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Molecular Cell Biology

Cancer Research

DAXX Suppresses Tumor-Initiating Cells in Estrogen Receptor–Positive Breast Cancer Following Endocrine Therapy

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Abstract

Estrogen receptor (ER)-positive breast cancer recurrence is thought to be driven by tumor-initiating cells (TIC). TICs are enriched by endocrine therapy through NOTCH signaling. Side effects have limited clinical trial testing of NOTCH-targeted therapies. Death-associated factor 6 (DAXX) is a newly identified marker whose RNA expression inversely correlates with NOTCH in human ER$^+$ breast tumor samples. In this study, knockdown and overexpression approaches were used to investigate the role of DAXX on stem/pluripotent gene expression, TIC survival *in vitro*, and TIC frequency *in vivo*, and the mechanism by which DAXX suppresses TICs in ER$^+$ breast cancer. 17β-Estradiol (E_2)-mediated ER activation stabilized the DAXX protein, which was required for repressing stem/pluripotent genes (*NOTCH4, SOX2, OCT4, NANOG*, and *ALDH1A1*), and TICs *in vitro* and *in vivo*. Conversely, endocrine therapy promoted rapid protein depletion due to increased proteasome activity. DAXX was enriched at promoters of stem/pluripotent genes, which was lost with endocrine therapy. Ectopic expression of DAXX decreased stem/pluripotent gene transcripts to levels similar to E_2 treatment. DAXX-mediated repression of stem/pluripotent genes and suppression of TICs was dependent on DNMT1. DAXX or DNMT1 was necessary to inhibit methylation of CpGs within the SOX2 promoter and moderately within the gene body of NOTCH4, NOTCH activation, and TIC survival. E_2-mediated stabilization of DAXX was necessary and sufficient to repress stem/pluripotent genes by recruiting DNMT1 to methylate some promoters and suppress TICs. These findings suggest that a combination of endocrine therapy and DAXX-stabilizing agents may inhibit ER$^+$ tumor recurrence.

Significance: Estradiol-mediated stabilization of DAXX is necessary and sufficient to repress genes associated with stemness, suggesting that the combination of endocrine therapy and DAXX-stabilizing agents may inhibit tumor recurrence in ER$^+$ breast cancer.

Introduction

Recurrent breast cancer during or following endocrine therapy may arise from a small population of cells referred to as tumor-initiating cells (TIC; refs. 1, 2). These TICs could be responsible for tumor recurrence during endocrine therapy due to stem-like properties. These properties include their small proportion in number, ability to self-renew and generate a heterogeneous tumor, and intrinsic resistance to conventional and targeted anticancer therapies, including endocrine therapy (3). Thus, anti-ER therapy effectively target the majority of a breast cancer, but the persisting TICs remain and are able to generate a more aggressive, endocrine therapy–resistant cancer (4). NOTCH signaling has been reported to promote survival and self-renewal of both normal mammary stem cells and TICs (5–8).

Overexpression of the active form of NOTCH in the mouse mammary gland promotes metastatic breast cancer (9, 10). Furthermore, endocrine therapy increases NOTCH signaling in ER$^+$ breast cancer (11), and this activation is required for TIC survival (4, 7). NOTCH receptors (NOTCH 1, 2, 3, 4) are expressed as heterodimeric proteins at the cell surface. Activation is initiated upon interaction with their ligands (Delta-like1, 3, 4 and Jagged1, 2) on adjacent cells. Ligand–receptor engagement triggers endocytosis of the extracellular NOTCH domain into the ligand-expressing cell (12–14). The remaining transmembrane domain is cleaved by the metalloproteinase ADAM10 or 17 to form the NOTCH external truncation (NEXT). Finally, the gamma (γ)-secretase complex cleaves NEXT releasing the NOTCH intracellular domain allowing for nuclear translocation and regulation of gene transcription (15). This last process can be inhibited by a γ-secretase inhibitor (GSI), inhibiting NOTCH signaling in breast cancer, and resulting in a reduction of TIC survival, tumorigenesis, and tumor recurrence *in vitro* and *in vivo* (7, 16, 17). Numerous GSIs have been developed and clinical trials are ongoing (17). GSI

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therapy has yet to result in clinical approval for the treatment of breast cancer. This is, in part, due to gastrointestinal toxicity and skin cancer development (18). Thus, there is a clinical need for a novel target and therapy that is able to inhibit TIC survival and frequency in ER^+ breast cancer and avoid toxicity.

To identify new therapeutic NOTCH biomarkers and targets, Albain and colleagues completed a biomarker presurgical window trial (ClinTrials.gov Identifier: NCT00756717; ref. 19). Death domain–associated protein 6 (DAXX) was identified as a novel NOTCH target with potential clinical significance in ER^+ breast cancer (manuscript in preparation). Its transcript expression was significantly upregulated in human breast cancers treated with endocrine therapy after a short exposure to a GSI. As NOTCH is required for TIC survival, and inhibited by GSI, we hypothesized that increased DAXX expression may downregulate TIC survival. We tested this by determining whether DAXX was necessary and/or sufficient to restrict TIC survival using ER^+ breast cancer cells: MCF7 (wild-type p53) and T47D (mutant p53) *in vitro* and *in vivo* and investigated mechanisms by which DAXX regulates TIC survival.

Materials and Methods

Cell culture

MCF7, T47D, BT474, MDA-MB-231, and MDA-MB-468 cells were purchased from ATCC. The BCM-5097 ER^+ PDX tumor was purchased from Dr. Michael Lewis (Baylor College of Medicine, Houston, TX). All cell lines were authenticated December 2018 by short tandem repeat allelic profiling (ATCC) and maintained at a low passage number (below 20 passages/cell line). Maintenance of cells in appropriate medium is provided in Supplementary Materials and Methods.

Chemicals

The 17β-estradiol (E_2; Sigma Aldrich, catalog no. E8875), fulvestrant (Selleck Chemicals), cycloheximide (gift from Dr. Charles Hemenway, Loyola University Chicago, Chicago, IL), MG132 (Sigma-Aldrich catalog no. M8699), 5-azacytidine (5-AZA) (Sigma Aldrich) were suspended in 100% ethanol or dimethyl sulfoxide (DMSO) to form stocks solution, stored in the dark, and maintained in −20°C. The stock solutions were diluted 1:1,000 vol/vol in growth medium to form working concentrations of 5 nmol/L E_2, 100 nmol/L fulvestrant, 10 μmol/L cycloheximide, 10 μmol/L MG132, and 10 μmol/L 5-AZA.

RNA interference and transfection reagents

A pool of four DAXX siRNAs were used to knockdown DAXX expression *in vitro* (Dharmacon GE Life Sciences). Nontargeting scrambled control siRNA (SCBi) was purchased from Qiagen. DNMT1 siRNA was purchased from Origene (catalog no. SR301244). The transfection reagent Lipofectamine RNAiMAX (catalog no. 13778150) was purchased from Thermo Fisher Scientific and used at a ratio of 1:1 ratio with 10 nmol/L of appropriate siRNA according to the manufacturer's protocol. Cells were incubated in transfection medium for 48 hours.

DAXX overexpression by transfection

A mammalian expression vector, pCMV6-entry, containing a human DAXX cDNA was purchased from Origene and used to transiently overexpress DAXX in cell lines.

Western blot analysis

The Western blot protocol is described in detail in the Supplementary Materials and Methods. The primary antibodies DAXX (1:1,000, Cell Signaling Technology), β-actin (1:2,000, Sigma Aldrich), NOTCH4 (1:1,000, Santa Cruz Biotechnology), DNMT1 (1:1,000, Santa Cruz Biotechnology), PARP-1 (1:1,000, Santa Cruz Biotechnology), ERα (1:1,000, Cell Signaling Technology) were diluted in 5% milk or 20% Roche and added to the membrane and incubated overnight at 4°C with constant agitation.

Real-time PCR

MCF7 and T47D cells were exposed to specified growth conditions, following which, total RNA was extracted according to the manufacturer's protocol using the RiboPure RNA Purification Kit (Ambion, catalog no. AM1924). RNA yield was determined using a NanoDrop Spectrophotometer (Thermo Fisher Scientific). RNA was reverse transcribed to cDNA using a reverse transcriptase enzyme and kit according to the manufacturer's instructions (Multiscribe Reverse Transcriptase Kit, Applied Biosystems, catalog no. N8080234). The reaction was performed 25°C for 10 minutes, 48°C for 30 minutes, 95°C for 5 minutes, and 25°C for 60 minutes. Real-time PCR was performed using iTaq SYBR Green Supermix (Bio-Rad) to detect transcript levels. The PCR conditions were: 10 minutes at 95°C, 40 cycles of 10 second at 95°C, then 45 seconds at 60°C. Following this, a melt curve was conducted for 40 cycles as a control as outlined by the manufacturer of the StepOnePlus Real-time PCR machine (Applied Biosystems). Hypoxanthine–guanine phosphoribosyltransferase (HPRT) was used as a loading control to normalize C_t expression values for each gene transcript. Relative fold-change in transcript expression between each sample was calculated using the $2^{(-\Delta\Delta C_t)}$ method as outlined: ΔC_t Experimental = (C_t value of experimental gene of experimental group-C_t value of HPRT of experimental group), ΔC_t control = (C_t value of experimental gene of control group-C_t value of HPRT of control group), $\Delta\Delta C_t$ = (ΔC_t experimental group-ΔC_t control group), relative quantity (RQ) = $2^{(-\Delta\Delta C_t)}$. Primer sequences are listed in Supplementary Table S1A.

Cycloheximide chase assay

MCF7 cells were grown in 0 or 5 nmol/L E_2 for 24 hours followed by addition of 10 μmol/L CHX in the presence or absence of 10 μmol/L MG132. Cells were incubated for their specified times (0–20 hours) and total protein was extracted and detected as described in the Western blot analysis methods.

Bulk cell proliferation

MCF7 or T47D cells at a density of 1×10^5 cells were plated in separate wells of a 6-well plate for 24 hours. Cells were then washed with PBS 2×, and specified growth medium was added containing 0 or 5 nmol/L E_2. The growth medium was changed daily up to 7 days. Cells were trypsinized and total viable cells were counted using a Countess Cell Counter. Fold increase in live cell number was calculated by dividing the final viable cells at day 7 by the number of cells initially plated at day 0.

Cell-cycle analysis

MCF7 or T47D cells at a density of 1×10^5 cells were plated in separate wells of a 6-well plate for 24 hours. Cells were washed with PBS 2× and specified growth medium was added to each

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well. Growth medium was changed daily up to 7 days. Cells were fixed in 100% ethanol and stained with propidium iodide. Cell-cycle analysis was conducted using flow cytometry according to the manufacturer's instructions (Cell Signaling Technology).

Mammosphere-forming assay

The protocol used was adapted from Shaw and colleagues (20). Briefly, DMEM-F12 medium (Gibco, catalog no. 11039021) was heated to 60°C and 2 g of methylcellulose was added to the solution. The contents were then continuously stirred at 60°C for 2.5 hours until the methylcellulose was uniformly dissolved. The medium was then stirred overnight at 4°C. The next day, 4 mL of B-27 supplement and 4 μL of recombinant human EGF (hEGF, Sigma Aldrich, catalog no. E-9644) was added and the solution was stirred for 30 minutes at 4°C. The medium was then transferred to centrifuge tubes and centrifuged at 9,500 rpm in a Beckman rotor at 4°C for 30 minutes to remove any precipitate. The solution (mammosphere medium) was transferred into 50 mL conical tubes and stored at −20°C until ready for use. Images were then taken after 7 days and transferred to Powerpoint and the mammospheres for each field were counted based on the scale of the image using a size cut off of 50 μm. The mammosphere-forming efficiency (MFE) was calculated using the following equation: %MFE = [(total number of mammospheres counted) × (dilution factor)]/(50,000 cells) × 100. Detailed protocol is provided in Supplementary Materials and Methods.

Tumor-initiating potential

The protocol for this animal study was approved by Loyola University Chicago's Institutional Animal Care and Use Committee. MCF7 cells were transfected with SCBi or DAXXi siRNA for 2 days. Cells were injected into the mammary fat pad of female, ovariectomized *FoxN1* nu/nu athymic nude mice (Harlan Sprague-Dawley) with 5 animals/cell dilution. Cells were injected at varying dilutions including 10,000, 1×10^5 or 1×10^6 cells/animal. Animals were also implanted with a silastic-release capsule containing E_2, allowing for a slow, sustained release of E_2 that mimics peri/postmenopausal levels (83.8 pg/mL) for up to 8 weeks (21). Cells were implanted into nude mice at a 1:1 ratio with Matrigel (Corning). After 8 weeks, the number of mice that developed tumors designated as having a tumor area greater than or equal to 40 mm^2 were counted as tumor-bearing mice. The estimated frequency of TICs based on the number of tumors that formed at each cell dilution was determined using extreme limiting dilution analysis (ELDA) software from Walter Eliza Hall Institute of Medical Research (http://bioinf.wehi.edu.au/software/elda/).

Cellular fractionation

Cells were grown in their specified experimental conditions for 3 days, trypsinized, and the protein within each cell compartment (cytoplasmic or nuclear) was isolated using the SubCellular Protein Fractionation Kit (Thermo Fisher Scientific) using the manufacturer's protocol. Protein levels were then visualized by Western blotting using actin as a cytoplasmic control and PARP-1 as a nuclear control.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were conducted using SimpleChIP Plus Kit (Cell Signaling Technology, catalog no. 9005). Briefly, cells were trypsinized, cross-linked with a 1% formaldehyde solution (Sigma Aldrich, catalog no. F8775) for 30 minutes and then quenched with 1.25 mol/L glycine followed by sonication using the Dismembrator (Model 100, Thermo Fisher Scientific). Samples for sonicated for 20 seconds at 30% power, placed on ice for 1 minutes, and resonicated six times. Chromatin was isolated following the manufacturer's protocol. Isolated chromatin was incubated with 2 μg of a DAXX-specific antibody (S-20, Santa Cruz Biotechnology) or 2 μg of a nonspecific rabbit IgG overnight at 4°C. Following DNA fragment isolation, quantitative PCR was performed using immunoprecipitated DNA with primers designed to flank promoter regions at AP-1 consensus sequences of selected genes. Primer sequences are provided in Supplementary Table S1B.

Bisulfite sequencing and CpG methylation status

MCF7 cells were transfected with siRNA (SCBi, DAXXi, or DNMT1i) and plated in growth conditions (0 or 5 nmol/L E_2) for 24 hours. DNA was isolated from cells using the Qiagen DNeasy Blood and Tissue Kit (Qiagen). DNA was converted to bisulfite-treated DNA using the EZ DNA Methylation-Gold Kit (Zymo Research) following the manufacturer's protocol. DNA was then amplified with primers specific to CpG islands within the SOX2 promoter or within intron 29 and exon 29 of the *NOTCH4* gene (SOX2: forward: 5′-AAAGATTTTAATAAGAGAGTGGAAGGAA-3′, reverse: 5′-CCAAAAACCCAAAAAAATAATTTTAAC-3′; NOTCH4: forward: 5′-TTTGGTTTTTAATTGGGGTAATAATT-'3, reverse: 5′TAACCCCTATCCCTTCAAACTTTA'3) under the following reaction conditions: 95°C for 30 seconds, 35 cycles (95°C for 20 seconds, 54°C for 45 seconds, 68°C for 30 seconds), 68°C for 5 minutes, using the EpiMark Hot Start Taw DNA Polymerase enzyme kit (New England BioLabs). The PCR product was then cleaned and purified using the UltraClean PCR Clean-Up Kit (Mo Bio Laboratories) and sequenced by Sanger Sequencing by ACGT Inc.

Kaplan-Meier plotter analysis

KMplot.com is a publicly available website with software that has compiled the RNA expression of human breast cancer based on tissue microarray and associated clinical outcomes such as recurrence-free survival (RFS; refs. 22, 23). RFS of patients with ER^+ breast cancer was interrogated on the basis of high versus low DAXX RNA expression. Median or quartile transcript expression was analyzed. From this database, RFS rates of patients with ER^+ breast cancer using parameters offered by the software were obtained. The parameters used to generate each survival curve and specific probe beeswarm plot are provided in Supplementary Table S1C.

Patient-derived xenografts

The protocol for this animal study was approved by Loyola University Chicago's Institutional Animal Care and Use Committee. The ER^+ PDX BCM-5097 was derived from a Caucasian female posttreatment with metastatic disease. It is ER and progesterone receptor (PR) positive and negative for overexpression of human epidermal growth receptor 2 (HER2; ref. 24). This tumor was passaged in female, ovariectomized NOD/SCID mice implanted with an E_2-containing capsule for two passages. Tumor bits (0.2 mm^2) were implanted into ovariectomized *FoxN1* nu/nu athymic nude mice-containing an E_2 capsule. After 8 weeks, a new capsule was implanted to a subset of animals, while the remaining animals were maintained on E_2-deprived conditions for 19 days.

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Tumors were then removed and total protein was isolated. ERα, DAXX, and NOTCH4 protein levels were detected by Western blot analysis as described previously.

Coexpression analysis of METABRIC DATA for DAXX

The Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) cohort has 1,980 women with primary, stages I and II invasive breast cancers (25). There were 1,518 patients with ER^+ breast cancer including node-negative, node-positive, $HER2^-$, and $HER2^+$ disease. Of the patients with ER^+ breast cancer, 1,088 received adjuvant hormonal therapy. A total of 430 patients received no adjuvant therapy. The METABRIC cohort was interrogated for coexpression of DAXX and TIC/pluripotent RNAs (ALDH1A1) in ER^+ breast cancer using the Breast Cancer Integrative Platform (http://www.omicsnet.org/bcancer/database; ref. 26).

Statistical analysis

All experiments were conducted in triplicate at a minimum and repeated three independent times, with results reported as mean ± SD. Comparisons between experimental groups were performed using ANOVA with a *post hoc* Tukey test using GraphPad Prism 6 software. The Kaplan–Meier curve was generated using GraphPad and statistical differences between the two experimental groups was calculated by the log-rank, Mantel–Cox test. Differences in TIC frequency between SCBi and DAXXi MCF7 tumors were calculated by the χ^2 test. Linear regression was used to determine the estimated half-life of DAXX for each individual cycloheximide chase experiment. All three half-life calculations were then pooled together for each group and statistical differences between groups was determined by ANOVA.

Results

Stabilization of the DAXX protein requires ER activation

The role of DAXX on TIC survival in ER^+ breast cancer is not known. We first determined whether the DAXX transcript and/or protein expression is modulated by E_2 or endocrine therapy. E_2 treatment increased DAXX protein expression while downregulating NOTCH4 protein in three ER^+ breast cancer cell lines (MCF7, BT474, and T47D; Fig. 1A). Endocrine therapy treatment either by E_2 deprivation or fulvestrant resulted in decreased DAXX protein but increased NOTCH4 protein expression as compared with E_2 treatment (Fig. 1A). This suggested that DAXX protein expression was dependent on ER activation and inversely correlated with NOTCH4. This ER dependence of DAXX was further supported by Western blot results, indicating DAXX protein levels did not change in triple-negative breast cancer cell lines MDA-MB-231 or MDA-MB-468, in response to E_2 (Fig. 1B). In addition, patient-derived ER^+ xenografts (PDX BCM-5097) grown in the presence of physiologic E_2 resulted in increased DAXX, but decreased NOTCH4 protein expression, which were reversed upon depletion of E_2 for 19 days (Fig. 1C). To assess whether the DAXX-NOTCH4 protein expression was detectable in TICs, protein expression from bulk cells and mammospheres were quantified and compared. While E_2 treatment increased DAXX,

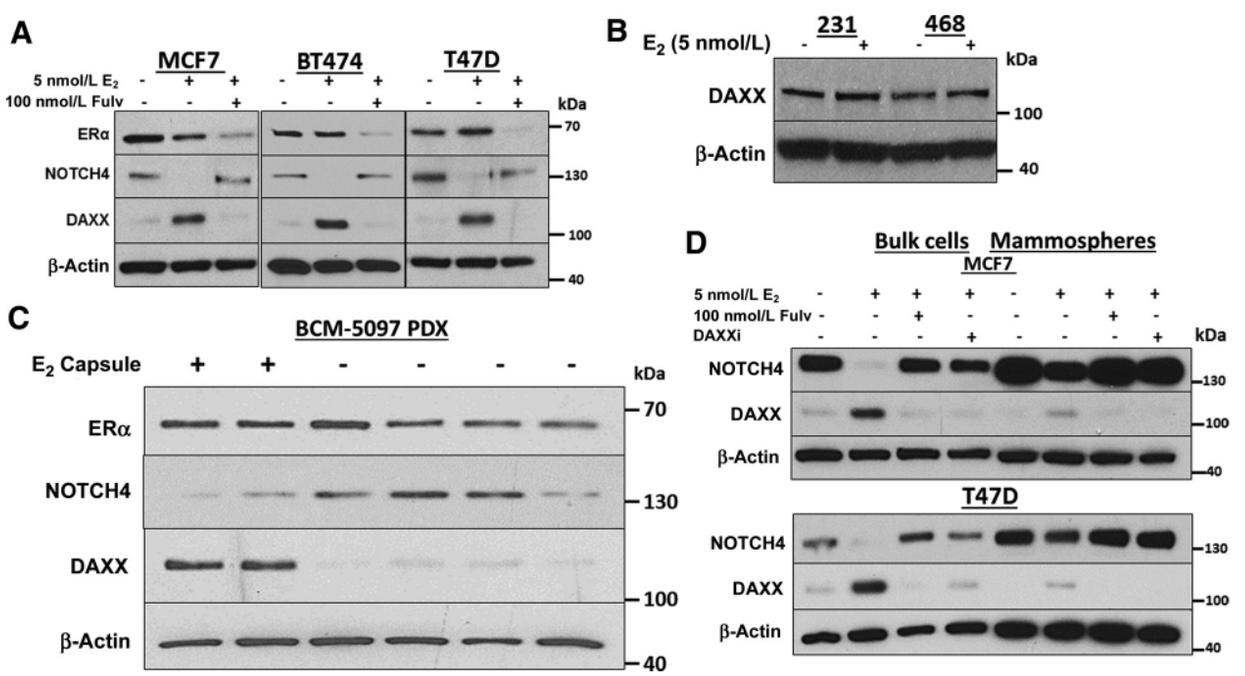

Figure 1.

E_2-mediated ER activation is required for DAXX protein expression. **A,** MCF7, BT474, and T47D cells were treated with ethanol (0 nmol/L E_2), 5 nmol/L E_2, or E_2 + fulvestrant (Fulv; 100 nmol/L) for 3 days. ERα, NOTCH4, and DAXX protein levels were detected by Western blotting. **B,** MDA-MB-231 (231) and MDA-MB-468 (468) cells were treated with ethanol (0 nmol/L E_2), 5 nmol/L E_2 for 3 days, followed by Western blotting for DAXX. **C,** PDX BCM-5097 tumors were harvested for detection of ERα, NOTCH4, and DAXX protein levels. **D,** MCF7 and T47D cells were transfected with nonspecific (SCBi) or DAXX-specific (DAXXi) siRNA for 2 days and then grown for 3 days with 0 nmol/L E_2, 5 nmol/L E_2, or 5 nmol/L E_2 + 100 nmol/L fulvestrant, followed by Western blotting to detect NOTCH4 and DAXX proteins. After 7 days, mammospheres were harvested by pooling together three wells and NOTCH4 and DAXX proteins were detected by Western blotting.

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but decreased NOTCH4 protein in bulk cells, mammospheres were enriched for NOTCH4 protein and had little detectable DAXX protein (Fig. 1D). However, E_2 treatment modestly increased DAXX and decreased NOTCH4 proteins in mammospheres (Fig. 1D). These findings suggest that ER activation by E_2 inhibits TICs by inducing the DAXX protein to repress NOTCH4.

To determine whether E_2-induced DAXX protein was due to increased RNA transcript levels, real-time PCR was performed to detect DAXX transcripts. Levels of DAXX transcripts did not change in response to increasing concentrations of E_2 (0, 0.50, 5.0, or 50 nmol/L; Fig. 2A). To confirm that ER is functional in both cell lines, transcript expression of a classical ER-inducible gene, *TIF1* (PS2), was measured in response to increasing concentrations of E_2. PS2 transcript levels increased in response to E_2 peaking at 5 nmol/L (Fig. 2A). On the basis of these results, E_2-mediated activation of the ER may increase DAXX levels by stabilizing the protein. The proteasome inhibitor, MG132, was used to assess whether proteasome activity is required for the decrease in DAXX protein in response to endocrine therapy. In the absence of MG132 (DMSO), DAXX protein expression was low in the absence of E_2 and increased in the presence of 5 nmol/L E_2 in both cell lines (Fig. 2B). In the presence of MG132, DAXX protein expression is higher with ET and remains relatively unchanged with E_2 treatment (Fig. 2B), suggesting endocrine therapy promotes degradation of DAXX protein by the proteasome. To determine whether the rate of DAXX protein stability is increased by E_2 and attenuated by endocrine therapy, cycloheximide pulse-chase experiments were conducted in the absence or presence of MG132 in a time-dependent manner. E_2 deprivation or fulvestrant resulted in a rapid decrease in DAXX protein levels compared with E_2 in MCF7 cells (Fig. 2C). This destabilization of DAXX protein is delayed by E_2 or is prevented by MG132 treatment (Fig. 2C). To achieve a more accurate half-life of DAXX protein decay by endocrine therapy, a shorter time course of 0 to 4 hours was used as shown in Fig. 2C. A DAXX protein decay curve summarized results of threeindependent experiments and was used to calculate the average half-life ($t_{1/2}$) of the DAXX protein by linear regression analysis (Fig. 2D). In summary, the half-life of the DAXX protein is 1.73 hours by E_2 deprivation or 1.59 hours by fulvestrant. E_2 treatment significantly increases the half-life of DAXX to 10.28 hours (Fig. 2D). This suggested DAXX requires E_2-mediated ER activation for its protein stabilization, and endocrine therapy depletes DAXX protein rapidly in ER^+ breast cancer cells.

DAXX is required to inhibit NOTCH4, NOTCH activation, and TIC survival *in vitro*

As NOTCH expression and activity have been shown to be repressed by ER and this repression is reversed by endocrine therapy (4, 7, 11, 27), we hypothesized that E_2-mediated ER activation may restrict NOTCH and TIC survival by increasing DAXX. To test this hypothesis, expression of NOTCH4 protein, canonical NOTCH gene targets (*DELTEX1*, *HES1*, and *HEY1*) and MFE were measured to assess NOTCH activity and TIC survival upon DAXX knockdown. MCF7 and T47D cells were depleted of

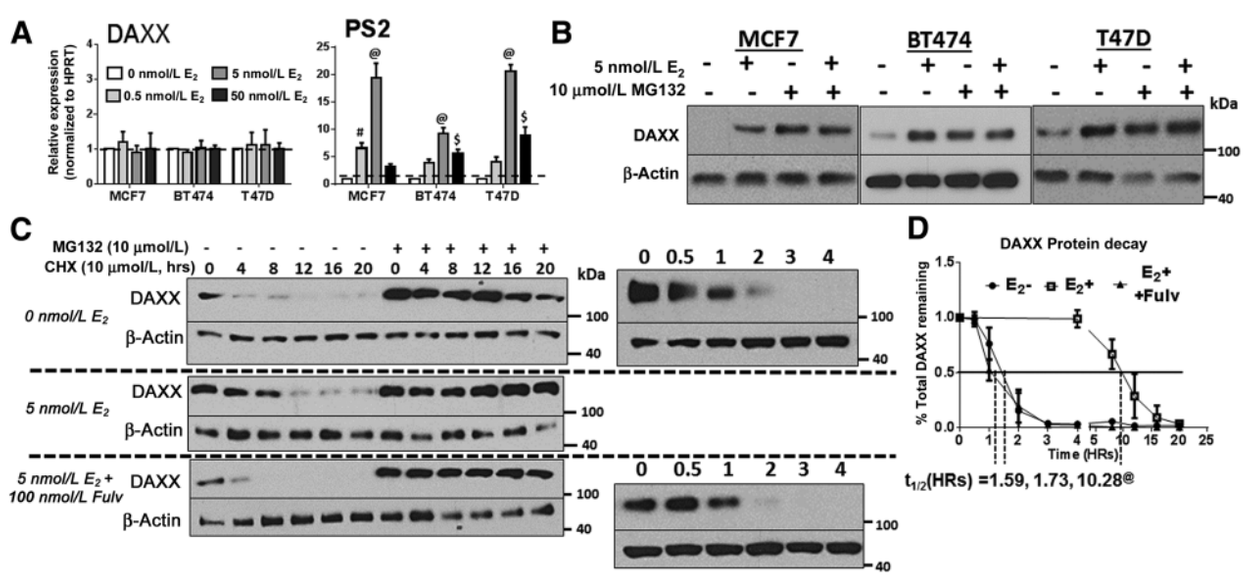

Figure 2.

DAXX protein stabilization is mediated by E_2-mediated ER activation. **A,** Real-time PCR was performed to measure relative transcript levels of DAXX and PS2 in MCF7, BT474, and T47D cells after 3 days of treatment with 0 or 5 nmol/L E_2. Bar graphs show mean ± SD values normalized to HPRT and compared with 0 nmol/L E_2 based on three independent experiments using the $2^{-\Delta\Delta C_t}$ calculation. A one-way ANOVA was performed on ΔC_t values after normalization to HPRT. **B,** The same cells were treated with 0 or 5 nmol/L E_2 for 3 days; 12 hours prior to harvesting, cells were treated with vehicle (DMSO) or 10 μmol/L MG132. Western blotting was performed to detect DAXX protein levels. **C,** MCF7 cells were grown in 0 nmol/L E_2, 5 nmol/L E_2, or E_2 + 100 nmol/L fulvestrant for 24 hours, following which, cells were treated with 10 μmol/L cycloheximide (CHX) for the indicated times. Western blotting was performed to detect DAXX protein levels. MCF7 cells treated with 0 nmol/L E_2 or 5 nmol/L E_2 + 100 nmol/L fulvestrant were treated with 10 μmol/L cycloheximide for a shorter time course. Western blotting was performed to detect DAXX protein levels. Blots are representative of three independent experiments. **D,** ImageJ was used to measure densitometry of DAXX protein levels as a ratio to β-actin. The estimated half-life ($t_{1/2}$) of the DAXX protein for each independent experiment was calculated by linear regression analysis. Means of DAXX protein half-life ± SD were statistically compared by a one-way ANOVA. #, $P < 0.05$; $, $P < 0.01$; @, $P < 0.001$.

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DAXX using a SmartPool of four DAXX-specific siRNA sequences. These sequences were first individually tested for their effects on DAXX protein expression by Western blot analysis (Supplementary Fig. S1A). Each individual siRNA in the SmartPool efficiently decreased DAXX protein with the pooled sample being the most efficient. The SmartPool was therefore used for subsequent experiments. E_2-mediated ER activation increased DAXX protein but decreased NOTCH4 protein (Fig. 3A). E_2-mediated decrease in NOTCH4 expression was reversed upon DAXX knockdown (Fig. 3A). Furthermore, E_2-mediated decrease in %MFE as a measure of TIC survival was almost completely rescued by DAXX knockdown using the SmartPool (Fig. 3B) or individual DAXX siRNAs (Supplementary Fig. S1B). Two forms of ET, E_2 deprivation or fulvestrant significantly increased %MFE compared with E_2 treatment (Fig. 3B). To assess whether changes in NOTCH4 expression and TIC survival by DAXX were due to alterations in classical E_2-mediated ER activation, expression of PS2 transcripts were measured. Figure 3C showed that while E_2 induced PS2 transcripts, this expression was not dependent on DAXX. Also, DAXX knockdown had little effect on E_2-induced total bulk cell proliferation (Supplementary Fig. S2A) and cell-cycle progression (Supplementary Fig. S2B). The effects of E_2-mediated DAXX expression and TIC survival were most probably due DAXX-mediated repression of canonical NOTCH gene targets as shown in Fig. 3D. Expression of NOTCH direct target genes, *DELTEX1*, *HES1*, and *HEY1* transcripts were decreased upon E2 treatment and this was reversed when DAXX was knocked down (Fig. 3D).

DAXX is required to suppress TIC frequency *in vivo*

The *in vitro* results indicated that E_2-mediated ER activation stabilizes DAXX to repress NOTCH signaling and suppress TIC survival. To determine whether DAXX is necessary to suppress TIC survival *in vivo*, an ELDA was performed using cells expressing or depleted for DAXX. DAXX protein expression was efficiently knocked down as shown in Fig. 4A. An E_2-containing silastic-release capsule was implanted for sustained release of physiologic E_2 for up to 8 weeks (21). Tumor incidence was higher in each dilution group when DAXX was depleted compared with control (Fig. 4B). A Kaplan–Meier curve of % tumor-free mice demonstrated that mice developed tumors faster from DAXX-depleted cells compared with DAXX-expressing cells (Fig. 4C). Kaplan–Meier curves for each individual cell dilution show statistical significance at the 1×10^6 cell dilution group (Supplementary Fig. S3A–S3C). Estimated TIC frequency based on overall tumor frequency was determined for each dilution group using ELDA software (http://bioinf.wehi.edu.au/software/elda), comparing animals injected with control versus DAXX-depleted cells. Analysis indicated an estimated TIC frequency of 1/589,536 for

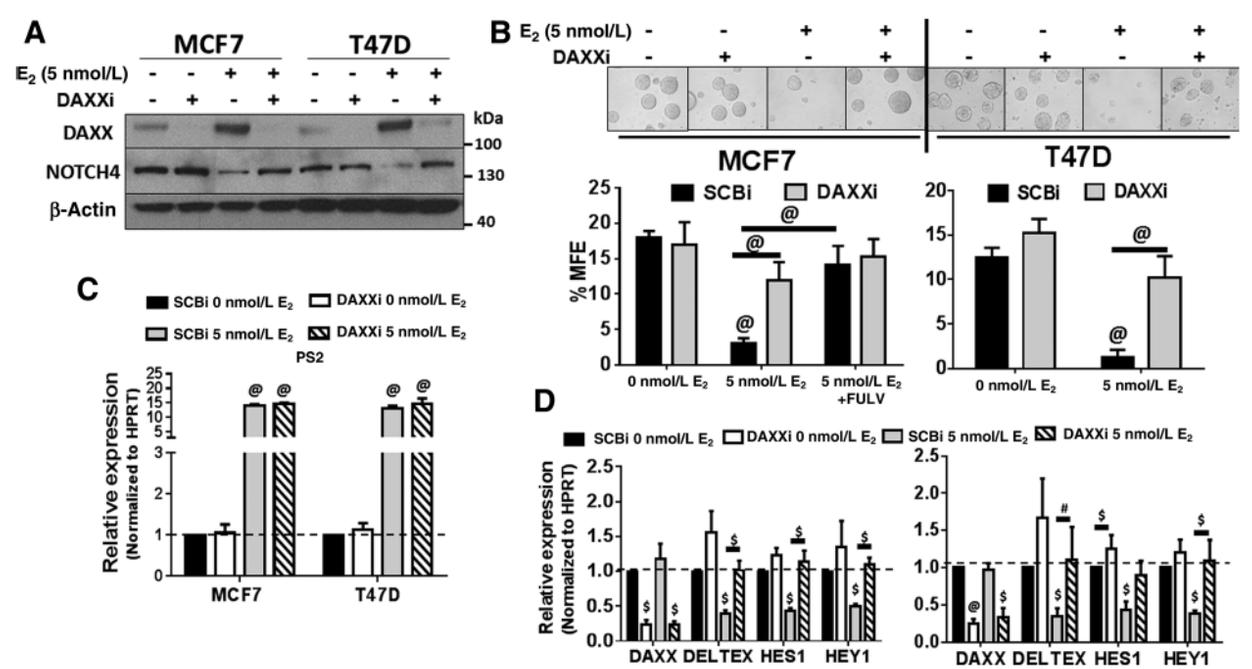

Figure 3.

DAXX is required to inhibit NOTCH4 and TIC-survival. **A,** MCF7 and T47D cells were transfected with a nonspecific (SCBi) or DAXX-specific (DAXXi) siRNA for 2 days and then treated with 0 or 5 nmol/L E_2 for 3 days. DAXX and NOTCH4 proteins were detected by Western blotting. **B,** A total of 50,000 cells were plated into an ultralow attachment plate containing methylcellulose mammosphere-forming medium. After 7 days, mammospheres were imaged at ×20 magnification, harvested, and %MFE calculated. Representative images of mammospheres taken by light microscopy are shown. Scale bars, 100 μm. Bar graphs show %MFE ± SD from three independent experiments. Statistical significance was calculated using a two-way ANOVA with a Tukey *post hoc* test for multiple comparisons. Symbols denote statistical significance between 5 nmol/L and 0 nmol/L E_2, SCBi and DAXXi, and E_2 and E_2+ fulvestrant (FUL). **C** and **D,** Real-time PCR was used to detect transcript levels of PS2 and DAXX (Fig. 1C) and NOTCH targets DELTEX, HES1, and HEY1 (Fig. 1D). Bar graphs show mean values ± SD of relative transcript expression normalized to HPRT and compared with SCBi + 0 nmol/L E_2 conditions from three independent experiments using the $2^{-\Delta\Delta C_t}$ calculation. A two-way ANOVA was performed on ΔC_t values after initial normalization to HPRT. Symbols denote statistical significance between SCBi and DAXXi and 0 and 5 nmol/L E_2. #, $P < 0.05$; $, $P < 0.01$; @, $P < 0.001$.

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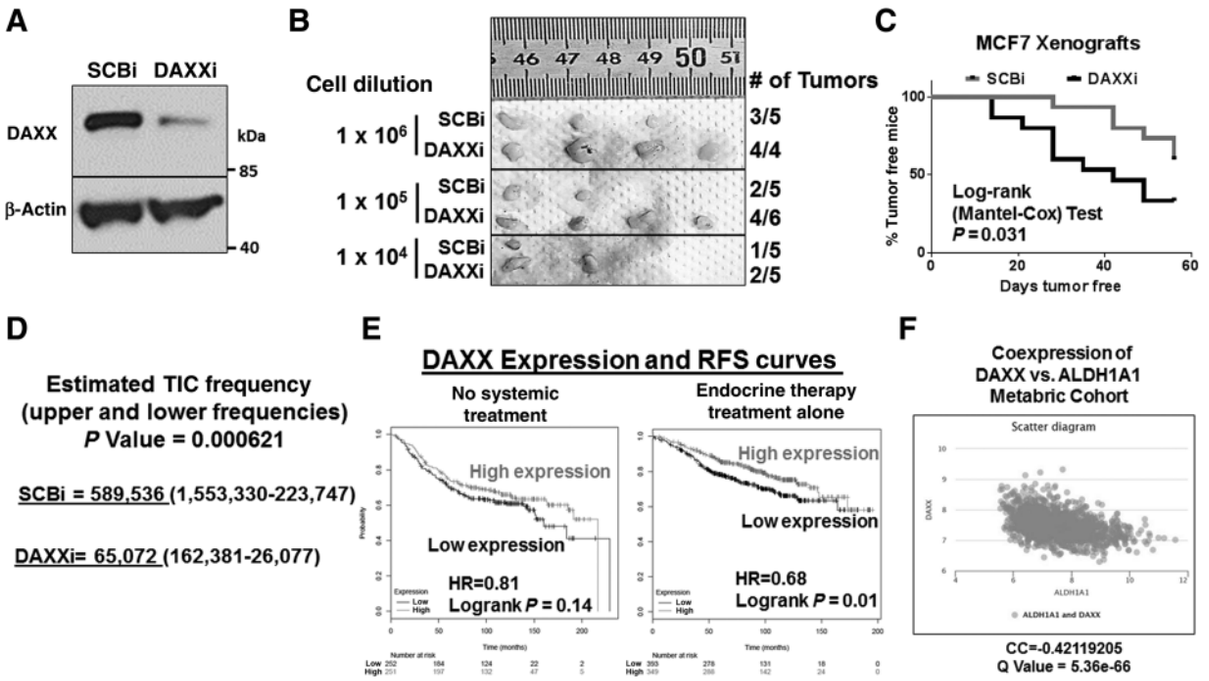

Figure 4.

DAXX is necessary to restrict TIC frequency *in vivo*. MCF7 cells were transfected with a nonspecific (SCBi) or DAXX-specific (DAXXi) siRNA for 2 days. 10,000 (1×10^4), 100,000 (1×10^5), or 1,000,000 (1×10^6) cells were resuspended into Matrigel solution (1:1 Matrigel:PBS) and injected into the mammary fat pads of 5 female, athymic, nude mice/dilution. Silastic capsules containing E_2 were also implanted for up to 8 weeks. **A,** DAXX protein levels were detected by Western blotting. **B,** Tumor area was measured weekly using Vernier calipers. Total number of tumors per group were counted. Tumor images were taken after 8 weeks. **C,** Kaplan–Meier curve represents the rate of tumor incidence across all cell dilutions. Statistical significance was calculated by the log-rank (Mantel–Cox) test. **D,** Estimate of TIC frequency for each cell dilution group was calculated using the ELDA software. SCBi and DAXXi TIC-frequency was compared by the χ^2 test to determine statistical significance. **E,** Using Kmplotter.com, RFS for women with ER^+ breast cancer was compared in DAXX high expressing tumors versus DAXX low expressing tumors by "best cutoff" as determined by the kmplotter software. The data represent the mean of two probes using the multigene analyzer. Two cohorts of patients were analyzed: systemically untreated (left) and endocrine therapy only (right; excluded for chemotherapy). All parameters used to generate these curves are summarized in Supplementary Table S1C. **F,** Coexpression analysis of the METABRIC cohort comparing DAXX RNA expression versus ALDH1A1 in patients with ER^+ breast cancer. DAXX versus ALDH1A1 RNA expression was evaluated by correlation coefficient (CC) and Q value.

DAXX-expressing cells versus a frequency of 1/65,072 for DAXX-depleted cells under E_2 treatment conditions (Fig. 4D). This was an over 9-fold increase in TIC frequency when DAXX was depleted.

These *in vivo* results suggested that DAXX is required for E_2-mediated suppression of TIC frequency, and that ER-targeted therapies deplete DAXX levels allowing for enrichment of TICs. As TICs maybe a primary contributor to cancer recurrence, we used the KMplotter software (22, 23) to determine whether DAXX RNA expression correlated with RFS in patients with ER^+, breast cancer. Patients were stratified by high or low median DAXX transcript expression separated using the best cutoff as defined by the KMplotter software, and compared the length of time of RFS of two cohorts of patients: those who were systemically untreated (Fig. 4E, left graph) or those treated with endocrine therapy, excluding chemotherapy (Fig. 4E, right graph). The data presented are the mean of two DAXX probes (201763_s_at and 216038_x_at) using the multigene analyzer. There were no differences in RFS for t systemically untreated patients (Fig. 4E, left graph). However, patients with tumors that expressed low DAXX RNA had a significantly shorter RFS compared with high DAXX RNA levels with endocrine therapy treatment alone (Fig. 4E, right graph).

Individual RFS data for each probe is provided in Supplementary Fig. S4. Both the best median cutoff and quartile RFS data are shown along with the Beeswarm plots for each probe (Supplementary Fig. S4A and S4B). RFS using individual DAXX probes showed similar results (Supplementary Fig. S4) as the mean of the two probes (Fig. 4E, right graph). In addition, as nodal status alone is prognostic for RFS, RFS was also determined for patients with ER^+ breast cancer based on DAXX RNA expression and nodal status. DAXX using median or quartile RNA expression in ER^+, node-negative patients was modestly prognostic for RFS using one probe (Supplementary Fig. S5A and S5B). Median or quartile expression of DAXX RNA was prognostic for better RFS in patients with ER^+, node-positive breast cancer using one probe (201763_s_at; Supplementary Fig. S5C), but not for the second probe (216038_x_at; Supplementary Fig. S5D). All parameters used to generate these survival curves are summarized in Supplementary Table S1C.

These findings are in agreement with expression of other TIC-associated markers including ALDH1A1 (Fig. 4F). DAXX RNA expression inversely correlates with ALDH1A1 expression when analyzing the METABRIC ER^+ breast cancer cohort for DAXX versus ALDH1A1 RNA expression (Fig. 4F). These results are in agreement with the current experimental data that indicate high

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DAXX expression suppresses TICs and may be a clinical biomarker for low rates of ER^+ tumor recurrence following endocrine therapy.

DAXX represses expression of developmental genes

To assess whether DAXX is a transcriptional repressor in ER^+ breast cancer, RNA sequencing was performed in E_2-treated MCF7 cells expressing or depleted for DAXX. Initial comparison of two independent experiments indicates high degree of similarity under parameters of FKPM = 10, fold increase > or = 1.5, and $P < 0.05$ (Supplementary Fig. S6A–S6C). Specifically, 18 genes are shown to be downregulated and 51 genes were upregulated (Supplementary Fig. S6A) upon DAXX depletion compared with control. Metscape Pathway Analysis identified a number of pathways that were downregulated and upregulated (Supplementary Fig. S6B) upon DAXX knockdown. Downregulated pathways include response to antineoplastic agents and response to steroid hormone pathways, both of clinical concern for the treatment of ER^+ breast cancer (Supplementary Fig. S6B). Three pathways significantly upregulated by DAXX depletion include the embryologic process of gastrulation, pre-NOTCH expression, and processing and the NOTCH signaling pathway (Supplementary Fig. S6B). Further analysis of the genes upregulated in the gastrulation pathway indicated a large number of genes associated with TIC survival, self-renewal, and cancer recurrence, including *SOX2* (28, 29), *OCT4* (30, 31), *NANOG* (32), *ALDH1A1* (33), and *NOTCH4* (6, 7). These results are summarized in a heatmap outlining all of the genes upregulated in the gastrulation pathway upon DAXX depletion (Supplementary Fig. S6C). The raw and calculated RNA-sequencing data were deposited into the GEO repository with the accession number: GSE134919. These results support a hypothesis that DAXX represses the NOTCH signaling pathway (Supplementary Fig. S6C). In addition, DAXX represses expression of pluripotent stem genes (Supplementary Fig. S6C) to suppress TIC survival.

DAXX is enriched on TIC-associated gene promoters and is required for gene repression

To determine whether DAXX is a nuclear regulator of pluripotent gene transcripts, cell fractionation was conducted to determine the cellular location of DAXX in response to E_2 treatment. Fractionation studies show that the DAXX protein is mostly localized in the nucleus of MCF7 and T47D cells, and this localization increases in response to E_2 treatment (Fig. 5A). To

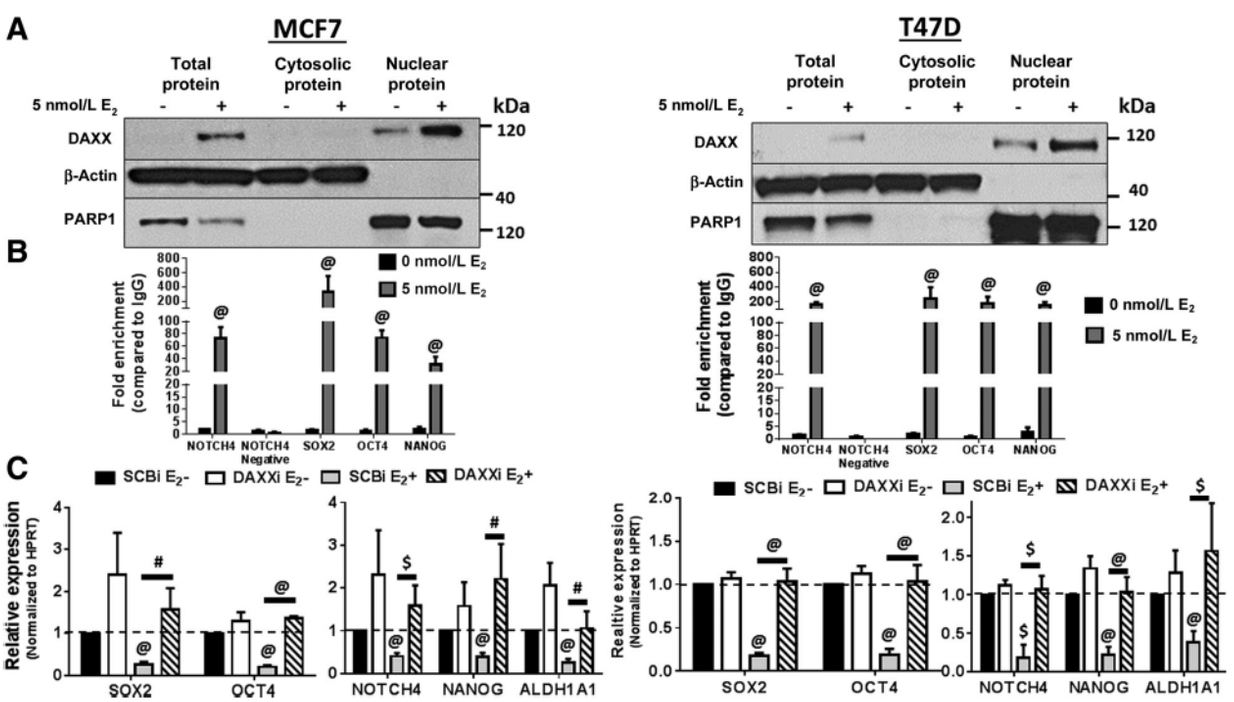

Figure 5.

Nuclear DAXX is enriched on TIC gene promoters and is necessary to repress TIC genes. **A,** MCF7 (left) and T47D (right) cells were treated with 0 or 5 nmol/L E_2 for 3 days. DAXX protein was detected by Western blot analysis in total lysates or cellular fractions. Actin was used as a cytosolic control and PARP1 as a nuclear control. **B,** MCF7 and T47D cells were treated with 0 or 5 nmol/L E_2 for 1 day. Cells were fixed, chromatin was isolated and probed using either a nonspecific IgG or DAXX-specific antibody (2 μg of antibody for each condition), and DNA fragments were isolated and purified. Fold DAXX enrichment as compared with IgG at TIC-associated gene promoters was quantified by real-time PCR. Fold-enrichment was determined by $2^{-\Delta C_t}$ calculation. Bar graphs show mean ± SD values of fold enrichment for each gene promoter measured. ΔC_t values for each gene promoter from cells treated with 0 nmol/L E_2 versus 5 nmol/L E_2 were compared and statistical significance was calculated using a nonpaired, two-sided Student *t* test. Symbols denote statistical significance between 0 nmol/L and 5 nmol/L E_2. **C,** MCF7 and T47D cells were transfected with a nonspecific (SBCi) or DAXX-specific siRNA for 2 days. Cells were treated with 0 or 5 nmol/L E_2 for 1 day. Transcript levels of SOX2, OCT4, NOTCH4, NANOG, and ALDH1A1 (ALDH) were detected by real-time PCR from MCF7 and T47D cells. Bar graphs show mean ± SD values of relative transcript expression normalized to HPRT and compared with SCBi 0 nmol/L +E_2 conditions from three independent experiments using the $2^{-\Delta\Delta C_t}$ calculation. A two-way ANOVA was performed using ΔC_t values after initial normalization to HPRT. Symbols denote statistical significance between 0 nmol/L and 5 nmol/L E2 and SCBi and DAXXi groups. #, $P < 0.05$; $, $P < 0.01$; @, $P < 0.001$.

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test whether DAXX is recruited to promoter regions of pluripotent genes that promote TIC survival, DAXX ChIP assays were conducted on genes identified by RNA-sequencing. A transcription factor desert region previously identified was used as a negative control (NOTCH4 Negative; ref. 34) as well as an isotype IgG control. Primer regions flanking regulatory regions of TIC-associated genes are provided in Supplementary Fig. S7A. Activator Protein 1 (AP-1) consensus regions were selected due to previous reports that AP-1 drives *NOTCH4* transcription (34, 35) and that DAXX binds AP-1 to repress AP-1–driven transcription (36). In the absence of E_2, when DAXX expression is low, little detectable DAXX is enriched on promoter regions of *NOTCH4*, *SOX2*, *OCT4*, and *NANOG* in either cells (Fig. 5B). Conversely, E_2 stimulates enrichment of DAXX on these promoter regions from 40 to nearly 400-fold over IgG (Fig. 5B). To test whether this enrichment of DAXX at the promoter of these genes is associated with changes in transcript levels, and to confirm RNA-sequencing results, real-time PCR was performed on RNA extracted from cells treated without and with E_2 and when DAXX was expressed or depleted. In both cell lines, E_2 significantly decreased SOX2, OCT4, NOTCH4, NANOG, and ALDH1A1 transcripts (Fig. 5C). This decrease by E_2 was almost completely prevented when DAXX was depleted similar to levels of E_2 deprivation (Fig. 5C). In addition, transcript levels of luminal/epithelial (FOXA1 and E-Cadherin) and mesenchymal (N-Cadherin, SNAIL, and SLUG) markers were measured by real-time PCR. DAXX was required for expression of epithelial markers FOXA1 and E-Cadherin (CDH1), while E_2 deprivation or DAXX depletion increased RNA expression of mesenchymal markers (N-Cadherin, SNAIL, and SLUG) in MCF7 and T47D cells (Supplementary Fig. S7B and S7C). Together, these results indicate that DAXX is a critical transcriptional repressor of pluripotent TIC and mesenchymal genes and suppressor of TIC survival when ER^+ breast cancer cells are exposed to E_2.

DAXX requires DNMT1 to restrict TIC survival and TIC gene expression

To determine whether DAXX-mediated repression of TIC-gene expression and TIC-survival required DNMT1, DNMT1 protein expression was first detected by Western blot in the absence or presence of E_2. DNMT1 protein expression was high in both ER+ breast cancer cell lines regardless of E_2 treatment (Fig. 6A). To assess whether CpG methylation or DNMT1 was required for TIC-survival or TIC-gene expression, 5-Azacytidine (AZA) treatment or

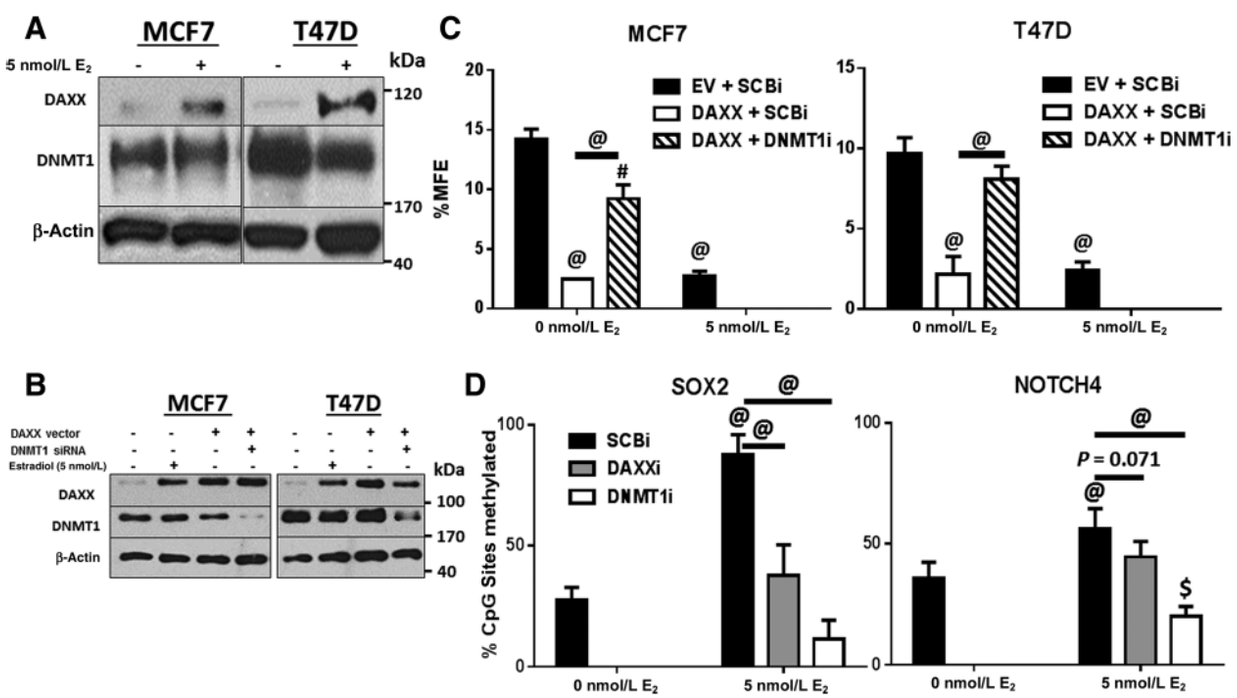

Figure 6.

DAXX requires DNMT1 to restrict TIC survival and transcript expression. **A,** MCF7 and T47D cells were treated with 0 or 5 nmol/L E_2 for 3 days. DNMT1 and DAXX protein levels were detected by Western blot analysis using actin as a loading control. **B** and **C,** MCF7 and T47D cells were transfected with a mock vector (EV) or the human DAXX cDNA (DAXX) for 2 days and then retransfected with the nonspecific (SCBi) or DNMT1-specific (DNMT1i) siRNA for an additional day. **B,** DAXX and DNMT1 proteins were detected by Western blotting. **C,** After 7 days, mammospheres were imaged, isolated, measured, and %MFE was calculated. Bar graphs show mean ± SD. %MFE from three independent experiments. Statistical significance was calculated using two-way ANOVA with a Tukey *post hoc* test for multiple comparisons. Symbols denote statistical significance between empty vector and DAXX, SCBi and DNMT1 under DAXX conditions, and 0 nmol/L and 5 nmol/L E_2. **D,** MCF7 cells were transfected with a nonspecific (SCBi), DAXX-specific (DAXXi), or DNMT1-specific (DNMT1i) siRNA for 2 days. Cells treated with 0 or 5 nmol/L E_2 for 1 day. Total DNA was isolated and subjected to bisulfite treatment. Bisulfite-converted DNA was amplified using SOX2 promoter–specific primers or NOTCH4-CpG island–specific primers that anneal to bisulfite-treated DNA. The PCR product was purified and sent for DNA sequencing. CpG sites that were read as "T" were considered unmethylated and sites read as "C" were considered methylated. Bar graphs show mean (total number of methylated CpG sites)/(total CpG sites read × 100) ± SD from five independent experiments. Statistical significance was calculated using two-way ANOVA with a Tukey *post hoc* test for multiple comparisons. Symbol denotes statistical significance between 0 nmol/L and 5 nmol/L E_2 and SCBi and DAXXi/DNMT1i groups. #, $P < 0.05$; @, $P < 0.001$.

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DNMT1 knockdown was conducted followed by measurement of %MFE or real-time PCR. E_2 decreased %MFE as a measure of TIC-survival (Supplementary Fig. S8A and S8B) and expression of TIC-gene transcripts (Supplementary Fig. S8C and S8D). AZA treatment almost completely prevented the decreased TIC survival and TIC-associated gene expression by E_2 (Supplementary Fig. S8A and S8C). Similarly, knockdown of DNMT1 by siRNA reversed E_2-mediated decrease in %MFE and expression of TIC gene transcripts (Supplementary Fig. S8B and S8D). These results indicated that DNMT1 protein levels were abundant in ER^+ breast cancer cells and DNMT1 was necessary to suppress TIC survival and repress TIC genes in response to E_2. To determine whether DAXX required DNMT1 to inhibit TIC-survival and associated gene expression, DAXX was overexpressed under conditions where DNMT1 was expressed or depleted. Dual transfection was effective at both increasing DAXX expression and decreasing DNMT1 expression (Fig. 6B). Ectopic expression of DAXX was sufficient to restrict TIC survival when both cell lines were deprived of E_2 similar to levels measured in the presence of E_2 (Fig. 6C). Conversely, ectopic DAXX expression suppressed TIC survival when DNMT1 was depleted (Fig. 6C), suggesting that DAXX required DNMT1 to suppress TIC survival. In agreement, ectopic DAXX expression decreased TIC gene transcripts in the absence of E_2 similar to levels detected in response to E_2 (Supplementary Fig. S9A). The decreased TIC gene transcripts by ectopic DAXX expression was reversed by DNMT1 knockdown (Supplementary Fig. S9A). These results suggest that DNMT1 is required for DAXX-mediated suppression of TIC survival and TIC gene expression.

E_2-mediated hypermethylation of the SOX2 promoter and the *NOTCH4* gene is dependent on DAXX or DNMT1

To determine whether DAXX or DNMT1 was necessary for hypermethylation of a pluripotent TIC gene region such as SOX2 and NOTCH4 in response to E_2, cells were depleted of DAXX or DNMT1 by siRNA in the absence or presence of E_2 followed by bisulfite treatment and DNA sequencing of the SOX2 promoter region or within the body of the *NOTCH4* gene containing 9 and 13 CpG sites, respectively. Representative images of each sequencing read of bisulfite-converted DNA are shown in Supplementary Fig. S9B. For SOX2, in the absence of E_2, approximately 26% of the 9 CpG sites were methylated (Fig. 6D; Supplementary Fig. S9B). Upon E_2 treatment, nearly 90% of the 9 CpG sites were methylated (Fig. 6D; Supplementary Fig. S9B). DAXX depletion reduced the methylation status to less than 40% (Fig. 6D; Supplementary Fig. S9B). DNMT1 depletion reduced methylation to 11% (Fig. 6D; Supplementary Fig. S9B). For NOTCH4, E_2 deprivation resulted in nearly 35% of the 13 CpG sites being methylated compared with 5 nmol/L E_2 conditions in which 55% of the sites were methylated (Fig. 6D; Supplementary Fig. S9B). Upon DAXX or DNMT1 depletion, methylation decreased to nearly 45% with DAXX knockdown and to 20% with DNMT1 knockdown, respectively (Fig. 6D; Supplementary Fig. S9B). Of note, DAXX depletion resulted in almost complete hypomethylated CpGs within intron 29, but did not affect CpGs within exon 29 (Supplementary Fig. S9B). Thus, DAXX may be required for partial methylation of this region of the *NOTCH4* gene while DNMT1 was required for most of the region. Together, these results suggested that DAXX or DNMT1 was necessary to hypermethylate the SOX2 promoter in response to E_2, while the epigenetic status of the NOTCH4 CpG island was partially regulated by DAXX.

DAXX is sufficient to restrict NOTCH signaling and TIC survival

We hypothesized that if DAXX can be increased under endocrine therapy conditions, then TIC survival will be inhibited. To test this hypothesis, DAXX was ectopically expressed using a mammalian expression vector (pCMV6-Entry) and then grown in the absence or presence of E_2. Ectopic expression of DAXX was maintained in the absence of E_2 (Fig. 7A). Upon DAXX expression, NOTCH4 protein was decreased in the absence of E_2 to similar levels detected in response to E_2 (Fig. 7A). Ectopic expression of DAXX significantly inhibited TIC survival as measured by %MFE under E_2 deprived conditions when TIC survival is normally high in both MCF7 and T47D cells (Fig. 7B). This restriction of TIC survival was similar to E_2 treatment (Fig. 7B). Conversely, ectopic expression of DAXX had no significant effect on total bulk cell proliferation regardless of E_2 treatment (Supplementary Fig. S10A), suggesting DAXX was a critical suppressor of TIC survival and not overall bulk cell survival. Similarly, DAXX overexpression had little effect on the classical ER-responsive gene, *PS2* (Supplementary Fig. S10B), while significantly decreasing NOTCH target gene transcripts, DELTEX1, HES1, and HEY1 (Supplementary Fig. S10B) and TIC-associated gene transcripts, SOX2, OCT4 (Fig. 7C), NOTCH4, NANOG, and ALDH1A1 (Fig. 7D). Furthermore, overexpression of DAXX was sufficient to induce expression of epithelial markers [FOXA1 and E-Cadherin (CDH1)], while inhibiting expression of mesenchymal transcripts including N-Cadherin (CDH2) and SLUG in MCF7 and T47D cells (Supplementary Fig. S11). Overall, these findings suggest that DAXX is a novel breast cancer TIC suppressor by potentially recruiting DNMT1 to hypermethylate some TIC-associated gene regions thus repressing their expression.

Summary of results

New findings showed that the DAXX protein restricted TIC survival and thus represents a novel target with agents that can stabilize the DAXX protein. The DAXX protein is stabilized E_2-mediated ER activation, and conversely ET rapidly destabilized the DAXX protein. ET-mediated depletion of DAXX increased TIC survival, while having little effect on bulk cell proliferation. Molecular analysis indicated DAXX restricted TIC survival by potentially binding promoters of pluripotent TIC-associated genes, facilitating hypermethylation of the SOX2 promoter and partly the *NOTCH4* gene, and repressing TIC transcript expression. Endocrine therapy depleted the DAXX protein, resulting in derepression of TIC-associated genes, enrichment of TICs, and possibly poor prognosis, as reflected in worse RFS in patients treated with ET whose ER^+ tumors expressed low DAXX transcript expression (Fig. 7E).

Discussion

In ER^+ breast cancer, TICs are thought to contribute to the development of recurrent and/or treatment-resistant breast cancer (1, 2). This is due to the intrinsic properties of these small percentage of cells, including resistance to endocrine therapy and their ability to regenerate a heterogeneous tumor (1, 2, 4, 8, 37, 38). One of the mechanisms by which the ER is thought to reduce TICs is through regulation of TIC-associated genes (*SOX2*, *NANOG*, *OCT4*) and inhibition of the NOTCH signaling pathway (1, 2, 4, 8, 28, 37, 38). ER has been shown to inhibit NOTCH signaling, which is required for TIC-survival and self-renewal in breast cancer (8, 39). Anti-Notch

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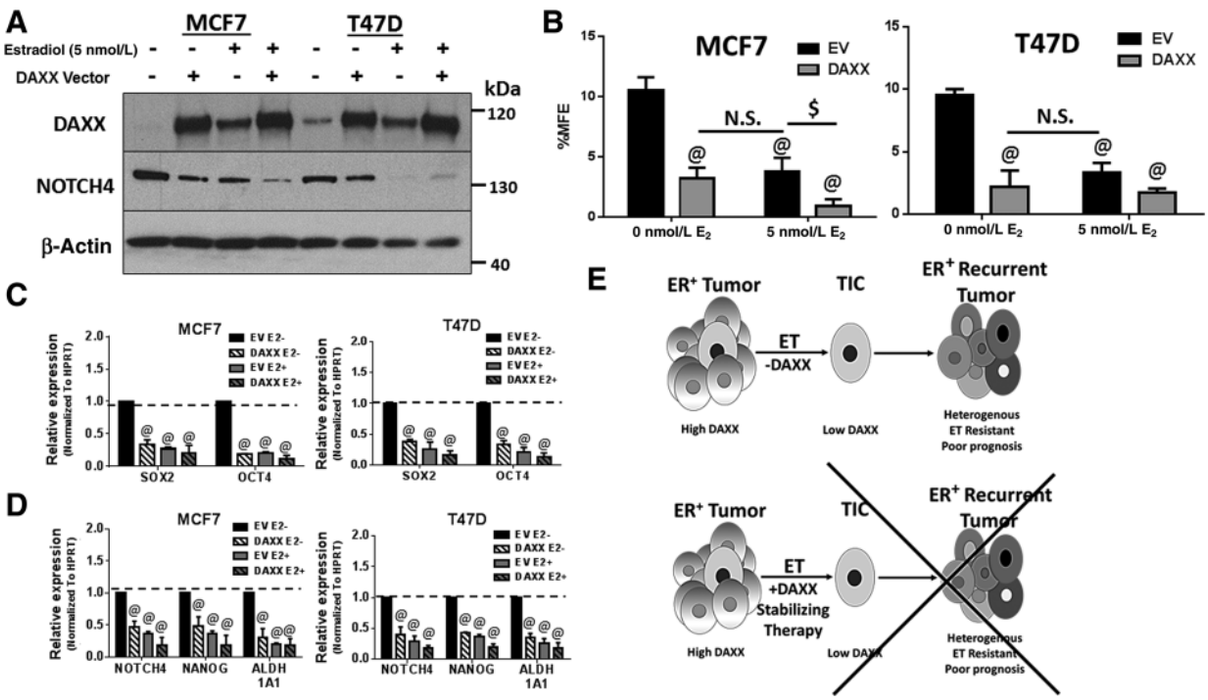

Figure 7.

DAXX is sufficient to restrict NOTCH4 and TIC-survival in response to endocrine therapy (ET). **A,** MCF7 and T47D cells were transfected with the empty vector (EV) or DAXX-containing vector using polyethylenimine for 2 days and then treated with 0 or 5 nmol/L E_2 for 3 additional days. DAXX and NOTCH4 protein levels were detected by Western blot analysis using β-actin as a loading control. **B** and **C,** A total of 50,000 MCF7 and T47D cells were plated in ultralow attachment plates containing mammosphere-forming medium for 7 days. Mammospheres were imaged, isolated, counted, and %MFE calculated from three independent experiments. Bar graphs show mean %MFE ± SD from three independent experiments. Statistical significance was calculated using two-way ANOVA with a Tukey *post hoc* test for multiple comparisons. Symbols denote statistical significance between empty vector and DAXX and 0 nmol/L and 5 nmol/L E_2 groups. MCF7 (**C**) and T47D (**D**) cells were transfected with the empty vector or DAXX-containing vector for 2 days and then treated with 0 or 5 nmol/L E_2 for 1 day. SOX2, OCT4, NOTCH4, NANOG, and ALDH1A1 transcripts were detected by real-time PCR. Bar graphs show mean values ± SD of relative transcript expression normalized to HPRT and compared with SCBi 0 nmol/L +E_2 from three independent experiments using the $2^{-\Delta\Delta C_t}$ calculation. Symbols denote statistical significance between empty vector and DAXX and 0 nmol/L and 5 nmol/L E_2. A two-way ANOVA was performed on ΔC_t values after initial normalization to HPRT. **E,** Working model that endocrine therapy selects for TICs by depleting DAXX protein levels. DAXX depletion results in derepression of TIC genes promoting TIC survival. TICs contribute to heterogeneous and resistant recurrent tumors. Therapies that stabilize DAXX may prevent TIC survival and prevent ER^+ breast cancer recurrence. #, $P < 0.05$; $, $P < 0.01$; @, $P < 0.001$. N.S., nonsignificant.

therapies such as GSIs are often associated with gastrointestinal toxicity and skin cancer (40). Thus, there is a critical need for new therapeutic targets to reduce TICs in ER^+ breast cancer to prevent resistance, tumor recurrence, tumor, and severe side effects. Through a biomarker clinical trial (ClinTrials.gov Identifier: NCT00756717), we identified DAXX as a novel gene whose transcript is upregulated after short exposure to a GSI during endocrine therapy preceding definitive surgery in a window biomarker trial (19). From publicly available expression data (kmplot.com), high DAXX RNA correlates with a longer RFS in women with ER^+ breast cancer following endocrine therapy (Fig. 4E), suggesting it may be a potential positive prognostic factor and a novel therapeutic target.

DAXX was initially characterized as a proapoptotic factor by interacting with the receptor TNF receptor superfamily member 6 (FAS, CD95; ref. 41). Initial studies showed that DAXX-mediated activation of JUN N-Terminal Kinase (JNK) was necessary to initiate caspase-independent apoptosis (42). Recently, DAXX has been shown to regulate promyelocytic leukemia protein (PML), to maintain the heterochromatin state, and act as a transcriptional repressor (43). In hormone-dependent prostate cancer, DAXX was shown to recruit DNMT1 for epigenetically silencing of its target genes (44). Furthermore, Morozov and colleagues showed that epigenetic silencing of the mesenchymal–epithelial transition (c-MET) requires DAXX (45). Together, these findings suggest that DAXX functions as a transcription repressor through recruitment of DNMT1.

Results from this study indicate that E_2-mediated ER activation stabilizes the DAXX protein. Stable DAXX protein binds to regulatory regions of pluripotent and other stem cell genes, possibly recruiting DNMT1 to hypermethylate promoter or gene body regions, resulting in repression of gene transcription. Thus, ER-mediated DAXX protein stability is potentially responsible for restricting TIC survival and frequency. Targeted inhibition of ER results in rapid depletion of the DAXX protein, loss of DAXX enrichment at regulatory regions of stem cell genes, hypomethylation of the SOX2 promoter and partly the *NOTCH4* gene, and increased TIC survival. DAXX could be a novel TIC suppressor as ectopic expression of DAXX reduced pluripotent and stem gene expression, NOTCH signaling, and TIC survival when cells were treated with endocrine therapy. Overall, these findings provide rationale to identify novel DAXX-promoting therapeutics that

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enhance or maintain DAXX levels to selectively target TICs in ER$^+$ breast cancer with the purpose of preventing tumor recurrence.

This notion that ER activation may have beneficial effects in regards to cancer recurrence while paradoxical, is not surprising. E_2-mediated ER activation drives both proliferation and differentiation of human mammary stem and progenitor cells (39). The problem is that luminal cells become tumorigenic and grow in response to ER activation (46). This is why initially most patients with ER$^+$ breast tumors respond favorably to endocrine therapy, and is why endocrine therapy is considered one of the most effective and least toxic anticancer therapies to date. A potential unintended consequence of inhibiting the ER long term in a subset of patients, however, is the loss of its beneficial properties, namely its ability to drive luminal cell differentiation (47). This has been reported by a number of groups demonstrating that the ER restricts TICs in part by preventing the expression of TIC-associated pluripotency genes and signaling pathways, including NOTCH (5, 48). The exact mechanism by which ER restricts pluripotency is not fully understood, but it is thought to be due, in part, to regulation of epigenetic modifiers. This is demonstrated from findings by Bloushtain-Qimron and colleagues that indicated cell-fate of normal breast and cancer cells is predominantly determined by their genome-wide methylation pattern (49). Furthermore, they showed that TICs have a similar methylation pattern to that of healthy adult mammary stem cells, which included areas of hypomethylation near genes associated with stemness or pluripotency (49). This would suggest that epigenetic modifiers are critical for maintaining a stem-like cell fate or a differentiated state and their dysregulation may be a characteristic of TICs. The current findings support a novel mechanism in which DAXX functions as a repressor of TIC-genes and mediates the actions of ER to suppress TIC survival. The exact mechanism by which ER stabilizes the DAXX protein is not known but currently under active investigation.

Although DAXX RNA is unchanged in response to E_2 treatment, DAXX RNA expression in human ER$^+$ tumors is prognostic. The data suggest that protein expression might be a better predictor of response to therapy or recurrence. It is our future goal to screen tumor tissue using a validated DAXX IHC protocol to assess response and recurrence. However, there may be a threshold of DAXX RNA expression that is sufficient to overcome DAXX protein destabilization by endocrine therapy. It is possible that some ER$^+$ tumors expressing high DAXX RNA levels may be able to maintain DAXX protein levels and avoid selection for TICs. Conversely a subset of patients (as demonstrated by the KMplotter data) may not reach this threshold, thus DAXX protein levels are decreased and this allows for selection of TICs. If this is correct, it would provide further evidence that it may be in fact better to compare the upper quartile (top 25%) and lower quartiles (bottom 25%) in terms of DAXX RNA expression in terms of recurrence-free survival in patients.

In translating these results to the clinical, the therapeutic challenge will be to test novel agents that stabilize the DAXX protein under endocrine therapy conditions to suppress TIC-survival but yet not stimulate tumor growth. The goal will be eliminating the resistant TIC sub-population while debulking the tumor to prevent tumor recurrence during or after ET.

Disclosure of Potential Conflicts of Interest

A.K. Dingwall has an unpaid consultant/advisory board relationship with the Leukemia Research Foundation. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: D.S. Peiffer, A. Zlobin, K.S. Albain, C. Osipo
Development of methodology: D.S. Peiffer , D. Wyatt, C. Osipo
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.S. Peiffer, D. Wyatt, A. Zlobin, A. Piracha, K.S. Albain
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.S. Peiffer, A. Zlobin, A. Piracha, J. Ng, A.K. Dingwall, K.S. Albain, C. Osipo
Writing, review, and/or revision of the manuscript: D.S. Peiffer, J. Ng, A.K. Dingwall, K.S. Albain, C. Osipo
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Wyatt
Study supervision: C. Osipo

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DAXX Suppresses Tumor-Initiating Cells in Estrogen Receptor–Positive Breast Cancer Following Endocrine Therapy

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