

Loss of Rho GDI α and Resistance to Tamoxifen via Effects on Estrogen Receptor α

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Background Estrogen receptor (ER) α is a successful therapeutic target in breast cancer, but patients eventually develop resistance to antiestrogens such as tamoxifen.

Methods To identify genes whose expression was associated with the development of tamoxifen resistance and metastasis, we used microarrays to compare gene expression in four primary tumors from tamoxifen-treated patients whose breast cancers did not recur vs five metastatic tumors from patients whose cancers progressed during adjuvant tamoxifen treatment. Because Rho guanine dissociation inhibitor (GDI) α was underexpressed in the tamoxifen-resistant group, we stably transfected ER α -positive MCF-7 breast cancer cells with a plasmid encoding a short hairpin (sh) RNA to silence Rho GDI α expression. We used immunoblots and transcription assays to examine the role of Rho GDI α in ER-related signaling and growth of cells in vitro and as xenografts in treated nude mice ($n = 8-9$ per group) to examine the effects of Rho GDI α blockade on hormone responsiveness and metastatic behavior. The time to tumor tripling as the time in weeks from randomization to a threefold increase in total tumor volume over baseline was examined in treated mice. The associations of Rho GDI α and MTA2 levels with tamoxifen resistance were examined in microarray data from patients. All statistical tests were two-sided.

Results Rho GDI α was expressed at lower levels in ER α -positive tumors that recurred during tamoxifen treatment than in ER α -positive tamoxifen-sensitive primary tumors. MCF-7 breast cancer cells in which Rho GDI α expression had been silenced were tamoxifen-resistant, had increased Rho GTPase and p21-activated kinase 1 activity, increased phosphorylation of ER α at serine 305, and enhanced tamoxifen-induced ER α transcriptional activity compared with control cells. MCF-7 cells in which Rho GDI α expression was silenced metastasized with high frequency when grown as tumor xenografts. When mice were treated with estrogen or estrogen withdrawal, tripling times for xenografts from cells with Rho GDI α silencing were similar to those from vector-containing control cells; however, tripling times were statistically significantly faster than control when mice were treated with tamoxifen (median tripling time for tumors with Rho GDI α small interfering RNA = 2.34 weeks; for control tumors = not reached, hazard ratio = 4.13, 95% confidence interval = 1.07 to 15.96, $P = .040$ [adjusted for multiple comparisons, $P = .119$]). Levels of the metastasis-associated protein MTA2 were also increased upon Rho GDI α silencing, and combined Rho GDI α and MTA2 levels were associated with recurrence in 250 tamoxifen-treated patients.

Conclusion Loss of Rho GDI α enhances metastasis and resistance to tamoxifen via effects on both ER α and MTA2 in models of ER α -positive breast cancer and in tumors of tamoxifen-treated patients.

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Tamoxifen and aromatase inhibitors are used to target estrogen receptor (ER) α in breast cancer patients (1). However, hormone resistance remains a clinical problem and a major cause of recurrence and mortality in ER α -positive disease (2,3). It is unlikely that simple loss of ER α signaling is the driving force for resistance because both metastatic tumors and tamoxifen-resistant breast cancer cell lines frequently retain ER α expression (4,5) and remain responsive to steroidal antiestrogens like fulvestrant.

Several mechanisms have been proposed for tamoxifen-resistance in ER α -positive breast cancer [reviewed in (6)]. Complications arising from resistant tumor metastases are the most common cause of patient death; however, only a few genes have been shown to be essential for metastatic behavior [eg, TWIST (7) and some members of the Rho signaling pathway (8,9)]. The Rho family of GTP-binding proteins (RhoA-C, Rac-1, and Cdc42) regulates the polymerization of actin to

produce stress fibers, lamellipodia, and filopodia in response to extracellular signals, proliferation, and apoptosis (10). The Rho guanine dissociation inhibitors (Rho GDIs) are negative regulators of all three Rho family proteins (11). Higher levels of Rho and lower levels of Rho GDI family members are associated with lymph node involvement and breast cancer metastasis (12); accordingly, Rho GDI β has been reported to be a metastasis suppressor in human bladder cancer cells (8).

In this article, we addressed the role of Rho GDI α in ER α action, tamoxifen resistance, and metastatic behavior in breast cancer. Using cell line models in which we reduced Rho GDI α levels using short hairpin (sh) RNA, we also examined the molecular mechanisms by which loss of Rho GDI α affects cell motility and invasiveness and alters hormone response. Resistance and metastatic potential were evaluated using tumor xenografts in mice, and Rho GDI α 's ability to predict outcomes in tamoxifen-treated patients was tested using survival analysis of a retrospective cohort.

Methods

Plasmids and Chemicals

The *Xenopus vitellogenin ERE2-tk-luciferase* reporter plasmid, pCMV- β -galactosidase, and plasmids expressing yellow fluorescence protein (YFP) fused with wild-type ER α (YFP-WT) or YFP fused with a serine 305 to alanine mutant of ER α (YFP-S305A ER α), orglutathione-S-transferase (GST) fused with the ER α hinge domain have been previously described (13). pcDNA3-Rho GDI α was kindly provided by Dr Michael Garabedian (New York University, NY), and the Rho GDI α insert cDNA was cut out with *Bam*HI and *Eco*RI and subcloned between the *Bgl*II and *Eco*RI sites of pEYFP-C1 (Clontech, Palo Alto, CA). Constructs expressing GST fused with the wild-type ER α hinge domain (residues 253–310) or to a mutant S305A ER α hinge domain were generated through polymerase chain reaction and inserted into the pGEX 4T-1 vector (Pharmacia, Piscataway, NJ) at the *Bam*HI and *Eco*RI cloning sites. To silence cellular Rho GDI α expression, a shRNA was designed using Clontech software to recognize the RhoGDI α coding region from 389 to 407 (5'-GAAGCAGTCGTTTGTGCTG-3', gi:76780068) and was cloned into the pSiren-Retro Q vector (Clontech). We also generated a rescue vector in which Rho GDI α was made resistant to the Rho GDI α shRNA vector using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA); the sense primer sequence used was 5'-TCAGAAGCAAGGTCCTTCGTCCT-GAAGGAGGAGG-3' (mutated nucleotides that provide resistance to shRNA are underlined), the template was the YFP-Rho GDI α plasmid, and the manufacturer's protocol was followed. A control RNAi oligonucleotide was purchased from Clontech. The sources of 17 β -estradiol, 4-hydroxy-tamoxifen, and ICI 182780 were as previously described (14). A selective inhibitor of the Rho-associated protein kinase ROK (Y27632) was purchased from Calbiochem (La Jolla, CA); puromycin was purchased from Sigma (St Louis, MO).

Tumor Specimens for Expression Microarray Analysis

Frozen breast tumor specimens from a cohort of nine patients who received adjuvant tamoxifen were selected from the tumor bank of the Breast Center, Baylor College of Medicine (Houston, TX) for use in the RNA analyses. This study was approved by the Baylor

CONTEXT AND CAVEATS

Prior knowledge

Tamoxifen is often used to treat estrogen receptor (ER)-positive breast cancers, but the mechanisms whereby some patients become resistant have been unclear.

Study design

When microarrays were used to analyze gene expression in tumors from tamoxifen-sensitive vs tamoxifen-resistant patients, Rho guanine dissociation inhibitor (GDI) α was identified as a possible mediator of tamoxifen sensitivity. MCF-7 cells containing silencing RNA were used to examine the effects of decreased Rho GDI α expression on tamoxifen-resistant cell growth, Rho signaling, and ER α activity. The same cells were used to establish tumors as xenografts in nude mice. Last, low Rho GDI α and high expression of the metastasis-associated protein MTA2 were examined as potential biomarkers of tamoxifen-resistance among 250 patient tumors.

Contribution

MCF-7 cells expressing Rho GDI α silencing RNA were tamoxifen-resistant and had higher levels of Rho signaling and tamoxifen-induced ER α activity than control cells. When implanted in nude mice, these cells grew tumors even in the presence of tamoxifen, unlike control cells, and metastasized more readily. Preliminary results indicate that low Rho GDI α and high MTA2 expression may be useful clinical markers of tamoxifen-resistance.

Implications

Increased Rho, PAK1, and ER α activity by virtue of Rho GDI α inactivation appear to confer tamoxifen resistance. Consequently, inhibitors of these pathways might be useful to reverse tamoxifen resistance.

Limitations

The mechanistic experiments here were done in a single cell line, and the clinical data were based on a single, retrospective analysis. Further work in additional cell lines and a prospective clinical study will be necessary.

From the Editors

College of Medicine Institutional Review Board in accordance with federal human research study guidelines. Samples were 1) metastatic tumors from five patients whose breast cancers recurred within 11 months of the start of tamoxifen treatment (tamoxifen-resistant) and 2) primary tumors, collected at the time of initial diagnosis, from four patients who were treated with tamoxifen and remained disease free for 93–123 months with a median follow-up of 106 months (tamoxifen-sensitive). The patients had received no treatments other than tamoxifen. Microarray analysis on this cohort has been previously described (14).

Cell Culture and Transfection

The ER α -positive T47D human breast cancer cell line and HeLa human cervical cancer cell line were obtained from American Type Tissue Culture Collection (Rockville, MD). ER α -positive MCF-7 human breast cancer cells were previously described (15). All cell lines were maintained in minimal essential medium (MEM; Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS; Summit Biotechnology, Fort Collins, CO),

0.1 nmol/L nonessential amino acids, 2 mmol/L L-glutamine, and 50 U/mL penicillin–streptomycin, at 37°C with 5% CO₂ and 95% air.

Tamoxifen-resistant MCF-7 cells were generated as described previously by Knowlden et al. (5) and Herman and Katzenellenbogen (16). Briefly, we cultured parental MCF-7 cells in phenol red-containing MEM medium supplemented with 5% FBS, antibiotics, and 10-64-hydroxy-tamoxifen (TR1 cells) or in phenol red-free MEM medium supplemented with 5% charcoal-stripped FBS, antibiotics, and 10-7M 4-hydroxy-tamoxifen (TR2 cells). Cells were continuously exposed to 4-hydroxy-tamoxifen for 6 months during which time the medium was replaced every 4–5 days. Initially, cell growth was slow, but gradually increased, at which time the cells were designated MCF-7-TR. TR1 cells were continuously maintained in 1 μM tamoxifen, and TR2 cells in 100 nM tamoxifen, for longer than 1 year.

To generate MCF-7 cells with stable silencing of Rho GDIα expression, subconfluent cells in 10-cm tissue culture dishes were transfected with 5 μg of the vector carrying shRNA against Rho GDIα or 5 μg of the control vector using Fugene 6 reagent and the manufacturer's protocol (Roche, Indianapolis, IN). Stable clones of cells that contained Rho GDIα shRNA or control plasmid were selected with 1 μg/mL puromycin for 2 weeks, and positive clones in which Rho GDIα expression was silenced were identified using immunoblot analysis with a rabbit polyclonal anti-Rho GDIα antibody (1:2000; Santa Cruz Biotechnology, Inc, Santa Cruz, CA). One single-cell stable clone and a pool of stable transfectants were used in experiments. In some experiments, we used pools of T47D or tamoxifen-resistant TR1 cells, both of which were stably transfected with YFP-vector and YFP-GDIα expression plasmids and selected for 1 week with G418 antibiotic (Invitrogen).

Immunoblot Analysis

Cell extracts or immunoprecipitated proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described (13). Antibodies used for immunoblotting were as follows: mouse monoclonal anti-ERα clone 6F11 (1:500; Vector Labs, Newcastle, UK); rabbit polyclonal anti-SRC1 (1:200; Santa Cruz Biotechnology, Inc); mouse monoclonal anti-TIF2 (1:200; BD Transduction Lab, Los Angeles, CA); mouse monoclonal anti-p190 to RASGRF1 (1:500; BD Transduction Laboratories, San Diego, CA); rabbit polyclonal anti-Rho GDIα (1:2000; Santa Cruz Biotechnology, Inc); rabbit anti-MTA2 (1:2000; Sigma); mouse monoclonal anti-β-actin AC-15 (1:10 000, Sigma); rabbit polyclonal anti-ROKα (1:1000; Upstate Biotechnology, Lake Placid, NY); rabbit anti-phospho-ROKα Thr249 (1:1000; Abcam, Cambridge, MA); and rabbit polyclonal anti-phospho ERα Ser305 (1:100; Millipore, Billerica, MA). Rabbit polyclonal anti-ERK1/2 (1:1000) and anti-phospho-ERK1/2 (1:500), rabbit polyclonal nuclear factor κB (NF-κB) p65 (1:1000) and rabbit polyclonal pNF-κB S536 (1:1000; 93H1) were from Cell Signaling, Beverly, MA. The proteins that were transferred to nitrocellulose membranes were blocked overnight in a blocking solution (Tris-buffered saline–Tween [0.1% Tween-20; 20 mM Tris, pH 8.0; 150 mM NaCl] with 5% powdered nonfat dried milk) then incubated with primary antibodies listed above overnight at 4°C and with secondary antibodies (goat anti-mouse [1:2000] or goat anti-rabbit [1:5000]

antiserum; Amersham Biosciences, Piscataway, NJ) for 1 hour at room temperature, and visualized with enhanced chemiluminescence reagents (Alpha Innotech, San Leandro, CA). Immunoblots show a single representative of at least two or three separate experiments.

Colony Formation and Soft Agar Assays

For colony formation assays, 2.5×10^5 TR1 or TR2 cells per well were placed in six-well tissue culture plates 24 hours before transfection. One microgram of DNA (as indicated in the individual figure legends) and Fugene 6 reagent (Roche) were used to transfect the cells in each well. Starting 24 hours after transfection, the cells were incubated for 14 days with 800 μg/mL G418 antibiotic in the specific medium used to initially generate the tamoxifen-resistant cells, the colonies were stained with 1% crystal violet, and photographed under a microscope.

For soft agar assays, 5000 cells per well were plated in 4 mL of 0.35% agarose, 5% charcoal-stripped FBS in phenol red-free MEM, with a 0.7% agarose base in six-well plates. Two days after plating, medium containing vehicle or treatments as indicated was added to the top of the layer and replaced every 2 days. After 14 days, 300 μL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma) were added to each well and incubated at 37°C for 4 hours. Plates were then placed at 4°C overnight, and colonies larger than 50 μm in diameter were counted.

Rho GTPase Activation Assays

Cells were seeded in 10-cm plates and grown for 2 days in estrogen-deprived medium (phenol red-free MEM with 5% charcoal-stripped FBS). Then, cells were washed with ice-cold PBS and lysed in MLB buffer (25 mM HEPES, pH 7.5; 150 mM NaCl, 1% IGEPAL CA-630 [Sigma], 10% glycerol, 25 mM NaF, 10 mM MgCl₂, 1 mM EDTA, 10 mg/mL leupeptin, 10 mg/mL aprotinin, and 1 mM sodium orthovanadate). Cell lysates were clarified by centrifugation at 13 000g at 4°C for 10 minutes, and 700–1000 μg of cellular proteins were incubated with 15 μg of PAK-1 p21 binding domain (PBD) or 20 μg Rhotekin Rho binding domain (RBD) agarose conjugate (Upstate, Lake Placid, NY) at 4°C for 90 minutes. The beads were centrifuged and washed four times with MLB buffer. GTP-bound activated Rho proteins were detected by immunoblotting using mouse monoclonal antibodies against Rac-1 (1:3000); cdc42 (1:100; BD Transduction Laboratories, San Jose, CA); or rabbit polyclonal antibodies to RhoA, RhoB, or RhoC (all 1:200; from Cell Signaling).

Immunoprecipitation Assays

Immunoprecipitation assays were performed as described (17) with some modifications. High salt cell lysis buffer (15) with 1:100 diluted complete proteinase inhibitor III solution (Roche) was used to prepare cellular extracts, which were then adjusted to 150 mM NaCl plus 1.0% NP-40 (binding buffer) and incubated with 1–2 μg of the appropriate antibody as indicated. After centrifugation and washing four times with the lysis buffer, the precipitated components were eluted by boiling in SDS loading buffer, resolved by SDS-PAGE, and analyzed by immunoblotting.

p21-Activated Kinase 1 (PAK1) Immunocomplex Kinase Assay

Cells (3×10^6) were plated in 10-cm dishes with MEM supplemented with 10% FBS and incubated for 24 hours at 37°C in 5% CO₂. Cells were then serum-starved for 48 hours in phenol red-free MEM before lysis in 50 mM HEPES (pH 7.0), 150 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, 10 mM NaPO₄, 1% glycerol (vol/vol), 0.1% Triton X-100 (vol/vol), 1 mM Na₃VO₄, and 1:100 protease inhibitor cocktail tablet. Immunocomplex kinase assays were done with PAK1 protein immunoprecipitated from 200 µg of total cellular protein using 2 µg of rabbit polyclonal anti-PAK1 antibody (Santa Cruz Biotechnology, Inc) with rotation at 4°C for 2 hours. Immunoprecipitated pellets were washed three times with immunocomplex kinase assay wash buffer (0.1% Triton X-100, 150 mM NaCl, and 10 mM NaPO₄ [pH 7.0]). Ten micrograms of purified GST-ERα hinge domain protein, described previously (13), was added to the immunoprecipitated pellets in 20 µL kinase reaction buffer containing 20 mM HEPES (pH 7.0), 6 mM MgCl₂, 20 mM Na₃VO₄, and 10 µCi [γ -³²P]-ATP (3000 Ci/mmol; NEN, Boston, MA) and incubated for 20 minutes at 22°C. The reaction was stopped by the addition of SDS loading buffer. The samples were resolved using SDS-PAGE and transferred onto nitrocellulose membranes. The input GST-ERα hinge domain proteins were visualized with Ponceau S staining, the levels of ERα phosphorylation were determined by autoradiography, and the immunoprecipitated PAK1 was detected by immunoblot with a rabbit polyclonal anti-PAK1 antibody (1:1000; Santa Cruz Biotechnology, Inc).

ERα Transactivation Assays

Cells were maintained in phenol red-free MEM supplemented with 5% charcoal-stripped FBS (Hyclone, Logan, UT) for 5–7 days. One day before transfection, 2.5×10^5 MCF-7 or T47D cells or 0.5×10^5 HeLa cells were plated in 2 mL medium per well in six-well plates and then transfected using Fugene 6 reagent (Roche) following the manufacturer's protocol. The cells in each well were transfected with ERE-luciferase reporter, pCMV-β-galactosidase vector, and YFP-GDIα and/or ERα expression vectors as indicated. After 24 hours, cells were treated with estrogen (10^{-9} M), 4-hydroxy-tamoxifen (10^{-7} M) or vehicle as indicated in the figure legend for an additional 18–24 hours. Luciferase activity was assessed using the Luciferase Assay System (Promega) following the manufacturer's protocol.

Xenograft Studies

MCF-7 vector control cells and a Rho GDIα shRNA stably transfected clone were established as xenografts in ovariectomized 5- to 6-week-old BALB/c athymic nude mice (Harlan Sprague Dawley, Madison, WI) by inoculating the mice subcutaneously with 5×10^6 cells in 100 µL of medium with 100 µL of Matrigel (BD Biosciences, San Jose, CA), as described previously (18). The mice were supplemented with 0.72 mg 60 day-release estradiol pellets that were implanted subcutaneously between the shoulder blades (Innovative Research, Sarasota, FL). Tumors were measured with calipers three times per week. When tumors reached approximately 250 mm³ (ie, in 1.5–2.5 weeks), the mice were randomly allocated to continue estradiol ($n = 8$ –9 mice per group) or to

estrogen withdrawal plus tamoxifen ($n = 7$ –8 mice per group; estradiol pellets were removed and 500 µg/d of tamoxifen was given to each mouse subcutaneously in peanut oil, on each of 5 d/wk) for another 30 days. Tumor growth was assessed, and tumor volumes were measured as described previously (18). Mice were killed when moribund or when the tumor burden exceeded 1 cm³. Animal care was maintained in accordance with institutional guidelines.

Immunohistochemistry

Immunohistochemistry was performed by the Breast Center Pathology Core facility at Baylor College of Medicine, Houston, TX. Lung metastases arising from xenografts of Rho GDIα shRNA-containing MCF-7 cells were fixed overnight in 10% formalin buffered in methanol, followed by paraffin embedding. Thin (5 µm) sections were stained with hematoxylin and eosin (H&E). For detection of ERα and progesterone receptor, thin sections of formalin-fixed paraffin-embedded tissues were deparaffinized and rehydrated from xylene through a graded series of ethanol washes. Antigen retrieval was carried out by boiling in Tris-Cl (pH 9.0) in a standard pressure cooker for 20 minutes. Endogenous peroxidases were blocked by treatment with 3% hydrogen peroxide in methanol. Protein blocking was performed with Avidin Solution A (A/B blocking Kit; Vector Laboratories, Burlingame, CA) according to manufacturer's protocols. Slides were incubated with mouse anti-ERα 6F11 antibody (1:200; Vector Labs) or mouse anti-PR (1:1600, PgR1294; Dako, Inc, Carpinteria, CA) for 1 hour at room temperature, followed by a horseradish peroxidase-conjugated secondary antibody, and horseradish peroxidase was detected with diaminobenzidine tetrahydrochloride (Invitrogen), and counterstained with Gills hematoxylin. Images were captured on a Zeiss Axioskop 2 plus microscope with a Canon Powershot G5 camera.

Invasion Assay

CTRL sh and GDIα sh cell invasion was determined using 24-well BD Biocoat invasion chambers (Bedford, MA) containing 8-µm porous membranes precoated with Matrigel. Cells were serum-starved for 2 days. The cells were removed from the plate with warm versene (Lonza, Switzerland) and 100 000 cells were seeded into the chambers in serum-free phenol red-free MEM (Invitrogen) with or without 17β-estradiol (E, 10^{-9} M), 4-hydroxy-tamoxifen (T, 10^{-7} M), or the ROK inhibitor Y-27631 (R, 10^{-8} M). Chemoattractant, or medium containing 10% FBS, was added to the lower compartment. After 22 hours incubation at 37°C, the noninvasive cells and Matrigel were removed from the inside of the wells with a cotton swab. The invading cells were then fixed with Diff-Quik Fixative and stained with Diff-Quik Stain Solution I and II (Siemens, Aktiengesellschaft Erlangen, Germany). Finally, the membranes were removed from the chambers using a scalpel blade and mounted onto glass microscope slides, and cell invasion was quantified by counting the number of cells per membrane at $\times 20$ magnification. Data were then plotted as mean number of cells in triplicate. Error bars correspond to 95% confidence intervals; *P* values were calculated using two-sided Student *t* tests.

Statistical Analyses

Three major types of statistical analysis were used to analyze data for this study: those for the cell line experiments, for xenograft

data, and for analysis of a breast tumor dataset that was previously described by others (see below). In all cases, *P* values less than .05 were considered to denote statistical significance, and all statistical tests were two-sided. For cell line experiments, statistical differences (*P* values) among groups were obtained using a two-sided Student *t* test with GraphPad Prism 5 software (GraphPad Software, San Diego, CA).

The xenograft experiments were analyzed using survival analysis methods to address the main question of whether, in the absence of tamoxifen, the tumors that arose from cells with Rho GDI α shRNA grew faster than those that arose from cells containing only the empty vector and whether they were resistant to tamoxifen. We tested the hypothesis that decreased Rho GDI α expression would render cells resistant to the growth inhibitory effects of tamoxifen. We calculated the time to tumor tripling as the time in weeks from randomization to a threefold increase in total tumor volume over baseline. Time to tumor tripling is a direct way to assess differences in tumor growth rate, without explicitly modeling tumor growth (which requires a common model structure across all groups). Survival curves were computed by the Kaplan–Meier method and compared with the two-sided log-rank test. Wald 95% confidence intervals for the proportion of mice that were tumor-tripling free at 4 and 8 weeks were computed using the standard error of the survival function at the maximum event time less than or equal to 4 (or 8) weeks. Individual comparisons, after rejection of the global null hypothesis, were tested using contrasts in the context of a Cox regression. We used visual inspection of a graphical examination of log-log(s) plots to feel comfortable that the assumptions of proportionality were confirmed for the Cox analysis. Exponentiation was used to calculate the upper and lower limits of Wald 95% confidence intervals of the corresponding regression coefficients. *P* values were adjusted by the method of Holm (19). Analyses were carried out using SAS (V9.2; Cary, NC) and R (Version 2.9.2) (20).

To test the effects of Rho GDI α and MTA2 in retrospective clinical samples, we used the dataset previously described by Loi et al. (21), available at the Gene Expression Omnibus (GEO) repository (<http://www.ncbi.nlm.nih.gov/geo>), accession number GSE6532. The probe sets 213606_s_at, 201168_x_at, 201167_x_at, and 211716_x_at were averaged to represent the expression of Rho GDI α , and 203444_s_at was used to represent the expression of MTA2. Clinical data were matched to the expression data for all 255 tamoxifen-treated samples in the Loi et al. training dataset in the GEO database and samples that were not ER α positive or that were missing expression data were excluded, resulting in the inclusion of samples from 250 ER-positive tamoxifen-treated patients. Optimum cut points for the analyses were calculated using a bootstrapping approach wherein the χ^2 statistic was calculated using the two-sided log-rank test for each of a range of cut points on each of 500 bootstrapped samples. From these χ^2 statistics, the median χ^2 statistic was obtained for each cut point and used to select the cut points presented here. Because this dataset contains samples from many institutions and studies (21), we compared the proportion of samples that were RhoGDI α low and MTA2 high in each study with that of all other patients using the two-sided χ^2 test and found no difference in study proportions in these populations, indicating that the observed survival difference was not caused by

a study bias. The statistical program R with the package Survival was used to analyze the Loi dataset (20). Survival curves were compared using log-rank tests.

Results

Rho GDI α Loss and the Acquisition of a Tamoxifen-Resistant Phenotype

To identify genes whose expression is associated with the development of tamoxifen resistance, we used microarrays to compare gene expression in four primary tumors from patients treated with tamoxifen whose breast cancers did not recur during extended follow-up (in >93 months), with that in five metastatic tumors from patients whose breast cancers progressed during tamoxifen treatment (in <11 months). We have already reported finding novel mechanisms of tamoxifen resistance in this cohort that involve mitogen-associated protein kinase phosphatase 3 (MKP3) and tamoxifen-induced oxidative stress (14) and overexpression of the androgen receptor (AR; 22). Accordingly, cluster analysis showed that both MKP3 and AR were overexpressed in these tamoxifen-resistant tumors (Figure 1, A).

Multiple resistance mechanisms could be operating in tamoxifen-resistant patients. Levels of Rho GDI α were statistically significantly reduced in tamoxifen-resistant tumors as compared with tamoxifen-sensitive tumors (*P* = .017; Figure 1, B). Because lower levels of the Rho GDIs have been associated with metastasis (12), we established clones of ER α -positive MCF-7 cells, which have low metastatic potential (23) that were stably transfected with a plasmid encoding a Rho GDI α shRNA (GDI α sh) or a scrambled control shRNA (CTRL sh). We also generated a pool of MCF-7 cells that stably expressed the Rho GDI α shRNA (GDI α sh POOL). Expression of GDI α protein was efficiently silenced in both the cloned and pooled GDI α sh-transfected cells (Figure 1, C). We examined anchorage-independent growth using soft agar assays (Figure 1, D); the growth of cells transfected with vector alone was enhanced with estrogen and inhibited with tamoxifen, whereas pooled or cloned cells containing GDI α shRNA grew better under basal hormone-independent conditions and were further growth-stimulated with either estrogen or tamoxifen, thus demonstrating tamoxifen insensitivity. Similar results were seen in cells grown in culture using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) growth assays (Supplementary Figure 1, available online).

We next generated a stable pool of cells in which we “rescued” GDI α expression by transfecting cloned GDI α sh-containing cells with a mutant version of Rho GDI α that was resistant to small interfering RNA interference (Figure 1, E). CTRL sh cells and rescued cells were growth-inhibited with tamoxifen, whereas GDI α sh cells were insensitive to tamoxifen-mediated growth inhibition (Figure 1, F), but still sensitive to growth inhibition by the anti-estrogen ICI 182780, also called fulvestrant. We hypothesized that the requirement of Rho GDI α expression for tamoxifen sensitivity may be dependent on sustained ER α expression because fulvestrant, which inhibits growth, degrades the ER α protein (24). Tamoxifen-resistant growth of the GDI α sh clone was blocked in the RESCUE cells, confirming the role of Rho GDI α in tamoxifen action.

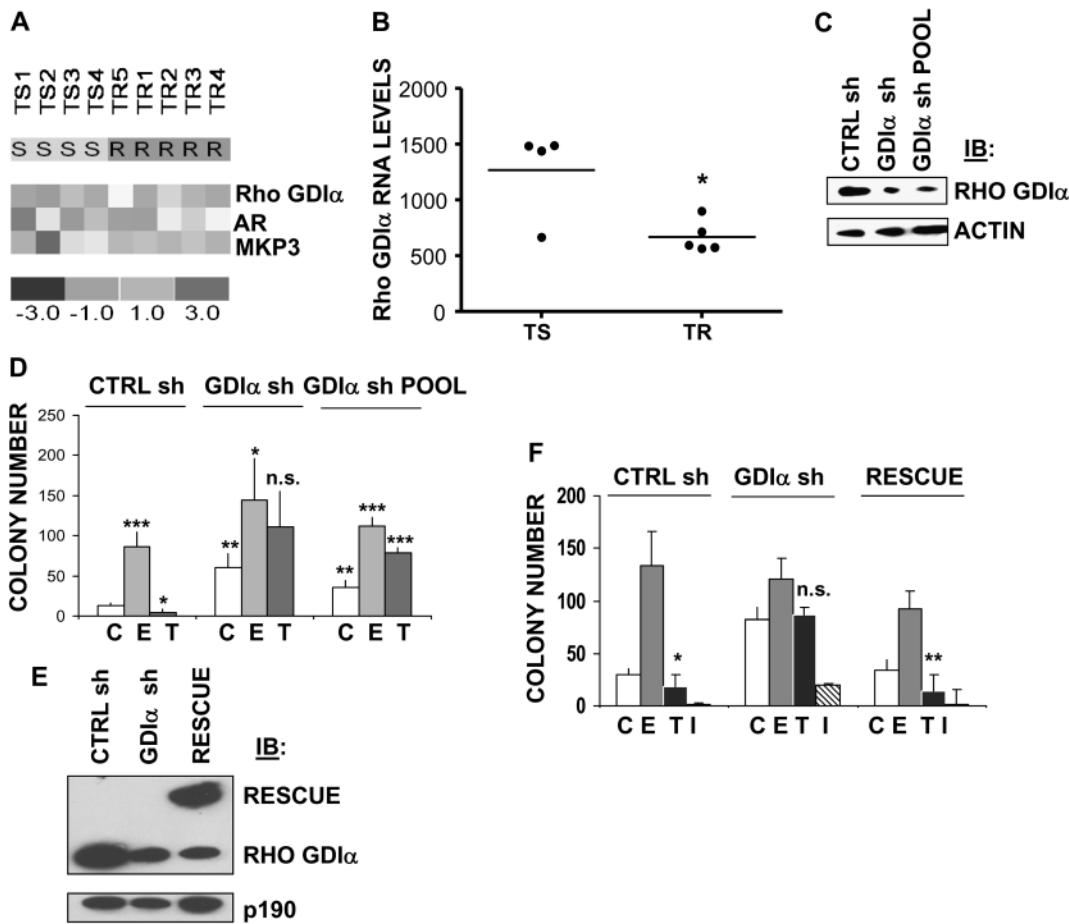


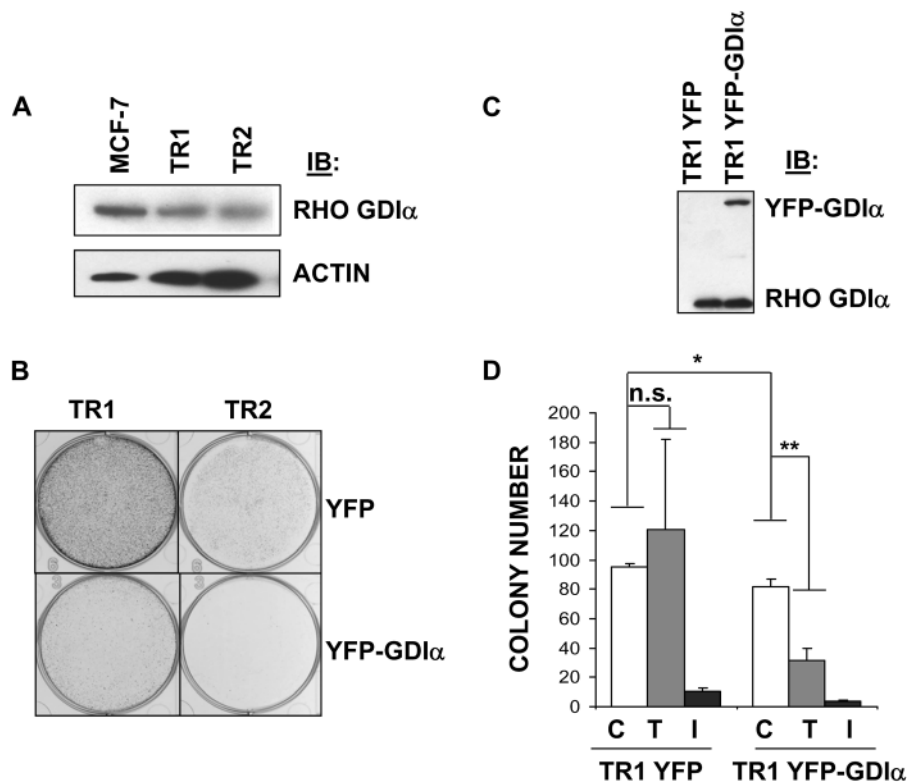
Figure 1. Rho guanine dissociation inhibitor (GDI) α expression and resistance to tamoxifen. **A**) Clustering analysis of selected gene expression patterns in tamoxifen-resistant vs tamoxifen-sensitive breast cancers. Rho GDI α , AR, and MKP3 expression were measured by microarray analysis of mRNA from primary breast cancer tissue from patients whose tumors were tamoxifen-sensitive (TS, $n = 4$) and from metastatic breast cancer tissue from patients whose tumors were tamoxifen-resistant (TR, $n = 5$). Relative gene expression between the two groups is represented by color, with red indicating high and blue indicating low levels of expression. **B**) Statistical analysis of Rho GDI α RNA expression in the four tamoxifen-sensitive and five tamoxifen-resistant breast tumors shown in (A). There was a statistically significant difference in Rho GDI α expression between tamoxifen-resistant and tamoxifen-sensitive cells ($P = .017$, two-sided t test). **C**) Silencing of Rho GDI α expression in MCF-7 cells. Immunoblot (IB) of whole-cell lysates from MCF-7 cells stably transfected with either a scrambled shRNA (CTRL sh) or Rho GDI α shRNA (GDI α sh), or from a pool of MCF-7 cells stably expressing the Rho GDI α shRNA (GDI α sh POOL). Blots were cut and rabbit polyclonal antibodies recognizing Rho GDI α were used to probe one portion of the membrane, and a mouse monoclonal antibody to β -actin (ACTIN), as a loading control, on the other. **D**) Anchorage-independent growth and tamoxifen sensitivity of MCF-7 cells with and without Rho GDI α silencing. CTRL sh, GDI α sh, and GDI α sh POOL cells were seeded (5000/well) in 0.35% agarose and then treated with 17 β -estradiol (E, 10^9 M), 4-hydroxy-tamoxifen (T, 10^7 M), or vehicle (C). Cells were allowed to grow for 14 days and the numbers

of colonies that were larger than 50 μ m were counted. The graphs represent the mean colony number on three plates from each of three independent experiments. Error bars correspond to 95% confidence intervals. P values were calculated using two-sided Student t tests. For C treatment between CTRL sh and GDI α sh, $**P = .004$ and between CTRL sh and GDI α sh POOL, $**P = .006$. For C vs E and C vs T in CTRL sh cells, $***P = .001$ and $*P = .020$, respectively. For C vs E and C vs T in GDI α sh cells, $*P = .027$ and n.s. (not significant), respectively. For C vs E and C vs T in GDI α sh POOL, $***P < .001$ and $***P < .001$, respectively. **E**) Rescue of Rho GDI α expression. GDI α sh-containing MCF-7 cells were transfected with a silencing-resistant version of Rho GDI α , YFP-GDI α . Whole-cell lysates from CTRL sh, GDI α sh, and the RESCUE clone were immunoblotted. Rabbit polyclonal antibodies recognizing Rho GDI α were used to evaluate its expression on one portion of the blot, and a mouse monoclonal antibody to p190 RASGRF1 was used to probe the other portion to ensure equal loading. **F**) Anchorage-independent growth and tamoxifen and fulvestrant sensitivity of GDI α sh vs rescue cells. CTRL sh, GDI α sh and RESCUE clones were plated in soft agar and then treated with 17 β -estradiol (E, 10^{-9} M), 4-hydroxy tamoxifen (T, 10^{-7} M), fulvestrant (ICI 182780, I, 10^{-8} M), or vehicle (C). Data are the mean colony number from three plates and are representative of two independent experiments. Error bars correspond to 95% confidence intervals. P values were calculated using two-sided Student t tests. For C vs T in CTRL sh, GDI α sh, and RESCUE cells, $*P = .01$, $P =$ n.s. (statistically nonsignificant), and $**P = .002$, respectively.

To explore the relationship between Rho GDI α levels and tamoxifen resistance, we generated two tamoxifen-resistant MCF-7 sublines (TR1 and TR2) using long-term exposure to tamoxifen. Rho GDI α levels were lower in both sublines compared with parental cells (Figure 2, A). We transfected both cell lines with YFP-vector or YFP-Rho GDI α plasmids and examined colony

formation efficiency (Figure 2, B). Both tamoxifen-resistant sublines formed colonies in the presence of tamoxifen, but when these cell lines were transfected to express YFP-Rho GDI α , the colonies were greatly reduced in size and numbers. We used growth assays to examine the properties of tamoxifen-resistant cells with stable reexpression of YFP-GDI α (Figure 2, C and D). TR1 cell growth

Figure 2. Rho guanine dissociation inhibitor (GDI) α expression in and growth properties of tamoxifen-resistant MCF-7 cells. **A)** Rho GDI α expression in two tamoxifen-resistant MCF-7-derived cell lines, TR1 and TR2. Cells were grown in the presence of 4-hydroxy-tamoxifen (1 μ M for TR1 and 100 nM for TR2) for longer than 1 year to generate pools of resistant cells. Whole-cell lysates from these and parental MCF-7 cells were immunoblotted and blots were cut and probed with rabbit polyclonal antibodies recognizing Rho GDI α or with mouse monoclonal antibodies to β -actin (ACTIN) to ensure equal loading. **B)** Effect of stable RhoGDI α expression on tamoxifen-resistant growth of TR1 and TR2 cells. TR1 and TR2 cells transfected with yellow fluorescence protein-vector (YFP) or YFP-Rho GDI α (YFP-GDI α) plasmids were used in colony formation assays as described in "Materials and Methods". Fourteen days after plating of the cells at low density, the colonies were stained with 1% crystal violet and then photographed under a microscope. The pictures are representative of two independent experiments. **C)** Stable transfection of TR1 cells with YFP-vector (TR1 YFP) or YFP-Rho GDI α (TR1 YFP-GDI α) for Rho GDI α expression. An immunoblot (IB) of whole-cell lysates from pools of transfected TR1 cells was probed with a rabbit polyclonal antibody against Rho GDI α . **D)** Anchorage-independent growth, tamoxifen and fulvestrant sensitivity of tamoxifen-resistant MCF-7 cells transfected with YFP-Rho GDI α . Pools of TR1 cells stably transfected with YFP-vector (TR1 YFP) or YFP-Rho GDI α (TR1 YFP-GDI α) were plated in soft agar and then treated with 4-hydroxy-tamoxifen (T, 10^{-6} M), fulvestrant (I, 10^{-8} M), or vehicle (C). Data are the mean colony number from three plates and are representative of three independent experiments.



Error bars correspond to 95% confidence intervals. *P* values were calculated using two-sided Student *t* tests. For vehicle-treated TR1 YFP cells, * *P* = .04. For C vs T in TR1 YFP and TR1 YFP-GDI α , n.s. = statistically nonsignificant, and ***P* = .007, respectively.

was not inhibited with tamoxifen. However, when Rho GDI α was stably reexpressed, the basal growth of TR1 cells was decreased and they became tamoxifen-sensitive. These results suggested that Rho GDI α levels may be a major determinant of tamoxifen resistance in ER α -positive cells.

Effect of Rho GDI α Silencing on Rho and ER α Signaling

To study mechanism, we first examined the activation of Rho family of GTPases using GST-pulldown assays (Figure 3, A and Supplementary Figure 2, available online). Increased levels of activated Cdc-42, Rac-1, and Rho A, B, and C were detected in cloned GDI α sh cells compared with CTRL sh cells, indicating increased Rho-GTPase activity in cells containing Rho GDI shRNA.

The Rho GTPases activate downstream effectors (25). Either activation or increased expression of one effector, PAK1, can render breast cancer cells tamoxifen-resistant (26,27). Protein kinase A and PAK1 kinase signal to and phosphorylate ER α at serine (S) 305 (13,28,29). Therefore, we examined PAK1 activation by immunoprecipitating PAK1 from cells with and without Rho GDI α expression and using purified GST-ER α protein (residues 253–310) as substrate (Figure 3, B). We found higher levels of phosphorylated ER α in Rho GDI α shRNA-containing cells. When we mutated the serine 305 residue of ER α to an alanine (S305A), we found no phosphorylation by PAK1. Expression of Rho GDI α shRNA also led to enhanced phosphorylation of ER α at S305 (Figure 3, C). Phosphorylation of ER α at S305 can enhance sensitivity to estrogen treatment

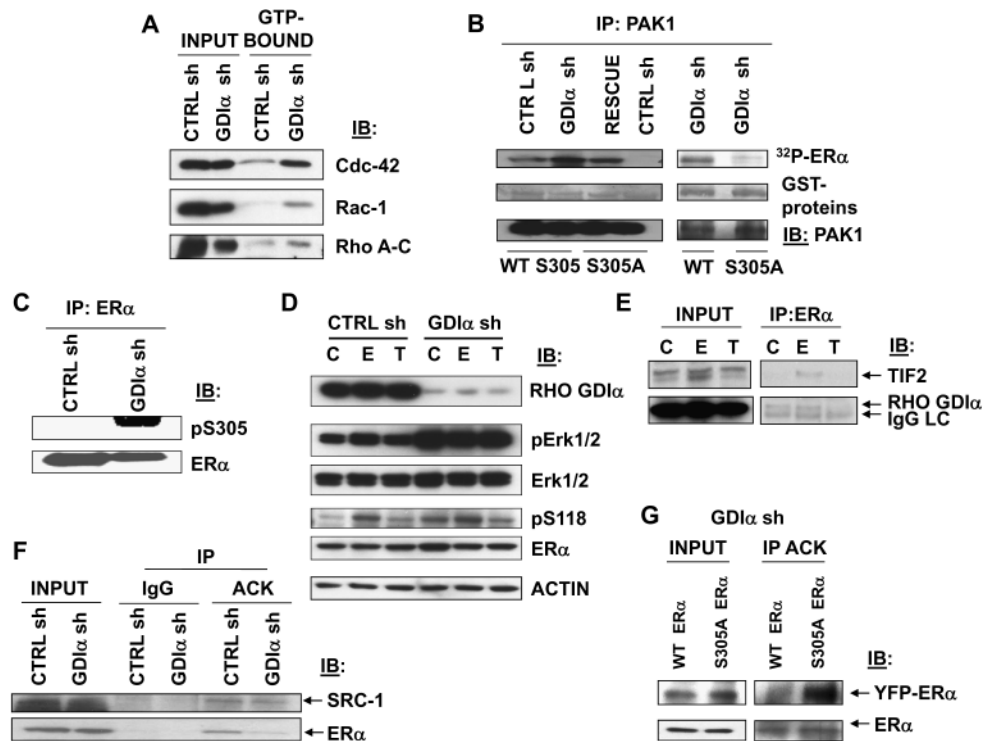
(13,29) and predicts poor response to tamoxifen in breast cancer patients (30). Thus, increased PAK1 activity in cells with decreased Rho GDI α expression may lead to enhanced phosphorylation of a site on ER α that is critical for resistance to tamoxifen.

Phosphorylation of ER α S305 by PAK1 and phosphorylation of ER α S118 by MAPK may be linked (28). We observed constitutive activation of pMAPK in GDI α sh cells, but not elevated levels of ER α pS118 with tamoxifen (Figure 3, D). We observed elevated pS118 levels in both cells with estrogen as expected (31). Thus, phosphorylation of ER α S305 and S118 are not coupled with tamoxifen treatment in our model, and elevated pMAPK levels might be a consequence of increased activation of PAK1 in cells with decreased GDI α expression.

Rho GDI α and ER α interact in MCF-7 cells (32). We next examined how tamoxifen affected their interaction by immunoprecipitation of ER α and immunoblotting for Rho GDI α in the presence of estrogen or tamoxifen (Figure 3, E). We observed an estrogen-dependent interaction between ER α and the transcription intermediary factor 2 (TIF2, an ER α coactivator protein used as a control) as reported previously (15). We observed an interaction between Rho GDI α and ER α under control and estrogen treatments but not with tamoxifen. We conclude that Rho GDI α probably does not enable tamoxifen sensitivity by direct binding to ER α in tamoxifen-treated cells.

Because phosphorylation of ER α at S305 has been reported to block acetylation of ER α at lysines (K) 302 and 303 (13), we also used

Figure 3. Effects of Rho guanine dissociation inhibitor (GDI) α silencing on Rho GTPase and estrogen receptor (ER) α signaling. **A)** Activation of cdc-42, Rac-1, and Rho (A–C) GTPases in cells with reduced Rho GDI α expression. Between 700 and 1000 μ g of cellular proteins from cloned CTRL sh and GDI α sh cells were incubated with 15 μ g of PAK-1 p21 binding domain (PBD) or 20 μ g Rhotekin Rho binding domain (RBD) agarose conjugate at 4°C for 90 minutes and separated by centrifugation. GTP-bound activated Rho proteins were detected by immunoblotting using mouse monoclonal antibodies against Rac-1 and cdc42; or rabbit polyclonal antibodies to RhoA, RhoB, or RhoC. Whole-cell lysates were used as input controls (INPUT). Blots are representative of three independent experiments. **B)** Activation of PAK1 in cells with reduced Rho GDI α expression. Immunocomplex kinase assays were performed using purified GST-ER α fragments (aa 253-310) as the substrate and using PAK1 that was immunoprecipitated (IP) from CTRL sh, GDI α sh, and RESCUE clones as the enzyme. An ER α GST-fragment in which the serine 305 phosphorylation site was converted to an alanine (S305A) was used as negative control. Phosphorylated ER α levels (32 P-ER α) were determined by autoradiography. The input GST-ER α hinge domain proteins (GST-proteins) were visualized with Ponceau S staining, and the immunoprecipitated PAK1 was detected by immunoblotting (IB) with a rabbit polyclonal anti-PAK1 antibody. **C)** Phosphorylation of ER α S305 in cells with reduced Rho GDI α expression. Cellular lysates from CTRL sh and GDI α sh cells were immunoprecipitated with a mouse monoclonal anti-ER α antibody and subsequently immunoblotted (IB) for detection of phosphorylated ER α S305 (rabbit polyclonal antibody to pS305 ER α) and total ER α protein. **D)** Phosphorylation of Erk 1 and 2 and ER α S118 in cells with reduced Rho GDI α expression. CTRL sh and GDI α sh cells were treated with 17 β -estradiol (E, 10 $^{-9}$ M), 4-hydroxy-tamoxifen (T, 10 $^{-7}$ M), or vehicle (C) for 5 minutes before lysis. Levels of Rho GDI α , phosphorylated (p) Erk1 and 2 (rabbit polyclonal antibody to Thr202 and Tyr204 of Erk1 and Thr185 and Tyr187 of Erk2), total Erk 1 and 2 (rabbit polyclonal antibody to Erk 1 and Erk2), phosphorylated ER α (mouse monoclonal antibody to pS118 ER α), and total ER α (mouse monoclonal antibody to ER α), proteins in cellular extracts were analyzed by immunoblot analysis (IB). Mouse monoclonal antibody to β -actin (ACTIN) was used as a control for equal loading and transfer. **E)** Binding of Rho GDI α to ER α in estrogen- and tamoxifen-treated MCF-7 cells. Cellular lysates from parental MCF-7 cells that were treated with 17 β -estradiol (E, 10 $^{-9}$ M), 4-hydroxy-



tamoxifen (T, 10 $^{-7}$ M), or vehicle (C) for 2 hours were immunoprecipitated with a mouse monoclonal anti-ER α antibody, and subsequently immunoblotted (IB) for detection of Rho GDI α (using a rabbit polyclonal antibody) or of the ER α coactivator TIF2, a positive control (mouse monoclonal anti-TIF2 antibody). Whole-cell lysates were used as input controls (INPUT). LC = light chain. **F)** Acetylation of ER α in cells with reduced Rho GDI α expression. Cellular lysates from CTRL sh and GDI α sh cells were immunoprecipitated with a rabbit polyclonal anti-acetylated lysine antibody (ACK) or IgG (negative control) and subsequently immunoblotted (IB) for detection of ER α (mouse monoclonal antibody to ER α) or its coactivator protein, SRC-1 (as a positive control, rabbit polyclonal antibody to SRC-1). Whole-cell lysates were used as input controls (INPUT). **G)** Acetylation of ER α in cells with the ER α S305A phosphorylation site mutation. GDI α sh cells were transfected with the yellow fluorescence protein (YFP)-ER WT or YFP-ER S305A plasmid constructs (encoding 96 kDa YFP-receptor fusion proteins) to evaluate changes in the acetylation status of ER α in the absence of phosphorylation (S305A). Cellular lysates were immunoprecipitated (IP) with a rabbit polyclonal anti-acetylated lysine antibody (ACK) and subsequently immunoblotted (IB) for detection of the ER α . Whole-cell lysates were used as input controls (INPUT). For all panels shown (A–G), immunoblots are representative of two or three separate experiments.

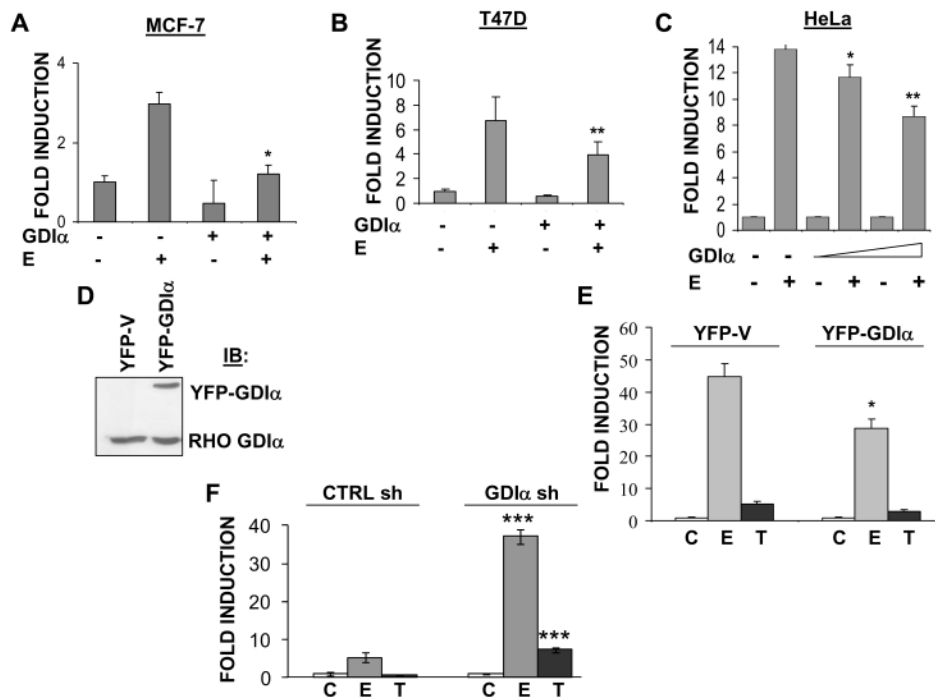
immunoblots to check whether ER α could be immunoprecipitated with an anti-acetyllysine antibody from lysates of cells with decreased Rho GDI α expression. We found lower levels of acetylated ER α in GDI α sh cells (Figure 3, F), whereas the acetylation of the ER α coactivator SRC-1 (a positive control) was unchanged. When Rho GDI α sh cells expressing ER α S305A were used in these experiments, acetylation of ER α was restored in the S305A mutant (Figure 3, G). These results suggest that the hinge region of ER α , which contains the important regulatory residues, K302, K303, and S305, is altered concomitant with Rho GDI α silencing.

Effect of Rho GDI α on ER α Transactivation

Because Rho GDI α overexpression and PAK1 activation had been reported to enhance ER α transcriptional activity (27,33), we transfected three human cell lines (MCF-7 and T47D breast cancer

cells, and HeLa cervical cancer cells) with YFP-GDI α and a luciferase reporter plasmid from which transcription could be activated through an estrogen response element. Because HeLa cells were ER-negative, they were also transfected with an ER α expression vector. In all cells tested, Rho GDI α overexpression decreased estrogen-induced transactivation (Figure 4, A–C). We also tested the effect of Rho GDI α expression on ER α activity in stable pools of T47D cells overexpressing YFP-Rho GDI α . ER α activity was decreased with estrogen but not affected by tamoxifen in these cells (Figure 4, D and E). In keeping with these results, estrogen- and tamoxifen-induced ER α activity was enhanced with Rho GDI α silencing (Figure 4, F). These data demonstrate that Rho GDI α expression is inversely related to ER α activity. The reason for the differences in our results compared with those of some others (32,33) is not presently understood; however, the cell lines

Figure 4. Effect of Rho guanine dissociation inhibitor (GDI) α expression on estrogen receptor (ER) α transcriptional activity. **A–C** Effect of transient Rho GDI α expression on estrogen-stimulated luciferase reporter activity. MCF-7 (A) and T47D (B) cells were cotransfected with an estrogen response element (ERE)-luciferase reporter, a pCMV- β -galactosidase vector, and the yellow fluorescence protein (YFP)-GDI α vector. The ER α -negative HeLa cells (C) were also cotransfected with a wild-type ER α expression vector. Cells were treated with estrogen (E, 10^{-9} M) and after 18–24 hours, cell lysates were harvested. ERE-luciferase activity was measured and normalized by dividing by the β -galactosidase activity to give relative luciferase units. The data are representative of three independent experiments, each performed in triplicate, and are reported as fold induction. For E treatment between GDI α and YFP-expressing MCF-7 cells (A), T47D cells (B) and HeLa cells (C), $*P = .004$, $**P = .041$, and $*P = .041$ and $*P = .003$, respectively. **D** Creation of stable pools of T47D cells overexpressing the YFP-vector (YFP-V) or YFP-Rho GDI α (YFP-GDI α). Whole-cell lysates were immunoblotted and probed with a rabbit polyclonal anti-Rho GDI α antibody. **E** Estrogen-stimulated luciferase reporter activity in T47D cells with and without YFP-Rho GDI α expression. ERE-luciferase activity was assayed in stable pools of T47D cells overexpressing the YFP-vector (YFP-V) or YFP-Rho GDI α (YFP-GDI α) and treated with 17β -estradiol (E, 10^{-9} M), 4-hydroxy-tamoxifen (T, 10^{-7} M), or vehicle (C). Data are representative of three independent experiments, each performed in triplicate, and reported as fold induction. For E treatment between YFP-V and YFP-GDI α stable pools, $*P = .012$. **F** Effect of Rho GDI α silencing on estrogen and tamoxifen-



were different and it is possible that the data were normalized differently.

Effect of Rho GDI α Silencing on Tamoxifen-Resistant Growth and Metastasis of Xenografts

To examine the effects of decreased Rho GDI α expression on tumor growth in vivo, we next injected MCF-7 cells containing Rho GDI α shRNA or the empty vector into athymic nude mice. Mice were implanted with 60-day release estrogen pellets, and at approximately 1.5–2.5 weeks, when tumors reached approximately 250 mm³, the mice were randomized to continue estrogen (E), to have estrogen withdrawn (ie, the pellet removed; N), or to have estrogen withdrawn and to begin tamoxifen treatment (T). Xenograft tumor growth is shown in Supplementary Table 1 (available online).

Our results were consistent with tamoxifen-resistance among tumors from cells with Rho GDI α silencing. Among mice that carried tumors derived from CTRL sh cells, the time to tumor tripling in the presence of tamoxifen treatment (VT) was not statistically significantly different from that in the presence of estrogen withdrawal (VN) (median tripling time of VT tumors = not reached, of VN tumors = not reached, hazard ratio [HR] = 1.36, 95% confidence interval [CI] = 0.274 to 6.80, $P = .704$ [with adjustment for multiple comparisons, $P = 1$]; Figure 5, A; Table 1). However, among mice that carried tumors derived from Rho GDI α shRNA-expressing cells, the time to tumor tripling in the presence of tamoxifen (RT) was not statistically different from that

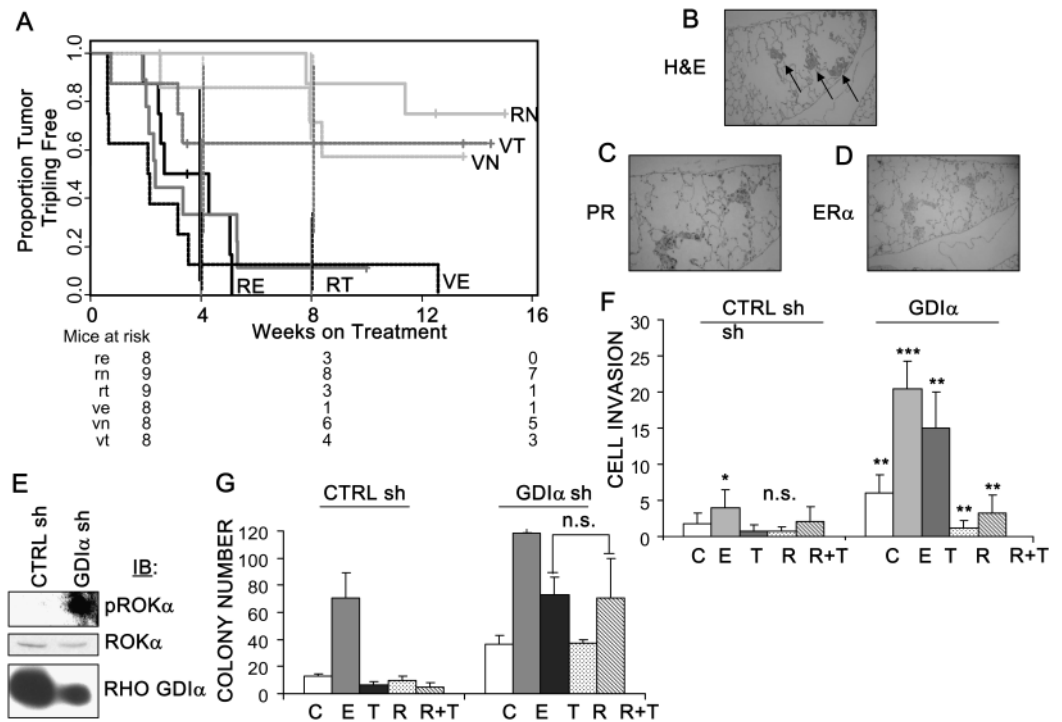
induced ER α transcriptional activity. ERE-luciferase activity was measured in stable CTRL sh and GDI α sh clones treated with 17β -estradiol (E, 10^{-9} M), 4-hydroxy-tamoxifen (T, 10^{-7} M), or vehicle (C). Data are representative of three independent experiments, each performed in triplicate, and are reported as fold induction. For E and T treatments between CTRL sh and GDI α sh, $***P < .001$. For panels (A–C), (E), and (F), error bars correspond to 95% confidence intervals and P values were calculated using two-sided Student t tests.

in mice treated with estrogen (RE), suggesting that tamoxifen was acting like estrogen to stimulate growth (tripling time of RT tumors = 2.34 weeks, of RE tumors = 3.48 weeks, HR = 0.953, 95% CI = 0.338 to 2.688, $P = .928$ [adjusted, 1]). Comparison of the two tamoxifen-treated groups, mice with tumors derived from vector vs GDI α sh-containing cells (RT vs VT), demonstrated a statistically significant difference in time to tumor tripling (tripling time of RT tumors = 2.34 weeks, of VT tumors = not reached, HR = 4.133, 95% CI = 1.07 to 15.96, $P = .040$ [adjusted, 0.119]).

To summarize, when mice were treated with estrogen or estrogen withdrawal, xenografts derived from Rho GDI α shRNA-expressing cells (RE or RN) tripled in size at about the same rate as those derived from vector control cells (VE or VN). However, when the mice were treated with tamoxifen, xenografts from cells with Rho GDI α silencing (RT) tripled in size at a statistically significantly faster rate compared with those derived from vector-containing cells (VT). These data provide strong evidence that Rho GDI α silencing can confer tamoxifen resistance in vivo.

Most strikingly, two of eight (25%) examined lungs from the tamoxifen-treated vs three of five (60%) estrogen-treated mice that carried tumors derived from GDI α sh cells carried metastases within 3 months. For example, in a representative H&E slide from the lung of an estrogen-treated mouse carrying a GDI α sh cell-derived tumor, three metastases can be seen in a single section (Figure 5, B, arrows). MCF-7 infrequently metastasizes to distant organs (23), and none of the mice carrying tumors derived from vector-only cells developed metastases during this experiment.

Figure 5. Loss of Rho guanine dissociation inhibitor (GDI) α expression and tamoxifen resistance *in vivo*. **A)** Overall survival in mice with MCF-7 xenografts expressing Rho GDI α shRNA. Stably transfected CTRL shRNA (V, dashed lines) and Rho GDI α shRNA (R, solid lines) containing cells were injected into the mammary fat pad of athymic nude mice. Mice were implanted with 0.72 mg 60-day release estradiol pellets at the time of xenograft injection. At 1.5–2.5 weeks, when tumors reached approximately 250 mm³, the mice were randomly allocated to continue estrogen treatment (VE [n = 8, tumor tripling events = 8], **black lines**) or to estrogen withdrawal (VN [n = 8, events = 3] and RN [n = 9, events = 2], **green lines**) or to estrogen withdrawal plus tamoxifen administration (VT [n = 8, events = 3] and RT [n = 9, events = 8], **red lines**) for another 30 days. Survival curves (shown as the proportion of mice in which tumors had not tripled in size) are graphed as the time in weeks from randomization to a threefold increase in total tumor volume over baseline (time to tumor tripling). Mice at risk in each group at 0, 4, and 8 weeks are shown beneath the survival curves, 95% confidence intervals for survival proportion are shown as vertical lines at 4 and 8 weeks. Curves were computed by the Kaplan–Meier method and compared using two-sided log-rank tests. **B)** Lung metastases from mice carrying xenografts of MCF-7 cells containing Rho GDI α shRNA. Histological analysis of hematoxylin and eosin (H&E)-stained sections from spontaneous lung metastases that arose in estrogen-treated mice carrying tumors derived from GDI α sh cells. **Arrows** point to metastatic lesions. **C** and **D)** Immunohistochemical detection of the progesterone receptor (PR, C) and estrogen receptor (ER) α (D) in the metastatic lesions; $\times 10$ magnification. **E)** ROK α expression in cells with Rho GDI α silencing. Immunoblot (IB) of whole-cell lysates from CTRL sh and GDI α sh cells probed with rabbit polyclonal antibodies to phosphorylated (p) or total ROK α protein, and to Rho GDI α . **F)** Invasiveness of cells containing Rho GDI α shRNA. *In vitro* invasion



Thus, Rho GDI α silencing enhanced metastasis. The metastases probably have an intact hormone response pathway, not only because their growth was stimulated by both estrogen and tamoxifen but also because tumors in the estrogen-treated group were positive for the estrogen-responsive progesterone receptor

Table 1. Statistical analysis of xenograft tumor growth

Group comparison*	<i>P</i> value†	Adjusted <i>P</i> value‡
VT vs VN	.704	1
RT vs RE	.928	1
RT vs VT	.039	.119
VT vs VE	.008	.032
RT vs RN	.003	.018
RE vs RN	.004	.019

* Vector control tumors (V); Rho GDI α sh tumors (R); treatment groups: no treatment (N), tamoxifen (T), estrogen (E).

† Wald χ^2 tests using contrasts in a Cox proportional hazards regression.

‡ *P* values adjusted for multiplicity by the Holm method (19).

chambers were used with CTRL sh and cloned GDI α sh cells treated with 17 β -estradiol (E, 10⁻⁹ M), 4-hydroxy-tamoxifen (T, 10⁻⁷ M), vehicle (C) and/or the ROK inhibitor Y-27632 (R, 10⁻⁸ M). Data are representative of three independent experiments, each performed in triplicate. Error bars correspond to 95% confidence intervals. *P* values were calculated using two-sided Student *t* tests. For C treatment between CTRL sh and GDI α sh, *****P* = .002**. For C vs E in CTRL sh cells, ****P* = .03**. For C vs T, C vs R, and T vs R+T in CTRL sh cells, **n.s.** = statistically nonsignificant. For C vs E in GDI α sh cells, ******P* < .001**. For C vs T, C vs R, and T vs R+T in GDI α sh cells, ******P* < .001**. **G)** Anchorage-independent growth, tamoxifen and Y-27632 sensitivity of GDI α sh vs CTRL sh cells. CTRL sh and GDI α sh clones were plated in soft agar and then treated with 17 β -estradiol (E, 10⁻⁹ M), 4-hydroxy-tamoxifen (T, 10⁻⁷ M), vehicle (C), and/or Y-27632 (R, 10⁻⁸ M). Data are the mean colony number of two wells. Error bars correspond to 95% confidence intervals. *P* values were calculated using two-sided Student *t* tests. For T vs R+T in GDI α sh cells, **n.s.** = statistically nonsignificant.

(Figure 5, C). Because estrogen treatment results in decreased ER α protein levels (34), we did not see ER α expression in the metastases (Figure 5, D). These results are important because there are few preclinical models of hormone-responsive metastases, and breast cancer metastasizes to the lung more frequently than to any other site but bone. About half of lung metastases are ER α -positive (35).

Among other downstream effectors of Rho signaling, the Rho-associated kinases (ROKs) and NF- κ B, are important for cell motility and invasion (36–39). Although we did not observe NF- κ B activation (Supplementary Figure 3, A and B, available online), we detected elevated levels of activated ROK α in cells containing Rho GDI α shRNA (Figure 5, E). It has been suggested that estrogen promotes an epithelial-to-mesenchymal-like transition that would foster cellular invasion (40). Consistent with this idea, when we measured cell invasion in modified Boyden chamber assays, we saw enhanced invasion by estrogen-treated CTRL sh cells compared with untreated cells (Figure 5, F). In cells with decreased Rho GDI α expression, we saw enhanced constitutive

and estrogen-stimulated invasion. Tamoxifen also enhanced invasion, suggesting that tamoxifen is acting as an ER α agonist. ROK inhibition by treatment with Y-27632 reduced basal growth and blocked tamoxifen-stimulated invasion only in cells in which Rho GDI α expression was decreased. It is known that many metastasis suppressor genes do not affect primary tumor growth, but only metastatic processes such as motility and invasion (9). Therefore, we tested the ROK inhibitor in anchorage-independent assays (Figure 5, G). Treatment with the ROK inhibitor Y-27632 did not affect basal growth cells with decreased Rho GDI α expression, and it did not restore tamoxifen-sensitive growth. This suggests that the ROK inhibitor's activities might be restricted to the invasive potential of these cells but not useful to block tamoxifen's agonist-like effects on growth.

Association of Combined Rho GDI α and MTA2 Status With Clinical Outcomes

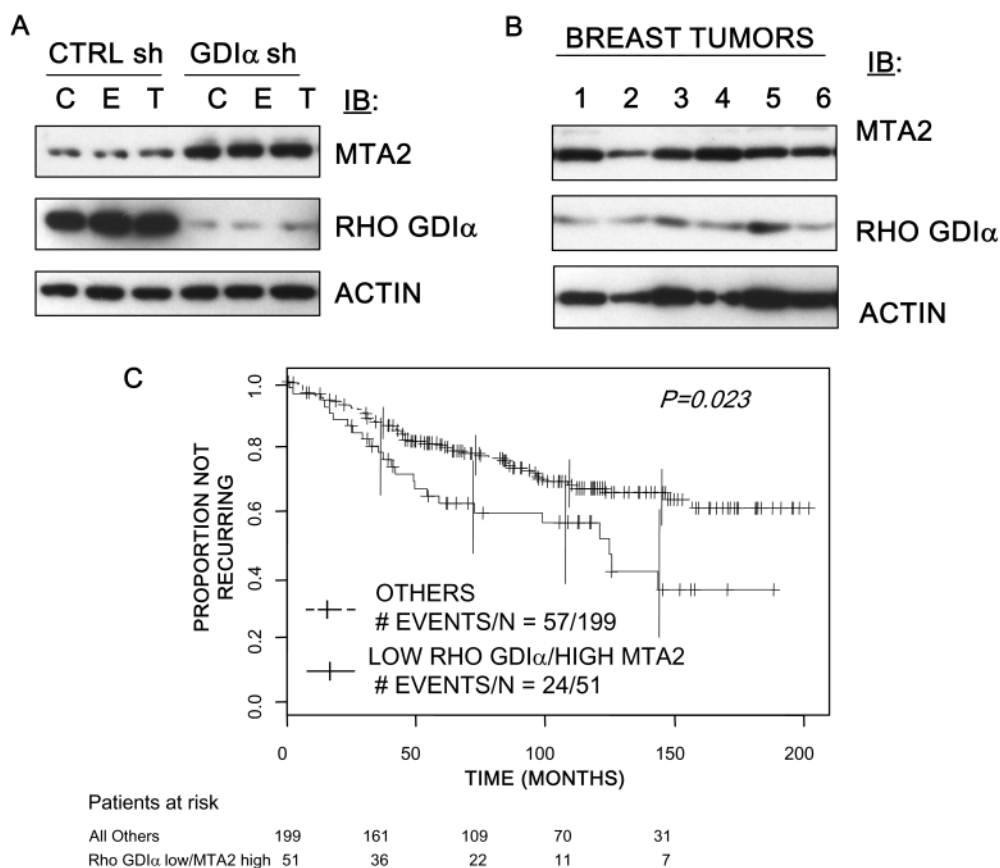
The metastasis-associated protein (MTA) family is associated with breast tumor progression and ER α activity (41). We demonstrated that MTA2 overexpression in ER α -positive cells generated a tamoxifen-resistant phenotype (42). To explore the metastatic phenotype associated with Rho GDI α silencing, we performed immunoblot analysis and found an increase in MTA2 protein levels in cells with decreased Rho GDI α expression compared with CTRL sh cells (Figure 6, A). The increase in MTA2 levels was independent of estrogen and tamoxifen treatment. An immunoblot

analysis of six primary breast tumors showed that levels of both proteins varied among the tumors (Figure 6, B).

To assess the clinical significance of Rho GDI α and MTA2 expression levels, we analyzed a microarray dataset described by Loi et al. (21), GSE6532. To determine the effects of Rho GDI α and MTA2 on the response to tamoxifen, only data from the 250 patients who were classified as ER+ and who received tamoxifen monotherapy were considered. On the basis of the microarray data, we subdivided patients in the tamoxifen-treated group into a Rho GDI α low and MTA2 high ("low/high") group vs all other groups using a bootstrapping approach to select optimum cut points. Of the 250 patients, 51 were classified as "Rho GDI α low and MTA2 high" and 199 were classified as "Others". The patients were similar in terms of nodal status, histological grade, age, and tumor size (Supplementary Table 2, available online). In the tamoxifen-treated group, the low/high patients (n = 51) had a statistically significantly poorer recurrence-free survival compared with all other patients (Figure 6, C, $P = .023$ [log-rank test, Cox-proportional hazards, HR = 5.84, 95% CI = 1.15 to 2.99]). No difference in survival was observed when patients were subdivided based on expression of Rho GDI α or MTA2 alone in the treated group. We performed the same analysis on the samples classified as tamoxifen-untreated also in the Loi dataset and found no difference in recurrence-free survival; however, the number of untreated patients was small precluding a definitive conclusion about prognosis (data not shown). We hypothesize that low levels of Rho

Figure 6. Rho guanine dissociation inhibitor (GDI) α and MTA2 expression and clinical tamoxifen resistance.

A) Rho GDI α and MTA2 expression in MCF-7 cells expressing short hairpin (sh) RNA to Rho GDI α (GDI α sh cells) or control shRNA (CTRL sh cells). CTRL sh and GDI α sh cells were treated with 17 β -estradiol (E, 10^{-9} M), 4-hydroxy-tamoxifen (T, 10^{-7} M), or vehicle (C) for 24 hours before lysis. Levels of Rho GDI α (rabbit polyclonal antibody to Rho GDI α) and rabbit polyclonal antibody to MTA2 were analyzed in cellular extracts by immunoblot analysis (IB). Mouse monoclonal antibody to β -actin (ACTIN) was used as a control for equal loading and transfer. The immunoblots are representative of three separate experiments. **B)** Rho GDI α and MTA2 expression in six breast tumors. Protein extracts from six breast tumors were analyzed for Rho GDI α and MTA2 expression using immunoblot analysis (IB). β -actin (ACTIN) was used as a control for equal loading and transfer. **C)** Rho GDI α and MTA2 expression and clinical outcome in tamoxifen-treated patients. The proportion of patients remaining disease-free is shown using Kaplan-Meier plots of survival data from tamoxifen-treated estrogen receptor α -positive patients from the Loi et al study (21) stratified by low Rho GDI α and high MTA2 RNA levels. The number of events and patients in each group are shown ($P = .011$, log-rank test, Cox-proportional hazards, hazard ratio = 5.84, 95% confidence interval = 1.149 to 2.988).



GDI α expression may predict poor outcome among tamoxifen-treated patients, potentially through its effects on metastatic pathways such as MTA2.

Our working model (Figure 7) is that loss of Rho GDI α expression enhances metastasis and tamoxifen resistance via effects on both ER α and MTA2. Decreased expression or loss of Rho GDI α subsequently causes release of bound Rho family members (RhoA-C, Cdc42, and Rac-1), leading to the subsequent activation of its downstream effectors, including PAK1 and ROK. Activated PAK1, either directly or indirectly, leads to ER α phosphorylation at S305, which inhibits ER α acetylation by shifting the conformation of ER α thus altering hormone response. ROK activation enhances cell motility, invasiveness, and metastasis. Rho GDI α loss also enhances MTA2, which can participate both in metastasis and tamoxifen resistance.

Discussion

We demonstrate that Rho GDI α silencing affects pathways that affect both the metastatic phenotype and hormone responsiveness of breast cancer cells. Despite recent advances with antihormonal therapies, the median survival of patients with metastatic breast cancer is very poor—only 2–3 years (43). Thus, resistance to hormonal therapies continues to be a major clinical problem. The ER α status of metastases does not always correlate with that of the primary tumor (4,44), but it has been shown that ER α expression was lost in only 17% of tamoxifen-resistant tumors as compared with expression in the primary tumor of each patient (45), so that sustained ER α expression and function are probably important for hormone resistance.

One major mechanism that has been proposed for resistance is enhanced bidirectional cross talk between ER α and growth factor receptor pathways, as ERBB2 (46). Another emerging mechanism

involves the activation of intracellular signaling molecules that can either circumvent growth dependence on ER α (47–49) or augment ER's function (49). Therefore, selectively inhibition of intracellular signal transduction is a promising new method to target tamoxifen-resistance in patients (50). In our pilot cohort of tamoxifen-resistant patients, it appeared that multiple mechanisms of resistance could occur simultaneously. Our identification of a role for Rho GDI α in ER α action, tamoxifen resistance, and metastatic behavior further underscores the potential for inhibitors of signal transduction to overcome tamoxifen resistance in breast cancer. If multiple mechanisms of resistance occur in patients, then a combination of therapeutics targeted to multiple pathways may be required for effective treatment.

The acquisition of a remodeled cytoskeleton and a motile invasive phenotype are important steps in metastasis, and the Rho GTPases mediate these processes [reviewed in (51)]. Several reports have demonstrated a connection between pathways that regulate the cytoskeleton and hormone resistance in breast cancer. Tamoxifen treatment has been reported to alter the localization of actin and fibronectin receptors, and actin skeleton remodeling in endometrial cells (52). Furthermore, overexpression of the Ras-GDP exchange factor AND-34/BCAR3, which associates with the focal adhesion protein p130Cas, has been reported to alter actin distribution, and to confer tamoxifen-resistant growth (53).

Here, we examined whether loss of a negative regulator of the Rho GTPases could affect hormone response. By comparing tamoxifen-sensitive primary tumors with metastatic tumors that recurred while the patients were undergoing tamoxifen treatment, we discovered lower levels of Rho GDI α RNA in the resistant tumors. By contrast to other laboratories that used tumors that developed before tamoxifen treatment (54,55), we used tumors that developed during tamoxifen treatment, with the hypothesis that mechanisms of resistance might differ under tamoxifen selection. Indeed, our study of one tumor from this cohort revealed that overexpression of the MAPK phosphatase MKP3 conferred tamoxifen resistance in breast cancer cells (14). We might not have identified MKP3 as a determinant of resistance if we had not screened tumors that appeared during tamoxifen treatment because tamoxifen induced altered MKP3 expression via induction of reactive oxygen species and altered MKP3 expression was among the genes associated with tamoxifen resistance in primary breast tumors (56,57).

It has been shown that the kinase PAK1 phosphorylates ER α and that nuclear PAK1 localization is associated with tamoxifen resistance in breast cancer patients (27,58). PAK1 expression might be intricately interwoven with the metastasis resistance phenotype associated with Rho GDI α shRNA expression. It is also possible that the ER α S305 site, a target of PAK1 phosphorylation, is a major determinant of tamoxifen resistance as has been suggested by Michalides et al. (29,58). The S305 site is a factor in resistance to both tamoxifen and aromatase inhibitors via cross talk with growth factor signaling pathways in cells expressing somatic ER α K303R mutations (59–61). This mutation was associated with poor outcomes in univariate analyses of tumors from untreated breast cancer patients, and its presence was correlated with older age, larger tumor size, and lymph node-positive disease. Molecular analyses of the K303R ER α mutation have shown that the mutated

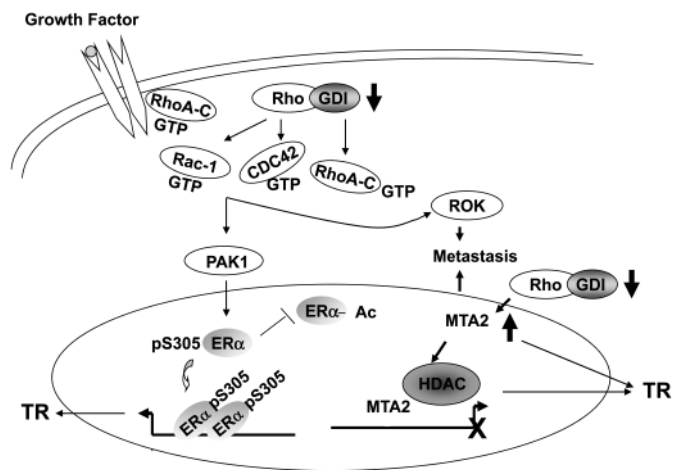


Figure 7. Suggested model for induction of tamoxifen resistance and metastasis by Rho guanine dissociation inhibitor (GDI) α silencing via effects on estrogen receptor (ER) α and MTA2. Loss of Rho GDI α leads to the release of bound Rho family members (Rho A, Cdc-42, and Rac-1) with the subsequent activation of downstream effectors PAK1 and ROK. Activated ROK may enhance cell motility and invasion. Activated PAK1 may phosphorylate ER α at serine (S) 305 and inhibit its acetylation, thus inducing tamoxifen resistance (TR). Loss of Rho GDI α is also associated with enhanced MTA2 expression, which can induce both metastasis and TR.

arginine at the 303 position allows ER α to be more highly phosphorylated by protein kinase A (PKA) and Akt kinase signaling (61,62) and alters the dynamic recruitment of coactivators and corepressors, such as BRCA-1 (63). Overexpression of the K303R mutation in ER α -positive MCF-7 breast cancer cells conferred increased sensitivity to subphysiological levels of estrogen and decreased sensitivity to tamoxifen treatment when engaged in cross talk with growth factor receptor signaling pathways (60). Expression of the K303R ER α mutation also conferred resistance to the nonsteroidal aromatase inhibitor anastrozole in ER α -positive cells (59,61). In these studies, blocking ER α S305 phosphorylation ablated tamoxifen resistance and cross talk between the ER α K303R mutant and the ERBB2 and IGFR signaling pathways (59,61). Our results suggest that the development of specific PAK1 (64) and ROK inhibitors might be useful to reverse tamoxifen resistance.

Low Rho GDI β is associated with increased metastasis risk and decreased survival in patients with locally advanced bladder cancer (65). Both endothelin-1 and neuromedin U are mediators of Rho GDI β effects on metastasis and tumor progression (66,67). Here, we report that MTA2 may be a mediator of Rho GDI α 's effects on metastasis and tumor progression in patients. The MTA family is important to ER function and ER-associated pathology [for a review, see (68)]. MTA2 can act as a negative corepressor of ER α action leading to hormone-independent growth (42). Expression of GDI α shRNA leads to a dramatic increase in MTA2 levels, and tamoxifen-treated breast cancer patients with low Rho GDI α and high MTA2 protein levels appear to exhibit a shorter disease-free survival time compared with all other patients. These are the first data to suggest a clinically important connection between the Rho GDI α and MTA2 pathways. Our data also suggest a possible mechanism, in which the loss of Rho GDI α function promotes distant progression of breast tumors by triggering downstream molecules, such as MTA2, with metastasis-promoting activities. If Rho GDI α works as a suppressor of prometastatic genes, then genes such as MTA2 and ROK would be activated and present as druggable targets for treatment of breast cancer patients with metastatic or tamoxifen-resistant disease. The association of combined Rho GDI α and MTA2 status with clinical outcome supports our hypothesis that Rho GDI α loss can cause resistance to tamoxifen.

There are limitations to this study. First, the hormone-resistant phenotype conferred via Rho GDI α silencing has been shown in only one human breast cancer cell line. For this finding to be generalizable, it will be important to test the effects of Rho GDI α in other ER-positive models. Second, the presented clinical data are based on a retrospective analysis, which may suffer from bias. Future prospective studies could clarify the role of Rho GDI α and MTA2 in breast cancer.

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