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Supporting Information

14-3-3τ drives estrogen receptor loss via ERα36 induction and GATA3 inhibition in breast cancer

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SI Materials and Methods

Cell culture

MCF7, HEK293T, MDA-MB-468 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (50 IU/ml), streptomycin (50 μg/ml) in a humidified incubator with 5% CO_2 at 37°C. T47D cells were grown in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% FBS, penicillin (50 IU/ml), streptomycin (50 μg/ml) in a humidified incubator with 5% CO_2 at 37°C.

Immunofluorescence staining

Immunofluorescence analysis was performed as described previously (1). Cells were fixed with 4% paraformaldehyde for 10 min, followed by permeabilization with 0.5% Triton-X 100 in 1X phosphate-buffered saline (PBS) for 10 min. The fixed cells were then subjected to blocking with 2% BSA in 1X PBS for two hours, followed by incubation with ERα66 antibody (Santa Cruz #543) overnight and the Texas Red X- conjugated secondary antibody (Invitrogen, #T6391) for one hour. The fixed cells were then incubated with FITC-FLAG conjugated primary antibody (GeneScript #89493-648) for 2 hours. The nuclei were stained with Hoechst 33258. Images were captured on a Zeiss fluorescence microscope (Axio Observer Inverted Microscope) equipped with ApoTome 2 (Zeiss).

Mesenchymal stem cells

Mesenchymal stem cells were maintained at >70% confluency in Hyclone MEM alpha Modification media supplemented with 10% FBS and differentiated to cancer-associated fibroblasts (CAF) with 1 ng/mL recombinant human transforming growth factor beta 1 (TGF-β1,

Invivogen) added to media daily for 3 days. Differentiation was confirmed by alpha-smooth muscle actin (αSMA) induction via Western blot analysis.

Plasmid Construction

pQCXIP-14-3-3τ-FLAG and sh14-3-3τ constructs have been described previously (2). The doxycycline-inducible pXP-YFP and pXP-GATA3 were constructed using a lentiviral cDNA delivery system from Dr. Xuewen Pan (Novartis, Cambridge, MA). The expression vectors for ERα36 shRNAs were constructed using the pLKO.1-TRC cloning vector (Addgene) digested with AgeI and EcoRI (New England Biolabs). The target sequences are presented in Supplementary Table 1.

Virus production and stable cell line generation

Retrovirus was produced in HEK293T cells. Media was changed 24 hours after transfection. At 72 hours and 96 hours after transfection, the virus-containing media was collected and filtered with a 0.45 μm PVDF filter. Stable cell lines were generated by transducing with appropriate virus concurrently with the addition of 8 μg/ml Polybrene (Sigma). Seventy-two hours after transduction, 1 μg/mL puromycin (Gibco, for 14-3-3τ constructs) or 400 μg/mL G418 (VWR International, for GATA3 constructs) was added to select for stable cell.

Tamoxifen treatment

Continuous low-dose tamoxifen (CLD-TAM) treated MCF7 and T47D cells were established by culturing ERα66-positive cell line MCF7 or T47D in medium plus 1 μM tamoxifen over 80 days, which was maintained in respective medium continuously.

Luciferase reporter assay

The *ESR1* promoter specific to ERα36 isoform was cloned using PCR of genomic DNA from -738 to +22 surrounding the TSS. PCR primers contain a XhoI site 5' to the forward cloning primer and a HindIII site 5' to the reverse cloning primer. The following primer pairs were used: forward primer 5'- ACCCTCGAGGGTACCCGCGCCCGC-3' and reverse primer 5'- CCCAAGCTTGGAGATTATTTTTAAGC-3'. PCR product was digested with XhoI and HindIII (Promega) and then ligated together with pGL3-Basic backbone digested with XhoI and HindIII. Luciferase reporter assays were performed using the Promega Dual-Luciferase Reporter Assay System per manufacturer's guidelines. Cells were transiently transfected with 1.5 μg ERα36 promoter luciferase reporter and 0.5 μg cytomegalovirus (CMV)-β-galactosidase transfection control using PolyJet (SignaGen Laboratories). After treatments, cells were lysed in passive lysis buffer supplemented with protease inhibitors. Samples were then assayed for firefly luciferase signal, followed by β-galactosidase activity assay. Data was analyzed by normalizing individual sample firefly luciferase activity to its respective β-galactosidase activity.

Western Blotting

Cells were harvested with SDS lysis buffer (10 mM Tris-HCl, 2 mM EDTA, 1% SDS) and sonicated briefly. Cell lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with described antibodies.

GST-pulldown assay

Cells were harvested in NP-40 lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10% glycerol, and 1% NP-40) with protease inhibitor cocktails and sonicated briefly. To detect *in vitro* binding, glutathione *S*-transferase (GST)–14-3-3τ protein was purified from Escherichia coli and coupled to glutathione-Sepharose beads (GE Healthcare) as described before (3). Cell lysates were incubated with GST or GST–14-3-3τ beads overnight at 4°C and then washed three times with cold PBS. The specific signals were detected with appropriate antibodies.

Proteasome degradation

Cells were grown in serum-free media for 24 hours and then treated with 10 μM SC-79 for 24 hours. Eighteen hours after SC-79 treatment, cells were treated with 10 μM MG-132 (Calbiochem) and harvested 6 hours later with SDS lysis buffer.

Antibodies

The following antibodies were used for Western blotting, immunofluorescence, co-immunoprecipitation, and ChIP assays: E-cadherin (BD Biosciences #610181), ERα66 (Santa Cruz #543), GAPDH (Santa Cruz #32233), Actin (Sigma #A2066), GST (Santa Cruz #138), 14-3-3τ (Santa Cruz #732), GATA3 (Cell Signaling #D13C9), GATA3pS308 (Invitrogen #PA5-104846), ESR1 antibody used to detect ERα36 (Rockland #600-401-BB9), FLAG (Sigma #F3165), IgG rabbit (Thermo), and αSMA (Sigma #A2547).

RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

RNA extraction was performed using the standard trizol method (Invitrogen) and qPCR was performed on a MX3000P thermal cycler with SYBR green and ROX reference dye. All primer pairs are described in Supplementary Table 2.

Colony Formation Assay

Six-well plates were seeded with $5x10^4$ either unconditioned or CLD-TAM conditioned cells in triplicate on Day 0. Starting Day 1, wells were treated daily with 5 μM TAM and media changed every other day until Day 14. The plates were gently washed with PBS, fixed with 37% formaldehyde for 45 minutes, stained with crystal violet for 1 hour, washed with distilled water five times, and allowed to dry overnight. The resulting colonies were counted and representative wells imaged.

Statistical Analysis

The Pearson correlation coefficient was calculated to evaluate correlations. The two-tailed probability (*P* values) for each Pearson correlation coefficient was calculated between two experimental groups. Two-tailed *t*-test was also used to analyze significant changes between experimental groups. *P* values of less than 0.05 were considered statistically significant and denoted by *. *P* values of less than 0.01 were denoted by **; *P* values < 0.001 were denoted by ***. RNA-Seq and RPPA data in TCGA database and RNA-Seq data in METABRIC breast cancer dataset were extracted from cBioportal server (https://www.cbioportal.org/) and GEPIA2 server (http://gepia2.cancer-pku.cn/#index) (4). Clinical data in the METABRIC breast cancer dataset were extracted from https://www.synapse.org/ server. Kaplan-Meier curves were generated using the R program or in KM Plotter. GSE27473 microarray dataset were extracted from NCBI GEO

server. Pan cancer data in TCGA database was extracted and analyzed using TNMplot (https://tnmplot.com/analysis/) (5).

Fig. S1

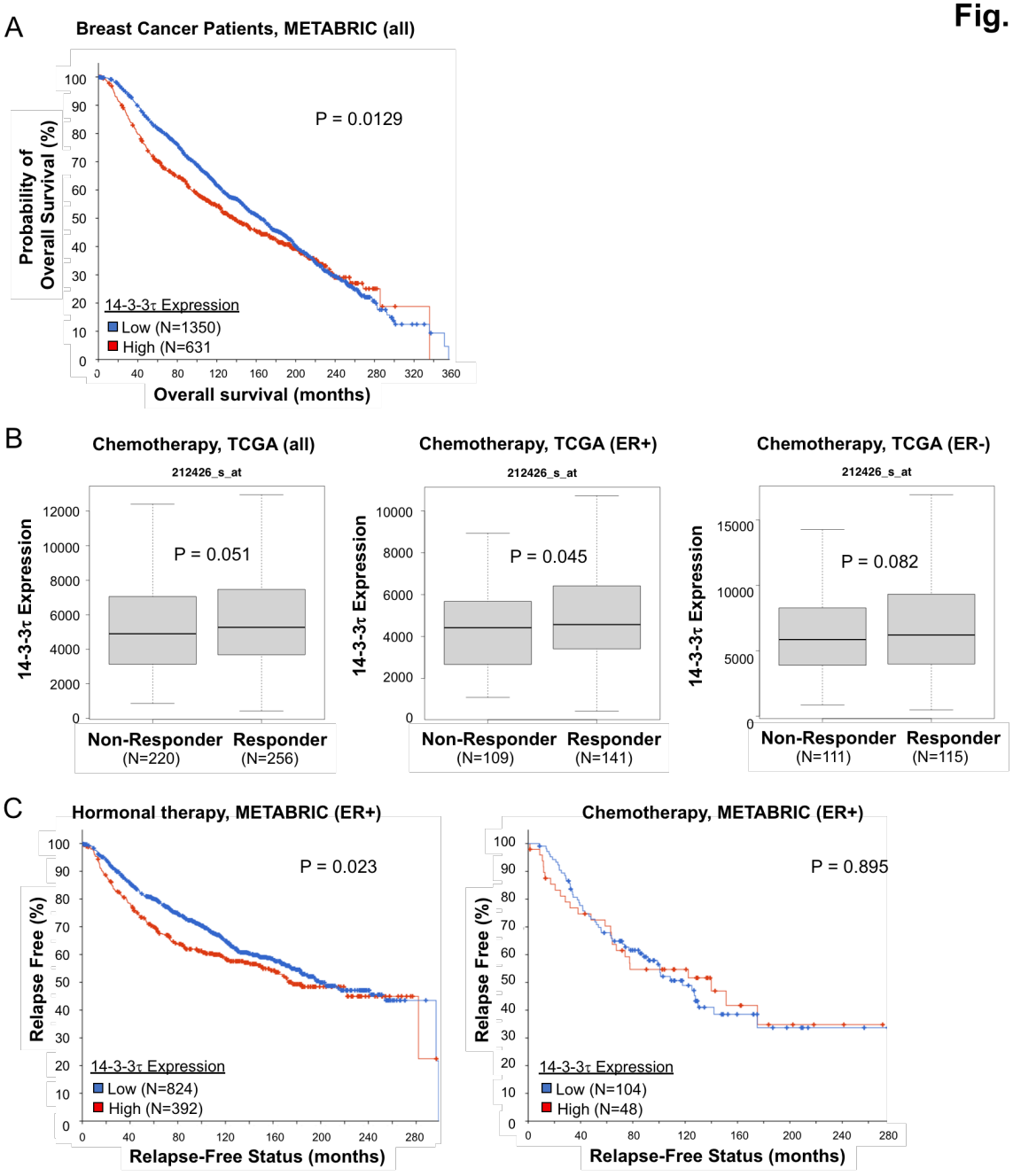


Figure S1.

A) METABRIC overall survival analysis showing breast cancer patients with high 14-3-3τ expression have shorter overall survival. Patients were split by upper tertile of 14-3-3τ expression in their breast cancers.

B) The responders to chemotherapy in TCGA breast cancer cohort have marginally higher levels of 14-3-3τ expression.

C) High 14-3-3τ expression (red) only significantly impacts relapse-free survival in METABRIC ER+ patients treated with endocrine therapy, but not with chemotherapy. Patients were split by upper tertile of 14-3-3τ expression in their breast cancers.

Fig. S2

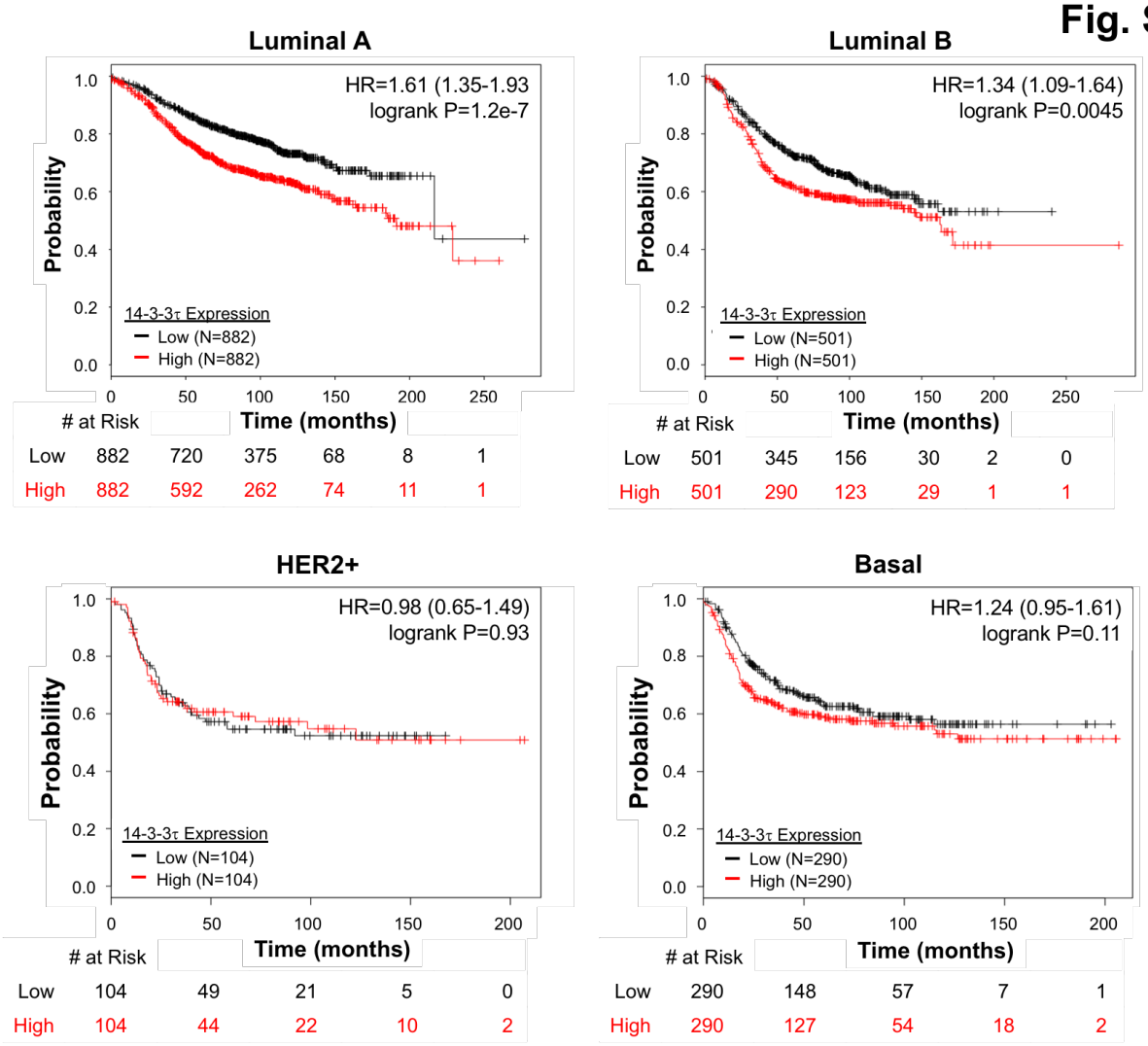


Figure S2. High 14-3-3τ expression is associated with decreased survival in ER+ (luminal A and luminal B) breast cancer but not in HER2 or basal-like breast cancers.

Relapse-free survival of breast cancer patients with low (black lines) or high (red lines) 14-3-3τ expression in primary tumors segregated based on molecular subtype. The effect of 14-3-3τ on the risk of relapse is highest in luminal A (Hazard ratio, HR 1.61), followed by luminal B (HR 1.34). The data are analyzed in an online meta-analysis server Kaplan-Meier Plotter (6) from a total of 3554 breast cancer patients. Patients were equally separated into two groups based on the expression of 14-3-3τ in their breast cancers. Most Luminal A and Luminal B breast cancer patients were treated with hormonal therapy. HR: hazard ratio.

Fig. S3

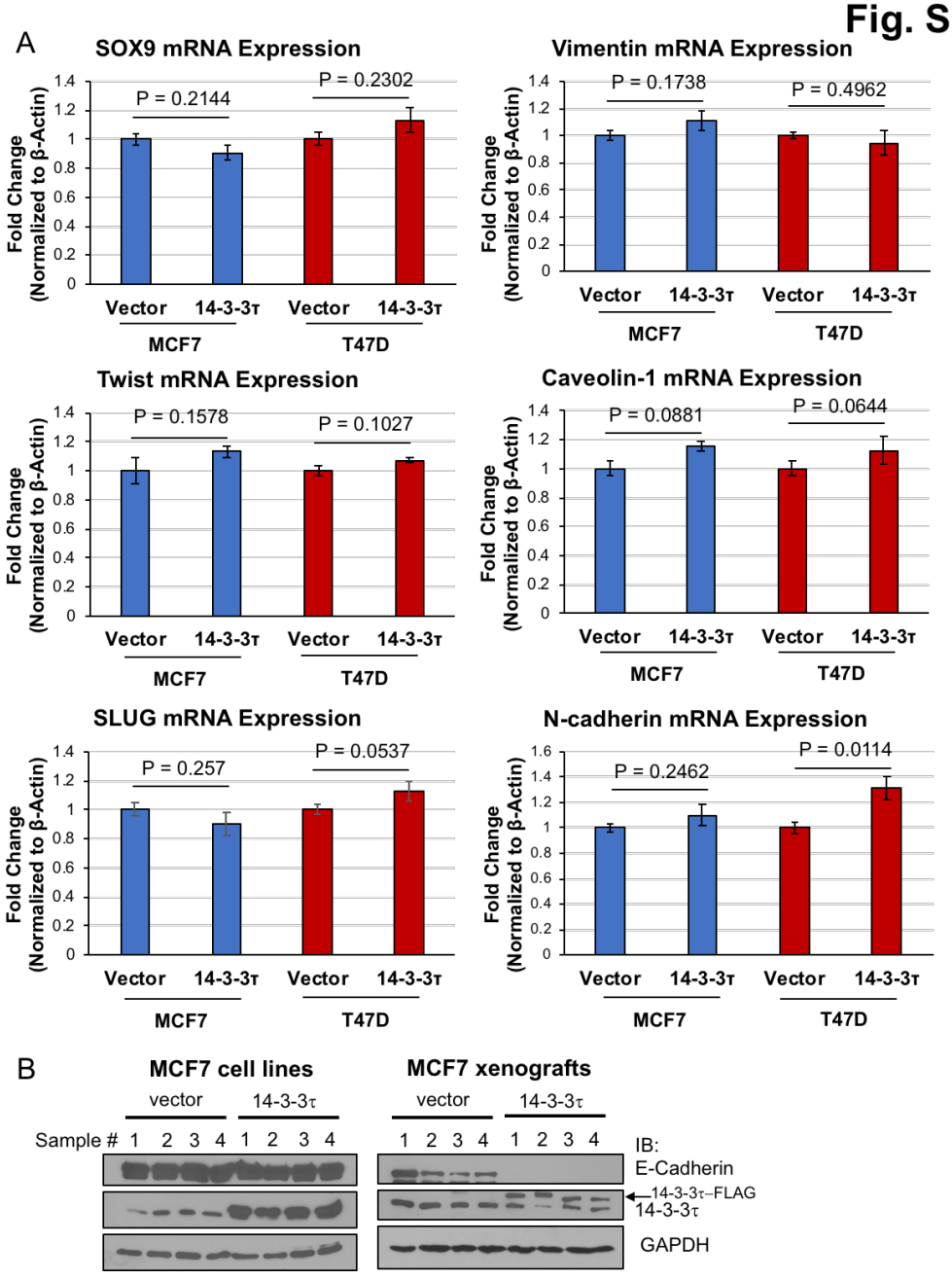


Figure S3. EMT marker analysis in 14-3-3τ-overexpressing cell lines and xenografts.

A) There is no pattern or significant difference in mRNA expression of EMT markers (SOX9, Vimentin, Twist, Caveolin-1, SLUG, N-cadherin) between MCF7- and T47D- vector and 14-3-3τ-overexpressing cell lines as analyzed by RT-qPCR. Shown are mean ± SD (n=3 technical replicates).

B) Western blot analysis demonstrates no change in E-cadherin protein expression between vector control and 14-3-3τ-overexpressing MCF7 cells (n=4 biological replicates per cell line). On the contrary, 14-3-3τ-overexpressing MCF7 xenografts completely lost the expression of E-cadherin.

Fig. S4

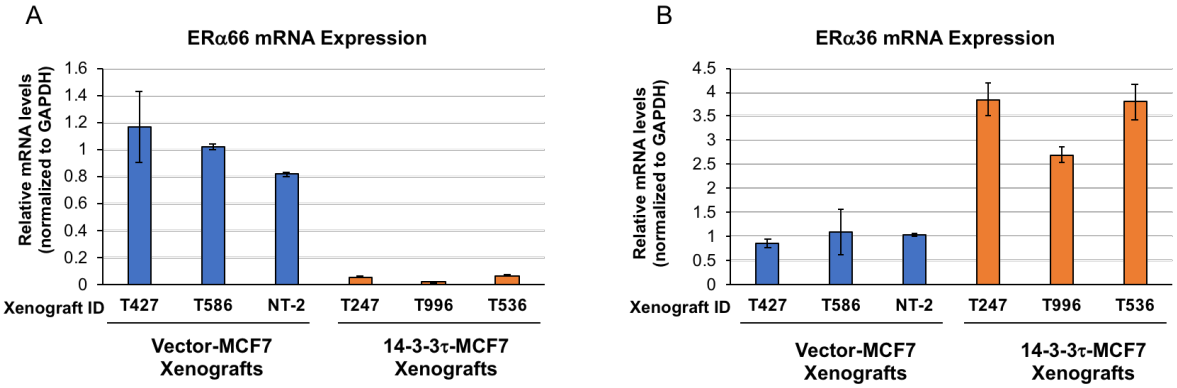
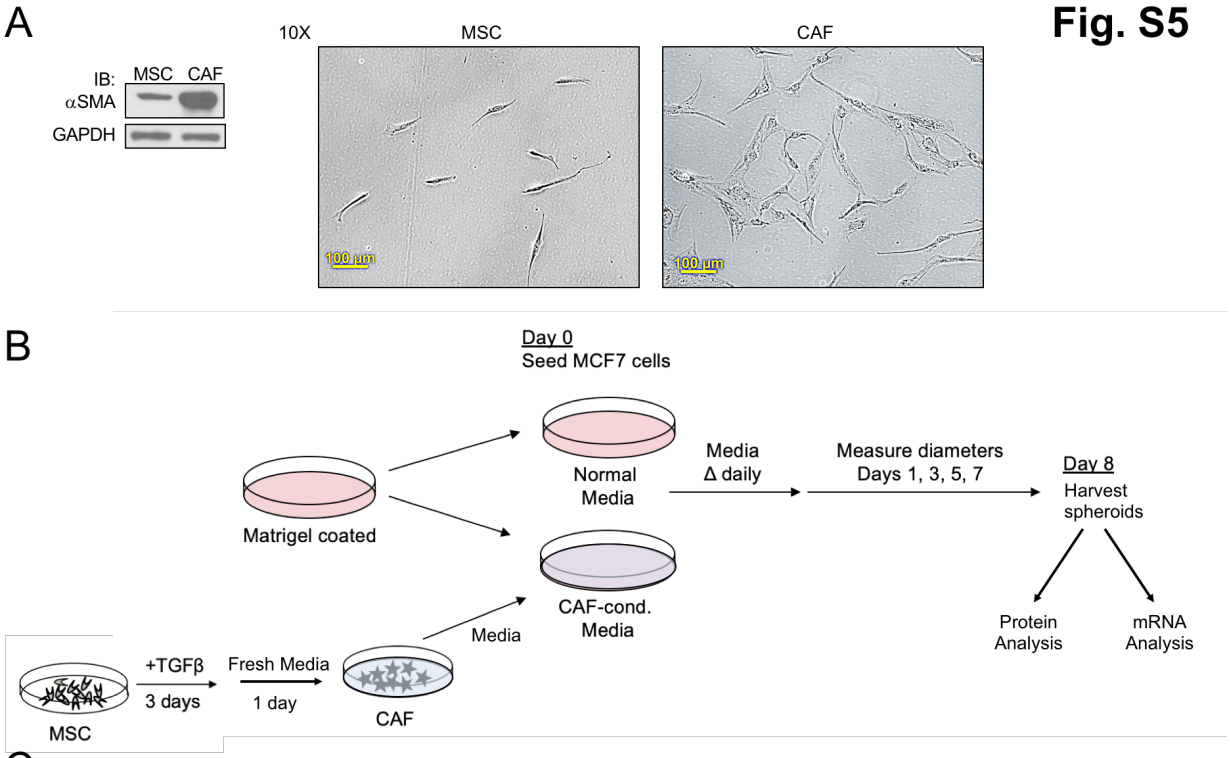


Figure S4. mRNA analyses of individual xenografts.
RT-qPCR of ERα66 and ERα36 in each xenograft. Shown are mean $\pm$ SD (n=3 technical replicates). Xenograft ID denotes each individual xenograft from the experiments described previously (2).



C

MCF7-NM (all n=103)		Day 1	Day 3	Day 5	Day 7
Vector	Average	20.65202	44.85644	57.1174	91.84856
	SD	1.562787	10.21203	11.34561	17.71607
14-3-3τ	Average	20.95519	48.24031	61.5149	164.3794
	SD	2.007883	8.823325	10.77502	28.28916
shScr	Average	19.4051	45.74456	52.90398	96.57117
	SD	1.52436	8.361239	8.994422	14.08103
sh14-3-3τ#1	Average	19.4051	43.41619	45.1579612	85.06952
	SD	1.52436	7.163723	7.75377357	10.55236
sh14-3-3τ#2	Average	19.41471	43.57155	49.41961	86.0005
	SD	1.916142	6.593583	7.983586	10.96011

MCF7-CCM (all n=103)		Day 1	Day 3	Day 5	Day 7
Vector	Average	20.13115	62.46481	88.64029	121.4098
	SD	1.440675	9.644939	16.95726	16.53388
14-3-3τ	Average	20.76125	71.55067	119.7824	180.4091
	SD	1.885296	17.33347	26.73541	28.52981
shScr	Average	20.1574	53.07913	74.44466	119.8172
	SD	2.269496	9.996342	12.85749	15.04166
sh14-3-3τ#1	Average	20.2575	49.8267	69.15301	102.831
	SD	1.699703	7.327564	12.84972	12.87823
sh14-3-3τ#2	Average	19.81058	48.80825	64.56184	98.57783
	SD	1.597761	8.8923	13.0654	10.76447

Figure S5. Spheroid model of 14-3-3τ-driven ERα66 loss and EMT induction using CAF-conditioned media.

A) Mesenchymal stem cells (MSC) were differentiated to cancer-associated fibroblast (CAF) by TGFβ treatment. Differentiation was validated by Western blot analysis of αSMA protein induction and morphological changes characteristic of fibroblasts.

B) Schematic of methodology for spheroid growth in normal media (NM) or CAF-conditioned media (CCM).

C) Summary table of average spheroid diameter by day (n=103). Left table is normal media (NM, black) average spheroid diameter and standard deviation, right table is CAF-conditioned media (CCM, gray).

Fig. S6

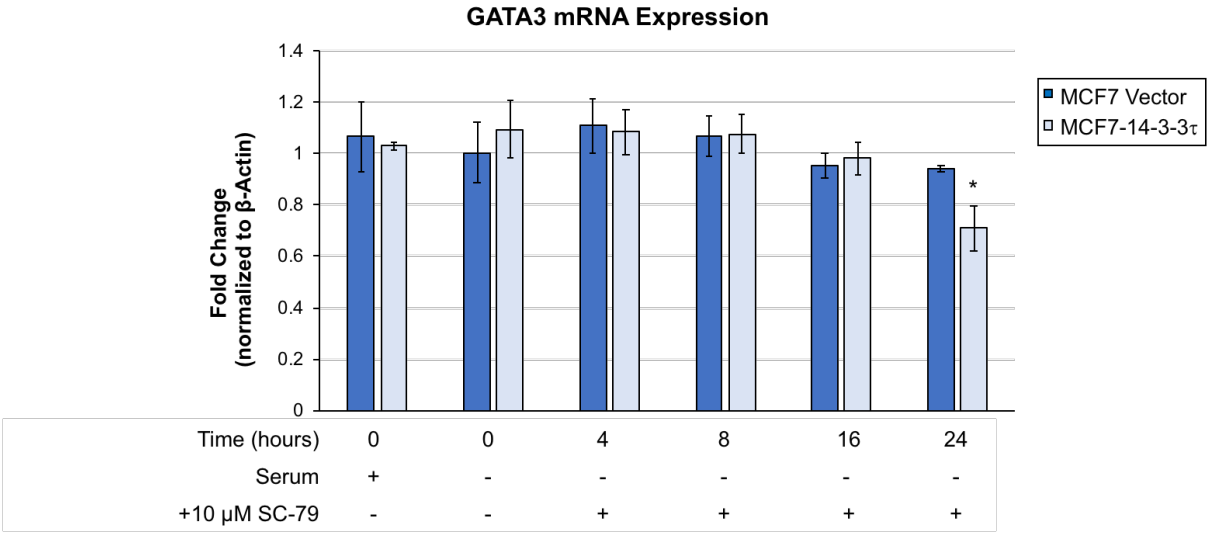


Figure S6. RT-qPCR analysis of MCF7 vector and MCF7-14-3-3τ + SC-79 timecourse. There is a significant reduction of GATA3 transcript in MCF7-14-3-3τ cells at 24 hours after SC-79 treatment. Data represent mean ± SD, (n=3 technical replicates); **P*<0.05, (two-tailed *t* test).

MCF7-14-3-3τ NM		Day 1	Day 3	Day 5	Day 7
shScr	**Average**	**20.43337**	**49.82837**	**64.27433**	**161.9161**
	SD	2.076836	7.857509	9.756186	24.58351
shERα36#1	**Average**	**19.42067**	**44.48417**	**51.28427**	**99.66152**
	SD	1.505185	5.496481	7.3723	12.14699
shERα36#2	**Average**	**19.36673**	**46.05223**	**53.4132**	**102.4822**
	SD	1.876936	5.495498	7.362252	12.14214

MCF7-14-3-3τ CCM		Day 1	Day 3	Day 5	Day 7
shScr	**Average**	**20.82721**	**75.64721**	**116.1449**	**183.9051**
	SD	1.847193	14.38955	19.7415	25.09211
shERα36#1	**Average**	**20.14856**	**52.92126**	**75.38923**	**121.4924**
	SD	1.410684	9.967237	11.03059	13.42058
shERα36#2	**Average**	**19.78173**	**50.11845**	**74.01558**	**120.072**
	SD	1.619247	10.08992	11.03944	13.44223

Figure S7. Summary of average spheroid diameter by day.
Left table is normal media (NM, black) average spheroid diameter and standard deviation, right table is CAF-conditioned media (CCM, gray).

Supplementary Tables:

Supplementary Table 1. Target nucleotide sequences in ERα36-specific exon for shERα36

	Sense	Antisense
shERα36-1	CTCACATGTAGAAGCAAAGAA	TTCTTTGCTTCTACATGTGAG
shERα36-2	ATGTAGAAGCAAAGAAGAGAA	TTCTCTTCTTTGCTTCTACAT

Supplementary Table 2. Primer pairs for RT-qPCR (5' to 3')

	Forward Primer	Reverse Primer
ERα66	CACTCAACAGCGTGTCTCCGA	CCAATCTTTCTCTGCCACCCTG
ERα36	CAAGTGGTTTCCTCGTGTCTA	TGTTGAGTGTTGGTTGCCAGG
β-Actin	CACCAACTGGGACGACAT	ACAGCCTGGATAGCAACG
Vimentin	ACATGCTGTTCCTGAATCTGAG	GTCTTGACCTTGAACGCAAAG
GATA3	ACCACAACCACACTCTGGAGGA	TCGGTTTCTGGTCTGGATGCCT
SOX9	AGGAAGCTCGCGGACCAGTAC	GGTGGTCCTTCTTGTGCTGCAC
Twist	CCAGGTACATCGACTTCCTCT	TCCATCCTCCAGACCGAGAAGG
Caveolin-1	TTCTCTTTGCCACCCTCAGCTG	GAAGCATCGTCCTACGCTCGTA
Claudin-7	GGAGACGACAAAGTGAAGAAGGC	AAAAGTCTGTGACAATCTGATGGC
SLUG	ATCTGCGGCAAGGCGTTTTCCA	GAGCCCTCAGATTTGACCTGTC

Supplementary Table 3. ChIP Primer Pairs for Gene Promoter Regions (5' to 3')

	Forward Primer	Reverse Primer

ERα66	CCGCCCCATTCTACCATTCT	TCCTTCCATAAACTGTCCGGT
ERα36	CAGGCAAATAAACACGGGGC	CTCTAAAGCCCCAGGACGTG
Gene Desert	TGAGCATTCCAGTGATTTATTG	AAGCAGGTAAAGGTCCATATTTC

SI References:

1. Ho SR, Mahanic CS, Lee YJ, & Lin WC (2014) RNF144A, an E3 ubiquitin ligase for DNA-PKcs, promotes apoptosis during DNA damage. *Proc Natl Acad Sci U S A* 111(26):E2646-2655.
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4. Tang Z, Kang B, Li C, Chen T, & Zhang Z (2019) GEPIA2: an enhanced web server for large-scale expression profiling and interactive analysis. *Nucleic Acids Res* 47(W1):W556-W560.
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