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EARLY RELEASE:

ERα-XPO1 crosstalk controls tamoxifen sensitivity in tumors by altering ERK5 cellular localization

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Most breast cancer deaths occur in women with recurrent, ERα (+), metastatic tumors. There is a critical need for therapeutic approaches that include novel, targetable mechanism-based strategies by which ERα (+) tumors can be resensitized to endocrine therapies. The objective of this study was to validate a group of nuclear transport genes as potential biomarkers to predict risk of endocrine therapy failure, and to evaluate the inhibition of XPO1, one of these genes as a novel means to enhance the effectiveness of endocrine therapies. Using advanced statistical methods, we found that expression levels of several of nuclear transport genes including XPO1 were associated with poor survival and predicted recurrence of tamoxifen-treated breast tumors in human breast cancer gene expression data sets. In mechanistic studies we showed that the expression of XPO1 determined the cellular localization of the key signalling proteins and the response to tamoxifen. We demonstrated that combined targeting of XPO1 and ERα in several tamoxifen resistant cell lines and tumor xenografts with XPO1 inhibitor, Selinexor (SXR) and tamoxifen restored tamoxifen sensitivity and prevented recurrence *in vivo*. The nuclear transport pathways have not previously been implicated in the development of endocrine resistance, and given the need for better strategies for selecting patients to receive endocrine modulatory reagents and improving therapy response of relapsed ERα(+) tumors, our findings show great promise for uncovering the role these pathways play in reducing cancer recurrences.

The nuclear hormone estrogen receptor alpha (ERα) is present in approximately 75% of human breast cancers and is considered one of the most critical predictive factor of breast cancer prognosis (1). ERα is targeted by endocrine therapies, which are generally well-tolerated (1). Tamoxifen is one of the most effective therapeutics when a patient is diagnosed with estrogen receptor positive (ERα (+)) breast cancer. Although several recent trials showed that a combination of aromatase inhibitors (AIs) and ovarian suppression was effective in premenopausal breast cancer treatment, AIs also have significant adverse side effects in some postmenopausal women, such as increased joint pain, bone fractures and cardiovascular disease risk (1–3) and the American Society for Clinical Oncology (ASCO) still recommends the use of tamoxifen for premenopausal women (3). Therefore, tamoxifen remains an important endocrine therapy agent in both pre- and postmenopausal women, and is expected to continue to be widely used for some time.

The benefit of endocrine therapies is limited, as demonstrated by tumor recurrence in 30% of ERα (+) patients. The recurrence of cancer in ERα (-) patients is

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Abbreviations:

higher in the first five years after the diagnosis, yet for ERα (+) patients there is a consistent long-term risk of death due to recurrent breast cancer and even a greater risk after 7 years (4). In fact, most breast cancer deaths occur in women with recurrent ERα (+) metastatic tumors (5, 6). Recurrence appears to result from the upregulation of cytoplasmic-initiated/ERα dependent kinase pathways that provide an alternative mechanism to support tumor cell proliferation and survival and renders tumor cells resistant to endocrine therapies (7–11). Hormonal regulation of breast cancer involves crucial inputs from estrogens, acting through ERs, and growth factors, operating through growth factor receptors that regulate downstream protein kinase pathways. The relative regulatory inputs to/from these two pathways are thought to underlie the degree to which the cancers remain more indolent and responsive to endocrine therapies vs. acquisition of resistance to these therapies. In the latter situation, the upregulation of protein kinases serves as a hallmark of endocrine-resistant breast cancer (12–15). Also, while the presence of ERα is usually associated with a more favorable prognosis, (16–18) it is increasingly appreciated that not all ERα (+) breast cancer patients have a good outcome. A significant subset of patients with ERα (+) breast cancers, specifically those patients characterized as having a luminal B molecular subtype, also have high levels of protein kinase activity, do not respond to tamoxifen as efficiently, and have much less favorable disease-free survival, despite often being treated with additional chemotherapy (19–22).

ERK5, a member of the MAPK family, is present in most human breast tumors and is overexpressed in ~20% of these tumors (23). It was also recently identified in a kinase screening study as a major factor that regulates circulating tumor cell (CTC) invasiveness (24). We have recently identified ERα as the key factor responsible for the activation and regulation of the subcellular localization of ERK5 (25). In this previous work, we showed that its nuclear localization is abrogated by treatment with ERK5 or ERα inhibitors. Based on this information, we used a combinatorial approach in which we took advantage of our tamoxifen sensitive and resistant cell culture models, an animal model and data from patient samples to delineate the role of nuclear transport pathways, particularly XPO1, in tamoxifen sensitivity and endocrine therapy resistance. We identified high levels of XPO1, the major nuclear exporter of the tumor suppressor proteins, as a biomarker for tamoxifen resistance, and evaluated its inhibition as a novel means to enhance the effectiveness of endocrine therapies. Our findings suggest that higher expression of selected nuclear export pathway proteins results in decreased residence times of important nuclear factors that might be involved in proper transcriptional responses to tamoxifen treatment, thus conferring resistance to tamoxifen. Enhanced export to the cytoplasm results in key proteins communicating with other components of the cancer cell machinery involved in cell motility and enhanced kinase signaling, which function to increase aggressiveness of these cells. Our results show that inhibition of nuclear export machinery would improve therapy responsiveness and delay the development of hormone targeted treatment resistance and recurrence.

Materials and Methods

Cell Culture, Adenovirus, siRNA and Ligand Treatments

MCF-7 cells were grown in Minimal Essential Medium (MEM) with NEAA salts (Sigma, St Louis, MO), supplemented with 10% calf serum (HyClone, Logan, UT), and 100 μg/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA) (26). T47D cells were grown in DMEM with 10% calf serum (HyClone), and 100 μg/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA). BT474 were cultured in ATCC recommended Hybri-care medium with 10% inactivated FBS, sodium bicarbonate and antibiotics. MDA-MB-468 and MDA-MB-134 cells were grown in Leibovitz's medium with 20% calf serum (HyClone), and 100 μg/ml penicillin/streptomycin (Invitrogen). HCC1500 cells were cultured in ATCC formulated RPMI1640 media with 10% FBS, sodium bicarbonate and antibiotics. All cell lines were obtained from American Type Culture Collection (ATCC). For experiments with 4-OH-Tam treatment, the cells were maintained in the corresponding phenol red-free medium for at least 3 days and were then seeded at a density of 3×10^5 cells per 10 cm tissue culture dish (Corning, NY) for 2 days before adenovirus infection. Recombinant adenoviruses were obtained from Applied Biological Materials Inc (Richmond, California) and were used to generate MCF-7 cells expressing dominant negative ERK5 (AdERK5-DN) or overexpression XPO1 (AdXPO1) as described previously (25). Adeno virus with CMV construct were used as infection control (AdCMV).

Gene expression in MCF7 and BT474 cells

Cells were seeded in 6-well plates in concentration of 2.5×10^4 per well in treatment medium (phenol red free medium with 5% charcoal-dextran FBS, NaHCO3 and antibiotics). Medium was changed on day 2 and 4 and cells were treated on day 6 for 24 hours with ligands: Veh (EtOH), E2 (10−8M), 4-OH TAM (10−6M); SXR (10^{-7}M) +Veh, SXR +E2, SXR + 4-OH TAM. Total RNA was extracted with TRIzol reagent (Life Technologies) according to manufacturer protocol. The concentration and quality of RNA was determined with BioTek Cytation 5 plate reader. Reverse transcription was conducted with M-MuLV Reverse Transcriptase (BioLabs). qPCR reactions were done with Fast Start Universal SYBR Green reagent (Roche) using Applied Biosystem Step One Plus qPCR System. The primer sequences were obtained from Integrated DNA Technology website. 36b4 was used to normalize the gene ex-

pression level. The relative difference in gene expression level was calculated using ΔΔCt method.

In vivo xenograft study in mice:

Tumor xenograft studies were performed using BT474 cell line in immunocompromised female mice based on previously reported protocols (27, 28). We used 6 weeks old BALB/c athymic, ovariectomized nude female mice from Taconic Biosciences. After 1 week of acclimatization, we implanted subcutaneously 0.72 mg, 60 day release 17-β-E2 pellets from Innovative Research of America to maintain uniform level of estrogen. Next day we injected subcutaneously into both right and left flank of each mouse 2.5×10^7 BT474 cells resuspended in 50% PBS and 50% Matrigel. Once all the animals harbored tumors of approximately 200 mm^3, we randomized 5 animals to each treatment group. Half of the mice were implanted with vehicle pellets and the other half were implanted with 25 mg, 60 day release TAM pellets. We then randomized each group for Vehicle or SXR injection. We performed biweekly injections (Monday and Friday) for 4 weeks. Each mouse was housed individually. Animals were monitored daily by the veterinarians for any signs of starvation, dehydration, stress and pain. We monitored total weight, food intake and tumor size using a digital caliper biweekly. Tumors were removed from sacrificed mice at the end of the experiment or at the time when tumor size reached 1000 mm^3 and then stored at −80°C for further analysis.

Immunofluorescence Microscopy and Data Analysis

MCF-7, MCF-7 TAM R and BT474 cells were treated with Veh (0.1% EtOH) or 1 μM 4-OH-Tam in the presence or absence of 100 nM SXR for indicated times. Cells were then washed in PBS and fixed on glass coverslips in 4% paraformaldehyde for 30 minutes, washed two times for 5 minutes in PBS. After incubation in acetone for 5 minutes another PBS wash was performed and then cells were incubated with antibodies against XPO-1 (Santa Cruz, 1:500), ERα (Santa Cruz, 1:1000), ERK5 (Bethyl, 1:2000) or phospho-ERK5 (Upstate, Millipore, 1:500). Next day, cells were incubated with Goat anti mouse Alexa 568 or goat anti rabbit Alexa 488 secondary antibodies. These slides were mounted, and stained using Prolong Gold antifade with 4,6-diamidino-2-phenylindole (Molecular Probes) to identify the nuclei.

BT474 Xenograft samples were paraffin embedded and sectioned (4–5 micron). After rehydration, antigen retrieval and blocking the slides were incubated with XPO1 antibody (Santa Cruz, 1:100). Next day, slides were incubated with Goat anti mouse Alexa 568 secondary antibody. These slides were mounted, and stained using Prolong Gold antifade with 4,6-diamidino-2-phenylindole (Molecular Probes) to identify the nuclei.

Samples were imaged using a 63×/1.4 Oil DIC M27 objective in a Zeiss LSM 700 or 710 laser scanning confocal microscope (Carl Zeiss). The images were obtained in a sequential manner using a 488 Ar (10 mW) laser line for pERK5 signal (500–550 nm emission) and 555 nm (10 mW) laser line for ERα (600–650 nm emission). The individual channels were obtained using a sequential scanning mode to prevent bleed-through of the excitation signal. Laser power, gain and offset were kept constant across the samples and scanned in a high resolution format of 512 × 512 or 1024 × 1024 pixels with 2/4 frames averaging.

Further quantification of the images was performed in Fiji software (http://fiji.sc/wiki/index.php/Fiji) (29). Briefly, images were converted to 8 bit for segmentation for each channel. Images for all channels were background subtracted using a rolling-ball method, with a pixel size of 100. Images were segmented using the DAPI channel. DAPI images were contrast enhanced using Otsu algorithm. In order to split touching nuclei and produce the final nuclear masks, watershed algorithm was used. Resulting objects that had an area of less than 20 pixels and were close to edges were considered noise, and were discarded. DAPI image was selected as the mask and the signal from pERK5 and/or ERK5 signal was quantified in the nucleus. 3 frames per treatment were quantified. Experiments were repeated 2 times.

Cell Proliferation, Cell Cycle Progression, Invasion, Motility and Soft Agar Assays

For proliferation assays, cells were seeded on 96-well plates in confluency 2000 cells per well (except MDA-MB-134:5000 cells per well and HCC1500: 7000 cells per well) in no-phenol red media with 5% CD FBS. Cells were treated on day 2 and day 5 with indicated concentrations of (Z)-4-Hydroxytamoxifen (Sigma-Aldrich, #H7904) and/or SXR (Selleckchem, #S7252). On day 7 WST1 assay (Roche) was used to quantify cell proliferation ratio. Absorbance was measured at 450 nm using BioTek, Cytation5 plate reader (30). Invasion assays used BD-BioCoat Matrigel invasion chambers (BD Biosciences, San Jose, CA) with 10% fetal bovine serum as chemoattractant in the lower chamber as described (30). Motility and soft agar colony formation assays were performed as described (30). Briefly, sterile 2x MEM was mixed 1:1 with 1.2% low melting point agarose solution and disposed to 6-well plates to form 1.5 ml bottom base agar layer. Plates were immediately placed into the fridge for half an hour. Cells were diluted in 2xMEM and mixed 1:1 with sterile 1% low melting point agarose suspension cooled down to 37°C. Then 1.5 ml of cell-agarose suspension was placed on top of the bottom base agar layer. The final concentration was 6500 cells per well. The plates were placed at 4°C for 15 minutes and then 1 ml of full growth media was added for cells to recover. 24 hours later the media was changed to treatment media (5% CD FBS, NaHCO3, antibiotics) with ligands: Veh (EtOH), E2 (10^{-8}M), TAM (10^{-6}M); SXR (10^{-7}M) +Veh, SXR+E2, SXR+ TAM. Cells were treated once or twice a week aspirating old media and adding the fresh one. When the colonies were big enough (14 to 28 days) 500 ul of Giemsa Stain was added for 1 minute to each well. Wells were washed 3 times with PBS. Colonies were counted under the microscope.

Cell cycle distribution was assessed by flow cytometry on ethanol-fixed, RNase-treated and PI-stained cells. 10^6 cells were seeded in 10 cm dishes in reduced serum media (5% CD FBS with antibiotics), left overnight to attach and treated next day with ligands (Veh (0.1% EtOH), E2 10^{-8} M, 4-OH-TAM 1 μM, SXR 10^{-7} M, SXR + E2, SXR + 4-OH-TAM) for 24 hours. After the treatment, cells were collected in PBS with 0.1% FBS, washed twice and fixed with 70% v/v ice-cold ethanol for 24 hours at –20°C. Next day, after washing twice with PBS cells were resuspended in 1 ml of PBS with 50 μg/ml propidium iodide and 0.5 μg/ml RNase A. After 30 minutes. incubation at

room temperature at least 10^4 cells were analyzed on BD LSR II flow cytometer. The percentage of cells in S, G1, G2 cell cycle phases was analyzed with FCS Express 4 Software.

Tumor sample processing and QRT-PCR analysis of *XPO1*

RNA extraction and RT-qPCR analysis: Formalin-fixed paraffin embedded (FFPE) human breast cancer patient tumor samples obtained at the time of surgical resection were retrieved from the Carle Foundation Hospital Department of Pathology archives in accordance with an Institutional Review Board approved research protocol. Total RNA was extracted from 40 patient samples using FFPE RNA isolation kit (Invitrogen) following manufacturer's protocol. RNA was reverse transcribed into cDNA using gene specific primers and Superscript reverse transcriptase (Invitrogen). 33 of the 40 samples were found to have an adequate quality and quantity of cDNA to permit further analysis. Molecular subtype assignment of samples was based on 55-gene qRT PCR analysis of the PAM50 gene panel using the StepOne Plus system (Life Technologies) as earlier described (31). Quantitative RT-PCR for the *XPO1* gene was performed in parallel on all 33 molecular subtyped human patient samples.

Western Blot analysis in cell lines

On day 1 cells were seeded on 10 cm plates in concentration 100k per plate in no-phenol red media with 5% CD FBS. The media was changed on day 3. On day 6 whole cell lysates were collected in RIPA buffer (Thermo Scientific) with 1x Complete Protease Inhibitor (Roche). Samples were sonicated and boiled in SDS-loading buffer, then resolved on precast gels (Bio-Rad) and transferred to nitrocellulose membrane. Membrane was blocked in Odyssey Blocking Buffer (Licor). Following antibodies are used: pERK5 Thr218/Tyr 220 (#3371, Cell signaling), Lamin B1 (ab16048, Abcam), NUP153 (A301–789A), KPNA3 (A301–626A), NUP205 (A303–935A), RANGAP1 (A302–026A), KPNA2 (A300–483A), XPO1 (A300–469A), ERK5 (A302–656A) all from Bethyl Laboratories and ERK2 antibody (D-2 sc-1647, Santa Cruz Biotechnology) were used in 1:1000 dilution. β-actin (Sigma SAB1305546) used in 1:10000 dilution. The secondary antibodies were obtained from Odyssey and used at a working concentration of 1:10000. The images of the membranes are obtained by Licor Odyssey CLx infrared imaging device and software (32). To compare the levels of nuclear receptor signature genes in different cell lines, we normalized the signal from the signature protein to β-actin signal. Average values and stander error of mean from 3 experiments were reported.

For preparation of nuclear/cytoplasmic fractions cells were seeded at 2×10^6 cells/10 cm dishes. The media was changed at Day 2. At Day 4, cells were treated with Veh (0.1% EtOH) or 1 μM 4-OH-Tam for 45 minutes. Whole cell samples were suspended in cytosolic extraction buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1% NP-40 and protease/phosphatase inhibitors) and they were centrifuged at 12 700 rpm for 10 minutes at 4°C. Cytosolic fractions were transferred into a fresh tube and kept on ice. The pellets were washed with cytosolic extraction buffer without NP-40 and phosphatase/protease inhibitors three times and dissolved in Lysis buffer (0.5 M EDTA, 1 M Tris HCl (pH: 8), 10% SDS, 10% Empigen and phosphatase/protease inhibitors). After 10-minute incubation on ice, nuclear fractions were sonicated once at 20% amplitude for 5 seconds and centrifuged at 12 700 rpm for 10 minutes. Supernatants (nuclear fractions) were transferred into fresh tubes.

Tumor Data Sets and Data Analysis

log2 median-centered intensity expression values for signature genes were obtained from Oncomine database (33). Hierarchical clustering of data was performed and displayed using Cluster3.0 (34) and Java Tree View (35) software for analysis and visualization. Patients were stratified according to average expression value of the genes in the signature and the top 30% and bottom 30% of patients were used for computation of Kaplan-Meier curves (36) by the Cox-Mantel log-rank test (37) and Gehan-Breslow-Wilcoxon Test (38) in Graphpad Prism version 6.04 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

Inclusion criteria for breast cancer data sets included report of at least one time-to-outcome event (survival or recurrence) as a continuous variable, reporting of estrogen receptor (ER) and Triple Negative (TN) status, and reporting of a form of staging or grading. If multiple forms of staging or grading were reported, N stage was used; if only grade was reported, the levels of the grade were set equal to the levels of staging. Menopause status and treatment were collected if reported.

Analysis consisted of a Weibull accelerated failure time model (39) fit singly for each of the gene expression values of interest and included ER status, TN status, and stage as covariates. The model considered either time to death from disease or time to recurrence, with right censoring for all other outcomes. Each model was fitted both within and across all studies; the latter models included study as a fixed effect covariate. Subset analyses were conducted to consider the interaction between the gene expression effect and, separately, menopausal status and treatment. As the number of data sets available for the subset analyses were small, combined model fitting included data source as a fixed effect only. Multivariate models were fitted using Bayesian information criterion (40) based backward selection. All models were fit using the *survival* package (41) in R 3.0.3 accessed through Revolution R Enterprise 7.2.0 (© 2014 Revolution Analytics, Inc.). To assess the specificity and sensitivity of the tested markers, receiver operating characteristic (ROC) curves were generated using the *verification* package (42) in R 3.0.3.

Results

ERK5 nuclear localization is reduced during the course of tamoxifen resistance

To characterize the state of cellular localization of active ERK5 upon 4-OH-Tam treatment, we monitored the localization of ERK5 and pERK5 in a cell culture model of breast cancer endocrine resistance. We grew the initially tamoxifen responsive MCF-7 cells in continuous 4-OH-tamoxifen (4-OH-tam) treatment for 100 weeks. We and others have earlier demonstrated that this model

mimics the development of endocrine resistance and for the characterization of molecular changes associated with tamoxifen-resistant phenotype (43). When we monitored localization of ERK5 in cells that were in 4-OH-Tam for 10, 50 and 100 weeks, we found that ERK5 and pERK5 localization to nucleus was lost progressively compared

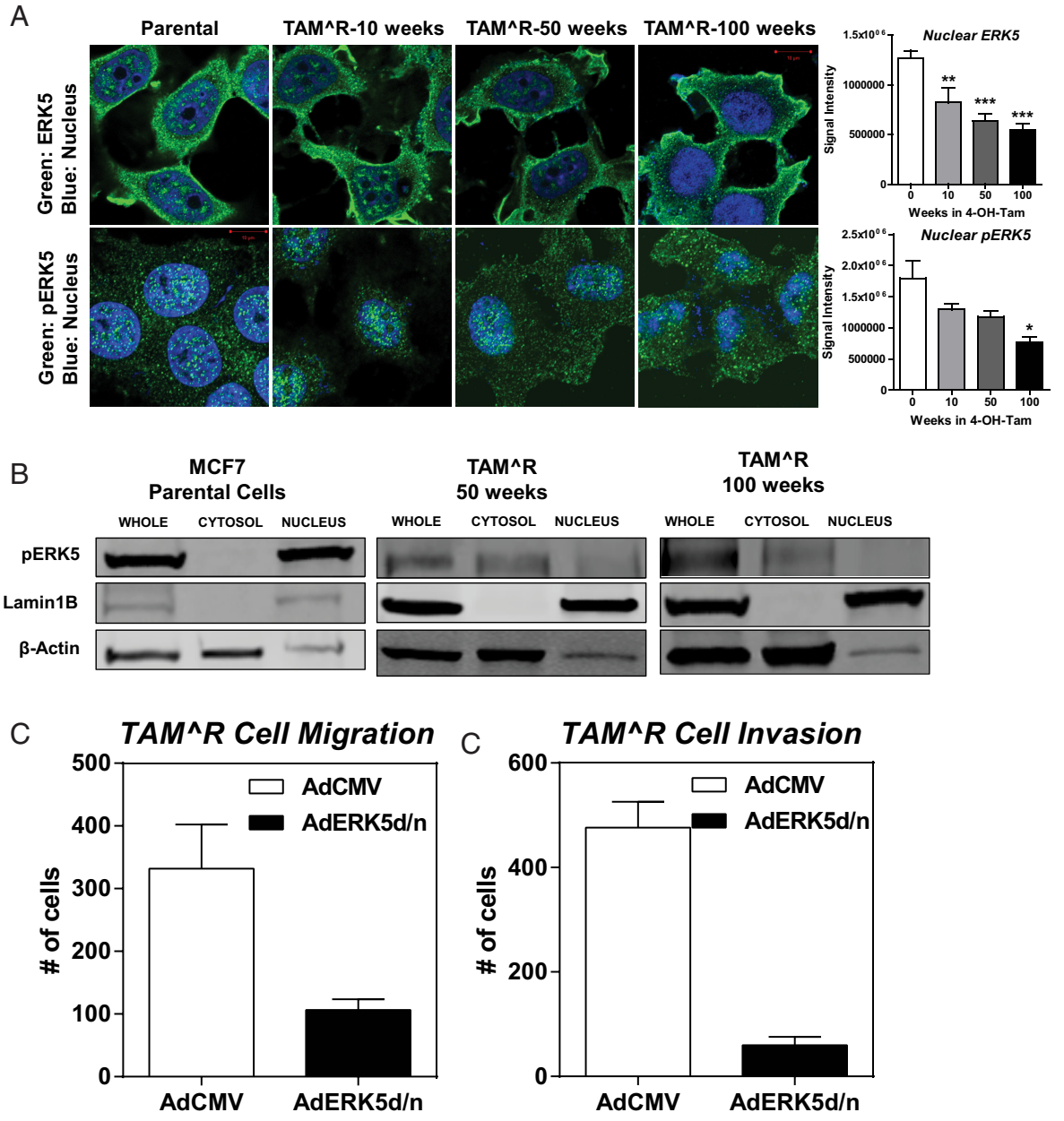

Figure 1. ERK5 nuclear localization is lost in MCF-7 cells that are Tamoxifen Resistant **(a)** ERK5 (upper panel) and pERK5 (lower panel) Immunostaining after 45 minutes of 4-OH-Tam treatment in tamoxifen sensitive MCF-7 cells and tamoxifen resistant MCF-7 TAM R cells that were kept in 4-OH-Tam for indicated times. MCF-7 cells or MCF-7 TAM R cells at different stage of resistance progression were treated with 1 μM 4-OH-Tam for 45 minutes and immunofluorescence microscopy was performed with an antibody specific to ERK5 or pERK5. Nuclei were stained with DAPI. 3 fields per slide were quantified. (N = 3). A one-way analysis of variance (ANOVA) model was fitted to assess the contribution of tamoxifen resistance progression on 4-OH-Tam treatment induced ERK5 or pERK5 nuclear localization. When the main effects were statistically significant at α=0.05, pairwise t-tests with a Newman-Keuls correction were employed to identify the time that ERK5 or pERK5 localization was significantly different from parental MCF-7 cells. *$P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$. **(b)** Nuclear localization of pERK5 decreases as tamoxifen resistance progresses. MCF-7 parental cells or MCF-7 TAM R cells that were kept in media containing 4-OH-Tam for 50 weeks or 100 weeks were fractionated and whole cell lysate and cytosolic or nuclear fractions were subjected to western blot analysis using pERK5, Lamin1b (as nuclear fraction marker) and β-actin (as cytosolic fraction marker) antibodies. **(c)** ERK5 activity regulated migratory potential in MCF-7 TAM R cells. MCF-7 TAM R cells were infected with AdCMV or dominant negative Ad-ERK5 for 24 hours and then seeded on the upper chamber of transwell system for migration assays (b) or invasion assays (c). Number of cells that migrated/invaded to the bottom side of the chambers were counted. Values are presented as mean ± SEM from 2 independent experiments.

to parental MCF-7 cells. (**Figure 1A**). This was consistent with increased cytosolic localization of pERK5 as the resistance progressed (**Figure 1B**). Moreover, ERK5 signaling contributes to motility and invasiveness in TAM resistant breast cancer cells similarly as in ERα (-) cell lines (**Figure 1C and D**), whereas in parental MCF-7 cells ERK5 mainly regulated cell cycle progression as we previously reported (25).

Derivation of nuclear transport signature

Because the lack of nuclear ERK5 localization in tamoxifen resistant cells suggested a potential role for nucleocytoplasmic transport machinery in the development of resistance, we monitored the expression of genes important for nuclear transport. To study the differential expression of nuclear transport genes that are most pertinent to clinical outcomes in breast cancer patients, we utilized the data from publicly available tumor data sets (**Figure 2A**). We first generated a comprehensive list of nuclear transport genes using web-based gene set enrichment analysis (GSEA) database. Next, we used Oncomine tumor data sets to select genes that were differentially expressed in ERα(+) vs. ERα(-) tumors (n = 49 genes). Interestingly expression of these 49 genes were overall lower in ERα(+) tumors. Furthermore, we used survival and outcome data analysis to generate a final list of 13 genes, some of which were previously shown to be modulated in different pathological conditions (**Figure 2B**).

Of the 13 selected genes, 9 encoded proteins that are either structural components of nuclear pore complex, such as NUP153 and TPR or other proteins that play a role in the transport of the cargo by interacting with the cargo in the cytoplasm or nucleus, such as XPO1 and KPNA2. XPO1 is an exportin that binds to nuclear export sequence (NES) of cargo proteins, among them the major tumor suppressor proteins like p53, p21, pRb, FOXO, survivin, IκB (44) and exports them out of the nucleus, and is already being evaluated in multiple later stage clinical trials in patients with relapsed and/or refractory he-

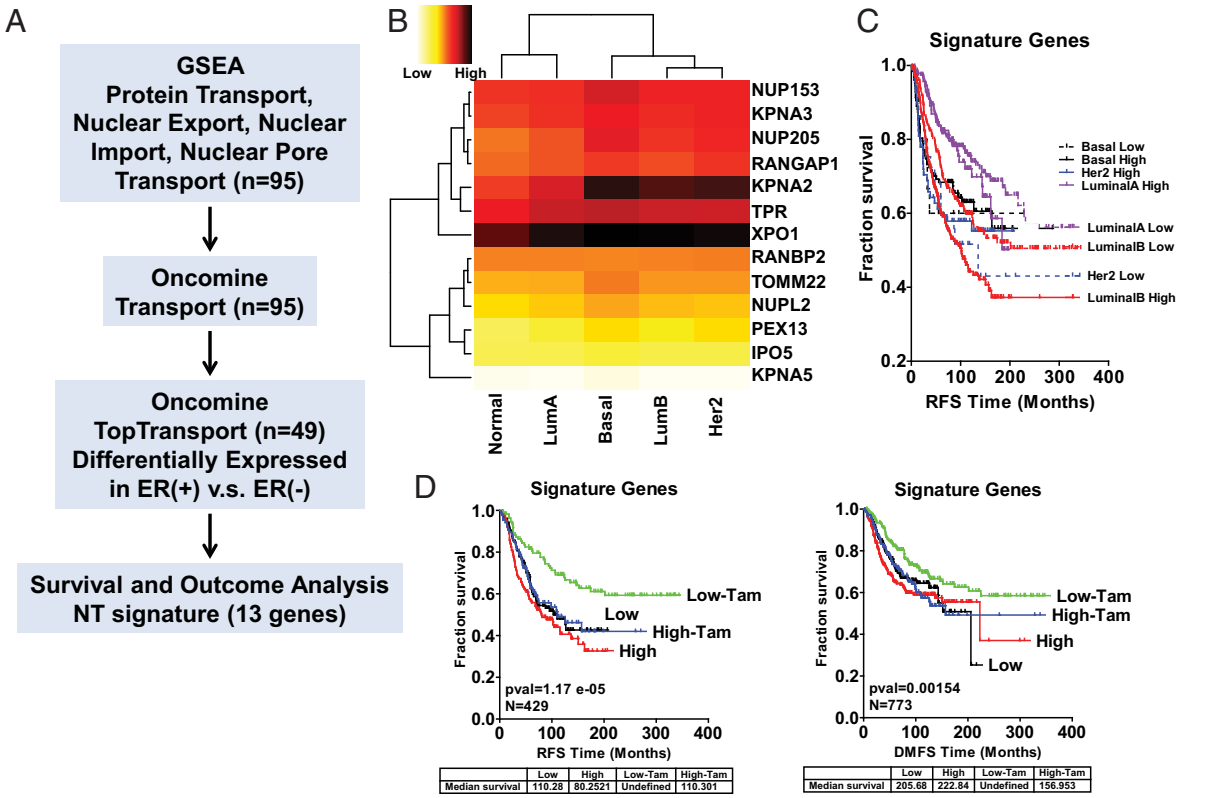

Figure 2. Derivation of nuclear transport signature and relation to tumor outcome. **(a)** Flow chart presenting derivation of nuclear transport signature. **(b)** List of nuclear transport signature genes and heat map of expression of each gene in different breast cancer subtype. The root tree shows similarities between different breast cancer subtypes when it comes to expression in signature pattern. The analysis of publicly available tumor data was performed using web-based Geneanalytics software using 13 genes as the input and with the following variables: 250 months, PAM50 subtype, Median split, compare tamoxifen, any chemo and end point recurrence-free survival (RFS) or distance metastasis-free survival (DMFS) **(c)** Comparison of recurrence-free survival of patients that are stratified depending on high and low expression of nuclear transport signature in different breast cancer subtypes using analysis from (b). **(d)** Nuclear transport signature predicts recurrence free and distant metastasis free survival (DMSF) of tamoxifen treated patients. Recurrence-free survival and metastasis-free survival analysis using our nuclear transport gene list and patient gene expression and survival/recurrence data from GSE2034(48), GSE20711(49), GSE6532 and GSE6532 1 (50), GSE7390(51), GSE9195 (52, 53) using Geneanalytics software and Graphpad software.

matological and solid tumor malignancies (45). KPNA2, 3 and 5 are karyopherins, belonging to the importin alpha family, and are involved in nuclear protein import. KPNA2 has recently been identified as a target of estrogen signaling in breast cancer cells (**46**). IPO5 is an importin that is important for nuclear transport of proteins. Several of these proteins, including NUP153 and TPR, were shown to exhibit transcription dependent mobility in the cell, suggesting an important role for these proteins in ERα mediated transcription (**47**). Expression level of all these genes was highest in basal like tumors followed by Her2 (+) tumors. Interestingly, luminal B type tumors, which are ERα (+) but more aggressive than luminal A tumors due to increased kinase signaling have similar overexpression of the signature genes as basal and Her2 (+) tumors which are also more aggressive, harder to treat and have poorer prognosis. (**Figure 2B**). More than 50% of breast cancer patients had high expression of nucleocytoplasmic transport signature in Lumina B, Her2 (+) and Basal subtypes (**S Figure 1A**).

Next, we monitored the predictive power of these 13 genes in 6 tumor data sets including GSE2034(48), GSE20711(49), GSE6532 and GSE6532 1 (50), GSE7390(51), GSE9195 (52, 53) using the Geneanalytics tool (http://geneanalytics.duhs.duke.edu/). High expression of signature genes predicted worse survival when we did not distinguish between the cancer subtypes. Next, we tested whether the impact of the gene signature on survival increased when we divided samples based on their subtypes. When we divided our data set based on PAM50 scores, luminal B subtype with high expression of these genes had the worst outcome (**Figure 2C**). Interestingly this was not the case for Her2 over expressing tumors as lower expression of the signature genes predicted a worst outcome in this subtype (**Figure 2C**). The predictive power of our gene signature improved further when we divided Luminal B samples based on tamoxifen treatment status (**Figure 2D**). Those patients that had low expression levels of nuclear transport genes (black line, Low) had survival similar to those of high expression (red line, High) but responded favorably to tamoxifen (green line, Low-Tam). However, those patients with high expression level of signature genes levels did worst (red line, High) and treatment with tamoxifen slightly improved survival, but the improvement was not statistically significant at $\alpha = 0.05$ (blue line, High-Tam). Recurrence and distant metastasis free survival of Luminal B patients with high expression level of nuclear transport signature genes did not improve with tamoxifen treatment. (**Figure 2D**). Thus, the average expression of these genes predicted those patients in the Luminal B subtype that would respond to tamoxifen well. These results show that we selected the most significant nuclear export regulators that have been implicated in disease-free survival and distant metastasis-free survival of tamoxifen resistant breast cancers. Based on these data we propose that during development of resistance to tamoxifen nuclear export components are selectively upregulated.

Characterization of XPO1 and the other signature genes in tumor data sets

To further understand the contribution of each gene to the predictive power of the identified nucleocytoplasmic transport signature, we characterized the effect of individual genes on patient outcomes using ten publicly available tumor data sets. The selection criteria, data sets and results for each gene individually are depicted in **S Figure 2** and **S Table 1**. The expression levels of the signature genes were highly correlated and the correlation levels of the genes are shown for the hormone-treated survival (**S Figure 3**) and recurrence (**S Figure 4**) data sets.

Survival. We used publicly available data from ten studies to assess the individual contribution of the expression of our set of 13 genes to overall survival time (**S Figure 2**). We used the two publically available data sets (54, 55) that reported any treatment information to assess the role of treatment in the relationship between gene expression values and survival. By fitting individual accelerated failure time models to each gene, we determined that increased XPO1 expression increased mortality rates in women receiving a combination of chemotherapy, hormone treatment, and radiation and also was associated with increased mortality in women receiving chemotherapy combined with tamoxifen treatment (**S Figure 5A**).

We then used these publicly available data to assess the influence tamoxifen treatment had on the association between expression of these 13 genes and overall survival. In two of these data sets from (54), 2450 women were treated with tamoxifen treatment, of which 732 women died of cancer, on average 2133 days after diagnosis. Using this subset of the data to fit individual accelerated failure time models for each gene, we obtained statistically significant associations between all genes and survival time (**S Figure 6**). XPO1 increased survival after tamoxifen in two different data sets (**S Figure 5B**). Thus, we conclude from the analysis of publicly available survival data that the specific genes whose expressions are significantly associated with survival depend on factors including hormone treatment and chemotherapy.

Recurrence. There were 12 studies providing information on time to recurrence (**S Figure 7**). Eight of the 13 signature genes, fitted individually in accelerated failure time

models with no covariates, were found by the fixed effect model (ie, the model fitted across the 12 studies) to have expression values significantly associated with time to recurrence (**S Figure 8**): IPO5, KPNA3, NUP153, RANBP2, RANGAP1, TOMM22, TPR, and XPO1. Two data sets included only women who received hormone therapy (HT) (50), both of which were used to analyze the effect of the 13 signature genes in tamoxifen-treated women. In these data sets, 164 women were included and 41 suffered a recurrence at, on average, 1970 days after diagnosis. Increased expression of XPO1 was associated with increased overall recurrence and recurrence after tamoxifen in different data sets (**S Figure 5C and D**). We conclude from the analysis of publicly available recurrence data that the specific genes whose expressions are significantly associated with recurrence depend on factors including hormone treatment and chemotherapy. While the list of all genes associated with recurrence differed from the list of all genes associated with survival, XPO1 was associated with both outcomes.

Nucleocytoplasmic transport signature genes are regulated by 4-OH-Tam treatment in ERα (+) breast cancer cell lines

To further characterize the molecular basis of the increase in expression of nuclear transport genes and their effect on tamoxifen response, we verified our findings in various cell lines that corresponded to different subtypes of breast cancer. To examine if ERα ligands induced expression of nucleocytoplasmic genes, we treated MCF-7 cells with 10 nM E2 or 1 μM 4-OH-Tam for 24 hours and examine mRNA expression of the genes using qPCR. 8 of the 13 signature genes were stimulated 1.5 fold or more at this time point by 4-OH-Tam treatment (**Figure 3A**). Moreover, using published ERα ChIP-Seq data (56) in MCF-7 cells and MCF-7 Tam R cells we showed that these genes had at least one ERα binding site within 100 kb of their promoters, suggesting that direct ERα recruitment after ligand treatment was responsible for the increase in gene expression (**Figure 3B**). In addition, short and long term treatment of MCF-7 cells with 4-OH-Tam increased protein expression of several of the signature genes including XPO1, RANGAP1, NUP205, NUP153 and KPNA2 (**Figure 3C**). When we compared expression of protein expression of same genes in tamoxifen sensitive MCF-7 and T47D cells with that of in tamoxifen resistant BT474, MDA-MB-134 and HCC-1500, we found that the protein level of 4 of these factors (XPO1, RANGAP1, NUP205 and NUP153) were higher in the tamoxifen-resistant cell lines consistently. (**Figure 3D**). Based on this evidence, we propose that nucleocytoplasmic transport signature is likely to be a marker for risk of failure of endocrine therapies and might also be a key element controlling the localization of key signaling molecules that underlie the development of endocrine resistance.

XPO1 inhibitor SXR resensitizes tamoxifen resistant breast cancer cell lines

To test feasibility of targeting the nuclear export pathways, we focused on XPO1, an exportin that binds to nuclear export sequence (NES) of cargo proteins and exports them out of the nucleus. In our analysis with tumor samples, high XPO1 values are associated with a poor outcome in all women who are treated with HT with or without chemotherapy. Moreover, XPO1 was previously indicated in nuclear export of ERK5(57). In addition XPO1 is already being targeted in other therapy resistant cancers, including leukemias (58) and prostate cancers (59, 60) with a highly specific small molecule inhibitor, Selinexor (SXR) (61). SXR is orally active and generally well tolerated. It has manageable side effects including nausea, fatigue and anorexia that improve over time on treatment. Even in patients that remained on therapy for more than eight months, no significant cumulative drug toxicities have been identified (62).

Because we found correlation between XPO1 expression and failure of the tamoxifen treatment in our preliminary analysis (**Figure 2D**), we hypothesized that the treatment of tamoxifen-resistant breast cancer cells with an XPO1 inhibitor would improve sensitivity of these cells to endocrine therapies. To test our hypothesis, we initially performed dose response studies in various cell lines to identify the ideal dose to treat these cells (**Figure 4A**). We also treated 4-OH-Tam responsive, MCF-7 and resistant, BT474, HCC 1500, MDA-MB-134 and MCF-7 TAM R cell lines with increasing doses of 4-OH-Tam and/or SXR. In all of the cell lines cotreatment with 4-OH-Tam and SXR caused a left shift in the nonlinear regression curves suggesting an improved response to any of the agents when the combination is applied (**Figure 4B**). Based on these results we used SXR at 10^{-7} M in the rest of our experiments. Next, we treated tamoxifen responsive MCF-7 and T47D cells and tamoxifen resistant MCF-7 TAM R, BT474, MDA-MB-134 and HCC-1500 cells with increasing doses of tamoxifen in the presence or absence of 10^{-7}M SXR. Inhibitor treatment decreased proliferation in tamoxifen sensitive cells and blocked tamoxifen induced proliferation in tamoxifen resistant cell lines (**Figure 4C**).

XPO1 is increased in Luminal B subtype patient tumor samples and human breast cancer cell lines and increased XPO1 levels increase cell proliferation of tamoxifen-resistant cell lines in the presence of 4-OH-Tam

First, we verified our findings in patient tumor samples (**Figure 5A**). In our studies in an independent cohort of human breast cancer patient tumor samples clinical samples, we found that XPO-1 and other signature genes had higher mRNA levels in the Luminal B molecular subtype of ER positive breast cancers that is characterized by significantly worse disease-free survival compared to ER positive cancers of the Luminal A molecular subtype(**Figure 5A**) (19–22). Our experiments in MCF-7 and BT474 cells indicated that XPO1 is important for G1-S phase transition in MCF-7 cells, where in BT474 XPO1 activity modulates 4-OH-Tam induced cell proliferation by regulating both G1-S and G2-M phase transition since SXR reduces percent of cells in both S and G2 stages (**Figure 5B**). Of note, 4-OH-Tam treatment increased percentage of cells in G2-M transition and treatment with SXR reduced number of cells in this phase (**Figure 5B**). When we tested if XPO1 inhibition modulated anchorage independent growth of BT474 cells, which proliferate in the presence of 4-OH-Tam, we observed that treatment with SXR decreased the number of colonies formed. The colonies that formed in the presence of E2 and 4-OH-Tam were bigger and had more dispersed shape, which was abrogated by SXR treatment (**Figure 5C**). Overexpression of XPO1 using an adenovirus system resulted in at least 2 fold increase in level of XPO1 mRNA and protein in MCF7 and BT474 cells increased active ERK5 levels in

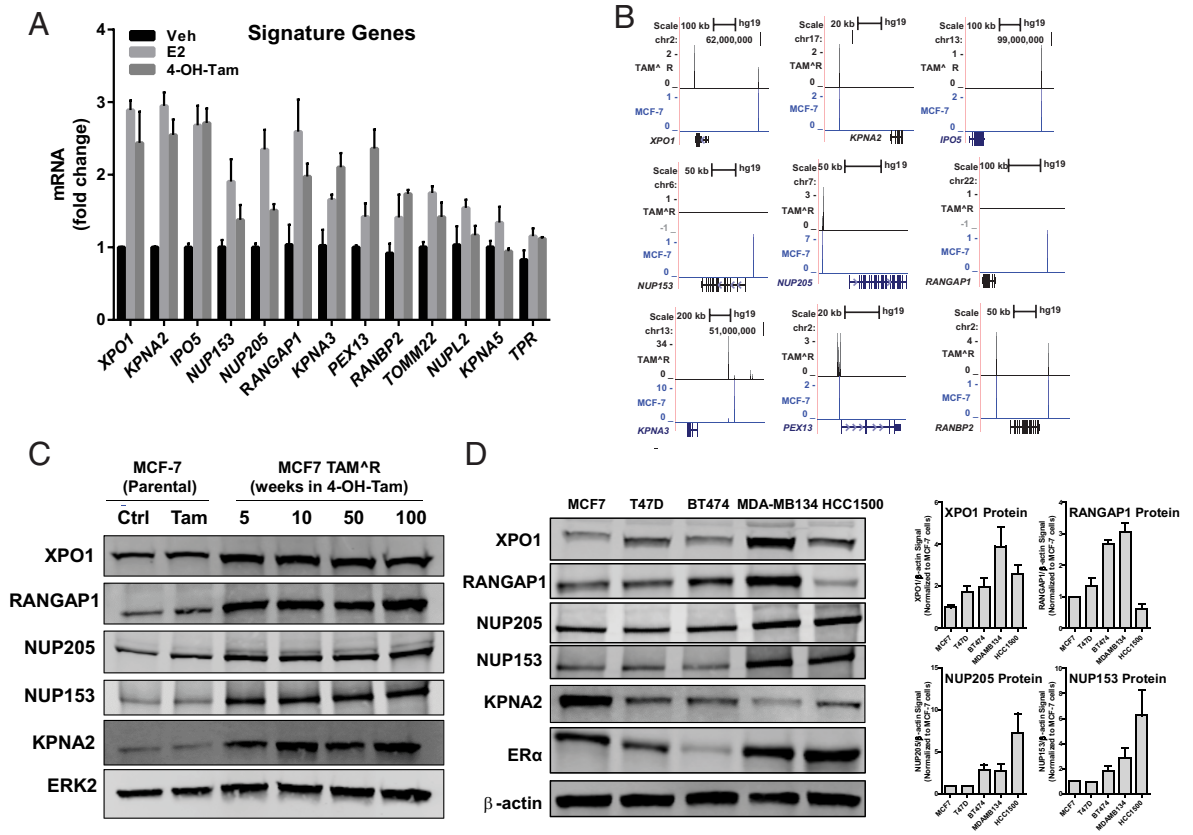

Figure 3. Expression of nuclear transport signature genes are stimulated by 4-OH-Tam in ERα (+) breast cancer cells. (a) ERα ligands increase mRNA expression of signature genes in MCF-7 cells. MCF-7 cells were treated with 10 nM E2 or 1 μM 4-OH-Tam for 24h. Total RNA was isolated and mRNA expression of signature genes were analyzed using qPCR. **(b)** ERα recruitment to gene regulatory regions of nuclear transport signature genes upon Tam treatment in MCF-7 cells or in MCF-7 TAM R cells. Dendograms for ERα occupancy in MCF-7 TAM R cells (black) and MCF-7 cells that are treated with 4-OH-Tam (blue) from UCSC (University of California, Santa Cruz) genome browser. BED files are obtained from (75). **(c)** Protein expression of nuclear transport signature genes are increased with ligand treatment in MCF-7 cells and MCF-7 TAM R cells. Western blot analysis of XPO1, RANGAP1, NUP205, NUP153, KPNA2 and ERK2 as the loading control for MCF-7 parental cells or MCF-7 TAM R cells that were kept in media with 4-OH-Tam for indicated times. The experiment was repeated 3 times. Representative results are shown. **(d)** Comparison of signature gene expression in different cell lines. ERα (+) MCF-7, T47D, BT474, MDA-MB-134 and HCC-1500 cells were cultured as described. Western blot analysis of XPO1, RANGAP1, NUP205, NUP153, KPNA2 and β-actin as the loading control for all cell lines was performed. Representative results from western are shown. Experiment was repeated three times. Signal from each antibody is calculated and normalized relative to β-actin signal. Level of proteins in cell lines is reported relative to MCF-7 cells. For proteins that increase in tamoxifen-resistant cells relative to MCF-7 cells average relative expression and SEM is plotted.

both of the cell lines (**Figure 5D**). Moreover XPO1 overexpression increased proliferation of both cell lines which was blocked by XPO1 inhibitor (**Figure 5E**).

XPO-1 activity is required for Tamoxifen preferential gene regulation in breast cancer cell lines and regulates subcellular localization of pERK5

To determine if XPO1 was required for ERα mediated gene transcription in response to estrogen and tamoxifen, we treated MCF-7 cells in the presence and absence of SXR a bioavailable small inhibitor that binds covalently to XPO1 and inhibits nuclear export. E2 mediated gene regulation events were not affected by the inhibitor treatment;however stimulation of tamoxifen preferential gene FOXM1 and YWHAZ (43) was lost in the presence of the inhibitor (**Figure 6A**). To test if 4-OH-Tam-mediated localization of pERK5 was different in tamoxifen resistant and sensitive cell lines, we treated MCF-7 cells and BT474 cells with Veh or 4-OH-Tam for 45 minutes and then isolated cytoplasmic or nuclear fractions. Western blot analysis of each fraction revealed that 4-OH-Tam treatment resulted an increase in nuclear pERK5 in MCF7 cells whereas active ERK5 was in cytoplasm in BT474 cells after 4-OH-Tam treatment (**Figure 6B**). To test if this localization disparity in two cell lines was due to XPO1 activity, we treated BT474 cells with Veh or 4-OH-Tam in the presence or absence of SXR for 45 minutes and then subjected cells to immunofluorescence analysis. This experiment showed that in the presence of 4-OH-Tam active ERK5 was completely extranuclear and treatment of the cells with the SXR relocated ERK5 back into the nucleus (**Figure 6C**).

XPO1 inhibitor SXR resensitizes tamoxifen resistant tumor xenografts to tamoxifen treatment

To verify our findings from cell line experiments in an in vivo system, we performed experiments using BT474 cell xenografts in immunocompromised mice as previously reported (27, 28). In this experiment BT474 cells formed bigger tumors in the animals that are treated with TAM compared to the tumors in animals treated with Veh (**Figure 7A, B and C**). In fact, XPO1 mRNA and protein expression was also higher in these tumors that

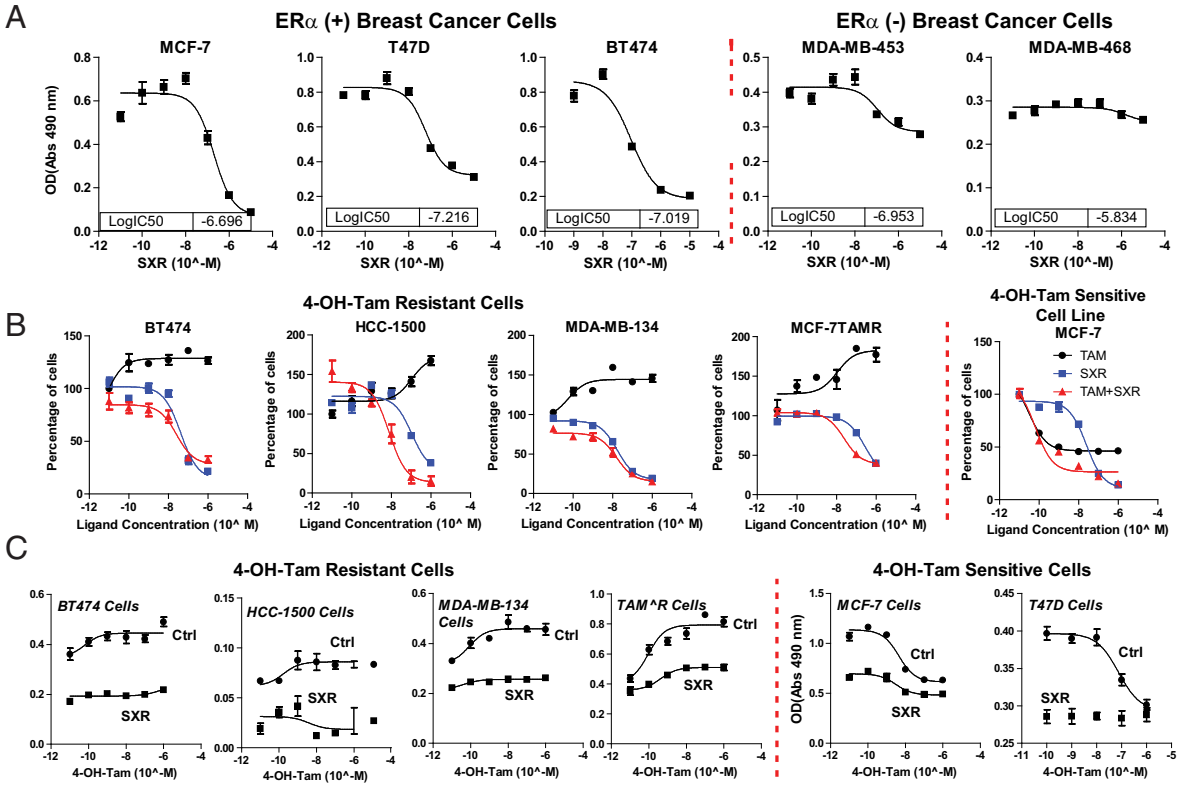

Figure 4. XPO1 inhibitor resensitizes tamoxifen resistant breast cancer cells to tamoxifen. **(a)** ERα (+) breast cancer cells are more sensitive to SXR treatment. Dose response for the SXR in ERα (+) MCF-7, T47D and BT474 cells and ERα (-) MDA-MB-453 and MDA-MB-468 cells. IC50 values were calculated using nonlinear regression analysis in Graphpad software. **(b)** Impact of single and combination of 4-OH-Tam and SXR treatments in various ERα (+) cell lines. Cells were treated with increasing doses of 4-OH-Tam and/or SXR. Cell numbers were examined using the WST1 assay at day 7. Values are the mean ± SEM from at least 2 independent experiments. **(c)** Impact of XPO1 inhibitor on cell proliferation. Cells were treated with increasing doses of 4-OH-Tamoxifen in the presence or absence of 10^{-7} M SXR. Cell numbers were examined using the MTS assay at day 7. Values are the mean ± SEM from at least 2 independent experiments.

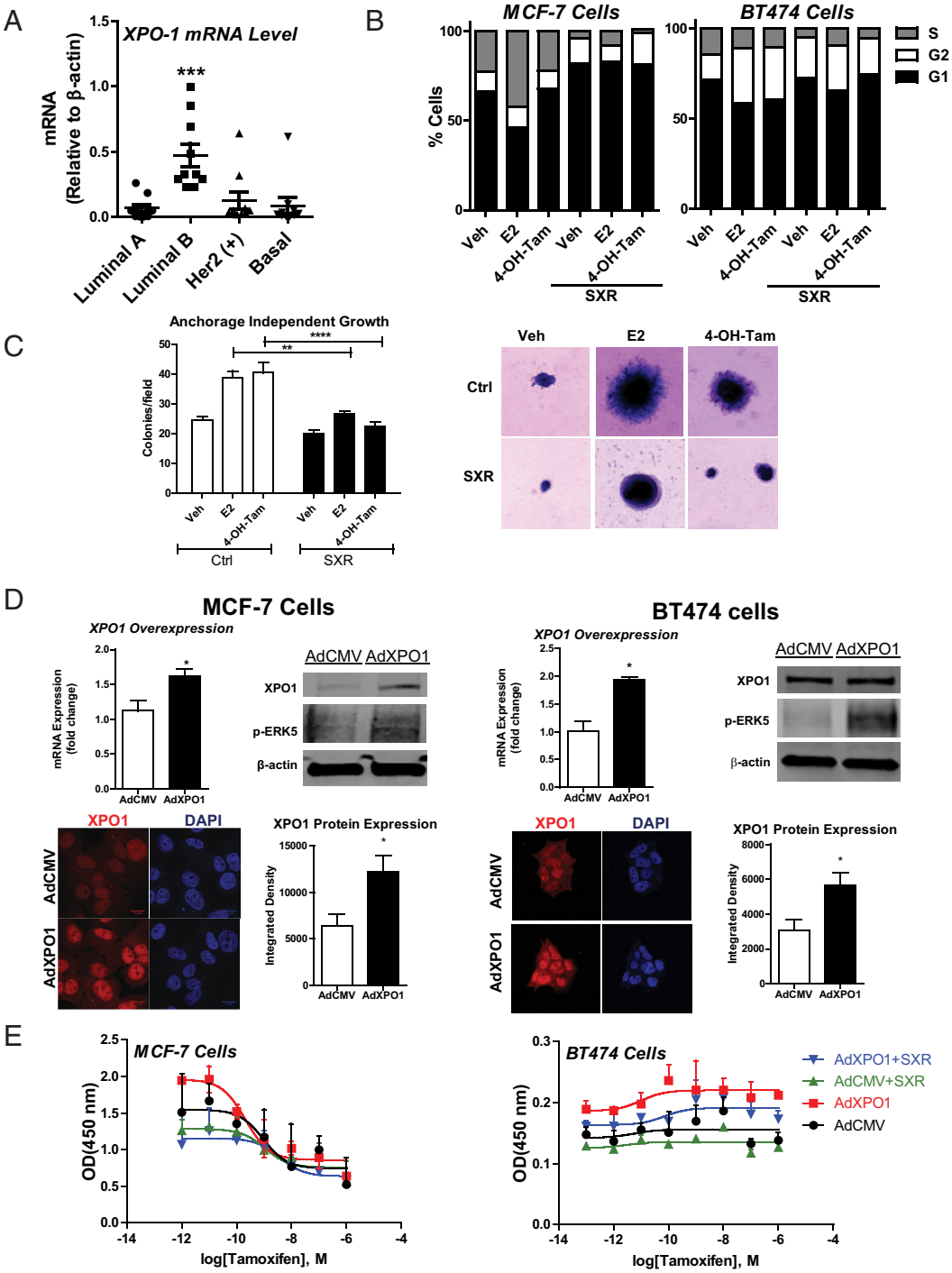

Figure 5. XPO1 is increased in Luminal B subtype patient tumor samples and human breast cancer cell lines and increased XPO1 levels renders cells insensitive to tamoxifen. **(a)** Verification of XPO-1 levels in patient tumor samples. mRNA from tumor FFPE samples were isolated. After quality of RNA was verified Q-PCR was run for XPO1 and 36B4 as control. A one-way ANOVA model was fitted to assess the contribution of subtype to expression of XPO1 mRNA. When the main effects were statistically significant at α=0.05, pairwise t-tests with a Newman-Keuls correction were employed to identify the subtype that had the highest level of XPO1 expression. ***, $P < .01$. **(b)** Impact of XPO1 inhibitor on cell cycle progression. Cells were treated with Veh or 1 μM 4-OH-Tam in the presence or absence of 10^{-7} M SXR. Cell numbers were examined using the FACS analysis. **(c)** Anchorage independent growth of tamoxifen resistant BT474 cells treated or nontreated with XPO1 inhibitor (SXR). Colony formation was visualized with Giemsa staining. On the right are representative pictures of spheroid, single colonies formed by BT474 cells. A two-way ANOVA model was fitted to assess the contribution of ligand (Veh, E2 or Tam) and inhibitor (Ctrl and SXR) treatment on anchorage dependent growth of BT474 cells. When the main effects were statistically significant at α=0.05, pairwise t-tests with a Bonferroni correction were employed to identify if treatment were statistically different from each other. ** $P < .01$, **** $P < .0001$. **(d)** XPO1 overexpression in MCF-7 and BT474 cell increase ERK5 activation. MCF7 cells (Left panel) or BT474 cells (Right panel) were infected with AdCMV as control or AdXPO1. *XPO1*mRNA overexpression was detected by Q-PCR. XPO1 protein overexpression was assessed by western blot of immunofluorescence analysis. *t* test was applied to assess if AdXPO1 virus infection resulted in statistically significant overexpression of XPO1 in

were treated with TAM (**Figure 7B and C**). Inhibition of XPO1 using biweekly SXR injections blocked tumor growth, but once the injections were stopped these tumors started to grow back (**Figure 7C, blue line**). Conversely, we saw complete disappearance of tumors in animals that received TAM+SXR, and these tumors did not come back weeks after the injections are stopped (**Figure 7C, purple line**), suggesting that a combination of SXR and TAM is more effective in resensitizing the tumor cells to tamoxifen. We also monitored food consumption and weight of these animals and did not see any statistically significant change in these parameters at $\alpha = 0.05$, which suggested that the effects that we observed are due to inhibition of XPO1 but not due to decrease in food consumption (**S Figure 9A**). Of note, the body weight of animals that received SXR+TAM treatment increased after we stopped injections suggesting improved overall health of these animals after tumor disappearance (**S Figure 9B**). These results showed feasibility of inhibiting XPO1 in wild type BT474 xenograft model. Based on this evidence, we believe that nucleocytoplasmic transport signature is likely to be an important element controlling the localization of key signaling molecules that underlie the development of endocrine resistance.

Legend to Figure 5 Continued. . .
each cell line. *$P < .05$ **(e)** XPO1 overexpression in MCF-7 and BT474 cells increase cell proliferation. MCF-7 cells (left panel) and BT474 cells (right panel) were infected with AdCMV as control or AdXPO1 and then cells were treated with increasing doses of 4-OH-Tam in the presence or absence of 100 nM SXR.

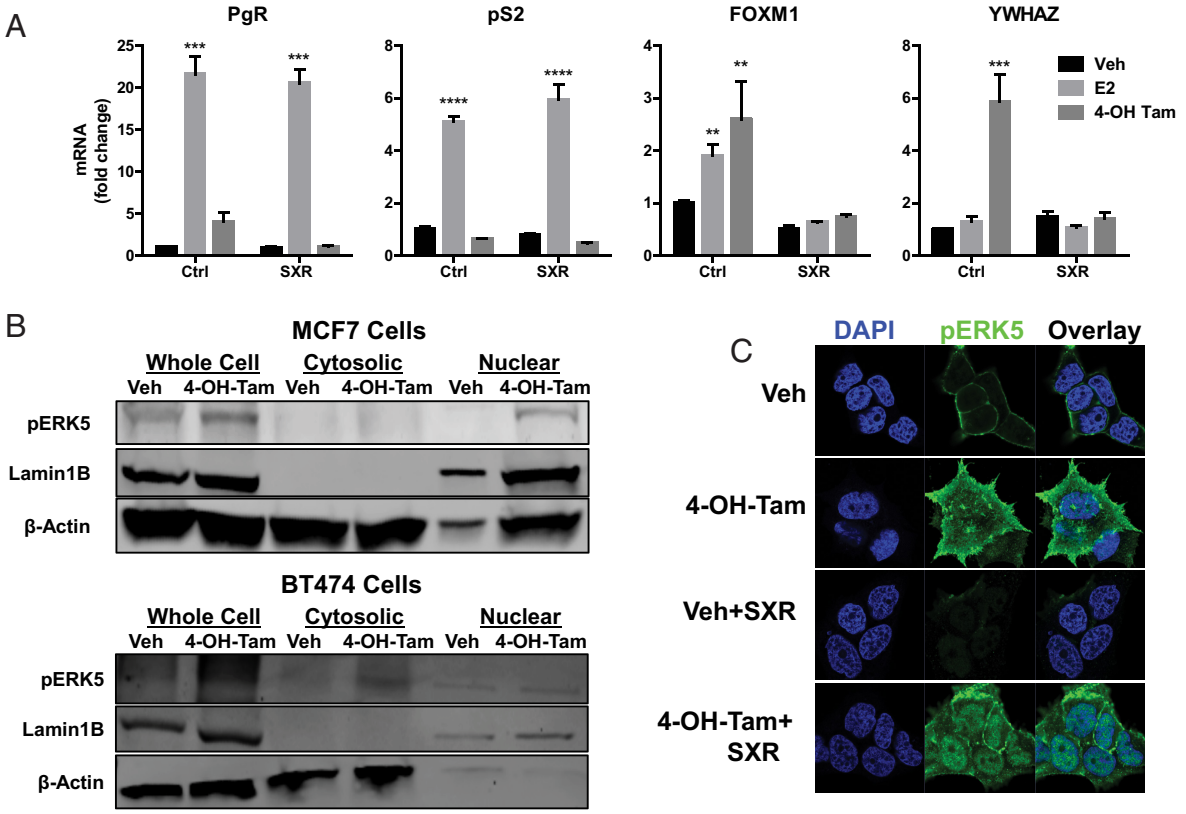

Figure 6. Impact of XPO1 activity on 4-OH-Tam preferential gene expression and subcellular localization of pERK5. (a) MCF-7 cells were treated with 10 nM E2 and 1 μM 4-OH-Tam for 24 hours. Expression of *PgR*, *pS2* and Tam preferential genes; *FOXM1* and *YWHAZ* was analyzed with real-time qPCR using 36b4 as internal control. The normalized fold expression was calculated with the ΔΔCt method relative to Veh samples. A two-way ANOVA model was fitted to assess the contribution of ligand (Veh, E2 or Tam) and inhibitor (Ctrl and SXR) treatment on gene expression. When the main effects were statistically significant at α=0.05, pairwise t-tests with a Bonferroni correction were employed to identify if treatment were statistically different from each other. ** $P < .01$, *** $P < .001$, **** $P < .0001$. **(b)** 4-OH-Tam treatment increases nuclear pERK5 in MCF-7 cells but cytoplasmic pERK5 in BT474 cells. MCF7 cells and BT474 cells were treated with Veh (0.1% EtOH) or 1 μM 4-OH-Tam for 45 minutes. Nuclear and cytoplasmic fractions were prepared as described. Increase in pERK5 levels in nuclear or cytoplasmic fractions were assessed by western blot analysis. Experiment was repeated 3 times and representative results are displayed. **(c)** XPO1 inhibition relocalizes pERK5 into nucleus in the presence of 4-OH-Tam in BT474 cells. BT474 cells were treated with Veh (0.1% EtOH) or 1 μM 4-OH-Tam in the presence or absence of 100 nM SXR for 45 minutes. Localization of pERK5 was monitored using immunofluorescence analysis.

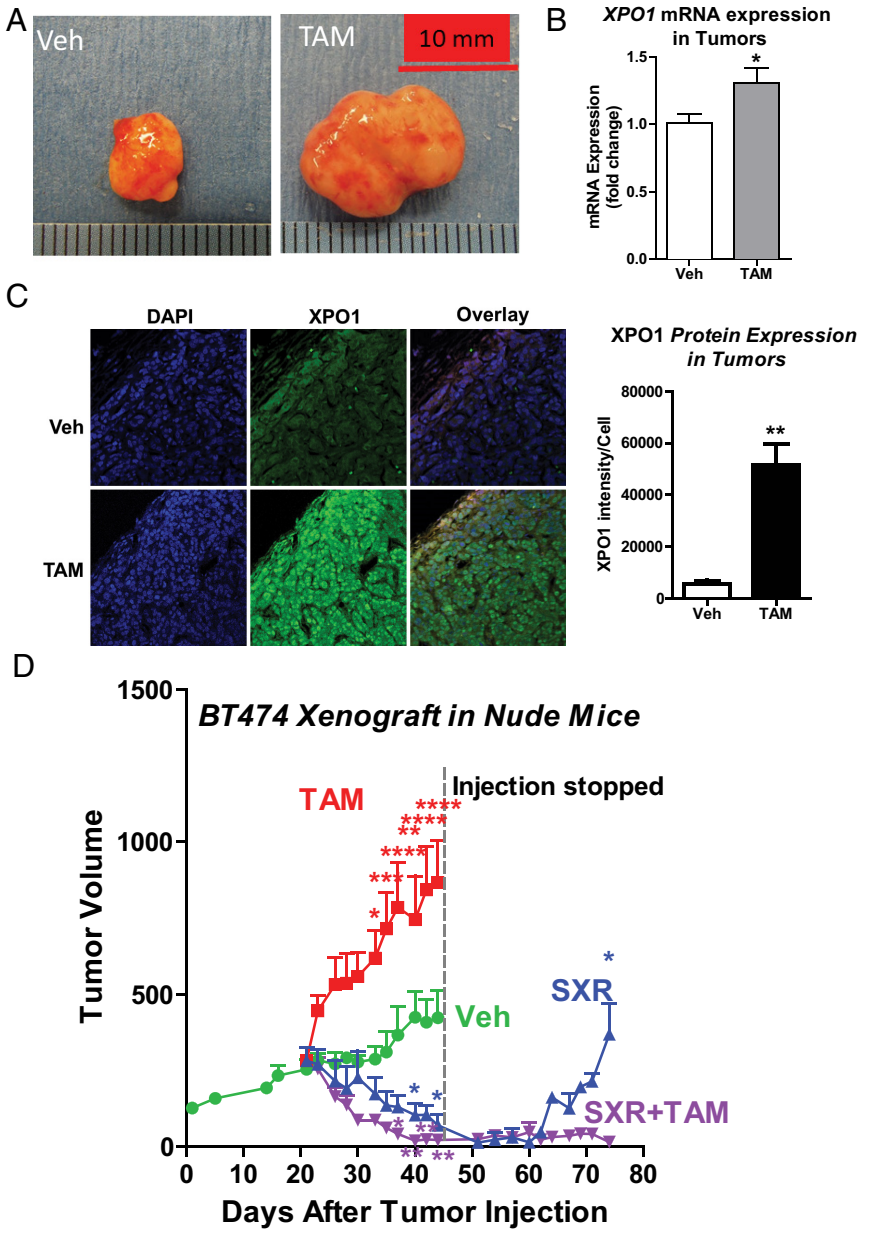

Figure 7. XPO-1 inhibitor SXR resensitizes BT474 xenograft tumors to treatment with tamoxifen 4–6 weeks old BALB/C nude mice were ovariectomized and after 1 week were implanted with E2 pellets. Next day animals were injected with 2.5×10^7 BT474 cells in 50% Matrigel subcutaneously. As tumors reached 200 mm3 animals Veh or Tam pellets were implanted subcutaneously. Starting from next day, animals were injected intraperitoneally with Ctrl or 25 mg/kg SXR biweekly for 4 weeks. (N = 5 animals per treatment group) **(a)** Tamoxifen induces growth of BT474 xenografts in nude mice. Picture of representative tumors from Veh and Tam treated animals. **(b)** Tamoxifen treatment increases XPO-1 expression in tumors. Tumors from Veh or Tam treated animals were harvested. RNA was extracted and cDNA was synthesized. Expression of XPO1 mRNA was assessed using Q-PCR assay. 36B4 primers were run as control. *t* test was used to assess if expression of *XPO1* mRNA in tumors from Veh or Tam treated mice were different. $*P < .05$ **(c)** Expression of XPO1 protein in tumors from Veh or Tam treated animals were assessed using immunofluorescence. XPO1 signal/cell intensity was calculated and normalized by dividing the total XPO1 signal by the cell number from each field. *t* test was used to assess if expression of XPO1 protein in tumors from Veh or Tam treated mice were different. $*P < .05$ **(d)** SXR resensitizes BT474 xenografts to tamoxifen treatment. Tumor size was measured biweekly using a caliper. The tumor volume was calculated using (Length X Width^2) X 3.14/6. A two-way ANOVA model was fitted to assess the time dependent contribution of ligand (Veh, Tam) and inhibitor (Ctrl and SXR) treatment on tumor volume. When the main effects were statistically significant at α=0.05, pairwise t-tests with a Bonferroni

Discussion

In our studies, we found that as ERα (+) breast tumors acquire resistance to tamoxifen, a group of nuclear transport proteins including XPO1 will be upregulated, increasing ERK5 export from nucleus. Thus, ERα, which is in the nucleus, will not have the partners to elicit proper transcriptional responses to tamoxifen and ERK5, which partners with other cytoplasmic proteins, will now contribute to tumorigenicity and tamoxifen resistance (**Figure 8**).

The development of tamoxifen resistance is a major limitation to the effectiveness of treatment of hormone-responsive breast cancer. While ERα presence is usually associated with a more favorable prognosis, it is increasingly appreciated that not all ERα (+) breast cancer patients have a good outcome. A significant subset of patients with ERα (+) breast cancers, such as those patients characterized as luminal B cancers that contain ER but also have high levels of protein kinase activity, have much less favorable disease-free survival. Blocking the activity of ER using selective estrogen receptor modulators (SERMs) such as tamoxifen or raloxifene, or the ER-degrading agents fulvestrant or aromatase inhibitors (AIs), which reduce estrogen production, has proven highly effective in targeted treatment of hormone-responsive breast cancers (1, 63, 64). Because of the inherent differences in subtypes, luminal B type tumors are less responsive to antitumor activities of tamoxifen. Our bioinformatics analysis suggest that in this subtype, nuclear transport proteins are upregulated and when combined with the higher kinase activity in these tumors, localization of key kinases to cytoplasm might explain reduced effectiveness of tamoxifen in these tumors.

Our findings suggest that expression of nuclear transport related genes in ERα (+) tumors might be used to select those patients that would favorably respond to tamoxifen. Moreover, based on our cell line and tumor xenograft studies, by combining XPO1 inhibitor with tamoxifen treatment we can improve the effectiveness and duration of tamoxifen treatment. Our approach was built upon our initial findings that estrogens increase nuclear localization of key signaling molecules like ERK5 and absence of ERα renders ERK5 extranuclear (25). When outside the nucleus, ERK5 enhances the actin cytoskeleton reorganization and thus contributes to cell aggressiveness and motility, which are characteristics of breast cancers that are resistant to endocrine therapies. This study validated the hypothesis that nuclear export proteins level could be used as a marker for risk of recurrence. Establishing XPO1 as a target for inhibition would enhance the effectiveness of endocrine therapies by maintaining tamoxifen sensitivity. Targeting localization of key signaling molecules to cellular localizations where they can be more efficiently utilized by ERα to increase efficiency of tamoxifen or other endocrine agents has a promise of higher efficacy and lower toxicity.

Cancer cells of different tumor types have been shown to be more sensitive to XPO1 inhibition than normal cells, including myeloma, where ratjadone, another XPO1 inhibitor, is showed to be selective and kill myeloma but not normal cells (65, 66) Inhibition of XPO1 in cervical cancer using another small molecule inhibitor, LMB, demonstrated the higher sensitivity of XPO1 inhibition in the cancer vs the normal cells (67).

Further research will be necessary to establish a prognostic test that can be used to identify those ERα (+) patients most likely to respond favorably to tamoxifen and allow identification of those patients who would benefit from XPO1 targeting agents to engender improved tamoxifen sensitivity. Gene expression and immunohistochemistry studies need to be performed to determine baseline values of XPO1 and how it relates to ERK5 localization and tamoxifen responsiveness in the tumors. In addition XPO1 has other targets in the tumor cells that might modulate responses to antiestrogens such as p53, p21, pRb or FOXO. XPO1 inhibitors might resensitize tamoxifen-responsive tumors to tamoxifen by modulating localization of these other factors as well. This could have a broad translational importance in the prevention and treatment of late stage cancers. For example, in cancers where ERK5 is localized to the cytoplasm, cellular aggressiveness can be down-regulated by pharmacological inhibition of XPO1, which results in decreased nuclear export, thus allowing return of ERK5 into nucleus, where it contributes to transcription and effective tamoxifen responsiveness. Thus, important advances in the therapy of late stage disease and avoidance of complications associated with broad kinase inhibitors could ultimately be expected. Further, our findings might be applicable to other cancers, including therapy resistant leukemia, prostate cancers and triple negative breast cancer, for which highly selective XPO1 inhibitors are already in clinical trials (45, 65, 68). In addition, the findings from our research might contribute to a broader understanding of how XPO1 might modulate localization of proteins important for activity of other nuclear receptors including androgen receptor and progesterone receptor.

Our research represents a new

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correction were employed to identify if treatment were statistically different from each other. *$P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$.

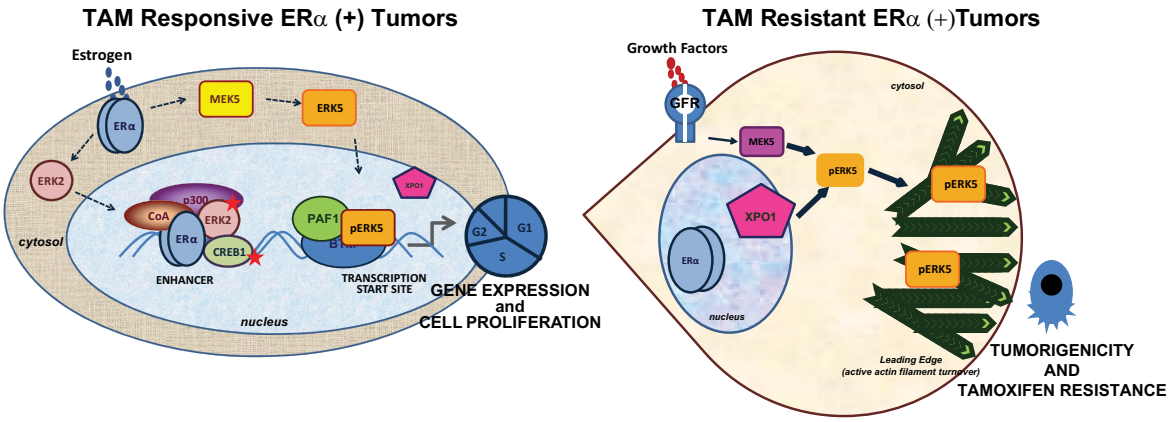

Figure 8. Model. As ERα (+) breast tumors acquire resistance to tamoxifen, a group of nuclear transport proteins including XPO1 will be upregulated, increasing ERK5 export from nucleus. Thus, **1)** ERα, which is in the nucleus, will not have the partners to elicit proper transcriptional responses to tamoxifen and 2) ERK5, which partners with other cytoplasmic proteins, will now contribute to increased tumorigenicity and tamoxifen resistance.

and substantive departure from the status quo by shifting focus to modulating the localization of key proteins rather than modulating their actual activity. Most current research efforts in the therapy resistance field have focused on a delineation of the underlying mechanisms that lead to increased activity of selective signaling pathways. Undoubtedly, interrogating and targeting the end-point kinases in tumors is highly relevant and these studies led to the development of combination therapies involving PI3K inhibitors or mTOR pathway inhibitors together with endocrine agents. However, resistance to these combination therapies also occurs, and in such cases, the cancer that develops is considerably more aggressive due to hyperactivation of compensatory mitogenic signaling pathways (69). Moreover, these kinase inhibitors have many adverse side effects. More recently, ERα mutations that decrease sensitivity of the receptor to selective estrogen receptor modulators (SERMs) and selective estrogen receptor degradors (SERDs) were identified in over 30% of the metastatic, but not primary, tumors (70–74). However, in two-thirds of ER-positive metastatic tumors, the mechanism of therapy resistance is not attributable to ER mutation and alterations often cannot be targeted effectively.

Our findings strongly suggest that this approach will be effective in allowing relocalization of key proteins, such as ERK5, to the nucleus to improve transcriptional response to tamoxifen that would otherwise function to regulate invasiveness and aggressiveness in the cytoplasm. This approach is expected to open new research horizons, particularly in the biology of luminal B and basal like breast cancers, which are more aggressive and resistant to current therapies. By using an integrative computational and experimental approach, we have generated evidence that XPO1 appears to play key roles in drug resistance in breast cancer. We have postulated that these pathways have not previously been focused on in breast cancer primarily because the effects that we describe pertain primarily to luminal B and basal like type breast cancers. Thus, specific breast cancer subtypes may well have been overlooked in previous studies designed primarily to analyze the overall effect in all breast cancers independent of the molecular subtype. Our findings delving into deciphering the mechanistic details of this relationship and testing the efficacy of targeting these pathways in the clinic show great promise for ultimately delivering novel diagnosis and treatment strategies for therapy-resistant ERα (+) luminal B and ER(-) basal subtype tumors. In summary, our study reported here is the first attempt in the field to define the causal role of the nuclear export pathways in tamoxifen resistance, and explore the feasibility of targeting these pathways to improve response to tamoxifen and decrease risk of recurrence.

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* These authors contributed to this work equally

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