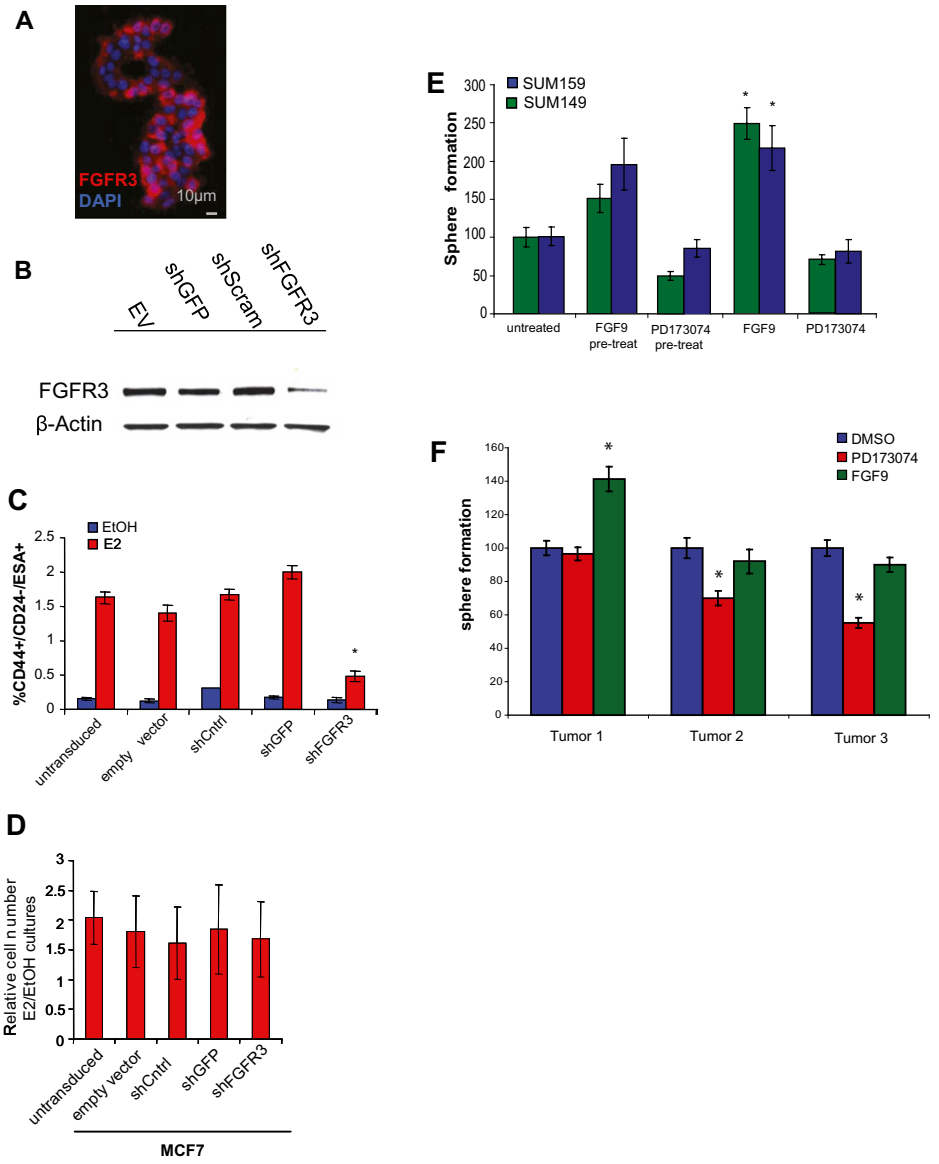


**Fig. S3.** (*A*) Immunofluorescence of FGFR3 (red) and DAPI (blue) of untreated MCF7 cultures. (*B*) Western blot of FGFR3 expression in MCF7 cells transduced lentivirus containing a small-hairpin directed to FGFR3. (*C*) Average percentage of $CD44^{+}/CD24^{-}/ESA^{+}$ cells in MCF7 cultures treated for 6 d with 1 nM 17-β-estradiol (E2) in cultures transduced with indicated small hairpins. *$P$ = 0.001 shFGFR3+E2 vs. shCntrl+E2. (*D*) Proliferation rates of FGFR3 knockdown MCF7 cell lines in response to 1 nM 17-β-estradiol (E2). Data are shown as total cell number in E2-treated cultures normalized to cell number in matched EtOH-treated cultures; $n$ = 4. (*E*) Tumorsphere formation of SUM159 or SUM149 cells pretreated with recombinant human FGF9 or FGFR inhibitor PD173074, or treated while making spheres with FGF9 or PD173074. Data collected at 6 d after seeding, *$P < 0.002$ FGF9 vs. vehicle. (*F*) Three different tumors derived from primary mammary epithelial cells transformed with MyrP110α, kRasG12V, p53R175H, and CCND1 and implanted into humanized mouse mammary glands were dissociated into single cells and plated at low dilution on super–low-attachment plates in filtered MEGM ± DMSO, 100 ng/mL FGF9, or 10 μM PD173074. Spheres were quantified on a Becton Dickonson Multisizer 8 d after seeding, and sphere formation was normalized to the DMSO condition; $n$ = 12.

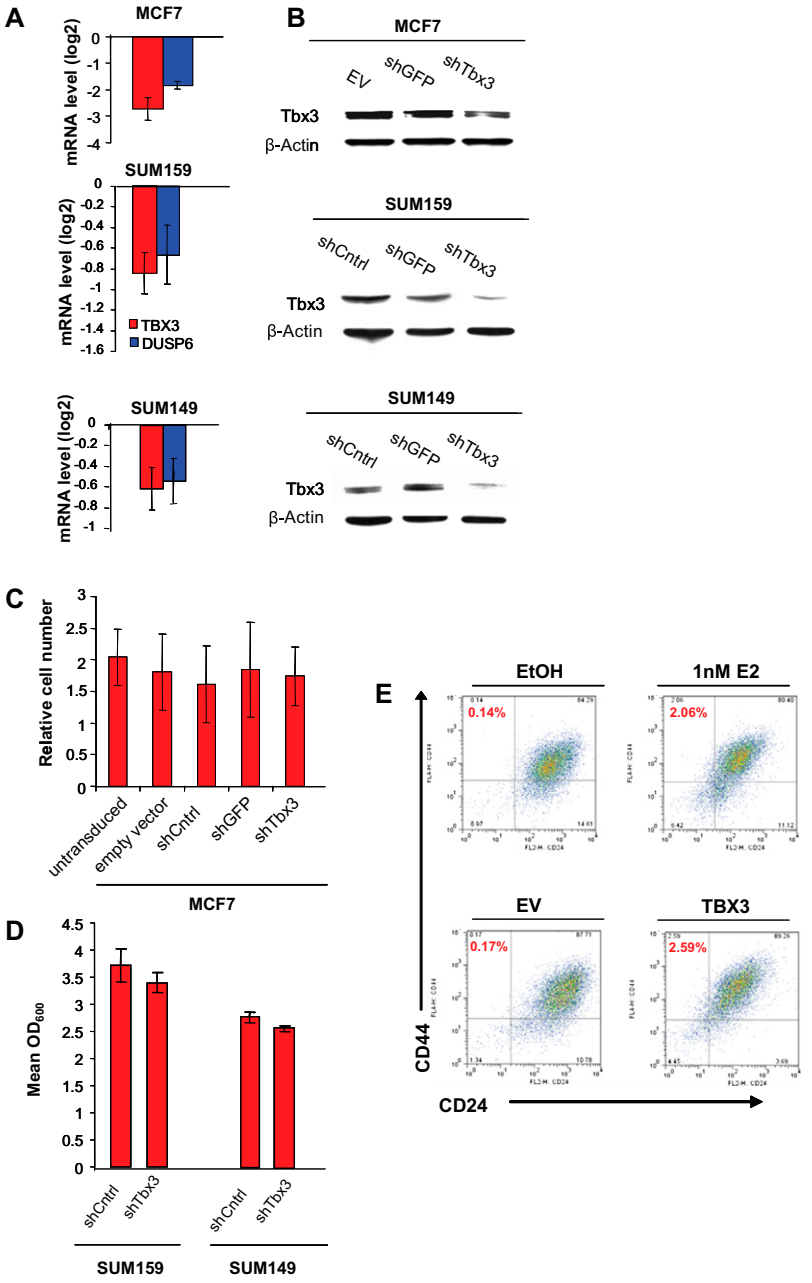


**Fig. S4.** RNAi-mediated knock down of Tbx3. (*A*) RT-PCR of Tbx3 and DUSP6 expression (relative to shCntrl). (*B*) Western blot of Tbx3 expression in MCF7, SUM159, and SUM149 cultures transduced with lentivirus containing short hairpins targeting a scrambled sequence (Cntrl), GFP, or Tbx3. (*C*) Proliferation rates of Tbx3 knockdown MCF7 cell lines in response to 1 nM 17-β-estradiol (E2). Data are shown as total cell number in E2-treated cultures normalized to the cell number in matched EtOH-treated cultures; $n = 4$. (*D*) Proliferation rates of SUM149 and SUM159 Tbx3 knockdown cultures as measured by Crystal Violet staining. Cells were stained 6 d after being equally plated, and $OD_{595}$ was measured on a spectrophotometer. Data are mean ± SEM. (*E*) Representative flow cytometric dot plots of CD44 and CD24 expression in MCF7 cultures over-\expressing Tbx3. Empty vector control-transduced cultures are shown for comparison.

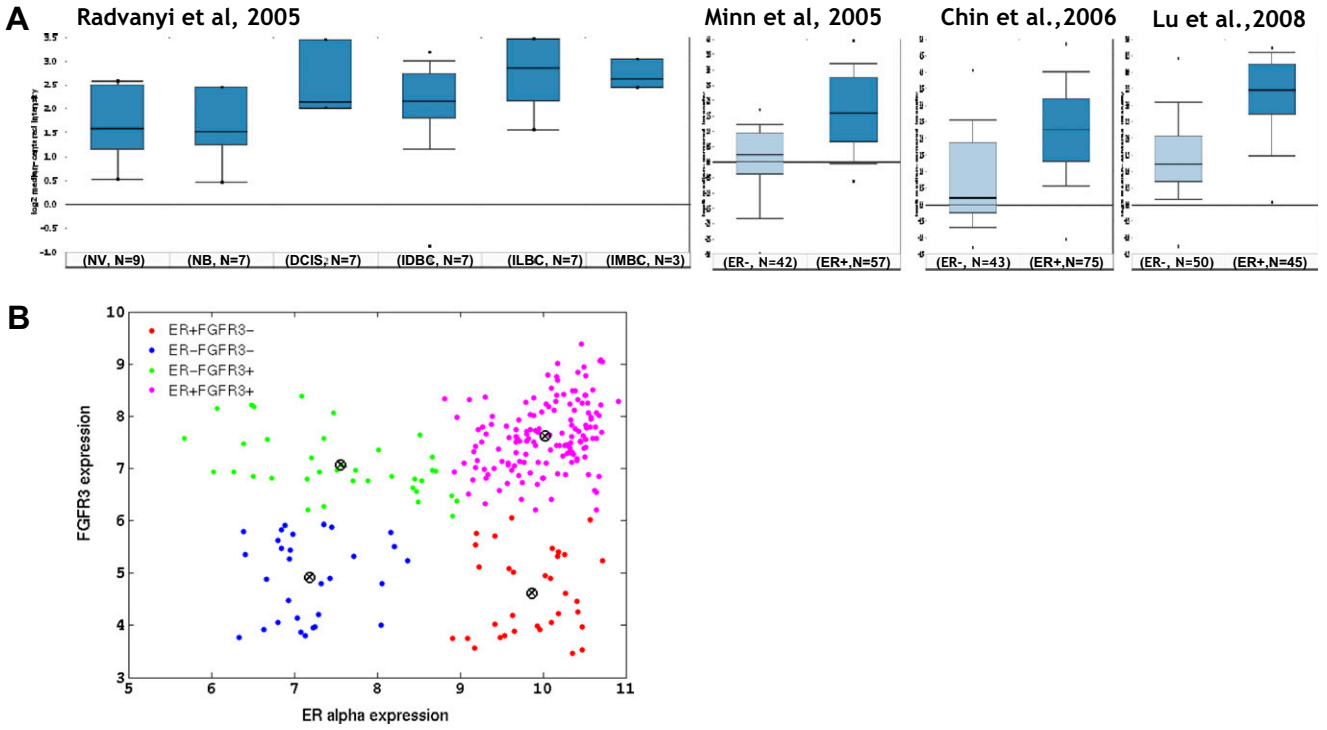


**Fig. S5.** TBX3 expression in human breast cancers. (*A*) Oncomine (Compendia Bioscience) was used for analysis and visualization of TBX3 expression in published microarray data sets. TBX3 overexpression was observed in many invasive breast cancer subtypes when compared with normal breast tissue, and correlates very highly with ER expression. DCIS, ductal breast carcinoma in situ; IDBC, invasive ductal breast carcinoma; ILBC, invasive lobular breast carcinoma; IMBC, invasive mixed breast carcinoma; NB, normal breast; NV, no value. (*B*) Gene expression correlation analysis in a data set shows that a large group of primary breast tumors coexpress high levels of ERα and FGFR3.

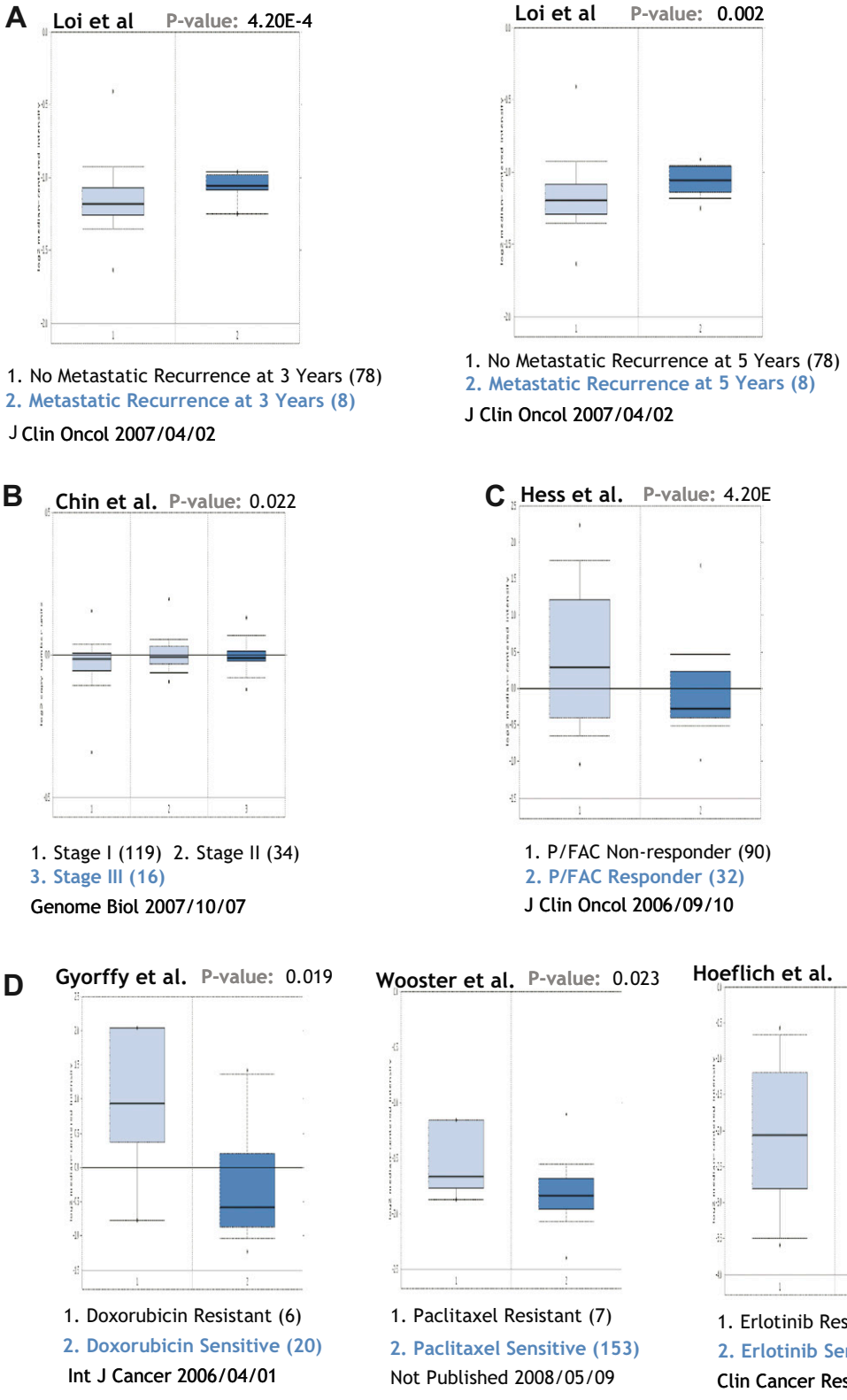


**Fig. S6.** TBX3 expression in tumors following response to therapy. Oncomine (Compendia Bioscience) was used for analysis and visualization of TBX3 expression in published microarray data sets grouped as follows: (*A*) metastasic recurrence at 3 and 5 y; (*B*) genomic amplification of Tbx3 locus and tumor stage; (*C*) pathological complete response to therapy; and (*D*) sensitivity of breast cancer cells to common breast cancer therapeutics in vitro.

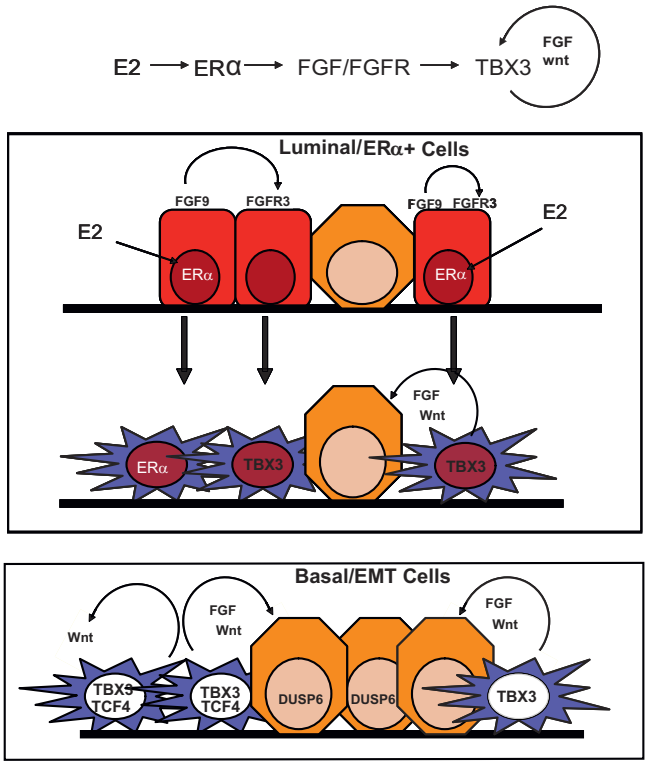


**Fig. S7.** Proposed model of paracrine signaling within breast cancer cell lines. In ERα$^{+}$ tumors, estrogen (E2) binds to the estrogen receptor in the non-CSC compartment to induce paracrine FGF9 secretion. FGF9 then binds to FGFR3 and induces Tbx3 expression. Expression of Tbx3 leads to further expression of Wnts and FGF/FGF signaling which promote CSC phenotypes. In ERα$^{-}$ tumors, it appears that FGFR/Tbx3 is active in the EMT-like cells, leading to stabilized paracrine signaling that regulates cancer stem cell (CSC) subpopulations.