

Physical and Functional Interactions between Cas and c-Src Induce Tamoxifen Resistance of Breast Cancer Cells through Pathways Involving Epidermal Growth Factor Receptor and Signal Transducer and Activator of Transcription 5b

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Abstract

High expression of the adaptor molecule Cas has been linked to resistance to the antiestrogen tamoxifen, both in tissue culture and in human tumors. The aim of this study was to elucidate the mechanism(s) by which overexpression of Cas confers resistance to tamoxifen. Cas overexpression in MCF-7 breast cancer cells was shown to alleviate both tamoxifen-mediated growth inhibition and induction of apoptosis. This enhancement of cell proliferation/survival occurred in the absence of detectable effects on estrogen receptor (ER) transcriptional activity under conditions where tamoxifen was present, indicating that Cas-dependent tamoxifen resistance is not the result of a switch to an ER-negative phenotype or enhanced responses to the partial agonist activity of tamoxifen. Instead, we present evidence, suggesting that Cas promotes tamoxifen resistance by deregulation of alternative cell proliferation pathways, particularly those mediated through enhanced c-Src protein tyrosine kinase activity arising from Cas/c-Src interactions. Overexpression of Cas was found to drive endogenous c-Src into complex with Cas, a process that has been shown previously to cause up-regulation of c-Src tyrosine kinase activity. MCF-7 cells overexpressing Cas exhibited increased phosphorylation of two c-Src substrates, Tyr⁸⁴⁵ in the kinase domain of the epidermal growth factor receptor (EGFR) and signal transducer and activator of transcription (STAT) 5b. Importantly, Cas-dependent protection from the antiproliferative effects of tamoxifen was reversed by the expression of dominant inhibitory variants of these substrates (Y845F EGFR and COOH-terminally truncated STAT5b). Based on these findings, we suggest that the Cas/c-Src/EGFR/STAT5 signaling axis is a major regulator of tamoxifen-resistant breast cancer cell growth and survival. (Cancer Res 2006; 66(14): 7007-15)

Introduction

This year, one in eight women in the United States will be diagnosed with breast cancer, which is second only to lung cancer

as the cause of cancer-related deaths in women (1). One of the most successful approaches for treating breast cancer has exploited the use of endocrine or antiestrogen therapies. Antiestrogens serve to prevent estrogens from binding the estrogen receptor (ER) and inducing cell proliferation (reviewed in refs. 2–4). Tamoxifen, which is historically the most frequently used antiestrogen, has been shown to reduce breast cancer recurrence rates and mortality in women with ER-positive disease (5). Unfortunately, resistance to endocrine therapy is a significant clinical problem in the management of breast cancer (2–4). Some 30% to 50% of women with ER-positive breast cancer do not respond to tamoxifen, displaying what is termed *de novo* or intrinsic resistance, whereas many patients who show an initial positive response ultimately suffer a recurrence indicative of acquired antiestrogen resistance. Because of this high failure rate, it is important to identify molecular mechanisms that contribute to antiestrogen resistance with the goal of being able to better predict responses and perhaps reverse resistant phenotypes.

Several molecules, signal transduction pathways, and mechanisms have been implicated in acquired antiestrogen resistance, including down-regulation of ER expression and/or function, increased cellular responses to the partial agonist activities of tamoxifen, and up-regulation of cell growth and survival pathways that are independent of ER transcriptional regulation. For example, up-regulation of expression and/or signaling of the human epidermal growth factor (EGF) receptor (EGFR) and other family members has been observed in resistant tumors and breast cancer cell lines (6, 7), as well as changes in the repertoire of proapoptotic and antiapoptotic gene expression (reviewed in ref. 8). The molecular events responsible for intrinsic or *de novo* antiestrogen resistance are less clear. In 1999, Brinkman et al. published a study showing that overexpression of breast cancer antiestrogen resistance 1 (BCAR1), the human homologue of the adaptor protein Cas, induced antiestrogen resistance in ZR-75-1 breast cancer cells (9). This same group also reported that high levels of Cas/BCAR1 expression in human breast tumors were associated with reduced overall survival and intrinsic resistance to tamoxifen (10). Through its ability to function as a scaffolding molecule that uses distinct protein-protein interaction domains to promote the formation of multicomponent protein complexes, Cas is a key participant in multiple signal transduction networks that affect cell proliferation, survival, and oncogenic transformation (reviewed in ref. 11).

In this study, we have investigated the mechanism(s) by which Cas mediates tamoxifen resistance in MCF-7 breast cancer cells.

Note: R.B. Riggins and K.S. Thomas contributed equally to this work.

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We show that Cas overexpression protects these cells from the antiproliferative and proapoptotic effects of tamoxifen without affecting ER- α expression. ER- β expression was found to be slightly elevated in cells overexpressing Cas, coincident with a decrease in 17 β estradiol (E_2)-dependent transcription of the endogenous *cyclin D1* gene. However, Cas overexpression had no effect on the transcription of estrogen response element (ERE)-containing reporter constructs or endogenous cyclin D1 mRNA in the presence of tamoxifen, indicating that Cas-dependent tamoxifen resistance is not the result of a switch to an ER-negative phenotype or enhanced responses to the partial agonist activity of tamoxifen. Instead, our data indicate that Cas can induce tamoxifen resistance by binding to and activating the c-Src protein tyrosine kinase (PTK), leading to phosphorylation of critical c-Src substrates, such as tyrosine 845 (Y845) in the kinase domain of the EGFR and signal transducer and activator of transcription (STAT) 5b. Expression of dominant inhibitory variants of either EGFR (Y845F) or STAT5b (COOH-terminally truncated STAT5b) abrogates the protective effect of Cas overexpression on tamoxifen-mediated growth inhibition, supporting a critical function for these molecules in this process. Based on these findings, we suggest that activation of the Cas/c-Src/EGFR/STAT5 signaling pathway is a critical event in Cas-mediated tamoxifen-resistant breast cancer cell growth and survival.

Materials and Methods

Cell culture. MCF-7 and COS-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Stable tetracycline-regulated MCF-7 clones containing either vector (pTre2-Pur) or Myc-Cas-pTre2-Pur were generated by transfecting plasmid DNA into "Tet-off" MCF-7 cells (BD Biosciences Clontech, Palo Alto, CA) and by selecting with 0.75 μ g/mL puromycin. Individual clones were picked and analyzed by immunoblot and immunofluorescence for regulated protein expression in the presence or absence of 1 to 2 μ g/mL doxycycline. Clones were routinely maintained in 0.75 μ g/mL puromycin. The tetracycline-regulated MCF-7 cells expressing wild-type (*WT*) EGFR have been described previously (12).

Mutagenesis, transfection, protein detection, and reagents. The c-Src-binding mutant of Cas (Cas^{P642A,Y668/670F}; triple mutant Cas) was generated by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) as described previously (13) and verified by DNA sequencing. Dominant-negative STAT5b (containing a 39-amino acid COOH-terminal truncation in the transactivation domain) has been described previously (14). Transient transfections were done either with Superfect (Qiagen, Valencia, CA) or by nucleofection (Amaxa Corporation, Gaithersburg, MD) as per manufacturer's specifications. Immunoprecipitation, immunoblotting, and immunofluorescence were done as described previously (13). Antibodies recognizing EGFR, cyclin D1, total extracellular signal-regulated kinase (ERK; Cell Signaling Technology, Beverly, MA), pY845 on the EGFR (BioSource, Camarillo, CA), ER- α (DakoCytomation, Glostrup, Denmark), hemagglutinin (HA; Covance, Berkeley, CA), Ran (BD PharMingen, San Diego, CA), and phosphorylated ERK (Sigma, St. Louis, MO) were obtained from the indicated sources. All other antibodies and reagents used for protein detection have been described previously (13, 15).

Bromodeoxyuridine incorporation. Bromodeoxyuridine (BrdUrd) incorporation assays were done as described previously (15). Under conditions of transient transfection, BrdUrd incorporation was measured only in cells expressing the protein of interest as detected by indirect immunofluorescence.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining. To assess terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining, 2.5×10^5 cells were plated onto fibronectin-coated coverslips (20 μ g/mL in PBS) in six-well dishes and cultured in the presence or absence of 1 μ g/mL doxycycline. On

the following day, cells were placed in phenol red-free DMEM supplemented with 10% dextran-stripped/charcoal-cleared FBS (CCS) and either ethanol, 10^{-8} mol/L E_2 , or 10^{-6} mol/L 4-hydroxytamoxifen (Sigma) in the presence or absence of 1 μ g/mL doxycycline. Forty-eight hours later, the cells were processed for TUNEL staining using an *in situ* cell death detection kit (Roche Applied Science, Penzberg, Germany) and analyzed by microscopy.

Transcriptional reporter assays. For ERE reporter assays, MCF-7 cells were plated in triplicate in six-well dishes and transfected with 3 μ g control (vector) or 1 to 3 μ g Myc-Cas-encoding plasmids together with 1 μ g pGL3-2ERE (kindly provided by Dr. Deborah Lanningan, University of Virginia, Charlottesville, VA). Following transfection, the cells were treated with vehicle (ethanol), 10^{-8} mol/L E_2 , 10^{-6} mol/L tamoxifen, or E_2 + tamoxifen for 25 hours. Luciferase assays were done as described previously and normalized to protein concentration (16). For STAT5 reporter assays, MCF-7 cells were seeded in 12-well dishes and then transfected with 0.4 μ g control (vector) or Myc-Cas-encoding plasmids together with 0.1 μ g pRL-SV40 encoding the Renilla luciferase (Promega, Madison, WI) and 0.3 μ g pSpi2.1-Luc (six copies of the STAT5-dependent γ -IFN-activated sequence (GAS)-like element of the *Spi2.1* gene; ref. 17). Assays were done in triplicate as described previously (18).

Real-time reverse transcription-PCR. Cells were plated at a density of 3×10^6 per well in four-well plates in DMEM with 5% CCS overnight in the presence or absence of 1 μ g/mL doxycycline. Cells were then serum starved in medium with or without doxycycline for another 24 hours. On the following day, cells were treated with either ethanol, 10^{-8} mol/L E_2 , 10^{-6} mol/L tamoxifen, or E_2 + tamoxifen in the presence or absence of 1 μ g/mL doxycycline for 4 hours. RNA was extracted using the RNeasy Mini kit (Qiagen), and reverse transcription of 5 μ L RNA (~ 3 μ g) was carried out using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). Real-time PCR was done using an iCycler iQ (Bio-Rad) in 96-well optical reaction plates. cDNA (3 μ L) was used as template in 25 μ L real-time PCR containing 3 mmol/L MgCl₂, 0.25 mmol/L deoxynucleotide triphosphates, 10 pmol primers, and 0.275 units JumpStart Taq DNA polymerase (Sigma). SYBR Green was used as a detector for monitoring the amplified dsDNA fragments. Threshold cycle values were obtained from the iCycler iQ Optical System software (version 1.0). Values for each mRNA are presented from two experiments with triplicate wells assayed at least twice. As a control, actin mRNA was determined for each sample and did not change under any condition. Variables for cyclin D1 mRNA amplification were (95°C, 59°C, and 72°C for 30 seconds) \times 40 cycles. Variables for actin mRNA amplification were (95°C for 30 seconds and 58°C for 60 seconds) \times 40 cycles. The forward and reverse primers for cyclin D1 and actin were as follows: cyclin D1 forward (CTACACGACAACACTCCATCC) and cyclin D1 reverse (TGTTCTCCTCCGCCTCTG); actin forward (AGGCATCAC-TATTGGCAACGA) and actin reverse (CACTTCATGATGGAATTGAATG-TAGTT).

Statistical analyses. Statistical analyses were done using the SigmaStat software package (Systat Software, Inc., Point Richmond, CA). Data with a normal distribution were subjected to one-way ANOVA, whereas data failing the normality test were log₁₀ transformed before doing ANOVA. Two-tailed Student's *t* tests were then used for the pair-wise comparison of experimental groups. Statistical significance was defined at $\geq 95\%$ confidence interval (95% CI) or $P \leq 0.05$. Bar graphs represent the mean \pm standard error (SE) for the number of independent experiments indicated in each figure legend.

Results

Cas overexpression alters the proliferative response of MCF-7 cells in the presence of tamoxifen. To investigate mechanisms by which Cas overexpression confers resistance to tamoxifen, stable cell lines were generated that express Myc-tagged Cas under the control of a tetracycline-regulated promoter in an MCF-7 background (Tet-off). Myc staining was observed in Cas-expressing cells grown in the absence but not presence of 1 to 2 μ g/mL doxycycline, showing the tight regulation of Myc-Cas expression

and the clonal nature of the cell lines (Fig. 1A, bottom and Fig. 1B). In the clone used for these studies, Myc-Cas was expressed at ~7-fold above endogenous levels in the absence of doxycycline (Fig. 1A and C, top). ER- α expression was largely unaffected by Cas expression or doxycycline treatment, indicating that Cas overexpression did not lead to the down-regulation of ER- α (Fig. 1C, second). In contrast, ER- β was up-regulated 3- to 8-fold above basal levels in cells overexpressing Cas as determined by densitometry (mean, 6.75; $P = 0.029$; Fig. 1C, third, compare lanes 3 and 4). This was not due to treatment with doxycycline, as the vector control cells showed no such up-regulation of ER- β (Fig. 1C, compare lanes 1 and 2).

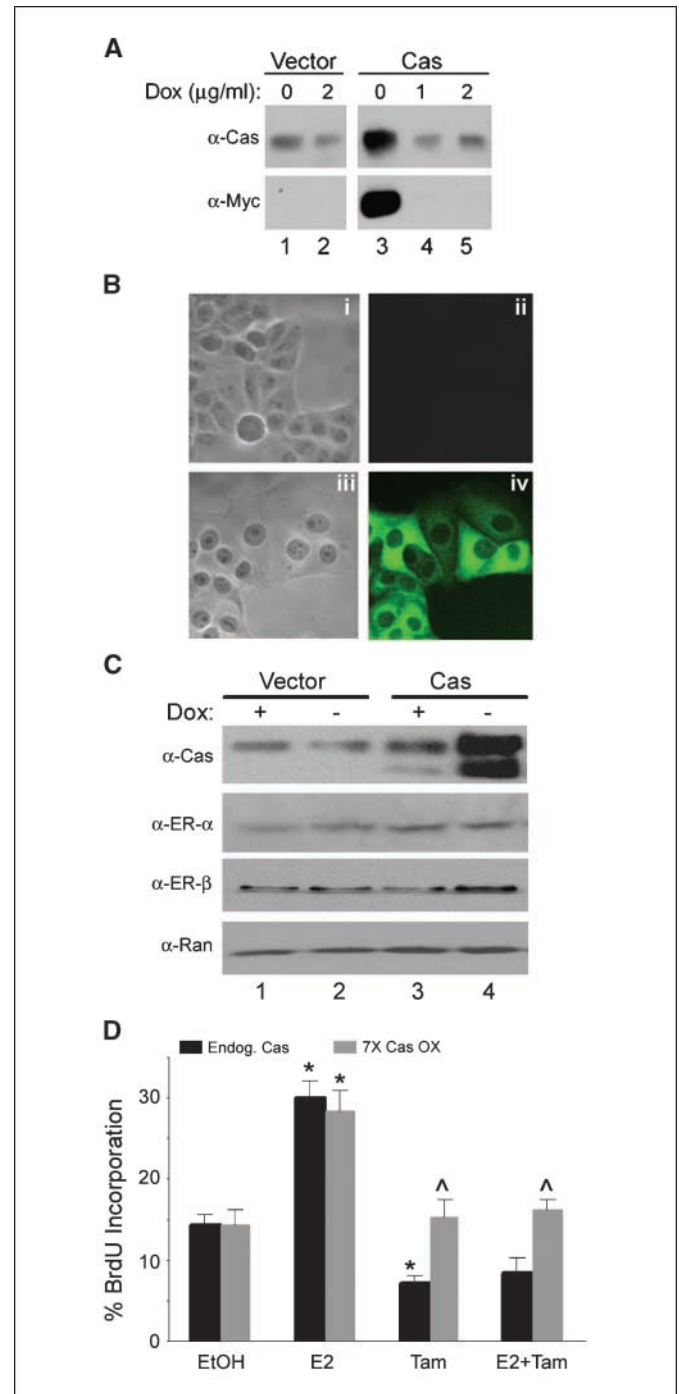
To determine the effect of Cas overexpression on cell proliferation, Cas-overexpressing MCF-7 cells were analyzed for BrdUrd incorporation under conditions where Cas was expressed at endogenous levels (+doxycycline) or 7-fold over endogenous levels (-doxycycline). In all cases, cells were cultured in medium depleted for estrogens (phenol red-free DMEM + 10% CCS) and supplemented with vehicle (ethanol), 10^{-8} mol/L E_2 , 10^{-6} mol/L 4-hydroxytamoxifen, or E_2 + tamoxifen. BrdUrd incorporation was elevated in cells treated with E_2 for 48 hours, irrespective of the level of Cas expression (Fig. 1D). However, although cells expressing endogenous levels of Cas showed a statistically significant decrease in BrdUrd incorporation in the presence of tamoxifen, cells overexpressing Cas showed no such inhibition. Similar results were observed in the presence of E_2 + tamoxifen, where cells overexpressing Cas again showed no inhibition of BrdUrd incorporation (Fig. 1D, E_2 + Tam, gray column). Together, these data indicate that Cas overexpression renders MCF-7 cells less sensitive to the antiproliferative effects of tamoxifen.

One mechanism through which Cas overexpression may render cells less sensitive to tamoxifen is modulation of the transcriptional activity of the ER. This was tested by measuring luciferase activity in cells containing a reporter plasmid (pGL3-2ERE) driven by a promoter containing two tandem EREs. Parental MCF-7 cells were transiently transfected with either control (vector) or Myc-Cas-encoding plasmids together with the luciferase reporter construct and then grown in the presence of vehicle (ethanol), 10^{-8} mol/L E_2 ,

10^{-6} mol/L 4-hydroxytamoxifen, or E_2 + tamoxifen for 25 hours. Cells treated with E_2 exhibited a significant increase in ERE-dependent transcription that was independent of the level of Cas expressed (Fig. 2A). No such increase was observed in the presence of tamoxifen or E_2 + tamoxifen, in either cells expressing endogenous Cas (Fig. 2A, black columns) or cells overexpressing Cas (Fig. 2A, gray columns). This shows that ERE-dependent transcription is regulated similarly by E_2 and tamoxifen in these cells, irrespective of the level of Cas expression.

To confirm that overexpression of Cas did not alter the levels of an endogenous ER target gene under these growth conditions, the

Figure 1. Doxycycline-regulated Cas overexpression induces tamoxifen resistance. **A**, ectopic Myc-Cas expression is regulated by doxycycline. Representative MCF-7 clones expressing vector (lanes 1 and 2) or Myc-Cas (lanes 3-5) were cultured in the presence or absence of the designated amount of doxycycline for 48 hours. Cell extracts (50 μ g) were separated on 8% SDS-PAGE and immunoblotted for total Cas (top) or Myc (bottom). Cas was expressed 7-fold above endogenous levels in the absence of doxycycline in the Myc-Cas clone according to densitometry. **B**, ectopic Myc-Cas is expressed in all cells in the population. The Cas-expressing clone analyzed in (A) was cultured in the presence (i and ii) or absence (iii and iv) of 2 μ g/mL doxycycline and immunostained to detect the Myc epitope on Cas (ii and iv). Phase images in (i) and (iii). **C**, ER- α levels are maintained, whereas ER- β levels are slightly elevated in Cas-overexpressing cells. Vector and Myc-Cas-expressing cells were grown in the presence or absence of 2 μ g/mL doxycycline for 48 hours and lysed. Cell proteins were separated by 8% SDS-PAGE and immunostained for Cas (top), ER- α and ER- β (second and third), or Ran as a loading control (bottom). Representative immunoblots (four independent experiments). **D**, Cas overexpression alters the proliferative response of MCF-7 cells to tamoxifen (Tam). Cas-expressing cells were cultured for 48 hours in the presence (black columns) or absence (gray columns) of 2 μ g/mL doxycycline in phenol red-free DMEM + 10% CCS supplemented with vehicle ethanol (EtOH), 10^{-8} mol/L E_2 , 10^{-6} mol/L 4-hydroxytamoxifen, or E_2 + tamoxifen (E_2 + Tam). BrdUrd (100 μ M) was added during the final 3 hours before fixation. Columns, mean of three to six independent experiments; bars, SE. *, significant difference from the mean at a $\geq 95\%$ CI relative to vehicle (ethanol)-treated cells expressing the same level of Cas; ^, significant difference from the mean at a $\geq 95\%$ CI relative to cells expressing endogenous levels of Cas (black columns) under the same treatment conditions.



Cas-inducible MCF-7 cells used in Fig. 1 were cultured in the presence (endogenous Cas) or absence (7-fold overexpression of Cas) of doxycycline and either vehicle (ethanol), E₂, tamoxifen, or E₂ + tamoxifen for 4 hours. Total RNA was extracted, reverse

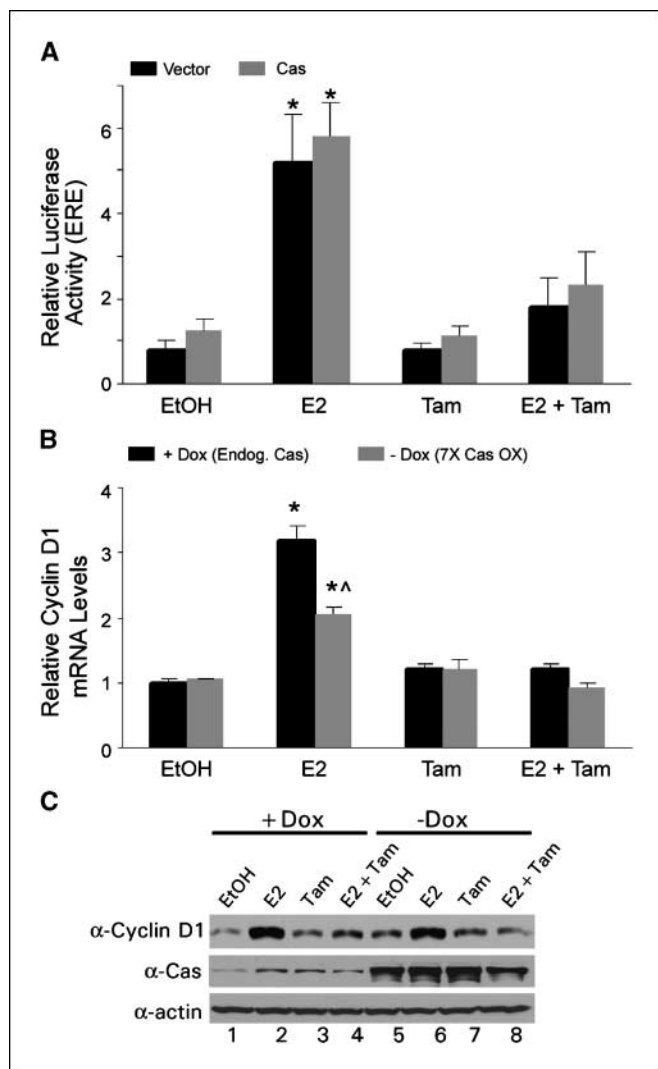


Figure 2. Cas overexpression has no effect on the expression of ERE-regulated genes. **A**, MCF-7 cells grown in normal growth medium (DMEM + 10% FBS) were transiently transfected in triplicate with vector (*black column*) or a plasmid encoding Myc-Cas (*gray column*) along with the luciferase reporter plasmid, pGL3-2ERE. Cells were then treated with ethanol, E₂, tamoxifen, or E₂ + tamoxifen and processed for luciferase assays 25 hours later. *Columns*, mean of three independent experiments, each done in triplicate; *bars*, SE. *, significant difference from the mean at a $\geq 95\%$ CI relative to vehicle (ethanol)-treated cells expressing the same level of Cas. **B**, doxycycline-inducible MCF-7 cells were grown in the presence or absence of 2 $\mu\text{g}/\text{mL}$ doxycycline for 24 hours in phenol red-free DMEM + 5% CCS and G418, serum-starved for 24 hours in phenol red-free DMEM + 0.1% BSA, and then incubated for 4 hours in phenol red-free DMEM + 5% CCS supplemented with ethanol, E₂, tamoxifen, or E₂ + tamoxifen. Total RNA was isolated, reverse transcribed, and analyzed by real-time RT-PCR using primers for cyclin D1 or actin as a control. *Columns*, mean of two independent experiments, each done in triplicate; *bars*, SE. *, significant difference from the mean at a $\geq 95\%$ CI relative to vehicle (ethanol)-treated cells expressing the same level of Cas; ^, significant difference from the mean at a $\geq 95\%$ CI relative to cells expressing endogenous levels of Cas (*black columns*) under the same treatment conditions. **C**, doxycycline-inducible MCF-7 cells were grown in the presence or absence of 2 $\mu\text{g}/\text{mL}$ doxycycline for 24 hours in phenol red-free DMEM + 5% CCS and G418, serum-starved for 24 hours in phenol red-free DMEM + 0.1% BSA, and then incubated for 12 hours in phenol red-free DMEM + 5% CCS supplemented with ethanol, E₂, tamoxifen, or E₂ + tamoxifen. Cell proteins were separated by 8% SDS-PAGE and immunoblotted for cyclin D1 (*top*), Cas (*middle*), and actin as a loading control (*bottom*).

transcribed, and analyzed by semiquantitative real-time reverse transcription-PCR (RT-PCR) using primers for cyclin D1, which is positively regulated by ER- α through cyclic AMP and activator protein response elements in the promoter (19). Relative to a housekeeping gene control (actin), cyclin D1 mRNA levels were induced by E₂ in either the presence or absence of doxycycline (Fig. 2B), although the level of induction was reduced in cells overexpressing Cas (Fig. 2B, E₂, *gray column*). This may be due to a reduced ER- α to ER- β ratio (see Fig. 1C), as ER- β has been shown to inhibit E₂-stimulated gene transcription in the presence of ER- α (20). Others have shown that antiestrogens can induce cyclin D1 expression in the presence of ER- β (21). However, cyclin D1 mRNA levels were not induced in the presence of tamoxifen or E₂ + tamoxifen, irrespective of the level of Cas expression (Fig. 2B). Cyclin D1 protein expression showed a similar pattern of regulation (Fig. 2C). These data thus show that overexpression of Cas does not modulate the transcriptional activity of ER or induce transcription of endogenous ER target genes under conditions where tamoxifen is present, suggesting that some other mechanism must account for the changes in sensitivity of Cas-overexpressing cells to tamoxifen.

c-Src and its substrates EGFR and STAT5b are required for Cas-dependent tamoxifen responses. Because ER transcriptional activity and target gene expression in the presence of tamoxifen were unaffected by Cas expression levels, we hypothesized that Cas may directly activate other signaling pathways independently of ER to modulate proliferation and survival in the presence of tamoxifen. Data from our group and others have established Cas as a potent regulator of c-Src PTK activity and growth/survival functions (13, 15, 22). Regulation of this activity is mediated through interactions between Cas and c-Src. To determine whether overexpression of Cas in MCF-7 cells served to drive more endogenous c-Src into complex with Cas, we measured Cas/c-Src interactions in the tetracycline-regulated Cas-overexpressing MCF-7 cells grown in the presence (endogenous Cas) or absence (7-fold Cas overexpression) of doxycycline. Doxycycline treatment had no effect on c-Src expression (Fig. 3A, *bottom*, compare *lane 1* with 2 and *lane 3* with 4). However, Cas/c-Src interactions were significantly increased under conditions of Cas overexpression (–doxycycline), as seen in both c-Src immune complexes where more Cas was associated with the same amount of c-Src (Fig. 3A, *top*, compare *lanes 3* and 4) and Cas immune complexes where immunoprecipitation of increased amounts of Cas resulted in the coprecipitation of more c-Src (Fig. 3A, *bottom*, compare *lanes 5* and 6). To test whether c-Src/Cas interactions were required to promote Cas-dependent changes in cellular responses to tamoxifen, we took advantage of a Cas mutant, in which the c-Src-binding sites were mutated (Cas^{P642A,Y668/Y670F}; triple mutant Cas; ref. 23). Substitution of these three amino acids effectively reduced c-Src binding as shown by the absence of triple mutant Cas in c-Src immune complexes (Fig. 3B, *top*, *lane 2*) and the concomitant lack of c-Src in Myc immune complexes containing triple mutant Cas (Fig. 3B, *bottom*, *lane 4*).

To determine whether expression of triple mutant Cas was capable of promoting BrdUrd incorporation in the presence of tamoxifen, despite its inability to bind to c-Src, MCF-7 cells were transiently transfected with control vectors or plasmids encoding either WT or triple mutant Cas. BrdUrd incorporation was then measured in transfected cells cultured in the presence of vehicle (ethanol), E₂, tamoxifen, or E₂ + tamoxifen as described above. BrdUrd incorporation was induced by E₂ in vector control (Fig. 3C, E₂, *black column*) and even more so in WT Cas-overexpressing cells

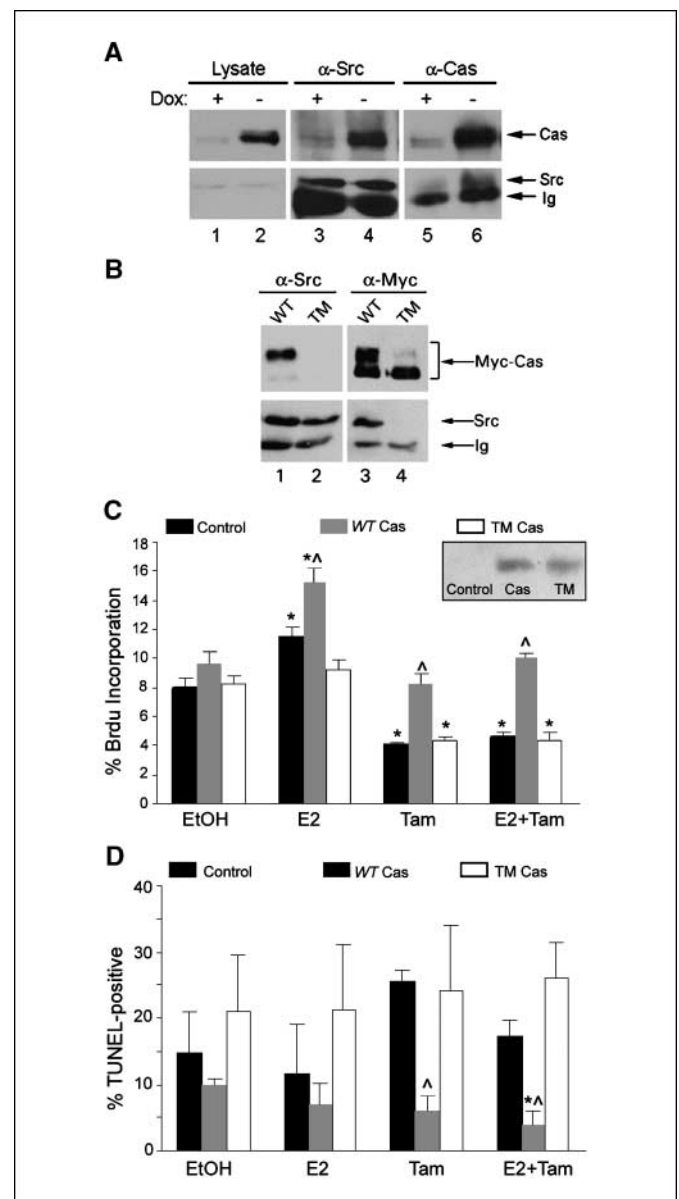
(Fig. 3C, E_2 gray column). However, cells expressing triple mutant Cas showed no such E_2 response (Fig. 3C, E_2 white column). Tamoxifen or E_2 + tamoxifen treatment resulted in decreased BrdUrd incorporation by control cells and cells expressing triple mutant Cas, whereas BrdUrd incorporation in cells overexpressing triple mutant Cas, whereas BrdUrd incorporation in cells overexpressing *WT* Cas was not significantly inhibited. *WT* but not triple mutant Cas overexpression was also found to protect cells from apoptosis in the presence of tamoxifen (Fig. 3D). Most notably, although control cells grown in tamoxifen exhibited an increased level of TUNEL staining in the presence of tamoxifen (Fig. 3D, Tam, black column), indicative of increased apoptosis, overexpression of *WT* Cas significantly inhibited this effect (Fig. 3D, Tam, gray column). In contrast, overexpression of triple mutant Cas was unable to inhibit the level of TUNEL staining in the presence of tamoxifen or E_2 + tamoxifen (Fig. 3D, Tam, white column; Fig. 3D, E_2 + Tam, white column). These data support a role for direct c-Src/Cas interactions in promoting resistance of MCF-7 cells to the growth-inhibitory and proapoptotic effects of tamoxifen.

We and others have shown that c-Src activity is elevated when it is associated with Cas (15, 22). In contrast, Cas constructs lacking the c-Src-binding sites, such as triple mutant Cas, do not promote c-Src activity and substrate phosphorylation (15). The finding that c-Src/Cas interactions were elevated in Cas-overexpressing cells and that these interactions were required for promotion of Cas-induced resistance to the inhibitory effects of tamoxifen on BrdUrd incorporation suggested that phosphorylation of c-Src substrates may be important in this pathway. One particularly attractive candidate c-Src target is tyrosine 845 (Y845) on the EGFR, which is phosphorylated by c-Src and contributes to EGF-coupled, G protein-coupled, and growth hormone-mediated cell proliferation (14, 24–27). To determine whether phosphorylation of Y845 could be elevated under conditions of Cas over-

expression, MCF-7 cells that overexpress *WT* EGFR in response to doxycycline (Tet-on) were transiently transfected with control plasmids (vector) or plasmids encoding either *WT* or triple mutant Cas. As expected, EGFR expression was induced upon doxycycline treatment (Fig. 4A, middle), and this was independent of Cas overexpression (Fig. 4A, bottom). Phosphorylation of Y845 was enhanced in cells overexpressing *WT* Cas (Fig. 4A, top, compare lanes 2 and 4) but to a lesser degree by triple mutant Cas (Fig. 4A, top, lane 6). Because the cells were not exposed to EGF under these conditions, this increase in phosphorylation of Y845 seems to be independent of ligand binding.

EGFR levels in parental MCF-7 cells are low (28), making it difficult to detect Y845 phosphorylation on the endogenous receptor. However, despite the low level of EGF receptors, MCF-7 cells are EGF responsive for proliferation (29). To confirm that EGFR signaling was intact in the Cas-inducible stable MCF-7 cells used in these studies, cells grown in the presence (endogenous Cas) or absence (7-fold Cas overexpression) of doxycycline for 48 hours

Figure 3. Cas-dependent effects on tamoxifen responses require the c-Src-binding sites on Cas. **A**, Cas overexpression drives endogenous c-Src into complex with Cas. Myc-Cas-expressing cells were grown in the presence or absence of 2 μ g/mL doxycycline for 48 hours. c-Src (lanes 3 and 4) or Cas (lanes 5 and 6) immune complexes were generated from 500 μ g cell extract, separated by 8% SDS-PAGE, and immunoblotted with Cas (top) or c-Src (bottom) antibodies. For comparison, 50 μ g cell extract was immunoblotted with the same antibodies (lanes 1 and 2). Exposure time for the Cas immunoblot of Cas immune complexes (top, lanes 5 and 6) was <60-fold the exposure time for the remaining Cas immunoblots (top, lanes 1–4). All exposures for c-Src immunoblots were equivalent. **B**, Cas^{P642A,Y668/670F} does not bind to endogenous c-Src. COS-7 cells were transfected with *WT* Cas-encoding or triple mutant Cas (TM Cas; Cas^{P642A,Y668/670F})-encoding plasmids and lysed 48 hours later. c-Src and Myc immune complexes were isolated and immunoblotted as described in (A). **C**, overexpression of triple mutant Cas does not function like *WT* Cas to promote BrdUrd incorporation in the presence of tamoxifen. MCF-7 cells were transfected with GFP-encoding plasmids, together with vector, *WT* Cas-encoding, or triple mutant Cas-encoding plasmids. Inset, Myc immunoblot of cell extracts. Following transfection, the medium was supplemented with 10% CCS and vehicle (ethanol), 10^{-8} mol/L E_2 , 10^{-6} mol/L 4-hydroxytamoxifen, or E_2 + tamoxifen for 48 hours. BrdUrd was added 3 hours before fixation as described for Fig. 1D. Columns, mean of three to eight independent experiments; bars, SE. *, significant difference from the mean at a $\geq 95\%$ CI relative to vehicle (ethanol)-treated cells expressing the same level of Cas; ^, significant difference from the mean at a $\geq 95\%$ CI relative to cells expressing endogenous levels of Cas (black columns) under the same treatment conditions. **D**, Cas, but not triple mutant Cas, protects cells from apoptosis in the presence of tamoxifen. MCF-7 cells transfected as described above were cultured for 48 hours in phenol red-free DMEM + 10% CCS in the presence of vehicle (ethanol), 10^{-8} mol/L E_2 , 10^{-6} mol/L 4-hydroxytamoxifen, or E_2 + tamoxifen and processed to detect TUNEL-positive cells as described in Materials and Methods. Columns, mean of three independent experiments; bars, SE. *, significant difference from the mean at a $\geq 95\%$ CI relative to vehicle (ethanol)-treated cells expressing the same level of Cas; ^, significant difference from the mean at a $\geq 95\%$ CI relative to cells expressing endogenous levels of Cas (black columns) under the same treatment conditions.



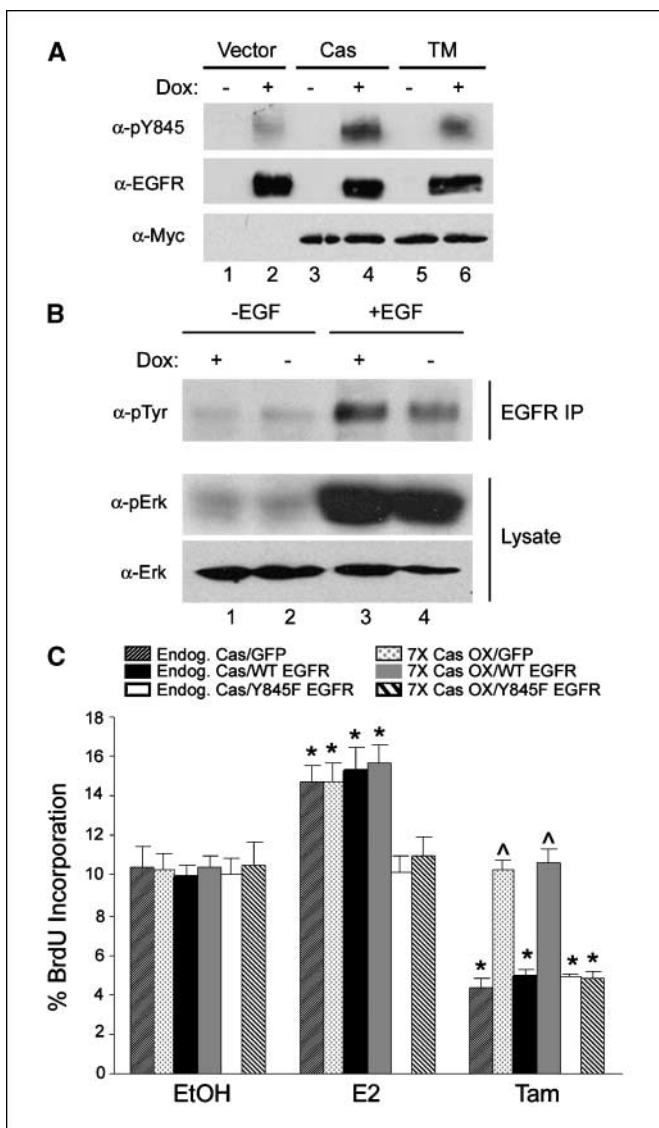


Figure 4. Functions emanating from Y845 on the EGFR play a role in Cas-mediated inhibition of tamoxifen effects. **A**, overexpression of Cas induces ligand-independent phosphorylation of Y845. MCF-7 cells expressing tetracycline-inducible WT EGFR were transfected with vector (*lanes 1 and 2*), Myc-Cas-encoding (*lanes 3 and 4*), or triple mutant Cas-encoding (*lanes 5 and 6*) plasmids and cultured for 48 hours in the presence or absence of 2 $\mu\text{g}/\text{mL}$ doxycycline to regulate EGFR expression. Total cell extract (50 μg) was separated by 8% SDS-PAGE and immunoblotted with antibodies recognizing the phosphorylated form of Y845 (*top*), EGFR (*middle*), or Myc (*bottom*). **B**, EGFR signaling is intact in Cas-expressing MCF-7 cells. Cas-overexpressing cells were cultured in the presence (endogenous Cas) or absence (7 \times -overexpressed Cas) of 2 $\mu\text{g}/\text{mL}$ doxycycline for 48 hours and stimulated with 100 ng/mL EGF for 15 minutes. EGFR immune complexes were generated from 500 μg cell extract, separated by 8% SDS-PAGE, and immunoblotted with pTyr (*top*) antibodies. Cell extract (50 μg) was immunoblotted with phosphorylated ERK and total ERK antibodies (*middle and bottom*). **C**, Cas-overexpressing cells were transfected with plasmids encoding GFP plus vector (*columns 1 and 2 in each set*), WT EGFR (*columns 3 and 4 in each set*), or Y845F-mutant EGFR (*columns 5 and 6 in each set*) and cultured in the presence (endogenous Cas) or absence (7 \times -overexpressed Cas) of 2 $\mu\text{g}/\text{mL}$ doxycycline in phenol red-free DMEM + 0.1% BSA. Twenty-four hours after transfection, the medium was supplemented with 100 $\mu\text{mol}/\text{L}$ BrdUrd and vehicle (ethanol), 10^{-8} mol/L E_2 , or 10^{-6} mol/L 4-hydroxytamoxifen for 8 hours. *Columns*, mean of five independent experiments; *bars*, SE. *, significant difference from the mean at a $\geq 95\%$ CI relative to vehicle (ethanol)-treated cells expressing the same level of Cas; \wedge , significant difference from the mean at a $\geq 95\%$ CI relative to cells expressing endogenous levels of Cas (*black and white columns*) under the same treatment conditions.

and stimulated with 100 ng/mL EGF for 15 minutes showed a marked EGF-dependent increase in EGFR phosphorylation (Fig. 4B, *top*) and ERK activation (Fig. 4B, *middle*). Cas expression levels did not seem to alter these EGF-dependent responses (Fig. 4B, compare *lanes 3 and 4*).

To test whether signals emanating from Y845 on the EGFR contributed to Cas-dependent resistance to the antiproliferative effects of tamoxifen, the tetracycline-regulated Myc-Cas MCF-7 cells were transiently transfected with plasmids encoding green fluorescent protein (GFP) and either, WT EGFR, or Y845F EGFR under conditions of maximal (–doxycycline; 7-fold overexpression of Cas) or endogenous (+doxycycline) levels of Cas. BrdUrd incorporation was then measured in transfected cells cultured in phenol red-free DMEM supplemented with 0.1% bovine serum albumin (BSA) and vehicle (ethanol), 10^{-8} mol/L E_2 , or 10^{-6} mol/L 4-hydroxytamoxifen as described above. As is the case for cells expressing endogenous EGFR (Fig. 4C, *columns 1 and 2 in each set*), cells overexpressing WT EGFR (Fig. 4C, *columns 3 and 4 in each set*) exhibited elevated BrdUrd incorporation in the presence of E_2 , irrespective of the level of Cas expression (Fig. 4C, E_2). BrdUrd incorporation was reduced following tamoxifen treatment unless Cas was overexpressed (Fig. 4C, *Tam, columns 1 and 2*). In contrast, expression of Y845F EGFR (Fig. 4C, *columns 5 and 6 in each set*) inhibited E_2 -dependent BrdUrd incorporation irrespective of Cas expression levels (Fig. 4C, E_2) and blocked the rescue of tamoxifen-mediated inhibition of BrdUrd incorporation by Cas overexpression (Fig. 4C, *Tam*). These data support a role for phosphorylated Y845 in promoting Cas-dependent resistance of MCF-7 cells to the growth-inhibitory activities of tamoxifen.

One of the molecules that has been shown to function downstream of Y845 in proliferative signaling pathways is STAT5b (14). To determine whether STAT5b phosphorylation was elevated in the presence of overexpressed Cas, parental MCF-7 cells were cotransfected with a plasmid encoding HA-tagged STAT5b together with either control (vector) or Myc-Cas-encoding plasmids. HA-STAT5b was then immunoprecipitated from cell lysates and immunoblotted with antibodies recognizing pTyr or HA. Under these conditions, Cas overexpression alone was sufficient to increase STAT5b phosphorylation (Fig. 5A, *top*). This also correlated with a 3.5-fold increase in STAT5 activity as measured by transcription from a reporter plasmid encoding luciferase under the control of a promoter containing six tandem repeats of a STAT5-responsive, GAS-like element (Fig. 5B; ref. 17). To determine whether STAT5b activities were required to promote resistance to the inhibitory effects of tamoxifen, either WT or COOH-terminally truncated dominant-negative STAT5b was expressed in the tetracycline-regulated Cas-overexpressing MCF-7 cells. BrdUrd incorporation was then measured in transfected cells grown in the presence or absence of doxycycline in phenol red-free DMEM supplemented with 0.1% BSA and vehicle (ethanol), 10^{-8} mol/L E_2 , or 10^{-6} mol/L 4-hydroxytamoxifen. As in previous experiments, E_2 treatment increased BrdUrd incorporation regardless of the level of Cas expression in WT STAT5b-overexpressing cells, and BrdUrd incorporation was decreased in tamoxifen-treated cells unless Cas was overexpressed (Fig. 5C; *Tam, black and gray columns*). In contrast, dominant-negative STAT5b inhibited E_2 -dependent BrdUrd incorporation and also prevented Cas overexpression from overcoming the inhibitory effects of tamoxifen (Fig. 5C; *Tam, white and hatched columns*). These data indicate that STAT5b is required for E_2 -dependent increases in BrdUrd incorporation and that it functions downstream of Cas to promote tamoxifen resistance.

Discussion

The aim of this study was to elucidate the mechanism(s) by which Cas overexpression confers resistance to the antiestrogen tamoxifen in MCF-7 breast cancer cells. Cas overexpression was shown to alleviate both tamoxifen-mediated growth inhibition and induction of apoptosis. Enhancement of cell proliferation/survival in the presence of tamoxifen through Cas overexpression occurred in the absence of detectable effects on ER- α expression or ERE-dependent transcriptional activity. This agrees with a study by Brinkman et al., who showed that mRNA levels of the estrogen-responsive gene *pS2* were down-regulated by tamoxifen in both parental and stable Cas-overexpressing ZR-75-1 breast cancer cell lines (9). Thus, in contrast to antiestrogen resistance induced by overexpression of the amplified in breast cancer 1 nuclear receptor coactivator (reviewed in ref. 30), Cas-dependent tamoxifen resistance is not the result of enhanced responses to the partial agonist activity of tamoxifen or a switch to an ER-negative phenotype. Interestingly, we observed a slight increase in ER- β protein expression (Fig. 1C), coincident with an attenuated induction of cyclin D1 mRNA by E₂ (Fig. 2B), when Cas was overexpressed in the Tet-inducible MCF-7 cell line. This could be due to a decreased ER- α to ER- β ratio, which has been shown to negatively regulate cyclin D1 expression (20). Although others have shown that ER- β stimulates cyclin D1 expression in the presence of antiestrogens (21), we do not believe that the increased ER- β levels are driving tamoxifen resistance in our cell model because Cas overexpression did not affect cyclin D1 mRNA or protein levels when tamoxifen was present (see Fig. 2B and C).

Rather than directly affecting ER transcriptional functions, our data indicate that Cas promotes tamoxifen resistance by deregulation of alternative cell proliferation pathways, particularly those mediated through c-Src PTK activity arising from Cas/c-Src protein complexes. In support of this model, overexpression of Cas was found to drive endogenous c-Src into complex with Cas, a process that has been shown previously to cause up-regulation of c-Src tyrosine kinase activity (15, 31). Expression of a Cas molecule deficient in c-Src binding was unable to alleviate tamoxifen-mediated growth inhibition. In fact, expression of triple mutant Cas also seemed to function as a dominant inhibitor of E₂-dependent BrdUrd incorporation (see Fig. 3C, E₂, white column), suggesting that signals emanating from Cas/c-Src complexes may be important for E₂-dependent proliferation. MCF-7 cells overexpressing Cas exhibited increased phosphorylation of two c-Src substrates, Y845 of the EGFR and STAT5b. Importantly, Cas-dependent protection from the antiproliferative effects of tamoxifen was reversed in the presence of mutated forms of these substrates that inhibit endogenous protein function (Y845F EGFR or dominant-negative STAT5b), confirming that these molecules play an important role in tamoxifen resistance induced by Cas overexpression.

Based on these findings, we propose a model whereby increased interactions between c-Src and Cas result in elevated Src PTK activity and phosphorylation of several important substrates that lead to proliferation and/or survival in the presence of tamoxifen ("tamoxifen resistance"; Fig. 6). This model suggests that tamoxifen resistance can be regulated, at least in part, by the degree with which Cas and c-Src are associated. Cas/c-Src interactions are controlled to a large extent by the expression levels of the component molecules (15). In this study, we have overexpressed Cas both stably and transiently in MCF-7 cells. Stable Tet-off MCF-7 cells overexpress Cas ~7-fold above endogenous levels (Fig. 1),

whereas transient transfection of Cas typically results in an ~20-fold overexpression (data not shown). The growth-inhibitory effects of tamoxifen were equivalently blocked under conditions of stable and transient Cas overexpression (Figs. 1D and 3C), indicating that

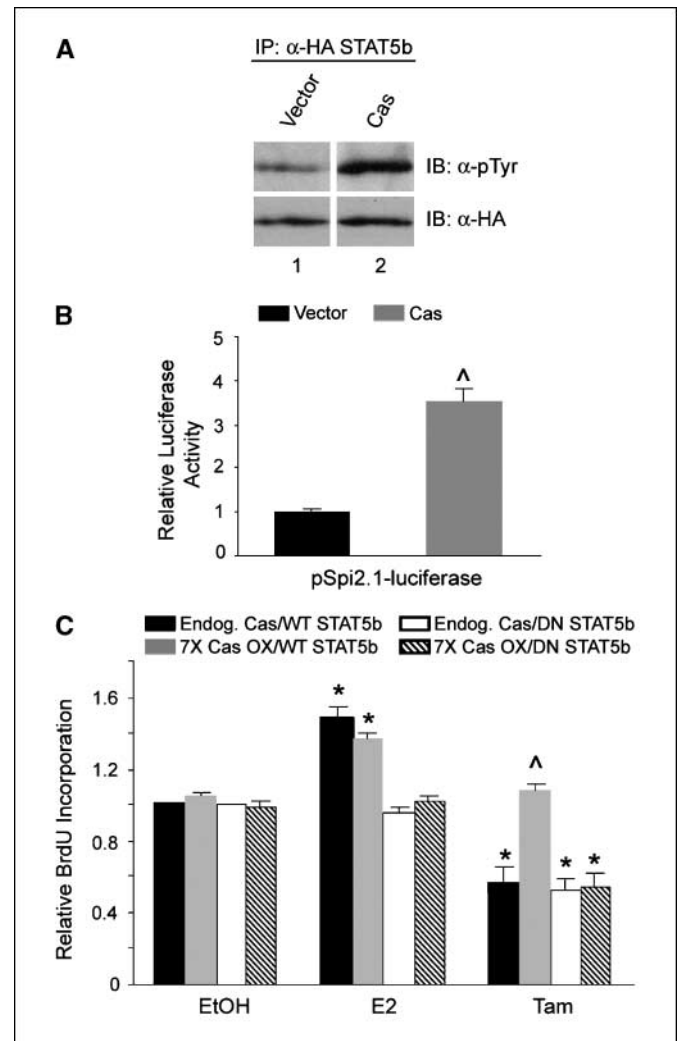


Figure 5. STAT5b functions are required for Cas-dependent effects on tamoxifen resistance. *A*, overexpression of Cas induces phosphorylation of STAT5b. MCF-7 cells were transfected with plasmids encoding HA-tagged STAT5b together with control (vector) or Myc-Cas-encoding plasmids. Forty-eight hours after transfection, cells were lysed, and HA immune complexes were generated from 1 mg cell extract. Immune complexes were separated by 8% SDS-PAGE and immunoblotted with antibodies recognizing pTyr (top) or HA (bottom). Images shown are the same exposure times. *B*, overexpression of Cas induces STAT5b transcriptional activity. MCF-7 cells grown in normal growth medium were transiently transfected in triplicate with vector (black column) or a plasmid encoding Myc-Cas (gray column) along with the luciferase reporter plasmids pSpi2.1-Luc (17) and pRL-SV40 (Renilla). Cells were processed for luciferase assays 48 hours later. Columns, mean of three independent experiments; bars, SE. ^, significant difference from the mean relative to vector-transfected cells (black column); expressing endogenous levels of Cas) at a $\geq 95\%$ CI. *C*, Cas-overexpressing cells were transfected with plasmids encoding either WT (black and gray columns) or COOH-terminally truncated STAT5b (dominant-negative STAT5b; white and hatched columns) and then cultured in the presence (black and white columns) or absence (gray and hatched columns) of 2 $\mu\text{g}/\text{mL}$ doxycycline in phenol red-free DMEM + 0.1% BSA. Twenty-four hours after transfection, the medium was supplemented with 100 $\mu\text{mol}/\text{L}$ BrdUrd and vehicle (ethanol), 10^{-8} mol/L E₂, or 10^{-5} mol/L 4-hydroxytamoxifen for 8 hours. *, significant difference from the mean at a $\geq 95\%$ CI relative to vehicle (ethanol)-treated cells expressing the same level of Cas; ^, significant difference from the mean at a $\geq 95\%$ CI relative to cells expressing endogenous levels of Cas (black and white columns) under the same treatment conditions.

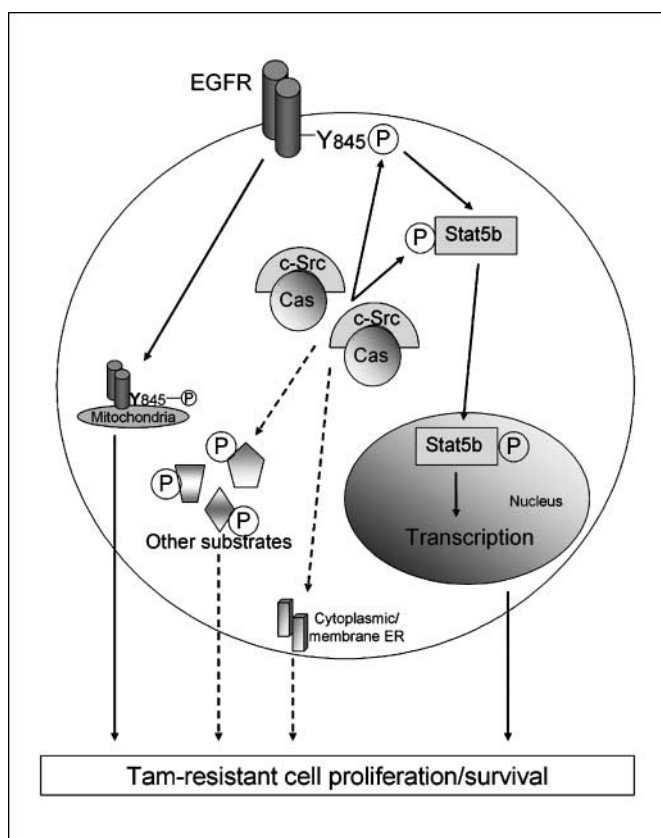


Figure 6. Model explaining how overexpression of Cas confers resistance of MCF-7 cells to the growth- and survival-inhibitory effects of tamoxifen.

7-fold overexpression is sufficient to maximally induce “tamoxifen resistance” as measured in these assays.

Because Cas/c-Src interactions can be regulated by the expression level of these proteins, it is important to note that c-Src mRNA and protein are elevated in as many as 70% of breast tumors compared with normal breast tissue, and this correlates with increased tyrosine kinase activity despite the fact that the c-Src in these tumors is not mutationally activated (28, 32–34). Cas has also been reported to be elevated to varying levels in a large percentage of breast tumors (67%; ref. 10). High Cas expression is associated with decreased overall and relapse-free survival as well as a poorer response to tamoxifen in the context of intrinsic/*de novo*, but not acquired, endocrine resistance (10, 35–37). Cas/c-Src complexes can be readily isolated from several breast cancer cell lines, including MCF-7 cells (Fig. 3; data not shown). Although tamoxifen-sensitive tumor cell lines, such as MCF-7 cells, require additional Cas (and by inference, Cas/c-Src complexes) to promote tamoxifen resistance, we postulate that, in at least a subset of human tumors that are intrinsically resistant to tamoxifen and express high levels of both c-Src and Cas, Cas/c-Src interactions may be sufficient to drive continued proliferation in the presence of tamoxifen.

Two c-Src substrates, Y845 on the EGFR and STAT5b, have been shown in this work to play a key role in Cas-mediated tamoxifen resistance. Phosphorylation of both substrates is elevated in the presence of overexpressed Cas, and expression of mutants that have been shown to dominantly inhibit the function of their endogenous counterparts impairs Cas-dependent protection from the growth-inhibitory effects of tamoxifen (Figs. 4 and 5). EGFR and

the related human EGFR 2 (HER-2) have both been implicated previously in tamoxifen resistance (reviewed in refs. 6, 7). Moreover, the EGFR-specific inhibitor gefitinib (Iressa, ZD1839) and the dual EGFR/HER-2 inhibitor lapatinib (GW572016) have been shown to enhance tamoxifen-mediated growth suppression and partially restore growth inhibition by tamoxifen to antiestrogen-resistant breast cancer cells, respectively (6, 38). This suggests that, at least in some instances, the catalytic activity of these molecules may be important for mediating tamoxifen resistance.

There are indications that noncatalytic activities of the EGFR may also contribute to cell proliferation and survival. Several recent studies suggest that signals stemming from phosphorylation of Y845 may be good candidates for these alternative pathways. First, phosphorylation of Y845 is not dependent on EGFR kinase activity, and it also does not affect the catalytic activity of the receptor or downstream activation of mitogen-activated protein kinase (MAPK; ref. 25). Second, expression of Y845F EGFR inhibits serum-mediated, EGF-mediated, and G protein-coupled receptor-mediated DNA synthesis and abrogates the protective effect of EGF on Adriamycin-induced apoptosis (24, 25, 27). Third, the EGFR has been shown to translocate to mitochondria following EGF stimulation, where phosphorylation of Y845 regulates its association with the mitochondrial protein cytochrome *c* oxidase II (Cox II; ref. 27). One or more of these noncatalytic functions of Y845 phosphorylation may play an important role in Cas-mediated c-Src activation and protection from the antiproliferative and proapoptotic effects of tamoxifen.

Recently, Dorssers et al. examined the gene expression profiles of ZR-75-1 breast cancer cells constitutively expressing high levels of Cas, and their data indicate that many of the genes commonly regulated by E₂ or EGF stimulation are not altered by Cas overexpression (39). Our data showing that ERE-dependent transcription is unaffected by Cas overexpression in MCF-7 cells (Fig. 2) support this conclusion for estrogen-regulated genes. With respect to EGF stimulation, data presented in this report indicate that the Cas/c-Src/EGFR(Y845)/STAT5b pathway may exploit functions of the EGF receptor that are independent of ligand activation and distinct from conventional EGF-stimulated proliferation pathways. For example, phosphorylation of Y845 in Cas-overexpressing cells is not dependent on the presence of EGF (Fig. 4A). This may help to explain why Cas overexpression and EGF stimulation may not coordinately regulate the same gene sets.

STAT5b, like Y845 on the EGFR, contributes to the synergistic collaboration between c-Src and EGFR in mediating cell proliferation (14). STAT5b becomes tyrosine phosphorylated in response to EGF in cells overexpressing EGFR, and this correlates with enhanced DNA-binding and STAT5b transcriptional activation. Furthermore, EGF-dependent phosphorylation of STAT5b requires both c-Src PTK activity and an intact Y845 on the EGFR. STAT5b can function in EGF-dependent proliferative pathways as shown by the fact that dominant-negative STAT5b abrogates EGF-induced DNA synthesis (40). Other recent findings suggest that the function of STAT5 in endocrine therapy responses may be quite broad. We have observed that transcriptional activation of β -casein, a STAT5 target, is ~10-fold elevated in ER-positive MCF-7/LCC9 breast cancer cells (R.B.R.; data not shown), which have acquired resistance to the steroidal antiestrogen Faslodex and cross-resistance to tamoxifen (41). Other groups have suggested that STAT5 is also a target of cytoplasmic (nongenomic) functions of the ER, which may include activation of c-Src, phosphatidylinositol 3-kinase, and/or MAPK (42). Our future efforts will be focused on

identifying additional components of the Cas/c-Src/EGFR/STAT5 signaling axis that can be exploited for improving sensitivity to tamoxifen and other antiestrogens in the laboratory and clinical setting.

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