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TITLE: Role of the Neddylation Enzyme Uba3, A New Estrogen
Receptor Corepressor, in Breast Cancer

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13. ABSTRACT (Maximum 200 Words) Estrogens play important roles in both the onset and malignant progression of breast cancer. The content of estrogen receptors in breast tumors is a valuable predictor of whether a patient will respond to therapy with antiestrogens, such as tamoxifen and fulvestrant (ICI 182,780). Expression and activity of ER can be lost or impaired in antiestrogen-resistant breast cancer. The proposed studies are designed to test the overall hypothesis that the ubiquitin-like NEDD8 protein modification pathway represses estrogen action by facilitating degradation of ER protein. Perturbation of this pathway may prove instrumental in breast tumor progression; alternatively, activation of this pathway may prove to be a valid target for novel therapeutics. This study on mechanisms that regulate ER levels and activity are highly relevant to the development and progression breast cancer, including tumor progression to states of hormone independence and antiestrogen resistance. Thus, understanding how the estrogen receptor is regulated is an area of research critical to understanding the tissue selective pharmacology of estrogens. In addition, tamoxifen and other selective estrogen receptor modulators target the estrogen receptor, and this study is of the utmost relevance to those important therapies.				
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INTRODUCTION

Estrogen regulates diverse biological processes through estrogen receptors (ER α and ER β) (1). Receptor levels and dynamics have a profound influence on target tissue responsiveness and sensitivity to estrogen, and receptor turnover rates provide estrogen target cells with the capacity for rapid regulation of receptor levels and thus dynamic hormone responses (2-5). Furthermore, several experimental results have recently demonstrated that receptor degradation is a key component of the response of cancer cells, including breast cancer cells, to antiestrogen therapy (6, 7). In advanced stage breast cancers, estrogen receptor expression and activity can be lost or impaired, and the tumors are often resistant to endocrine therapies, such as the steroidal antiestrogens, ICI 182,780 and ICI 164,384 (6, 7). Our findings during the funding period have raised the intriguing possibility for a role of ubiquitin and ubiquitin-like pathways, including the NEDD8 pathway, in ER α ubiquitination and degradation and suggest that disruptions in such pathways may contribute to the development of antiestrogen-resistance in human breast cancer. The overall hypothesis that ubiquitin protein modification pathways repress estrogen action by facilitating degradation of ER protein was tested. Our experimental results suggest that perturbation of this pathway may prove instrumental in breast tumor progression; alternatively, activation of ubiquitin protein modification pathways may prove to be valid targets for novel therapeutics.

BODY

Task 1 was to determine the effect of Uba3 on breast cancer cell proliferation. We attempted to generate breast cancer cell lines stably expressing the dominant negative Uba3 (C216S), a mutant that we had used previously to block the NEDD8 pathway (8). However, blocking this pathway in MCF7 breast cancer cells was lethal and the cells died. We then attempted to an inducible promoter to control C216S expression levels, but these efforts were similarly unsuccessful. We conclude that the NEDD8 is essential for cell survival.

To further address this task, we generated a breast cancer cell line stably expressing a dominant negative Ubc12. The results of this investigation are described in Fan et al. (8 and manuscript in appendix), and some of the key findings are highlighted here. We established the stable cell line MCF7/ Ubc12C111S, which contains an impaired NEDD8 pathway and examined the effect of the antiestrogen on ER α degradation in these cells. Expression of Ubc12C111S inhibited ICI 182,780-induced ER α down-regulation (Fig. 5A). We then examined the growth inhibitory effect of ICI 182,780 in MCF7/C111S cells. No significant difference was observed in basal cell proliferation rates between MCF7/C111S and MCF7/Vec cells in hormone-free medium (data not shown). Treatment with the antiestrogen inhibited basal cell growth of MCF7 and MCF7/Vec cells (Fig. 6A). In contrast, MCF7/C111S cells were partially resistant to ICI 182,780 (Fig. 6A, Left panel). Dose-response analysis showed that MCF7/C111S cells were resistant to a broad range of ICI 182,780 concentrations (Fig. 6A, Right panel). On the other hand, estradiol-induced proliferation of MCF7/C111S and control cells was similar (2-fold increase in cell number over a 6-day treatment period; data not shown). The effect of 4-OHT on MCF7/C111S and MCF7/Vec cell proliferation was examined in a time- and dose-response analysis. The response of the cell lines to 4-OHT was similar (Fig. 6B), suggesting that Ubc12C111S expression did not confer cells resistance to growth inhibitory effect of antiestrogens in general. These results suggest that the expression of Ubc12C111S conferred resistance of MCF7 cells to the growth inhibitory effects of ICI 182,780, but disrupting the NEDD8 pathway had no effect on the mitogenic response of MCF7 breast cancer cells to estradiol or the growth inhibitory effects of 4-OHT. Task one has been completed.

The second task of the project was to determine the molecular mechanisms of ER α corepression by the NEDD8 pathway. Toward this goal, we constructed Uba3 deletion constructs lacking one or both of the presumptive nuclear receptor interacting motifs (the NR boxes). GST-pulldown assays were conducted to determine which receptor domains mediate the interactions between ER α with Uba3. We were unable to detect direct interaction of the deletion mutant constructs with estrogen receptor (data not shown), suggesting that the NR boxes are essential for Uba3-ER interaction. However, this could also be due to important changes in protein conformation due to the removal of amino acid sequences. Thus, we took an alternative approach and generated point mutations within the NR boxes and then proposed to examine direct interactions of the mutant proteins with ER. Constructs were made and sequenced. However, we were unable to express proteins from the new constructs, for reasons that are unclear at this time. We speculate that perhaps the mutations made the protein unstable. Nonetheless, although mostly negative results were obtained, Task 2 has been completed.

Task 3 was to determine if ER α and ER β function is modified by APP-BP1 and Ubc12 and an NEDD8 target protein. First, we took a direct approach and determined if ER α is an NEDD8 target protein using co-transfection experiments, co-immunoprecipitation assays and Western blot analysis and looked for NEDD8-ER conjugates. We included various other components of the NEDD8 pathway, including co-transfecting Uba3, APPBP1, Ubc12 and various Cullin family members. We were unable to detect neddylated receptor (data not shown); therefore, we concluded that ER is not a direct substrate for modification by NEDD8. Next, we tested the hypothesis that the neddylation pathway may act to restrict

ER α activity by indirectly modulating receptor degradation. The results of this investigation are described in the manuscript in the appendix (8), and some of the key findings are highlighted below.

Coexpression of Uba3 decreased ER α protein level (Fig. 1A), and treatment with MG132, a specific proteasome inhibitor, blocked Uba3-stimulated down-regulation of ER α (Fig. 1B), confirming that the Uba3-induced ER α degradation is through the 26S proteasome. Overexpression of APP-BP1 or Ubc12 had no significant effect on ER α protein levels (data not shown), a result consistent with our previous observation that Uba3 is the limiting factor in neddylation-associated inhibition of ER α transcriptional activity (25). Next, to test the hypothesis that the neddylation pathway is required for ligand-mediated degradation of ER α , we used the dominant negative mutant of Ubc12, Ubc12C111S. Treatment of ER α transfected HeLa cells with estradiol resulted in a time-dependent decrease in ER α protein levels (Fig. 2A). In contrast, the effects of estradiol on receptor levels were less dramatic in cells expressing Ubc12C111S (Fig. 2A). Consistent with this observation, Uba3C216S, a dominant negative mutant of Uba3, also inhibited estradiol-induced ER α down regulation (Fig. 2B). Addition of the proteasome inhibitor MG132 prior to estradiol treatment completely abolished ligand-induced down-regulation of ER α (Fig. 2B), confirming that ER α undergoes proteasome-dependent degradation in response to estradiol. Collectively, these results demonstrate that a functional NEDD8 pathway is required for efficient, ligand-induced, proteasome-mediated degradation of ER α . Having established a role for the NEDD8 pathway in ER α down-regulation, we examined the effect of NEDD8 on receptor ubiquitination. Expression of dominant negative Ubc12C111S or Uba3C216S markedly decreased ER α ubiquitination in either the absence (Fig. 3, left panel) or presence of estradiol and MG132 (Fig. 3, right panel), compared to cells transfected with control vector or wild type Ubc12 or Uba3. These results suggest that a functional neddylation pathway is required for the efficient ubiquitination of ER α .

Having completed task 3, we continued to perform further investigations into the roles of ubiquitin-like pathway NEDD8 in the responses to estradiol and antiestrogens (deemed a logical extension of the SOW and within the scope of the fundamental questions underlying the SOW). Thus, the role of the ubiquitin-proteasome pathway in ER α -mediated transcriptional responses in breast cancer cells was investigated. Genetic and pharmacologic approaches were utilized to disrupt ER α ubiquitination, proteasome-mediated proteolysis and thus ER α degradation, including a dominant negative mutant of the NEDD8 conjugation enzyme (Ubc12C111S), the 20S proteasome inhibitor MG132, a ubiquitin mutant with all of its lysines mutated to arginine (UbK0), and the partial agonist/antagonist tamoxifen. To determine the effect of blocking ER α degradation on estradiol-induced transcriptional responses, estrogen receptor-responsive reporter assays and expression of endogenous ER-target genes in MCF7 human breast cancer cells were utilized. The results of this study are described in *Fan et al.* (9; manuscript in appendix), and some of the key findings are highlighted below.

We show that proteasomal degradation is not essential for transcriptional activity of ER α and suggest that the ubiquitin-proteasome system functions to limit estradiol-induced transcriptional output. The results demonstrate that blocking polyubiquitination of ER α stabilizes the receptor, resulting in the prolonged expression of ER α -responsive genes (Fig. 1B,C). Inhibiting the proteasome enhanced ER α transcriptional activity in MCF7 human breast cancer cells (Fig. 5A,B), indicating that ER α degradation plays a key role in limiting estradiol-induced transcriptional responses in these cells. The results further suggest that in cells containing low levels of ER α , proteasome-mediated receptor degradation plays a role in limiting estradiol-induced transcriptional responsiveness (Figure 1B). While blocking ER α degradation increased the magnitude of estradiol-induced gene transcription, no effect on hormone sensitivity was observed (Fig. 2). However, inhibiting the proteasome increased both the magnitude and duration of estradiol-induced expression of an ER α -target gene in breast cancer cells (Fig. 5A). Overall, the data support the hypothesis that proteasome-mediated degradation of ER α serves as a means to limit the duration of estradiol signaling in receptor positive breast cancer cells. The important implication of this study is that the estradiol-induced transcriptional response is limited by receptor degradation through the ubiquitin-proteasome system, and defects in proteasome-mediated degradation of ER α could lead to an enhanced cellular response to estradiol in breast cancer cells.

Abnormal expression of ER α has long been associated with both the initiation and progression of breast cancer (10). An increase in the number of ER α -positive cells, as well as increased individual cell ER α content, have frequently been detected in malignant breast tumors (11). Furthermore, increased ER α content has been shown to augment the magnitude of estrogen-stimulated gene expression, providing a growth advantage to breast cancer cells (2, 8, 9, 12). Collectively, these observations indicate that alterations in ER α degradation pathways may contribute to deregulation of ER α , perhaps leading to enhanced estrogen action in breast tumors.

We (described above) and others have clearly shown degradation of unliganded ER α is mediated by the ubiquitin-proteasome pathway, regulation of this pathway, at the molecular level, remains unclear. One potential mechanism involves CHIP, the carboxyl terminus of Hsc70-interacting protein, previously shown to target Hsp90 interacting proteins for ubiquitination and proteasomal degradation. We investigated a role for CHIP in degradation of unliganded ER α (13;

manuscript attached). In HeLa cells transfected with ER α and CHIP, ER α is downregulated through a ubiquitination dependent pathway, while ER α -mediated gene transcription decreased (Fig. 1 and Fig 2A). In contrast, siRNA inhibition of CHIP expression resulted in increased ER α accumulation and reporter gene transactivation (Fig 1B and Fig 2B). Transfection of mutant CHIP constructs demonstrated that both the U-box (containing ubiquitin ligase activity) and the tetratricopeptide repeat (TPR, essential for chaperone binding) CHIP domains are required for CHIP-mediated ER α downregulation (Fig 3). In addition, coimmunoprecipitation assays demonstrated that ER α and CHIP associate through the CHIP TPR domain (Fig 3). In ER α -positive breast cancer MCF7 cells, CHIP overexpression resulted in decreased levels of endogenous ER α protein and attenuation of ER α -mediated gene expression (Fig 4 and Fig 5). Furthermore, ER α -CHIP interaction was induced by the Hsp90 inhibitor geldanamycin (GA), resulting in enhanced ER α degradation; this GA effect was further enhanced by CHIP overexpression, but was abolished by CHIP-siRNA (Fig 6-7). Finally, ER α dissociation from CHIP by various ER α ligands, including estradiol, tamoxifen, and ICI 182,780 interrupted CHIP-mediated ER α degradation (Fig 8). These results demonstrate a role for CHIP in both basal and GA-induced ER α degradation. Furthermore, based on our observations that CHIP promotes ER α degradation and attenuates receptor-mediated gene transcription, we suggest that CHIP, by modulating ER α stability, contributes to the regulation of functional receptor levels, and thus hormone responsiveness, in estrogen target cells. Thus, based on our results, the chaperone/CHIP pathway, by regulating ER α levels, likely contributes to the development/progression of breast cancer. We believe that such a possible role for CHIP in breast cancer merits further examination.

In addition, during the funding period, we contributed to collaborative projects on the regulation of ER target genes in breast cancer, resulting in a co-authored publication (14; manuscript in appendix).

KEY RESEARCH ACCOMPLISHMENTS

- Showed that the neddylation pathway is required for ligand-mediated degradation of ER α
- Discovered that the NEDD8 pathway is required for efficient ubiquitination of ER α
- Established that disrupting the NEDD8 pathway confers antiestrogen resistance in breast cancer cells
- Provided evidence that allowed us to speculate that disruptions in the NEDD8 pathway may provide a mechanism by which breast cancer cells acquire ICI 182,780 resistance while retaining expression of ER α .
- Showed that ER α degradation plays a key role in limiting estradiol-induced transcriptional responses in MCF7 human breast cancer cells.
- Demonstrated that inhibiting the proteasome increased estradiol-induced expression of an ER α -target gene in breast cancer cells.
- Determined that proteasomal degradation is not essential for transcriptional activity of ER α and that the ubiquitin-proteasome system appears to function to limit estradiol-induced transcriptional output.
- Provided evidence to suggest that defects in proteasome-mediated degradation of ER α could lead to an enhanced cellular response to estradiol in breast cancer cells.
- Demonstrated that CHIP promotes ER α degradation and attenuates receptor-mediated gene transcription.
- Provided evidence to suggest that CHIP, by modulating ER α stability, contributes to the regulation of functional receptor levels, and thus hormone responsiveness, in estrogen target cells.
- Provided evidence to suggest the chaperone/CHIP pathway, by regulating ER α levels, may contribute to the development/progression of breast cancer.

REPORTABLE OUTCOMES

Manuscripts

1. *Fan M, Bigsby RM, **Nephew KP** 2003 The NEDD8 pathway is required for proteasome mediated degradation of human estrogen receptor- α and essential for the antiproliferation activity of ICI 182,780 in ER-positive breast cancer cells *Mol Endocrinol* 17:356-365 (cover article)
2. *Fan M, Nakshatri H, **Nephew KP**. 2004. Inhibiting proteasomal proteolysis sustains estrogen receptor-alpha activation. *Mol Endocrinol* 18:2603-2615
3. *Fan M, Park A, **Nephew KP**. Interactions between estrogen receptor and the COOH terminus of the Hsp70-interacting protein (CHIP) (*Mol Endocrinol* under revision)
4. *Leu YW, Yan PS, Fan W, Jin VX, Liu CJ, Curran EM, Welshons WV, Wei HS, Davuluri RV, Plass C, **Nephew KP**, Huang TH-M. 2004. Loss of estrogen signaling triggers epigenetic silencing of downstream targets *Cancer Res* 64:8184-8192 (cover article).

*This DOD award is acknowledged in these publications.

Présentations

1. Fan M, Long X, Bailey JA, Reed CA, Gize EA, Osborne E, Kirk EA, Bigsby RM, **Nephew KP** The activating enzyme of NEDD8 inhibits steroid receptor function. Keystone Symposium on Nuclear Receptor Superfamily, April, 2002
2. Fan M, Bigsby RM, **Nephew KP** 2002 Role for the neddylation pathway in estrogen receptor ubiquitination and degradation. 84th Annual Meeting of the Endocrine Society, June 19-22, San Francisco, CA (platform talk)
3. Fan M, Bigsby RM, **Nephew KP** 2002 Role for the neddylation pathway in estrogen receptor ubiquitination and degradation. Midwest Regional Molecular Endocrinology Conference, Indiana University, Bloomington, IN (platform talk)
4. Fan M, Nakshatri H, **Nephew KP** The role of proteasome-mediated estrogen receptor- α (ER) degradation in estrogen responsiveness 94th annual meeting of the American Association for Cancer Research, Toronto, Ontario, Canada (poster/discussion).
5. Fan M, Nakshatri H, **Nephew KP** 2003 The role of proteasome-mediated degradation of estrogen receptor- α in estrogen-induced transcriptional response. Elwood Jensen Symposium on Nuclear Receptors and Endocrine Disorders. University of Cincinnati, December 5-7 (platform talk).
6. Fan M, Park A, **Nephew KP** 2005 CHIP (Carboxyl Terminus of Hsc70-Interacting Protein) promotes basal and geldanamycin-induced degradation of estrogen receptor- α . 87th annual Meeting of The Endocrine Society, San Diego, CA (platform talk).

CONCLUSIONS

The antiestrogen ICI 182,780 is a drug is used as a second-line endocrine agent in patients who have developed tamoxifen-resistant breast cancer. Despite its potent antitumor effects, the drug does not circumvent the development of antiestrogen resistance (15-17). Moreover, the fact that most tumors acquiring ICI 182,780 resistance do so while retaining expression of ER α and estrogen responsiveness (18-20), suggests that administration of the antiestrogen may possibly lead to the selection of tumor cells defective in ER α down-regulation pathway(s), which in turn may confer a proliferative advantage in either the presence or absence of estrogens. In this context, mechanism underlying persistent expression of ER α in tumors with acquired resistance, such as disruptions in the NEDD8, CHIP or other ubiquitin or ubiquitin-associated/protein receptor degradation pathways, may thus present an important therapeutic target for future drug intervention.

For the "so what section" (evaluates the knowledge as a scientific or medical product to also be included in the conclusion of this report), the loss of ER α degradation pathway(s) may provide a mechanism by which breast cancer cells acquire antiestrogen resistance while retaining expression of ER α . Pathways that utilize the ubiquitin-proteasome system could serve as a therapeutic targets for breast cancer.

In summary, all three tasks have been completed. .

List of personnel receiving pay from the research effort: Kenneth P. Nephew, Ph.D., Principal Investigator; Meiyun Fan, Ph.D., Postdoctoral Fellow; Teresa Craft, M.S., Research Associate, Annie Park, B.S., Research Associate, Xinghua Long, Graduate Student.

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13. Fan M, Park A, **Nephew KP**. Interactions between estrogen receptor and the COOH terminus of the Hsp70-interacting protein (CHIP) (*Mol Endocrinol* under revision)
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APPENDICES

Reprints:

1. Fan M, Bigsby RM, **Nephew KP** 2003 The NEDD8 pathway is required for proteasome mediated degradation of human estrogen receptor- α and essential for the antiproliferation activity of ICI 182,780 in ER-positive breast cancer cells *Mol Endocrinol* 17:356-365 (cover article)
2. Fan M, Nakshatri H, **Nephew KP**. 2004. Inhibiting proteasomal proteolysis sustains estrogen receptor-alpha activation. *Mol Endocrinol* 18:2603-2615
3. Fan M, Park A, **Nephew KP**. Interactions between estrogen receptor and the COOH terminus of the Hsp70-interacting protein (CHIP) (*Mol Endocrinol* under revision)
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APPENDIX 1

The NEDD8 Pathway Is Required for Proteasome-Mediated Degradation of Human Estrogen Receptor (ER)- α and Essential for the Antiproliferative Activity of ICI 182,780 in ER α -Positive Breast Cancer Cells

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Steroid hormone receptors, including estrogen receptor- α (ER α), are ligand-activated transcription factors, and hormone binding leads to depletion of receptor levels via proteasome-mediated degradation. NEDD8 (neural precursor cell-expressed developmentally down-regulated) is an ubiquitin-like protein essential for protein processing and cell cycle progression. We recently demonstrated that ubiquitin-activating enzyme (Uba)3, the catalytic subunit of the NEDD8-activating enzyme, inhibits ER α transcriptional activity. Here we report that Uba3-mediated inhibition of ER α transactivation function is due to increased receptor protein turnover. Coexpression of Uba3 with ER α increased receptor degradation by the 26S proteasome. Inhibition of NEDD8 activation and conjugation diminished polyubiquitination of ER α and blocked proteasome-mediated degradation of receptor protein. The antiestrogen ICI 182,780 is known to

induce ER degradation. In human MCF7 breast cancer cells modified to contain a disrupted NEDD8 pathway, ICI 182,780 degradation of ER α was impaired, and the antiestrogen was ineffective at inhibiting cell proliferation. This study provides the first evidence linking nuclear receptor degradation with the NEDD8 pathway and the ubiquitin-proteasome system, suggesting that the two pathways can act together to modulate ER α turnover and cellular responses to estrogens. Based on our observation that an intact NEDD8 pathway is essential for the antiproliferation activity of the ICI 182,780 in ER α positive breast cancer cells, we propose that disruptions in the NEDD8 pathway provide a mechanism by which breast cancer cells acquire antiestrogen resistance while retaining expression of ER α . (*Molecular Endocrinology* 17: 356-365, 2003)

ESTROGEN REGULATES DIVERSE biological processes through estrogen receptors (ER α and ER β) (1). Receptor levels and dynamics have a profound influence on target tissue responsiveness and sensitivity to estrogen (2). ER α is a short-lived protein with a half-life of about 4 h, which is reduced to 3 h by 17 β -estradiol (estradiol), and to less than 1 h by the steroidal antiestrogens, ICI 182,780 and ICI 164,384 (3, 4). Receptor turnover rates provide estrogen target cells with the capacity for rapid regulation of receptor levels and thus dynamic hormone responses. An attenuated transcriptional response has been associated with down-regulation of ER α , and receptor up-regulation has been shown

Abbreviations: APP-BP1, Amyloid precursor protein-binding protein; AR, androgen receptor; csFBS, charcoal-stripped FBS; E2, ubiquitin conjugation enzyme; E3, ubiquitin ligase; ER, estrogen receptor; estradiol, 17 β -estradiol; FBS, fetal bovine serum; HA, hemagglutinin; GAPDH, glyceraldehyde phosphate dehydrogenase; GFP, green fluorescent protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NEDD8, neural precursor cell-expressed developmentally down-regulated; 4-OHT, 4-hydroxytamoxifen; PR, progesterone receptor; Uba, ubiquitin-activating enzyme; Ubc, ubiquitin-conjugation enzyme.

to enhance the cellular response to estrogen (2). Nonetheless, mechanisms governing ER α protein levels remain poorly understood.

It has recently been shown that degradation of ER α and other members of the nuclear receptor superfamily occurs through the ubiquitin-proteasome pathway (5). Ubiquitination is a multistep process involving the action of a ubiquitin-activating enzyme (E1 or Uba), a ubiquitin conjugation enzyme (E2 or Ubc), and a ubiquitin ligase (E3) (6). Because the high specificity for target proteins is primarily conferred by E3, regulation of E3 activity may play a crucial role in governing protein degradation *in vivo*. A large number of E3s are cullin-based ubiquitin ligases (7), including SCF (Skp1/Cul1/F-box/ROC1) and VCB (von Hippel-Lindau-Cul2/elongin B/elongin C) complexes. One important level of regulation of these cullin-based ubiquitin ligases involves modification of the cullin subunit with NEDD8, an ubiquitin-like protein (7).

NEDD8 conjugation (neddylation) resembles ubiquitination and involves the action of amyloid precursor protein-binding protein (APP-BP1)/Uba3, a heterodimeric E1-like enzyme, and Ubc12, an E2-like enzyme (8).

Whether a ligase is required for neddylation is unknown. To date, the only known substrates of NEDD8 are cullin family members (9, 10). Cullin neddylation is conserved and plays an important regulatory role for cullin-based E3 activity in yeast, plant, and mammalian cells (7, 11–13). Interrupting NEDD8 modification of cullins in mammalian cells has been shown to block ubiquitination of certain proteins involved in different cellular functions, including p27, I κ B α , HIF α , and NF κ B precursor p105 (14–19). Recent studies have revealed that cullin neddylation is a tightly controlled dynamic process (20–24), and the effect of neddylation on protein polyubiquitination appears to be specific (17, 18).

We recently identified the NEDD8 activating enzyme, Uba3 as an ER-interacting protein and inhibitor of transactivation by steroid nuclear receptors (25). We further demonstrated that an intact neddylation pathway is required for Uba3-mediated inhibition of ER transcriptional activity (25). Taken together with recent reports linking the ubiquitin and NEDD8 pathways (7), our findings raise the intriguing possibility for a role of neddylation in ER α ubiquitination and degradation. Here we show that Uba3 enhances ER α degradation by the 26S proteasome, and expression of dominant-negative mutants of Uba3 or Ubc12 impaired ER α ubiquitination and ligand-induced ER α degradation. Blocking the neddylation pathway with the dominant-negative Ubc in ER α -positive human breast cancer cells inhibited both receptor degradation and the growth inhibitory effect of the antiestrogen ICI 182,780 (known clinically as Faslodex or Fulvestrant). Collectively, these data show that the NEDD8 pathway plays an essential role in ubiquitination and proteasomal degradation of ER α and indicate that disruptions in the pathway may contribute to the development of antiestrogen resistance in human breast cancer.

RESULTS

Uba3 Enhances Proteasomal Degradation of ER α

To test the hypothesis that the neddylation pathway restricts ER α activity by modulating receptor degradation, we transfected HeLa cells with ER α , alone or in combination with an expression vector for Uba3, APP-BP1, or Ubc12, or with an empty vector (pcDNA3.1, Invitrogen, Carlsbad, CA); a green fluorescence protein (GFP) expression vector was cotransfected to serve as a means of normalizing transfection efficiency and sample preparations. Steady-state levels of ER α protein were determined by Western blot analysis. Coexpression of Uba3 decreased ER α protein level but had no effect on GFP expression (Fig. 1A). Treatment of the transfected HeLa cells with MG132, a specific proteasome inhibitor, blocked Uba3-stimulated down-regulation of ER α (Fig. 1B), confirming that the Uba3-induced ER α degradation is through the 26S proteasome. Overexpression of APP-BP1 or Ubc12 had no significant effect on ER α protein levels (data not shown), a result consistent with our previous observation that Uba3 is the limiting factor in ned-

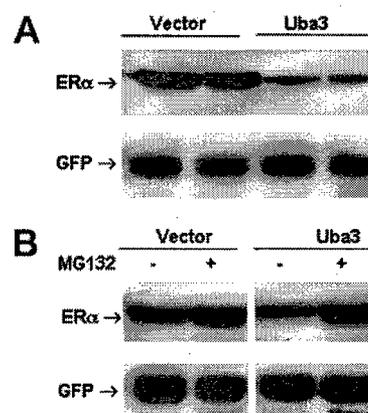


Fig. 1. Uba3 Enhances Proteasomal Degradation of ER α

A, Coexpression of Uba3 decreases ER α protein level in transfected HeLa cells. HeLa cells were transfected with pSG5-ER and pcDNA-Uba3 or pcDNA vector. Whole cell extracts were prepared 24 h post transfection and analyzed by Western blotting to determine ER α protein level. B, Proteasome inhibitor MG132 restores expression level of ER α in cells transfected with Uba3. Transfected HeLa cells (same as in A) were treated with 20 μ M MG132 for 6 h before protein extracts and ER α level analysis. GFP was used as an internal control to correct for transfection efficiency and SDS-PAGE loading. Representative results of three independent experiments are shown.

dylation-associated inhibition of ER α transcriptional activity (25).

The Neddylation Pathway Is Required for Ligand-Mediated Degradation of ER α

Estradiol stimulates ER α degradation through the ubiquitin-proteasome pathway (26–30). Having established a role for Uba3 in this process, it was important to assess whether neddylation pathway is required for ligand-induced degradation of ER α . To address this issue, we used a dominant-negative mutant of Ubc12 (Ubc12C111S). Due to a single Cys-to-Ser substitution at the active Cys residue, Ubc12C111S forms a stable complex with NEDD8, resulting in sequestration of NEDD8 and inhibition of subsequent NEDD8 conjugation (31, 32). Dominant-negative inhibition of NEDD8 conjugation by Ubc12C111S has been shown to impair efficient ubiquitination and protein degradation (14, 15, 17, 18). Treatment of ER α -transfected HeLa cells with estradiol resulted in a time-dependent decrease in ER α protein levels; receptor levels were reduced by 80% at 6–8 h (Fig. 2A). In contrast, the effects of estradiol on receptor levels were less dramatic in cells expressing Ubc12C111S, producing a reduction of only 40% by 6–8 h (Fig. 2A). Consistent with this observation, Uba3C216S, a dominant-negative mutant of Uba3 (31, 32), also inhibited estradiol-induced ER α down-regulation (Fig. 2B). Addition of the proteasome inhibitor MG132 before estradiol treatment completely abolished ligand-induced down-regulation of ER α (Fig. 2B), con-

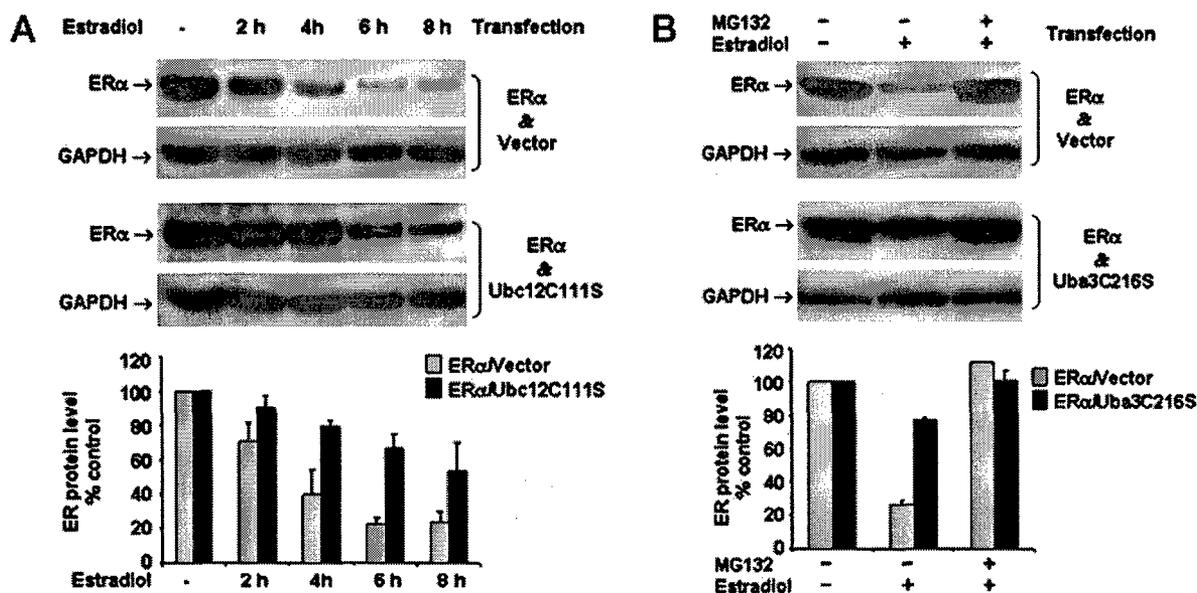


Fig. 2. Expression of Ubc12C111S or Uba3C216S Inhibits Ligand-Induced ER α Degradation

A, HeLa cells were transfected with pSG5-ER and pcDNA vector (*upper panel*) or pcDNA-Ubc12C111S (*lower panel*). Twenty-four hours after transfection, cells were treated with 100 nM estradiol for the indicated times and analyzed for ER α protein level using Western blotting. Relative ER α levels in cells cotransfected with vector (*gray*) or Ubc12C111S (*black*) from two independent experiments are shown in corresponding histogram. B, HeLa cells were transfected with pSG5-ER and pcDNA vector (*upper panel*) or pcDNA-Uba3C216S (*lower panel*). Twenty-four hours after transfection, cells were treated with vehicle or 20 μ M MG132 for 1 h followed by incubation with vehicle or 100 nM estradiol for 6 h, as indicated. ER α protein levels were analyzed by immunoblotting. Relative ER α levels in cells cotransfected with vector (*gray*) or Uba3C216S (*black*) from three independent experiments are shown in corresponding histogram. GAPDH was used as an internal control to correct SDS-PAGE loading.

firming that exogenous ER α in HeLa cells undergoes proteasome-dependent degradation in response to estradiol. Collectively, these results demonstrate that a functional NEDD8 pathway is required for efficient, ligand-induced, proteasome-mediated degradation of ER α .

The NEDD8 Pathway Is Required for Efficient Ubiquitination of ER α

Having established a role for Uba3 and Ubc12 in ER α down-regulation, it was important to examine the effect of NEDD8 on receptor ubiquitination. HeLa cells were cotransfected with ER α and hemagglutinin (HA)-tagged ubiquitin, along with wild-type Ubc12 or Uba3 or the corresponding mutant forms of these neddylation enzymes (Ubc12C111S or Uba3C216S). At 24 h post transfection, cells were treated with MG132 or vehicle, followed by estradiol treatment. Immunoprecipitation assays using an anti-ER α antibody were performed and the levels of ubiquitinated ER α in the precipitated immunocomplex were assessed by Western blotting with an anti-HA antibody. The polyubiquitinated ER α exhibited a ladder of higher molecular weight species on the blot membrane (Fig. 3). Expression of dominant-negative Ubc12C111S or Uba3C216S markedly decreased ER α ubiquitination in either the absence (Fig. 3, *left panel*) or presence of estradiol and MG132 (Fig. 3, *right panel*), compared

with cells transfected with control vector or wild-type Ubc12 or Uba3. These results suggest that a functional neddylation pathway is required for the efficient ubiquitination of ER α .

ER α Protein Levels in MCF7 Breast Cancer Cell Lines Stably Expressing Dominant-Negative Ubc12C111S

MCF7 human breast cancer cells express high levels of ER α and proliferate in response to estrogen treatment (33, 34), providing a model to study endogenous ER α function. To further investigate the role of neddylation in ER α function under physiological relevant conditions, we transfected Ubc12C111S into MCF7 cells and established the stable cell line MCF7/C111S. As a control, MCF7/Vec (MCF7 cells stably transfected with empty vector) was also established. Expression of the Ubc12C111S mutant protein in MCF7/C111S cells was confirmed by Western blotting and, consistent with a previous report (31), the mutant was detected as 26- and 31-kDa proteins (Fig. 4, lanes 3–8). In the regular growth medium containing phenol red and 10% fetal bovine serum (FBS), the level of ER α in MCF7/Vec cells was very low; after 3 d of culture in hormone-free medium containing 3% dextran-coated charcoal-stripped FBS (cs-FBS) and no phenol red, ER α expression was dramatically increased (Fig. 4, lanes 1 and 2). The culture medium (regular growth medium vs. hormone-free me-

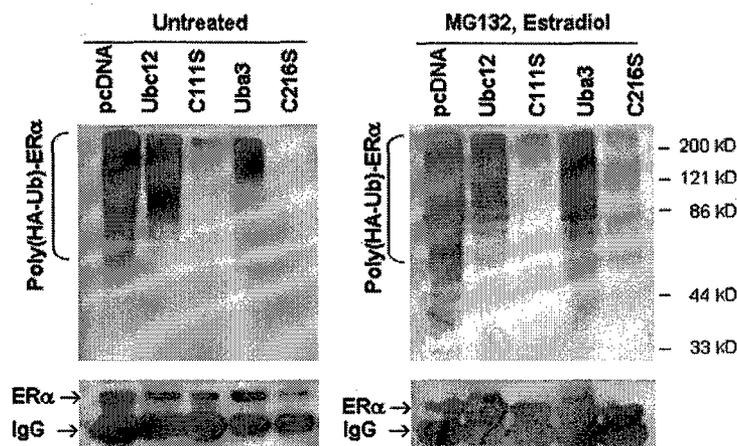


Fig. 3. An Intact NEDD8 Pathway Is Required for Efficient ER α Ubiquitination

HeLa cells were transfected with pSG5-ER, and pcDNA-HA-Ubiquitin, alone with indicated construct. Twenty-four hours after transfection, cells were either untreated (*left panel*) or treated with 20 μ M MG132 for 1 h followed by 100 nM estradiol exposure for 3 h (*right panel*). Protein extracts were prepared and subjected to immunoprecipitation using anti-ER α antibody. Polyubiquitinated ER α was detected by Western blotting using anti-HA antibody, and was visualized as a ladder of higher molecular weight species on the blot. The blot was striped and reprobbed by anti-ER α antibody to assess the amount of precipitated ER α (*lower panels*). The heavy chain of the anti-ER α IgG used for immunoprecipitation exhibits a 57-kDa band in the ER α blot. Representative results of three independent experiments are shown.

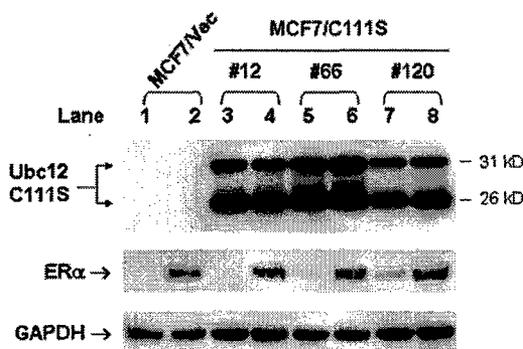


Fig. 4. The Expression of Ubc12/C111S and ER α in Three Independent MCF7/C111S Clones

MCF7/C111S cells stably expressing mutant Ubc12/C111S were maintained in growth medium (lanes 1, 3, 5, and 7) or hormone-free medium for 3 d (lanes 2, 4, 6, and 8) and analyzed by immunoblotting using anti-HA (*upper panel*) or anti-ER α (*lower panel*) antibodies, respectively. GAPDH was used as an internal control to correct for SDS-PAGE loading.

dium) showed no effect on the expression level of Ubc12/C111S. In three MCF7/C111S clones, receptor levels varied among the clones and, when cultured in growth medium, detectable ER α was seen in two of the three clones (Fig. 4, lanes 5 and 7). When cultured in estrogen-free medium, however, ER α levels were high in all three clones (Fig. 4, lanes 4, 6, 8).

Ubc12/C111S Inhibits ICI 182,780-Induced Down-Regulation of ER α

In contrast to estradiol, which down-regulates ER α in target tissues through both transcriptional and

posttranslational mechanism (35, 36), the pure antiestrogen ICI 182,780 causes ER α protein degradation without affecting ER α mRNA levels (3, 36). Based on our observations that the NEDD8 pathway is essential for ER α degradation in transfected HeLa cells (Fig. 2), it was of interest to examine the effect of the antiestrogen on ER α degradation in MCF7/C111S cells. Cells were cultured in hormone-free medium for 3 d before ICI 182,780 treatment. Under this condition, comparable amounts of ER α were observed in MCF7/C111S and MCF7/Vec cells (compare 0-h lanes in Fig. 5A). Treatment with ICI 182,780 rapidly (by 1 h) decreased ER α levels in the MCF7/Vec cells; by 4 h post treatment, the levels of ER α were reduced by 95% (Fig. 5A). In the MCF7/C111S cells, the effects of ICI 182,780 on ER α levels were much less dramatic (Fig. 5A). Thus, although ER degradation was not completely inhibited by expression of the dominant-negative Ubc12/C111S, these results confirm our observations using transient transfection in HeLa cells and further suggest that the NEDD8 pathway is required for efficient degradation of endogenous ER α . To examine the effect of another antiestrogen on ER α degradation in this system, cells were cultured in the presence of various doses of 4-hydroxytamoxifen (4-OHT) and ER α levels were examined. In both MCF7/Vec and MCF7/C111S cells, ER α levels remained unchanged or were slightly increased after treatment with 4-OHT (Fig. 5B). Stabilization of ER α by tamoxifen has been reported by others (30), perhaps due to inhibition of the basal rate of ER degradation by the antiestrogen.

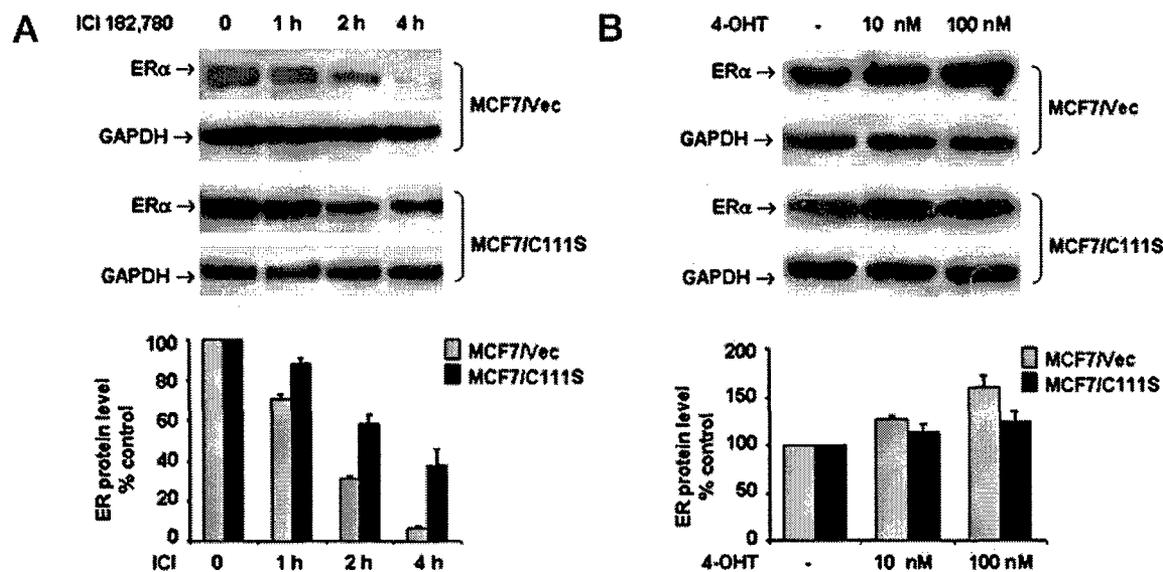


Fig. 5. ER α Degradation Is Impaired in MCF7/C111S Cells

A, ICI 182,780-induced ER α degradation is impaired in MCF7/C111S cells. MCF7/Vec (upper panel) and MCF7/C111S cells (lower panel) were cultured in hormone-free medium for 3 d and treated with 1 nM ICI 182,780 for the indicated times. B, 4-OHT does not cause ER α degradation in MCF7 cells. MCF7/Vec (upper panel) and MCF7/C111S cells (lower panel) were cultured in hormone-free medium for 3 d and treated with indicated doses of 4-OHT for 6 h. ER α protein levels were determined by Western blotting with anti-ER α antibody. The histogram shows the relative ER α levels after ICI 182,780 or 4-OHT treatment. Relative ER α levels in MCF7/vec (gray) from three independent experiments or MCF7/C111S (black) from three independent MCF7/C111S clones are shown in corresponding histogram. GAPDH was used as an internal control to correct SDS-PAGE loading.

Disrupting the NEDD8 Pathway Confers Antiestrogen Resistance in Breast Cancer Cells

Estradiol is mitogenic in MCF7 cells and stimulates cell proliferation through activation of ER α (37). The pure antiestrogen ICI 182,780, on the other hand, blocks ER α -mediated transactivation and induces ER α protein degradation, resulting in growth inhibition of breast cancer cells (38). Because expression of Ubc12C111S inhibited ICI 182,780-induced ER α down-regulation (Fig. 5A), we examined the growth inhibitory effect of ICI 182,780 in MCF7/C111S cells. No significant difference was observed in basal cell proliferation rates between MCF7/C111S and MCF7/Vec cells in hormone-free medium (data not shown). Treatment with the antiestrogen (1 nM) inhibited the basal cell growth of MCF7 and MCF7/Vec cells (Fig. 6A). In contrast, MCF7/C111S cells were partially resistant to ICI 182,780. Specifically, over an 8-d period, the antiestrogen inhibited the growth of control cells by 50% compared with 20–25% growth inhibition of the MCF7/C111S cells (Fig. 6A, left panel). Dose-response analysis showed that MCF7/C111S cells were resistant to a broad range (0.01–10 nM) of ICI 182,780 concentrations (Fig. 6A, right panel). On the other hand, estradiol-induced proliferation of MCF7/C111S and control cells was similar (2-fold increase in cell number over a 6-d treatment period; data not shown). The effect of 4-OHT on MCF7/C111S and MCF7/Vec cell proliferation was examined in a time- and dose-response analysis. The response of the cell

lines to 4-OHT was similar (Fig. 6B), suggesting that Ubc12C111S expression did not confer cells resistance to growth inhibitory effect of antiestrogens in general. These results suggest that the expression of Ubc12C111S conferred resistance of MCF7 cells to the growth inhibitory effects of ICI 182,780, but disrupting the NEDD8 pathway had no effect on the mitogenic response of MCF7 breast cancer cells to estradiol or the growth inhibitory effects of 4-OHT.

DISCUSSION

ER α is a short-lived protein whose degradation is primarily mediated by the ubiquitin-proteasome pathway (26–30). The recently described ubiquitin-like pathways, including the NEDD8 and SUMO (small ubiquitin-like modifier) conjugation systems (39), have been implicated in nuclear receptor regulation (40–44) and the NEDD8 pathway has been shown to enhance protein polyubiquitination (12, 14–19, 45–47). Our previous investigation into the role of the NEDD8 pathway in nuclear hormone receptor regulation showed that Uba3, the catalytic subunit of the NEDD8 activating enzyme complex, interacts with ER α and inhibits receptor function (25). Here we report that Uba3-mediated inhibition of ER α transactivation is due to increased receptor turnover and that an intact neddylation pathway is essential for ER α ubiquitination and degradation. By impairing the NEDD8 path-

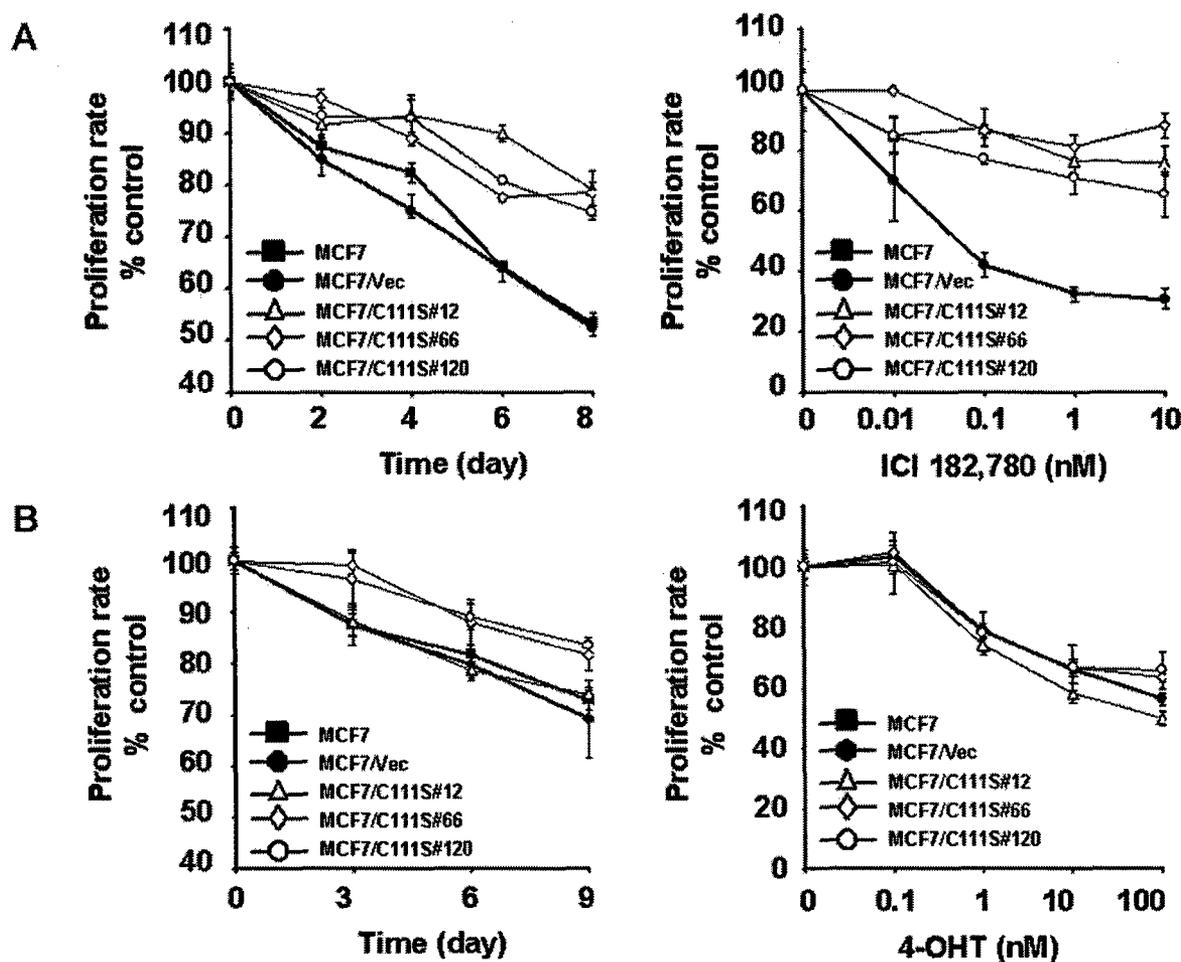


Fig. 6. Interruption of the NEDD8 Pathway Confers Resistance to ICI 182,780 in Human Breast Cancer Cells

A, Time- and dose-dependent growth inhibition of ICI 182,780. For time-response analysis, cells were treated with 1 nM ICI 182,780 and cell numbers were determined 0, 2, 4, 6, and 8 d after drug exposure. For dose-response assay, cells were treated with indicated doses of ICI 182,780 and cell numbers were determined on d 7. B, Time- and dose-dependent antiproliferative effect of 4-OHT. For time-response analysis, cells were treated with 10 nM 4-OHT and cell numbers were determined 0, 3, 6, and 9 d later. For the dose-response assay, cells were treated with indicated doses of 4-OHT and cell numbers were determined on d 7. For all assays, cells were cultured in hormone-free medium for 3 d before treatment and cell numbers were determined by MTT assay. Relative proliferation rate was expressed as percentage of cells grown in hormone-free medium. Each experiment was repeated three times in quadruplicate.

way in human MCF7 breast cancer cells, we demonstrated that the cells became resistant to the growth inhibitory effects of ICI 182,780. Thus, our data suggest that neddylation plays an important role in ER α degradation and we speculate that alterations in the NEDD8 pathway may provide a mechanism by which tumors can acquire antiestrogen resistance.

Several recent studies have focused on the role of the ubiquitin-proteasome pathway in nuclear receptor down-regulation (26–30). Enhancement of ER α ubiquitination by estradiol was first reported by Nirmala and Thampan (48), and Nawaz *et al.* (27) showed that a functional ubiquitin-proteasome system is required for ER α degradation. Both basal and ligand-induced ER α ubiquitination occurs at the nuclear matrix (49), but how ER α is targeted for ubiquitination has not

been fully established. Previously, we had shown that Uba3 interacts directly with ER and that this interaction is augmented by estradiol (25). Here, we show that overexpression of Uba3 enhanced degradation of ER α and that disruption of Uba3 activity reduces estradiol-induced receptor degradation. Taken together, these data support a role for Uba3 in the regulation of basal as well as ligand-induced ER α turnover.

The present study is the first to link the NEDD8 pathway to ubiquitination of ER α . The exact mechanism connecting the two pathways, however, remains unclear. The only known substrates for direct neddylation are members of the cullin family (10). Some of the cullins have been identified as core subunits of specific ubiquitin ligase complexes (7). Mechanistically, conjugation of NEDD8 to cullins may up-regulate

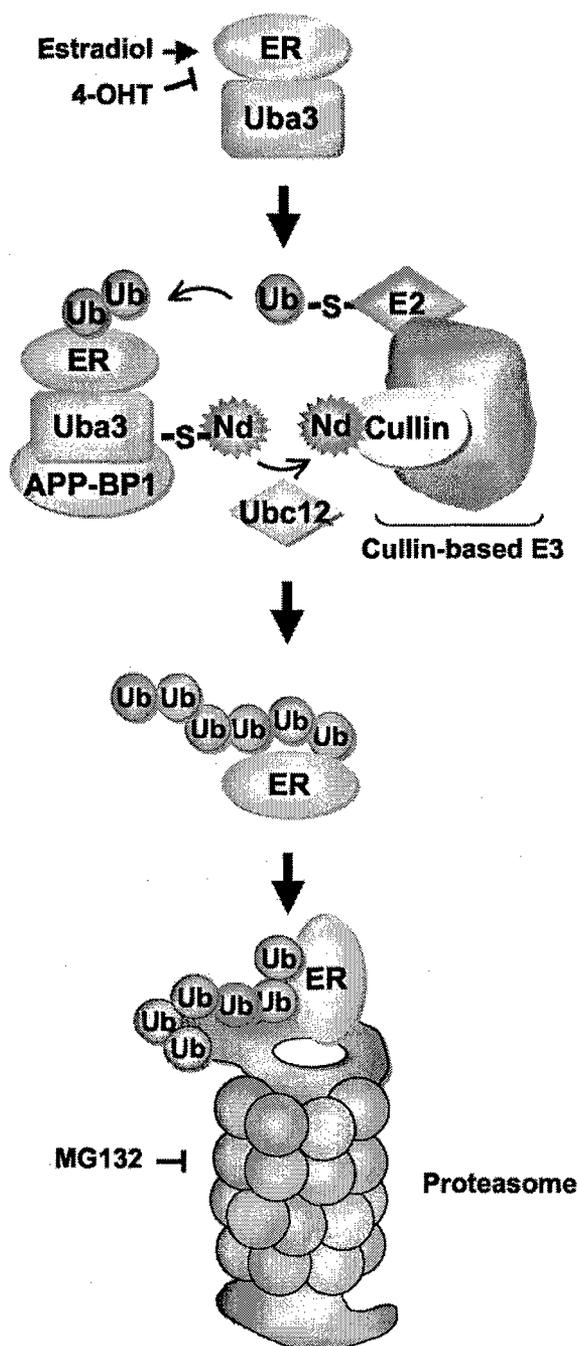


Fig. 7. Hypothetical Model Depicting the Role of Neddylation Pathway in Proteasome-Mediated Degradation of ER α

The physical interaction between Uba3 and ER α promotes the functional recruitment and activation of a cullin-based ubiquitin-protein ligase to augment receptor polyubiquitination. Uba3 and APP-BP1, the heterodimeric activating enzyme for NEDD8, and Ubc12, the NEDD8 conjugating enzyme, promote cullin NEDD8 modification of specific ubiquitin E3 ligases. Neddylation enhances the formation and activity of the ubiquitin E2-E3 complex. The potency of ER α -Uba3 interaction appears to correlate with ER α turnover rate. In the absence of ligand, ER α interacts weakly with Uba3, resulting in basal ubiquitination and degradation of ER α ; however, estradiol augments the ER α -Uba3 interaction

ubiquitin ligase activity of specific E3s by facilitating the formation of an ubiquitin E2-E3 complex (45). In this regard, the interaction between Uba3 and ER α could result in the functional recruitment and activation of a cullin-based ubiquitin-protein ligase, which, in turn, targets ER α for degradation by the ubiquitin-proteasome system. The hypothetical model depicting the role of neddylation pathway in proteasome-mediated degradation of ER α is shown in Fig. 7. Together with our previously reported data (25), these observations indicate that such targeted degradation of ER α leads to reduced hormonal responsiveness.

In addition to its effect on ER α , Uba3 inhibits the transactivation function of other steroid receptors, ER β , androgen receptor (AR) and progesterone receptor (PR) (25). Others have reported that NEDD8 interacts with aryl hydrocarbon receptor and the interaction affects the transcriptional activity and stability of the receptor protein (40). Furthermore, the NEDD8 protein has been found to colocalize with AR (50). Together with the observations that turnover of ER, AR, PR, and aryl hydrocarbon receptor occurs via degradation by the 26S proteasome (28, 51-53), these results provide compelling evidence for integration of the neddylation and ubiquitin-proteasome pathways in steroid hormone action. Because receptor levels can have a profound influence on target tissue responsiveness to hormone, NEDD8 and ubiquitin pathways, by modulating receptor protein turnover, could play important roles in determining and perhaps limiting cellular responses to steroid hormones and anti-hormones.

The antiestrogen ICI 182,780 is a 7 α -alkylsulfinyl analog of estradiol lacking agonist activity (54). The drug is used as a second-line endocrine agent in patients who have developed tamoxifen-resistant breast cancer (38). Although the drug clearly displays complex pharmacology, rapid degradation of ER α protein has been associated with the antiproliferative effects of ICI 182,780 on breast cancer cells (38, 54). Despite its potent antitumor effects, the drug does not circumvent the development of antiestrogen resistance (55-58). Moreover, the fact that most tumors acquiring ICI 182,780 resistance do so while retaining expression of ER α and estrogen responsiveness (55, 59) suggests that administration of the antiestrogen may possibly lead to the selection of tumor cells defective in ER α down-regulation pathway(s), which in turn may confer a proliferative advantage in either the presence or absence of estrogens. Mechanism underlying persistent expression of ER α in tumors with acquired resis-

to enhance ER α ubiquitination. On the other hand, 4-OHT interrupts the ER α -Uba3 interaction and stabilizes ER α , and MG132 blocks ER α degradation by inhibiting proteasome activity. APP-BP1, Amyloid precursor protein-binding protein; E2, ubiquitin conjugation enzyme; E3, ubiquitin protein ligase; estradiol, 17 β -estradiol; Nd, neural precursor cell-expressed developmentally down-regulated (NEDD8); \downarrow and \uparrow , Stimulation and inhibition, respectively.

tance may thus present an important therapeutic target for future drug intervention. In this context, the loss of NEDD8 expression during malignant transformation of prostate cancer was recently reported (60). Because our results show an intact NEDD8 pathway is essential for ER α ubiquitination and degradation, we speculate that disruptions in the NEDD8 pathway may provide a mechanism by which breast cancer cells acquire ICI 182,780 resistance while retaining expression of ER α .

MATERIALS AND METHODS

Materials

The following antibodies and reagents were used in this study: anti-ER (HC20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-HA (3F10; Roche Molecular Biochemicals, Indianapolis, IN); anti-GFP (GFP01, NeoMarkers, Inc., Fremont, CA); anti-GAPDH (glyceraldehyde phosphate dehydrogenase; Chemicon International, Inc., Temecula, CA); anti-rabbit IgG and protein G-agarose beads (Oncogene Research Products, San Diego, CA); SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL); protease inhibitor cocktail set III (Calbiochem-Novabiochem Corp., San Diego, CA); Bio-Rad Laboratories, Inc. (Hercules, CA) protein assay kit; FBS and csFBS (HyClone Laboratories, Inc., Logan, UT); LipofectAMINE Plus Reagent, geneticin, and other cell culture reagents were from Life Technologies, Inc. (Rockville, MD). Estradiol, 4-OHT, MG132, and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma (St. Louis, MO). ICI 182,780 was purchased from Tocris Cookson Ltd. (Ellisville, MO).

Plasmid Construction

The construction of pSG5-ER(HEGO), pcDNA-Uba3, pcDNA-HA-Uba3C216S, pcDNA-HA-Ubc12, and pcDNA-HA-Ubc12C111S was described previously (25). The pcDNA-HA-ubiquitin was kindly provided by Y. Xiong (61). The pCMV (cytomegalovirus)-GFP was purchased (Promega Corp., Madison, WI).

Cell Lines

The human cervical carcinoma cell line, HeLa, and the breast cancer cell line, MCF-7 were purchased from ATCC (Manassas, VA). HeLa cells were maintained in MEM with 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 50 U/ml penicillin, 50 μ g/ml streptomycin, and 10% FBS. MCF7 cells were maintained in MEM with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 50 U/ml penicillin, 50 μ g/ml streptomycin, 6 ng/ml insulin, and 10% FBS. Before experiments involving in transient transfection and hormone treatment, cells were cultured in hormone-free medium (phenol red-free MEM with 3% csFBS) for 3 d.

Transient Transfection Assays

HeLa cells were cultured in hormone-free medium for 3 d and transfected with equal amount of total plasmid DNA (adjusted by corresponding empty vectors) by using LipofectAMINE Plus Reagent according to the manufacturer's guidelines. Five hours later, the DNA/LipofectAMINE mixture was re-

moved and cells were cultured in hormone-free medium. All cells were also cotransfected with pCMV-GFP as internal control to correct for transfection efficiency and SDS-PAGE loading.

Stable Transfection

MCF7 cells were transfected with pcDNA-HA-Ubc12C111S or empty vector by using LipofectAMINE Plus Reagent and selected in growth medium containing 0.5 mg/ml geneticin for 3 wk. Drug-resistant colonies were chosen and expanded in growth medium containing 0.3 mg/ml geneticin. The expression of HA-Ubc12C111S in the stable cell lines (MCF7/C111S) was detected by Western blotting with anti-HA antibody. Geneticin-resistant clones from vector transfectants (MCF7/Vec) were pooled, maintained in growth medium containing 0.3 mg/ml geneticin, and used as control cells.

Preparation of Cell Extracts and Immunoblotting

Whole cell extracts were prepared by suspending cells ($\sim 2 \times 10^6$) in 0.1 ml of ice-cold lysis buffer (25 mM HEPES, pH 7.5; 0.3 M NaCl; 0.2% sodium dodecyl sulfate; 0.5% sodium deoxycholate; 0.2 mM EDTA; 0.5 mM dithiothreitol; 0.1% Triton X-100; 10 μ l protease inhibitor cocktail set III). After 15 min on ice, extracts were sonicated (3 \times 10 sec), insoluble material was removed by centrifugation (15 min at 12,000 \times g), and protein concentration in the supernatant was determined using the Bio-Rad Laboratories, Inc. protein assay kit. The protein extracts were mixed with 1/4 vol of 5 \times electrophoresis sample buffer and boiled for 5 min at 90 C. Protein extract (50 μ g per lane) was then fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with antibodies. Primary antibody was detected by horseradish peroxidase-conjugated second antibody and visualized using enhanced SuperSignal West Pico Chemiluminescent Substrate. The band density of exposed films was evaluated with ImageJ software (<http://rsb.info.nih.gov/ij/>).

Immunoprecipitation

For immunoprecipitation, 500 μ g whole cell extract was diluted to protein concentration of 1 μ g/ μ l using PBS containing protease inhibitor cocktail and incubated with 5 μ l anti-rabbit IgG and 20 μ l protein G-agarose beads for 1 h at 4 C. After centrifugation at 12,000 \times g for 15 sec, the precleared supernatants were incubated with 5 μ l anti-ER antibody overnight at 4 C, followed by another 1-h incubation with 30 μ l protein G-agarose beads. The beads were then pelleted by brief centrifugation, washed three times with PBS and once with PBS containing 0.4 M NaCl, and resuspended in 30 μ l SDS-PAGE loading buffer for SDS-PAGE and Western blotting.

Cell Proliferation Assays

To assess the effects of estradiol, ICI 182,780, or 4-OHT on cell proliferation, cells (1000/well) were plated in 96-well dishes in hormone-free medium for 3 d before drug exposure. For time-response analysis, cell numbers were determined by MTT assay (62) at indicated times after drug treatment; and for dose-response analysis, cell number was determined by MTT assay at d 7.

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Inhibiting Proteasomal Proteolysis Sustains Estrogen Receptor- α Activation

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Estrogen receptor- α (ER α) is a ligand-dependent transcription factor that mediates physiological responses to 17 β -estradiol (E2). Ligand binding rapidly down-regulates ER α levels through proteasomal proteolysis, but the functional impact of receptor degradation on cellular responses to E2 has not been fully established. In this study, we investigated the effect of blocking the ubiquitin-proteasome pathway on ER α -mediated transcriptional responses. In HeLa cells transfected with ER α , blocking either ubiquitination or proteasomal degradation markedly increased E2-induced expression of an ER-responsive reporter. Time course studies further demonstrated that blocking ligand-induced degradation of ER α resulted in prolonged stimulation of ER-responsive gene transcription. In breast cancer MCF7 cells containing endogenous ER α , proteasome inhibition enhanced E2-induced expression of endogenous pS2 and cathepsin D. However, inhibiting the proteasome decreased expression of progesterone receptor (PR),

presumably due to the heterogeneity of the PR promoter, which contains multiple regulatory elements. In addition, in endometrial cancer Ishikawa cells overexpressing steroid receptor coactivator 1, 4-hydroxytamoxifen displayed full agonist activity and stimulated ER α -mediated transcription without inducing receptor degradation. Collectively, these results demonstrate that proteasomal degradation is not essential for ER α transcriptional activity and functions to limit E2-induced transcriptional output. The results further indicate that promoter context must be considered when evaluating the relationship between ER α transcription and proteasome inhibition. We suggest that the transcription of a gene driven predominantly by an estrogen-responsive element, such as pS2, is a more reliable indicator of ER α transcription activity than a gene like PR, which contains a complex promoter requiring cooperation between ER α and other transcription factors. (*Molecular Endocrinology* 18: 2603-2615, 2004)

THE ACTIONS OF estrogens are mediated primarily through estrogen receptors (ER α and ER β) (1), ligand-dependent transcription factors that interact directly with estrogen response elements (EREs) in the promoters of target genes (1). Cellular levels of ER α (2), along with a large number of receptor coregulator complexes (3), play key roles in controlling appropriate physiological responses in estrogen target tissues, such as breast and uterus. Levels of ER α mRNA and protein are regulated primarily by its cognate ligand, 17 β -estradiol (E2) (4-6). E2 binding results in rapid turnover of ER α protein through the ubiquitin (Ub)-

proteasome pathway (7-11), which has been implicated in both the overall control of gene transcription (12-16) and transactivation function of ER α and other nuclear receptors (7, 17-24).

The Ub-proteasome system consists of the 26S proteasome, a complex composed of a 20S catalytic core for protein proteolysis and two ATPase-containing 19S regulatory particles that recognize polyubiquitin-tagged substrates (25). Like many other transcription factors, stimulation of ER α transcriptional activation appears to be associated with receptor ubiquitination and proteasomal degradation (11, 26). Several proteins possessing Ub ligase activity (e.g. E6AP, p300, BRCA1, and MDM2), as well as SUG1, a component of the 19S proteasome, have been shown to associate with ER α and modulate receptor signaling (27-34). These observations suggest that proteasome-mediated receptor degradation is important for ER function.

Recent studies have demonstrated that inhibiting proteasomal degradation increases transcriptional activity of many, but not all, nuclear receptors, indicating a receptor-specific effect of proteasome inhibition (17-24). Blocking ER α turnover by a proteasome-

Abbreviations: CAT, Chloramphenicol acetyltransferase; DMSO, dimethylsulfoxide; E2, 17 β -estradiol; ER, estrogen receptor; ERE, estrogen response elements; GR, glucocorticoid receptor; hnRNA, heterogeneous nuclear RNA; Luc, firefly luciferase; 4-OHT, 4-hydroxytamoxifen; p-Pol II, phosphorylated RNA pol II; PR, progesterone receptor; Q-PCR, real-time quantitative reverse transcription-PCR; RSV, Rous sarcoma virus; SRC, steroid receptor coactivator; SV40, simian virus 40; Ub, ubiquitin; Ubc, ubiquitin-conjugation enzyme; Vit, vitellogenin.

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specific inhibitor, MG132, results in decreased expression of an ER α -responsive luciferase reporter, implicating that proteasomal degradation of ER α is required for its transactivation function (7, 35). However, MG132, and other proteasome inhibitors, have recently been shown to deleteriously affect production of a functional firefly luciferase enzyme (36), complicating the assessment of studies utilizing only ER α -responsive reporters expressing luciferase, in combination with 20S proteasome inhibitors. In addition, several studies have recently suggested that receptor degradation may not be required for ER α -mediated transcription. Frasor *et al.* (11, 37) reported that the partial agonist/antagonist 4-hydroxytamoxifen (4-OHT), which protects ER α from proteasomal degradation, stimulates ER-mediated transcription of a group of genes in MCF7 cells (38). Dissociation of ER α activation from degradation has also been reported in pituitary tumor cells (39, 40).

In the present study, we investigated the role of the Ub-proteasome pathway in ER α -mediated transcriptional responses. Genetic and pharmacological approaches were used to disrupt ER α ubiquitination, proteasome-mediated proteolysis, and thus ER α degradation, including the 20S proteasome inhibitor MG132, a dominant-negative mutant of the NEDD8 conjugation enzyme (Ubc12C111S) (41, 42), a Ub mutant with all of its lysines mutated to arginine (UbK0) (43), and the partial agonist/antagonist 4-OHT. To determine the effect of blocking ER α degradation on E2-induced transcriptional responses, ER-responsive reporter assays and expression of endogenous ER-target genes were used. The results demonstrate that proteasomal degradation is not essential for transcriptional activity of ER α and indicate that the Ub-proteasome system functions to limit E2-induced transcriptional output.

RESULTS

Inhibiting the Proteasome Increases ER α Transcriptional Output

The enzymatic activity of chloramphenicol acetyltransferase (CAT), luciferase (Luc) or β -galactosidase (Gal) reporter proteins is commonly used for assessing transcriptional activity of nuclear receptors in the presence of proteasome inhibitors. Recent studies with breast cancer T47D cells revealed that proteasome inhibitors (MG132, lactacystin, and proteasome inhibitor I) interfere with the production of luciferase and galactosidase proteins by a posttranscriptional mechanism, whereas the enzymatic activity of CAT remains unaffected (36). To verify these observations in our experimental systems, we examined the effect of MG132 on expression of these reporter enzymes from constitutively active constructs in cervical carcinoma HeLa and breast cancer MCF-7 cells. Cells were transfected with Rous sarcoma virus (RSV)-CAT, simian virus 40

(SV40)-Luc, or cytomegalovirus (pCMV)- β -gal and then treated with vehicle [dimethylsulfoxide (DMSO)] or MG132 (1 μ M) for 24 h. Reporter enzyme activity was determined using standard assays for luciferase, CAT, and galactosidase. Treatment of HeLa cells with MG132 had no effect on CAT activity but decreased luciferase and galactosidase activity by 80% and 30%, respectively (Fig. 1A, *left panel*). Essentially similar results were obtained using MCF7 cells (Fig. 1A, *right panel*). These results agree with a previous report demonstrating that proteasome inhibitors have deleterious effects on the enzymatic activities of luciferase and galactosidase reporter proteins (36).

Previously, we and others showed that E2 induces ER α degradation in transiently transfected HeLa cells and MG132 abolishes such degradation (8, 9, 42). Based on the above results, we further investigated the relationship between ER α turnover and E2-induced transcriptional response using an E2-responsive CAT reporter. HeLa cells were transiently transfected with ERE-vitellogenin (Vit)-CAT and different doses of ER α -expressing construct (0.1–5 ng pSG5-ER α /10⁵ cells). Cells were treated with vehicle (DMSO) or MG132 (1 μ M) for 1 h followed by E2 (10 nM). CAT activity was measured 24 h after E2 treatment. Basal CAT activity increased, proportional to the amount of pSG5-ER α (Fig. 1B; *open bars*). As expected, E2 markedly induced CAT activity (Fig. 1B; *gray bars*); however, treatment with MG132 plus E2 resulted in greater CAT activity, compared with E2 alone (Fig. 1B; *black vs. gray bars*). Cells treated with MG132 alone exhibited slightly higher CAT activity than the DMSO control (Fig. 1B, *hatched bars*). A synergistic effect of MG132 plus E2 was observed in cells transfected with lower levels of ER α (0.1–0.3 ng pSG5-ER α /10⁵ cells). For example, the combined treatment of MG132 and E2 increased ERE-CAT activity by about 7.4-fold in cells transfected with 0.1 ng pSG5-ER α /10⁵ cells, whereas MG132 or E2 alone increased ERE-CAT activity by 1.82- or 3.10-fold, respectively (*table* in Fig. 2B). Immunoblot analysis showed that pretreatment with MG132 effectively blocked E2-induced ER α down-regulation in HeLa cells (Fig. 1C). Taken together, these observations demonstrate that ER α retains the capacity to activate transcription in the absence of proteasomal degradation, and blocking ER α turnover increases E2-induced transcriptional output. The results further suggest that, in cells containing low levels of ER α , proteasome-mediated receptor degradation plays a role in limiting E2-induced transcriptional responsiveness.

Effect of Inhibiting the Proteasome on E2 Sensitivity

Based on the observation that preventing receptor protein turnover increases ER α -mediated transcription, we examined the effect of inhibiting the proteasome on hormone sensitivity. HeLa cells were transfected with ERE-Vit-CAT and pSG5-ER α ,

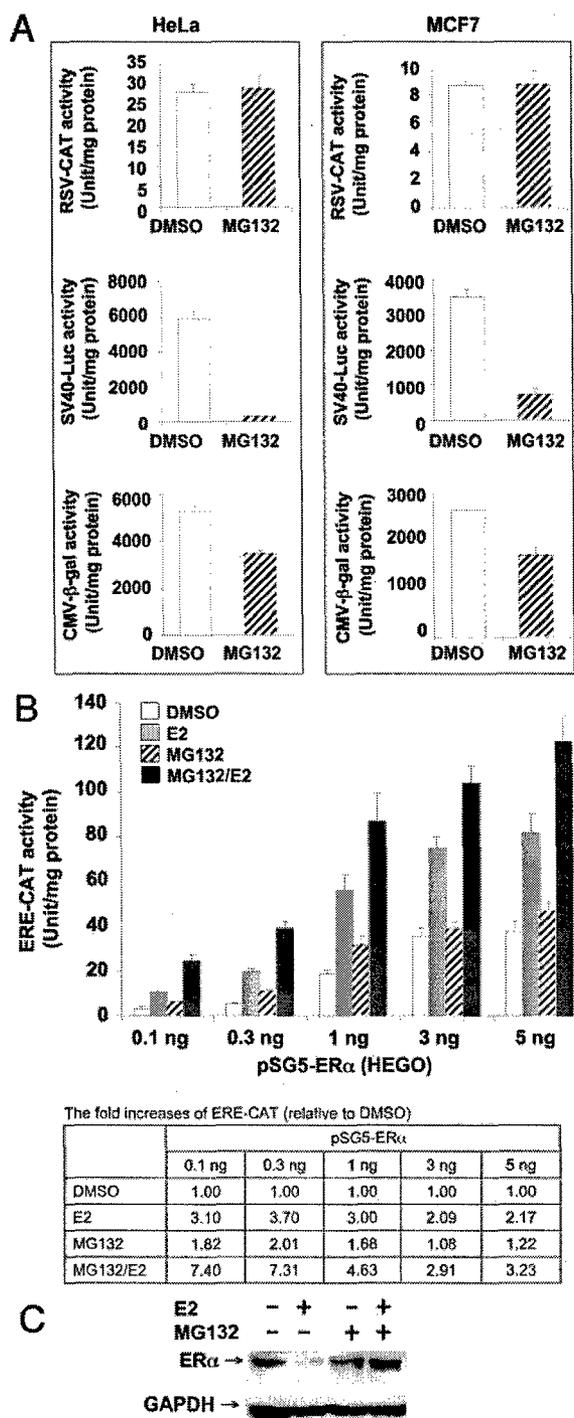


Fig. 1. Proteasome Inhibition Enhances E2-Induced CAT Reporter Gene Expression in HeLa Cells Transfected with ER α
A, Effect of proteasome inhibition by MG132 on expression of reporter enzymes from constitutively active promoters. HeLa cells (*left panel*) were plated on 12-well dishes at a density of 1×10^5 cells per well and cultured in hormone-free medium for 3 d. The cells were transfected with 100 ng RSV-CAT, 100 ng SV40-Luc, or 5 ng pCMV- β -gal using LipofectAMINE Plus Reagent. The DNA/LipofectAMINE mixture was removed 5 h later and cells were placed in hormone free medium containing either 0.1% vehicle (DMSO) or $1 \mu\text{M}$ MG132 for 24 h. Similarly, MCF7 cells (*right panel*) were

treated with DMSO or MG132 for 1 h, and then treated with various doses of E2 (1×10^{-15} to 1×10^{-8} M). CAT activity was determined 24 h after the addition of ligand. In cells transfected with 0.3 ng (Fig. 2A) or 1 ng pSG5-ER α (Fig. 2B), a hyperbolic dose response to E2 was observed; the lowest dose of hormone that induced CAT activity was 1×10^{-11} M E2. Increasing ER α expression (0.3 ng vs. 1 ng pSG5-ER α) and pretreatment with MG132 augmented maximal CAT induction by E2, but no effect on E2 sensitivity was observed. The minimal dose of E2 required to induce CAT was 1×10^{-11} M under all experiment conditions, and the EC $_{50}$ was not different (Fig. 2). These results demonstrate that blocking ER α degradation increases the magnitude of E2-induced gene transcription but has no effect on hormone sensitivity.

Inhibiting the Proteasome Extends the Duration of E2-Induced Gene Transcription

The results of the above experiments suggest that inhibiting the proteasome may extend the half-life of ligand-activated ER α and thus increase receptor transcriptional output. To test the possibility that MG132 treatment would subsequently extend the duration of an E2-induced transcriptional response, we performed a time course analysis using luciferase as a reporter protein. The half-life of CAT in mammalian cells is about 50 h (44);

plated at a density of 1.2×10^5 cells per well, transfected with 250 ng RSV-CAT, 250 ng SV40-Luc, or 10 ng pCMV- β -gal and then treated with DMSO or MG132 for 24 h. Reporter enzyme activities were normalized against total cellular protein and expressed as the mean \pm SD from three independent experiments, each in triplicate. **B**, Effect of MG132 on ER α -mediated CAT expression. HeLa cells were plated in 12-well dishes at a density of 1×10^5 cells per well and cultured in hormone-free medium for 2 d. The cells were transfected with 100 ng ERE-Vit-CAT and the indicated amount of pSG5-ER α using LipofectAMINE Plus reagent. The DNA/LipofectAMINE mixture was removed 5 h later and cells were placed in hormone-free medium for 24 h. Transfected cells were treated with DMSO or MG132 ($1 \mu\text{M}$) for 1 h and then treated with 10 nM E2 for 24 h. CAT activity was determined using the colorimetric CAT ELISA kit and normalized against total cellular protein. CAT activity is expressed as the mean \pm SD of three independent experiments, each performed in triplicate. Fold increases in ERE-CAT in the presence of E2 \pm MG132 are presented in the *table*. **C**, Effect of MG132 on E2-induced down-regulation of ER α . HeLa cells were plated in 60-mm dishes at a density of 3×10^5 cells per dish and cultured in hormone-free medium for 2 d. Cells were transfected with 100 ng pSG5-ER α using LipofectAMINE Plus reagent. The DNA/LipofectAMINE mixture was removed 5 h later, and cells were placed in hormone-free medium for 24 h. The transfected cells were treated with DMSO or MG132 ($1 \mu\text{M}$) for 1 h and then treated with 10 nM E2 for 8 h. Whole-cell lysates were prepared and subjected to immunoblotting analysis using an anti-ER α antibody (Chemicon, Temecula, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

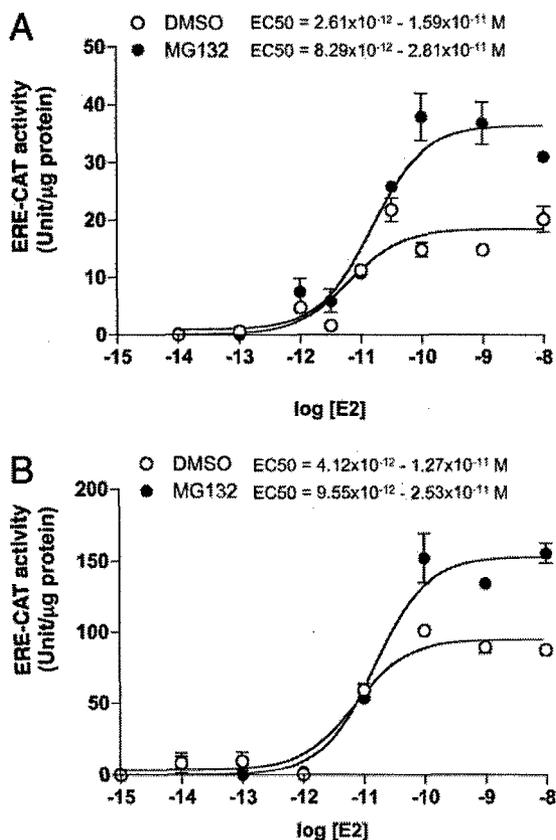


Fig. 2. Effect of MG132 on E2 Dose-Dependent Induction of Reporter Gene Expression in HeLa Cells

HeLa cells were plated in 12-well dishes at a density of 1×10^5 cells per well and cultured in hormone-free medium for 2 d. The cells were transfected with 100 ng ERE-Vit-CAT and 0.3 ng (A) or 1 ng (B) of pSG5-ER α using LipofectAMINE Plus reagent. The DNA/LipofectAMINE mixture was removed 5 h later and cells were placed in hormone-free medium for 24 h. The transfected cells were treated with DMSO or MG132 (1 μ M) for 1 h and then treated with the indicated concentration of E2 for 24 h. CAT activities were normalized against total cellular protein and expressed as mean \pm SD of three independent experiments, each performed in triplicate. EC₅₀ range was calculated with a 95% confidence.

In contrast, luciferase has an intracellular half-life of about 3 h (44), making it well suited for performing a dynamic analysis of promoter activation. Thus, we used HeLa cells transfected with ER α and ERE-pS2-Luc to study the effect of proteasome inhibition on E2-induced transcription in a time-dependent manner. In transfected HeLa cells, E2 induced a transient induction of luciferase activity, maximal at 6 h (Fig. 3A, *solid circles*). Pretreatment with MG132 decreased E2-induced luciferase expression at the early time points (1.5–6 h), but markedly increased E2-induced luciferase expression from 9–20 h (Fig. 3A, *solid triangles*).

As mentioned above, MG132 can inhibit luciferase production. To determine the effect of MG132 on luciferase synthesis in general, we transfected HeLa cells with a constitutively active luciferase construct (SV40-

Luc). In contrast to what we observed using ERE-pS2-Luc, MG132 consistently decreased the expression of SV40-Luc during the 20-h period (Fig. 4B), excluding the possibility that MG132 enhances ERE-luc activity by stabilizing luciferase protein. To subtract the general inhibitory effect of MG132 on luciferase synthesis, at each time point shown in Fig. 3C, ER α -mediated luciferase expression in the presence of MG132 was normalized to luciferase activity from the SV40-Luc construct [normalized ERE-Luc activity in the presence of MG132 = ERE-Luc activity in the presence of MG132 \times (SV40-Luc activity/SV40-Luc activity in the presence of MG132)]. The adjusted results clearly demonstrate that blocking receptor degradation with MG132 increases both the magnitude and duration of E2-induced gene transcription, suggesting that the duration of gene transcription induced by E2 is limited by ER α degradation through the 26S proteasome.

Inhibiting ER α Ubiquitination Prolongs E2-Induced Gene Transcription

In a previous study, we used a dominant-negative mutant of the NEDD8 conjugation enzyme, Ubc12C111S, to inhibit ER α ubiquitination and degradation (42). Here we used Ubc12C111S as a means to investigate the role of ER α turnover in ER α transactivation function and to corroborate our observations using MG132. The impact of Ubc12C111S on the time-dependent induction of a reporter gene by ER α was investigated. HeLa cells were transfected with pSG5-ER α and ERE-pS2-Luc, along with a control vector (pcDNA) or a construct expressing the mutant Ubc12 (pcDNA-Ubc12C111S). In cells transfected with pcDNA, E2 transiently induced luciferase expression, and maximal induction was observed at 5 h (Fig. 3D, *solid circles*). However, in cells transfected with pcDNA-Ubc12C111S, a delay in peak expression of E2-induced luciferase activity was observed (9 h; Fig. 3D, *solid triangles*), and luciferase expression remained elevated, even 20 h after E2 treatment. No effect of Ubc12C111S on maximal E2-induced luciferase activity was observed (Fig. 3D, *solid circles* vs. *solid triangles*). To confirm that the observed effect of Ubc12C111S on ER α -mediated luciferase expression was specific, luciferase activity in cells cotransfected with SV40-Luc and Ubc12C111S was assessed over time. No effect of Ubc12C111S on SV40-Luc expression was seen at 6 and 12 h after transfection; a slight increase in luciferase expression was observed at 20 h (1.3-fold; Fig. 3E). Overall, these results demonstrate that inhibiting ER α ubiquitination prolongs ER α -mediated transcription, supporting the hypothesis that proteasome-mediated degradation of ER α serves as a means to limit the duration of E2 signaling.

Blocking Polyubiquitination Sustains E2-Induced Gene Expression

To determine the effect of blocking polyubiquitination on ER α -mediated transcription, we used a Ub mutant,

UbK0, which has all of its lysines replaced by arginine. This mutant competes with endogenous ubiquitin and terminates ubiquitin chains, resulting in the accumu-

lation of short ubiquitin conjugates that cannot be degraded efficiently by the proteasome (43). First, we examined the effect of overexpressing UbK0 on E2-

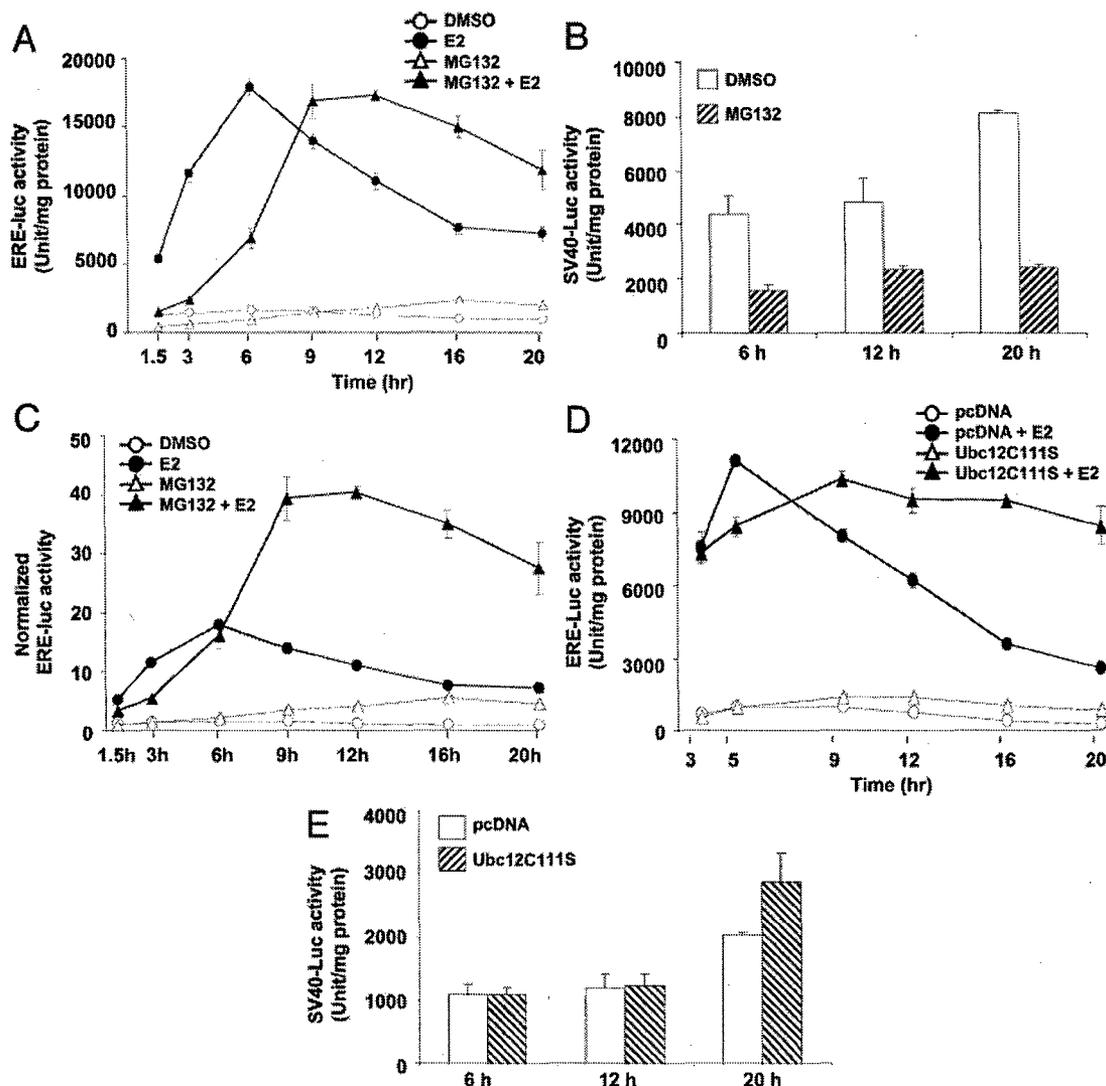


Fig. 3. Effect of Blocking ER α Turnover on Time-Dependent Induction of Reporter Gene Expression by E2 in HeLa Cells

A, Effect of MG132 on E2-induced expression of reporter gene. HeLa cells were plated in 12-well dishes at a density of 1×10^5 cells per well and cultured in hormone-free medium for 2 d. The cells were transfected with 250 ng ERE-pS2-Luc and 1 ng of pSG5-ER α using LipofectAMINE Plus reagent. The DNA/LipofectAMINE mixture was removed 5 h later, and cells were placed in hormone-free medium for 24 h. The transfected cells were treated with DMSO or MG132 ($5 \mu\text{M}$) for 1 h and then treated with 10 nM E2 for the indicated time period. Luciferase activity was determined using the Luciferase Assay System, normalized against total cellular protein. B, Effect of MG132 on SV40-Luc expression. HeLa cells were transfected with 100 ng SV40-Luc. The DNA/LipofectAMINE mixture was removed 5 h later and cells were placed in hormone-free medium containing either 0.1% vehicle (DMSO) or MG132 ($5 \mu\text{M}$) for the indicated time period. Luciferase activity was determined and normalized against total cellular protein. C, Normalized ERE-Luc activities. ER α -mediated luciferase activity in the presence of MG132 was normalized to luciferase activity from the SV40-Luc construct [Normalized ERE-Luc activity in the presence of MG132 = ERE-Luc activity in the presence of MG132 \times (SV40-Luc activity/SV40-Luc activity in the presence of MG132)]. D, Effect of overexpressing Ubc12C111S on E2-induced reporter gene expression. HeLa cells were transfected with 250 ng ERE-pS2-Luc, 1 ng pSG5-ER α , along with 100 ng pcDNA or pcDNA-Ubc12C111S, and treated with 10 nM E2 for the indicated period of time. Luc activities were normalized against total cellular protein. E, Effect of overexpressing Ubc12C111S on SV40-Luc expression. HeLa cells were transfected with 100 ng SV40-Luc, along with 100 ng pcDNA-Ubc12C111S or control vector pcDNA. The DNA/LipofectAMINE mixture was removed 5 h later, and cells were placed in hormone-free medium for the indicated time period. Luc activities were normalized against total cellular protein. For all assays, Luc activities are expressed as mean \pm SD from three independent experiments, each performed in triplicate.

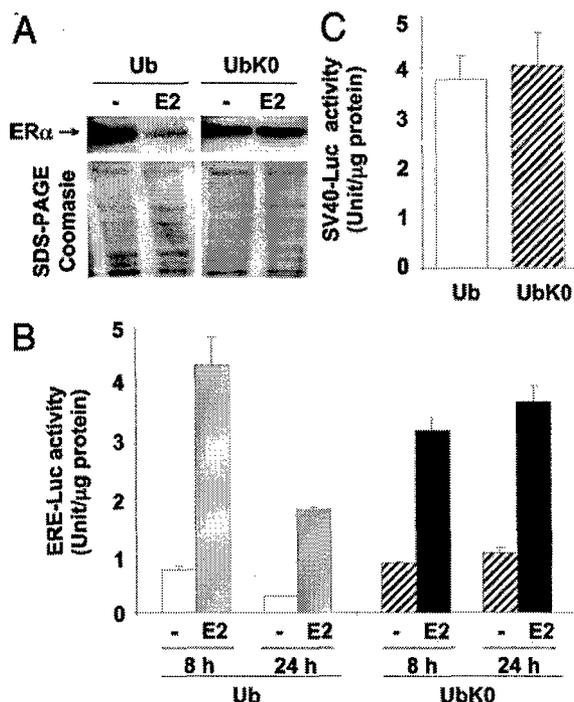


Fig. 4. Ub Mutant Blocks ER Degradation and Sustained E2-Induced Gene Expression

A, Overexpression of UbK0 blocks E2-induced ER α degradation. HeLa cells were plated in 60-mm dishes at a density of 3×10^5 cells per dish and cultured in hormone-free medium for 2 d. The cells were transfected with 150 ng pSG5-ER α , along with 150 ng pcDNA-Ub or pCS2-UbK0, using LipofectAMINE Plus reagent. The DNA/LipofectAMINE mixture was removed 5 h later, and cells were placed in hormone-free medium for 24 h before treatment with DMSO or 10 nM E2 for 8 h. Whole-cell lysates were prepared and subjected to immunoblotting analysis using an anti-ER α antibody. The Coomassie-stained SDS-PAGE gels show that equal amounts of cell lysates were loaded. **B,** Effect of UbK0 on ER α -mediated luciferase expression. HeLa cells stably transfected with ER α were plated in 12-well dishes at a density of 1×10^5 cells per well and cultured in hormone-free medium for 2 d. The cells were transfected with 250 ng ERE-pS2-Luc, along with 100 ng pcDNA-Ub or pCS2-UbK0 as indicated, using LipofectAMINE Plus reagent. The DNA/LipofectAMINE mixture was removed 5 h later, and cells were placed in hormone-free medium for 24 h before treatment with DMSO or 10 nM E2 for the indicated time period. **C,** Effect of UbK0 on luciferase expression from SV40-Luc. HeLa cells stably transfected with ER α were transfected with 100 ng SV40-Luc, along with 100 ng pcDNA-Ub or pCS2-UbK0. Five hours later, the DNA/LipofectAMINE mixture was removed, and cells were placed in hormone-free medium for the indicated time period. Luciferase activity was normalized against total cellular protein and expressed as the mean \pm SD from three independent experiments, each performed in triplicate.

induced ER α degradation. In HeLa cells cotransfected with wild-type Ub and ER α , the level of receptor protein decreased markedly after E2 treatment (Fig. 4A), accompanied by transient E2-induced expression of an ER-responsive luciferase reporter gene (Fig. 4B, 8 h

vs. 24 h). In contrast, cells transfected with UbK0 showed sustained E2-induced luciferase expression (Fig. 4B), and no decrease in ER α protein levels was observed (Fig. 4A). Furthermore, the effect of UbK0 on ER α -induced luciferase was specific, as UbK0 showed no effect on expression of the SV40-Luc construct (Fig. 4C). These results demonstrate that blocking polyubiquitination of ER α stabilizes the receptor, resulting in the prolonged expression of an ER α -responsive gene.

Proteasome Inhibition Enhances ER α -Mediated Transcription in MCF7 Breast Cancer Cells

To further investigate the role of ER α degradation in receptor transactivation ability under physiologically relevant conditions, we examined the effect of inhibiting the proteasome in MCF7 breast cancer cells, which endogenously express ER α . First, we examined the effect of MG132 on ERE-Vit-CAT expression in MCF7 cells. MCF7 cells were transiently transfected with ERE-Vit-CAT and then treated with DMSO or MG132 (1 μ M) for 1 h before E2 (10 nM) treatment. CAT activity was determined 24 h after E2 treatment. A 17.8 ± 1.7 fold increase in CAT expression was seen in MCF7 cells treated with E2, compared with the control; treatment with MG132 further increased E2-induced CAT activity to 25.6 ± 2.5 fold. Therefore, inhibiting the proteasome enhanced ER α transcriptional activity in MCF7 cells, indicating that ER α degradation plays a key role in limiting E2-induced transcriptional responses in breast cancer cells.

To determine the effect of proteasome inhibition on transcription of ER α -target genes in breast cancer cells, we pretreated MCF7 cells with MG132 and examined E2-induced pS2 gene expression. ER α regulates pS2 transcription through an imperfect palindromic ERE at position -405 to -393 of its promoter region (45); pS2 expression is considered a reliable indicator of ER α transcriptional activity (46). Time-dependent effects of MG132 on heterogeneous nuclear pS2 RNA (pS2 hnRNA) levels, which reflect the rates of pS2 gene transcription (47–50), were examined. Primers amplifying the adjoining sequence between the first intron and second exon of the pS2 gene were used, and expression of pS2 hnRNA was assessed by real-time quantitative RT-PCR (Q-PCR). After administration of E2, levels of pS2 hnRNA increased by 3 h, peaked at 12 h, and then declined by 70% during the next 8 h (Fig. 5A, gray bars). However, at all time points examined, E2-induced expression of pS2 hnRNA was markedly enhanced by pretreatment with MG132 (Fig. 5A, black vs. gray bars), and pS2 hnRNA levels declined only by 15% from 12–20 h after the combined treatment (Fig. 5A, black bars). MG132 alone showed no effect on basal pS2 hnRNA expression (Fig. 5A, hatched bars). In agreement with what we observed with pS2 hnRNA, the combined treatment of MG132 plus E2 resulted in greater expression of pS2 mRNA after 6 h, compared with E2 treatment

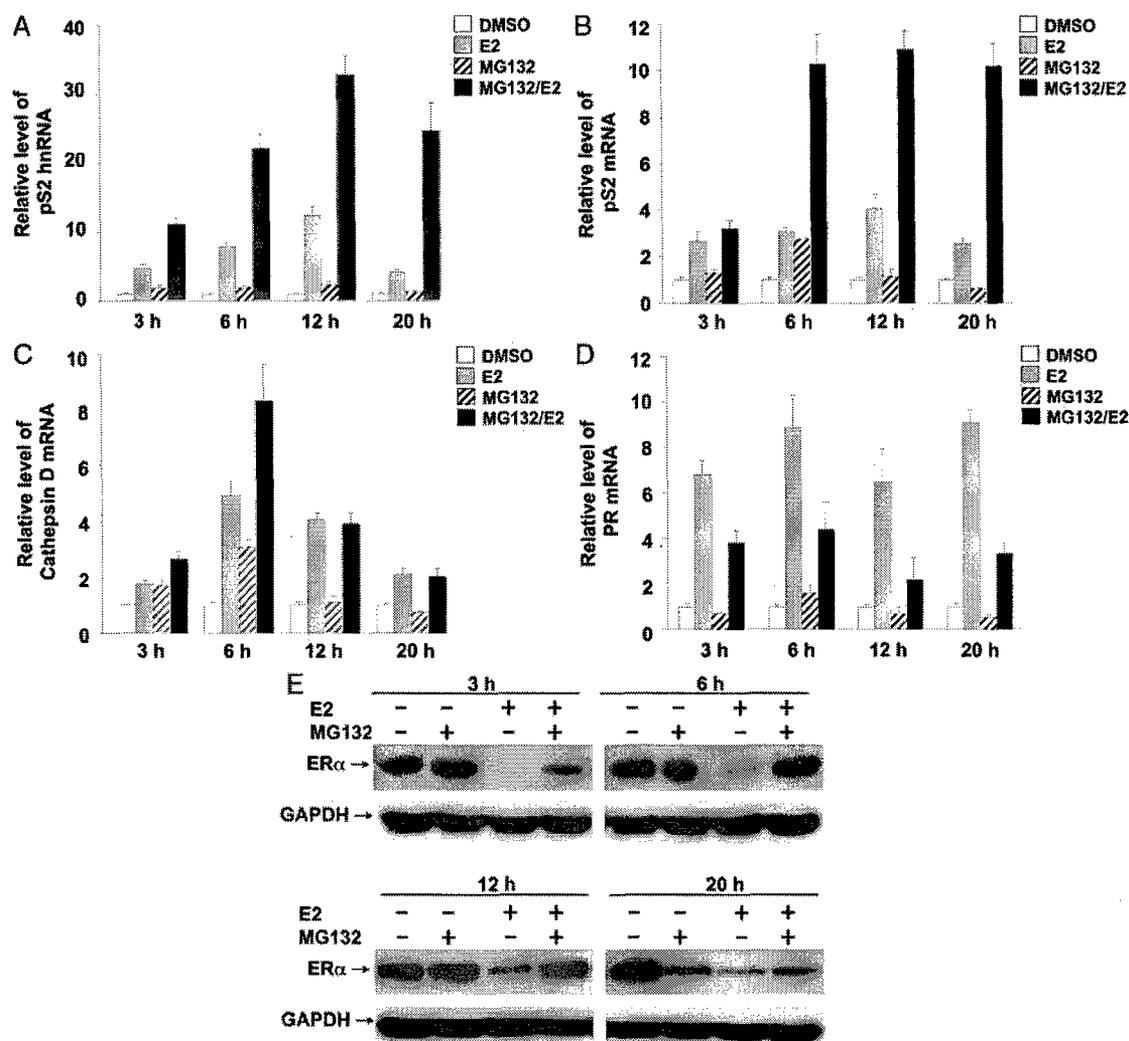


Fig. 5. Effects of MG132 on ER α -Mediated Transcription of Endogenous Target Genes in MCF7 Cells

MCF7 cells were plated at a density of 3×10^6 per 10-cm dish and allowed to grow in hormone-free medium for 3 d. The cells were pretreated with MG132 (5 μ M) for 1 h and then treated with 10 nM E2 for the indicated time periods. Total RNA was prepared and subjected to Q-PCR analysis to determine the expression levels of pS2 hnRNA (A), pS2 mRNA (B), cathepsin D mRNA (C), and PR mRNA (D). For all Q-PCR assays, the relative levels of mRNA were normalized with β -actin mRNA and standardized such that values obtained in cells treated with vehicle (DMSO) only were set to 1. The results were expressed as mean \pm SD from two independent experiments, each in duplicate. To determine the effect of MG132 on E2-induced ER degradation, MCF7 cells were treated as in panel A and subjected to whole-cell lysate preparation and immunoblotting with an anti-ER antibody (E). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

alone (Fig. 5B, *black vs. gray bars*); pS2 mRNA levels remained markedly elevated up to 20 h, the last time point examined (Fig. 5B, *black bars*). The coordinate increase in E2-induced expression of both pS2 hnRNA and pS2 mRNA by MG132 excludes the possibility that MG132 inhibits the hnRNA splicing process or stabilizes pS2 mRNA. Therefore, it seems reasonable to conclude that blocking the proteasome with MG132 enhances E2-induced pS2 transcription initiation. Together, these results demonstrate that inhibiting the proteasome increases both the magnitude and duration of E2-induced expression of the endogenous pS2 gene in breast cancer cells.

We also examined the effect of MG132 on mRNA expression of cathepsin D and progesterone receptor (PR), two well-known E2-regulated genes, in MCF7 cells. As shown in Fig. 5C, a transient increase in cathepsin D mRNA expression was observed after treatment with E2. Pretreatment with MG132 enhanced both basal and E2-induced cathepsin D expression at 3 and 6 h (Fig. 5C, *black vs. gray bars*); however, at 12 and 24 h, the effect of MG132 was no longer apparent. Treatment of MCF7 cells with E2 increased PR mRNA levels 7-fold by 3 h, and PR mRNA levels remained elevated throughout the experiment period (Fig. 5D, *gray bars*). MG132 pretreatment

decreased E2-induced expression of PR mRNA by more than 50% at all time points examined (Fig. 5D, *black vs. gray bars*), which agrees with a recent report that MG132 inhibits ER α -induced increase in PR protein levels (7). The differential effects of MG132 on these ER α -target genes demonstrate that promoter context must be considered when evaluating MG132 regulation of ER α -mediated transcription. Immunoblotting analysis showed that pretreatment with MG132 efficiently blocked E2-induced ER α down-regulation in MCF7 cells (Fig. 5E).

4-OHT Stimulates ER α -Mediated Transcription without Inducing ER α Degradation

The antiestrogen 4-OHT has been shown to up-regulate ER α levels by blocking ER α degradation (37), and previous studies have shown that 4-OHT functions as an ER α agonist in Ishikawa endometrial cancer cells (51, 52). To further examine the relationship between receptor stability and ER α -mediated transcription, we stably transfected ER α -negative Ishikawa cells with

ER α . The ER α (+) Ishikawa cells were then transfected with a luciferase reporter construct containing the human C3 promoter (C3T1-Luc) and then treated with either E2 (10 nM) or 4-OHT (1 μ M) for 16 h. After E2 administration, a 2-fold increase in luciferase activity was observed (Fig. 6A), accompanied by a marked decrease in ER α protein level (Fig. 6B). Treatment with 4-OHT also stimulated expression of luciferase (80% of E2-stimulated luciferase expression) (Fig. 6A), but the antiestrogen did not down-regulate ER α (Fig. 6B). Thus, these results demonstrate that the partial agonist activity of 4-OHT and ER α degradation are not coupled in endometrial cancer cells. It has been reported that steroid receptor coactivator 1 (SRC-1), by stimulating transcription activity of 4-OHT liganded ER α (53), can convert 4-OHT to a full agonist. We reasoned that if receptor degradation is essential for ER α to initiate transcription, SRC1 should enhance 4-OHT-stimulated ER α transactivation activity and, in parallel, induce proteasomal degradation of 4-OHT-liganded ER α . To test this reasoning, the ER α (+)Ishikawa cells

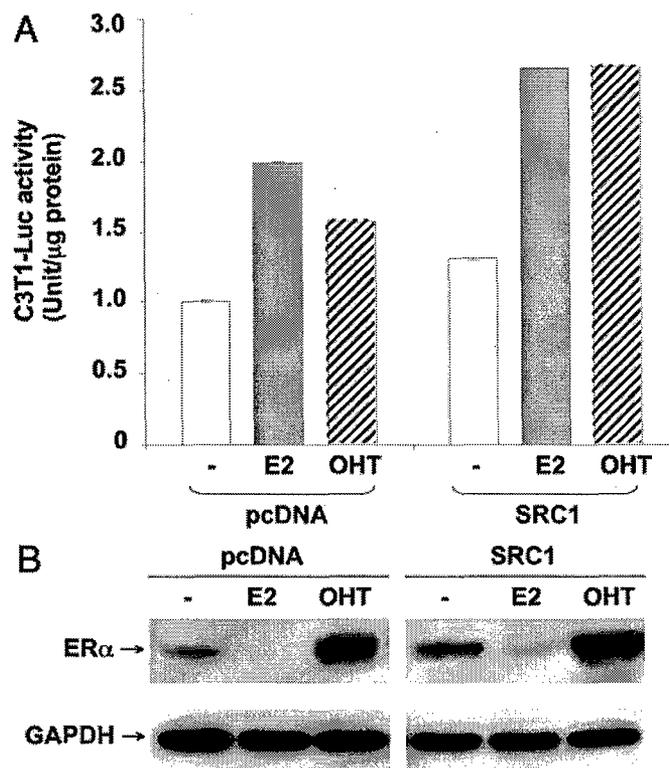


Fig. 6. Uncoupling of 4-OHT-Induced ER α Activation and ER α Degradation

A, 4-OHT stimulates ER α -mediated gene expression in Ishikawa cells. Ishikawa cells stably transfected with ER α were plated in 12-well dishes at a density of 1×10^5 cells per well and cultured in hormone-free medium for 2 d. The cells were transfected with 250 ng C3T1-Luc, along with 100 ng pcDNA or pcDNA-SRC1, using LipofectAMINE Plus reagent. The DNA/LipofectAMINE mixture was removed 5 h later, and cells were placed in hormone-free medium for 24 h before treatment with 10 nM E2 or 1 μ M 4-OHT for 16 h. Luciferase activity was normalized against total cellular protein and expressed as mean \pm sd from three independent experiments, each performed in triplicate. B, Effect of 4-OHT on ER α protein level. Ishikawa cells stably transfected with ER α were plated in 60-mm dishes at a density of 3×10^5 cells per dish and cultured in hormone-free medium for 3 d before treatment with 10 nM E2 or 1 μ M 4-OHT for 16 h. Whole-cell lysates were prepared and subjected to immunoblotting analysis using an anti-ER antibody. GAPDH was used as a loading control.

were cotransfected with a construct expressing SRC1 and C3T1-Luc and then treated with either E2 (10 nM) or 4-OHT (1 μ M) for 16 h. As expected, overexpressing SRC1 resulted in similar 4-OHT- and E2-stimulated ER α activity (Fig. 6A); however, 4-OHT did not induce receptor down-regulation (Fig. 6B). Thus, under these experimental conditions, 4-OHT, even when behaving as a full agonist in the presence of an increased level of SRC-1, did not induce ER α degradation. Taken together, these results demonstrate that ER α -mediated gene transactivation can be uncoupled from receptor degradation.

DISCUSSION

Like other rapidly turned over transcription factors, engagement of ER α in transactivation is coupled to ER α degradation by the Ub-proteasome pathway (7–11, 35). However, the functional impact of ER α degradation on cellular responses to E2 has not been well established. In this study, we analyzed the effect of blocking ER α degradation on E2-induced transcriptional output. We demonstrate that blocking ER α turnover prolongs the ability of ER α to transactivate target genes and increases the output of E2-induced gene transcription. We also show that 4-OHT can act as a full agonist in Ishikawa cells overexpressing SRC-1 to stimulate ER α transcriptional activity, without inducing receptor degradation. Furthermore, proteasome inhibition by MG132 increases ER α -mediated reporter gene expression, as well as expression of endogenous ER α -target genes (pS2 and cathepsin D), in MCF7 breast cancer cells. These data demonstrate that proteasomal degradation is not essential for ER α transcriptional activity; ER α remains functional after escaping ubiquitination and proteasomal proteolysis. An important implication of this study is that the E2-induced transcriptional response is limited by receptor degradation through the Ub-proteasome system, and defects in proteasome-mediated degradation of ER α could lead to an enhanced cellular response to E2.

In this study, several approaches targeting different steps in ubiquitination/proteasome proteolysis were used to block ER α degradation. MG132 was used to inhibit ER α proteolysis by specifically blocking activity of the 20S proteasome. A dominant-negative mutant (Ubc12C111S) of the NEDD8 conjugation enzyme was used to block ER α ubiquitination by inhibiting Ub ligase activity (41, 42). A Ub mutant with all of its lysines mutated to arginine (UbK0) was used to block ER α polyubiquitination by terminating polyubiquitin chains (43). One concern regarding the use of these approaches is a lack of specificity, such that the observed effect on enhanced E2-induced transcriptional output could be due to stabilization of multiple regulatory proteins, in addition to ER α . However, several observations suggest that this is not the case. MG132, Ubc12C111S, and UbK0 substantially enhance E2-induced, but not basal, expression of ERE reporter

genes or the endogenous pS2 gene, suggesting that the effect of these inhibitors on ER α target gene expression is hormone dependent and thus receptor dependent. Furthermore, a time-dependent effect on E2-induced gene transcription was observed, which agrees with the ability of these inhibitors to block ligand-induced ER α degradation. Finally, no time-dependent effect on SV40-Luc expression was observed, in contrast to ERE-Luc, suggesting that these inhibitors do not broadly affect gene transcription in a time-dependent manner. Therefore, we conclude that MG132, Ubc12C111S, and UbK0 enhance E2-induced gene transcription primarily by extending the lifetime of functional ER α .

Consistent with our ER α findings, proteasome inhibition has been shown to enhance the transcriptional response mediated by other nuclear receptors, including the glucocorticoid receptor (GR) (17, 24), aryl hydrocarbon receptor (18), peroxisome proliferator-activated receptor α (19), retinoid receptors (20), and the vitamin D₃ receptor (21). However, it has also been reported that MG132 decreases transcriptional activity of PR and androgen receptor (22, 23), indicating that the effect of proteasome inhibition on transcriptional activity could be receptor specific. This is presumably due to the involvement of mechanisms other than modulation of receptor levels; for example, MG132 inhibited androgen receptor activity by eliminating androgen-induced nuclear translocation and coactivator recruitment (22, 23).

In MCF7 cells, we observed differential effects of MG132 on E2-induced transcription of endogenous pS2, cathepsin D, and PR gene, suggesting that proteasome inhibition can have promoter-specific effects on gene transcription. Although the reason for this is not clear, these observations raise the intriguing possibility of a differential requirement of ER α turnover in gene transcription, such that ER α degradation is required for PR transcription, but not for pS2 and cathepsin D. However, another attractive possibility is that multiple regulatory elements, other than an ERE, could be differentially regulated by proteasome inhibition; the different structures of the PR, pS2, and cathepsin D promoters may favor this possibility. For endogenous genes, the effect of estrogen is usually mediated through cross-talk between the ERE and nearby regulatory elements, and there appears to be an inverse correlation between the influence of nearby elements and the strength of the ERE (54). The ERE sequence in pS2 promoter deviates from the consensus palindromic ERE by 1 bp and, when isolated from surrounding sequences, is able to mediate estrogen responsiveness (45); however, for the cathepsin D promoter, although the ERE-like sequence deviates from the consensus ERE by only 2 bp, it is unable to confer estrogen regulation alone and must cooperate with other regulatory elements (54). In the case of the PR promoter, only a half-site ERE is found, and estrogen induction of PR appears to require cooperation with nearby Sp1 and AP-1 sites (55). Based on the obser-

vation that ERE-Vit-CAT (Fig. 1B) and ERE-pS2-Luc (Fig. 2) activities correlate with cellular concentrations of ER α , we suggest that ER α levels are the determining factor for the transcription activity of genes controlled exclusively by ERE. We further suggest that transcriptional activity of endogenous genes driven predominantly by an ERE (e.g. pS2) may depend upon the availability of ER α . In contrast, the level of ER α is unlikely to be the sole determining factor for the transcription of genes without a consensus ERE in their complex promoters (e.g. PR). In support of this notion, it has been reported that E2-induced transcription of the PR gene does not parallel ER α occupancy (55). Therefore, it is possible that MG132 inhibits PR expression through other protein factors, either directly or indirectly. In this respect, when evaluating the transcriptional activity of ER α , after escaping proteasome degradation, promoter context must be considered. Based on our own and the results of others (50), it is plausible that the transcription rate of a gene driven predominantly by an ERE is a more reliable readout of ER α transcription activity than a gene containing a complex promoter requiring ER α plus other transcription factors.

Our results differ from a previous study by Reid *et al.* (35), showing that MG132 prevented recruitment of phosphorylated RNA pol II (p-Pol II) to the pS2 promoter. This is most likely due to different experimental conditions and endpoints used in the two studies. For example, in their study Reid *et al.* used a higher dose (10 μ M) and longer pretreatment (7 h) with MG132. However, under that condition, it is not clear whether the drug had any effect on p-Pol II recruitment to non-estrogen-responsive promoters. In addition, although α -amanitin was used to clean the pS2 promoter before p-Pol II recruitment analysis, it is not clear that gene transcription resumed immediately (within a 2 h period) after α -amanitin treatment. Thus, whether the differential recruitment of p-Pol II, in the absence or presence of MG132 after α -amanitin pretreatment, is correlated with pS2 gene transcription remains an open question. However, the observation by Reid *et al.* (35) that the 20S proteolytic subunit does not associate with the pS2 promoter in response to E2 stimulation, agrees with numerous studies showing that the 20S proteasome subunit is not required for transcription initiation and elongation (56–60). Our observation further shows that 20S proteasome activity is not essential for ER α -mediated gene transcription.

Although the mechanism(s) by which the proteasome modulates ER α -mediated transactivation remains to be fully elucidated, chromatin immunoprecipitation assays have demonstrated that both unliganded and liganded receptors constantly cycle on and off estrogen-responsive promoters (35). MG132 appears to halt this cyclic interaction, leading to prolonged occupancy of ER α on EREs (35). The cyclic turnover of ER α could be a mechanism used by cells to prevent multiple rounds of transcription initia-

tion from a single promoter, thus ensuring an appropriate cellular response to changes in circulating concentrations of hormone. To support this explanation, recent studies of GR show that proteasome inhibition dramatically increases both the residence time of GR on its target promoter and transcriptional output (24). In addition to extending the half-life of ligand-activated ER α , other factors, such as increased cellular concentration of receptor coactivators, could contribute to the enhancement of transcription by proteasome inhibition. Several ER α coactivators, including the steroid receptor coactivator family members (SRC1, SRC2, and SRC3) and cAMP response element binding protein (CREB)-binding protein/p300, are substrates of proteasomal degradation; proteasome inhibition appears to increase cellular concentrations of these coactivators (61).

We found that blocking ER α degradation (using MG132, Ubc12C111S, or Ubk0) decreases E2-induced ERE-pS2-Luc expression at earlier time points (1.5–6 h) after E2 treatment (Figs. 3 and 4). Although the reason for this is unknown, one possibility is that ubiquitination and 20S proteasome activity are required for optimal ER α activation, perhaps by facilitating the release of ER α from preexisting corepressor complexes. To fully elucidate the physiological role(s) of ubiquitination, identification of the primary Ub ligase(s) for ER α , as well as the ubiquitination site(s) in this receptor, will be necessary.

In target tissues where ER α levels are limiting, the magnitude of the response to E2 is correlated with cellular ER α concentrations (2, 62). The Ub-proteasome pathway, by modulating receptor protein turnover, could play an important role in determining cellular responses to circulating E2 levels. Our results indicate that both the magnitude and duration of E2-induced gene transcription are limited by proteasome-mediated degradation of ER α ; therefore, it seems reasonable to speculate that defects in ER α degradation could lead to enhanced cellular responsiveness to estrogens. In support of this possibility, it has been demonstrated that thyroid hormone and insulin, by blocking ligand-induced ER α degradation, can augment E2-stimulated cell proliferation (39, 63). Therefore, our future studies will examine the functional impact of proteasome-mediated ER α degradation on complex biological responses to estrogens, such as mammary gland development. In addition, aberrant ER α expression and estrogen responsiveness have been linked to breast tumor pathogenesis and development (64–66). Our previous studies demonstrate that blocking ER α degradation render breast cancer cells insensitive to the growth-inhibitory effects of ICI 182,780, a potent ER α down-regulator (42). Whether defects in the ER α degradation pathway contribute to deregulated estrogen signaling in breast cancer cells and play a role in disease progression to antiestrogen resistance remains to be elucidated.

MATERIALS AND METHODS

Plasmid Construction

The construction of pSG5-ER α (HEGO), ERE2-pS2-Luc, pcDNA-HA-Ubc12C111S, C3T1-Luc, pcDNA-SRC1, pCS2-UbK0, and ERE-Vit-CAT has been described previously (43, 67, 68).

Cell Lines

The human cervical carcinoma cell line HeLa and the breast cancer cell line MCF-7 were purchased from ATCC (Manassas, VA). The ER α -negative endometrial Ishikawa cell line was kindly provided by Dr. S. Hyder (University of Missouri, Columbia, MO). HeLa and Ishikawa cells were maintained in MEM with 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 50 U/ml penicillin, 50 μ g/ml streptomycin, and 10% fetal bovine serum. MCF7 cells were maintained in the same medium with the addition of 6 ng/ml insulin. Before experiments involving hormone treatment, cells were cultured in hormone-free medium (phenol red-free MEM with 3% dextran-coated charcoal-stripped fetal bovine serum) for 3 d.

Transient Transfection and Reporter Enzyme Assays

Cells (80% confluence) were transfected with an equal amount of total plasmid DNA (adjusted by corresponding empty vectors) by using LipofectAMINE Plus Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's guidelines. The DNA/LipofectAMINE mixture was removed 5 h later and cells were placed in hormone-free medium. Unless stated otherwise, 24 h after transfection, cells were treated with vehicle (DMSO) or MG132 (Sigma Chemical Co., St. Louis, MO) for 1 h before E2 (Sigma) treatment. At the end of the experiment, cell lysates were prepared for reporter enzyme assays. Luciferase activity was determined using the Luciferase Assay System (Promega Corp., Madison, WI). Gal activity was determined using a chemiluminescent reporter assay (PE Applied Biosystems, Foster City, CA), and CAT activity was determined using the colorimetric CAT ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN). Total cellular protein was determined by using the Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA). Reporter activities were expressed as relative light units normalized to total cellular protein.

Q-PCR

MCF7 cells were plated at a density of 3×10^6 per 10-cm dish and allowed to grow in hormone-free medium for 3 d. The cells were pretreated with MG132 (5 μ M) for 1 h before E2 (10 nM) treatment. Total RNA was prepared by a RNeasy Mini Kit (QIAGEN, Valencia, CA), according to the manufacturer's protocol. RNA (2 μ g) was reverse transcribed in a total volume of 40 μ l containing 400 U Moloney murine leukemia virus (M-MLV) reverse transcriptase (New England Biolabs, Beverly, MA), 400 ng random hexamers (Promega), 80 U RNase Inhibitor, and 1 mM deoxynucleotide triphosphates. The resulting cDNA was used in subsequent Q-PCR reactions, performed in 1 \times iQ SYBR Green Supermix (Bio-Rad) with 5 pmol forward and reverse primers. The primers used in the Q-PCR were, for pS2 mRNA: forward primer, 5'-ATAC-CATCGACGTCCCTCCA-3'; and reverse primer, 5'-AAGCGTGTCTGAGGTGTCCG-3' (69); for pS2 hnRNA: forward primer, 5'-TTGGAGAAGGAAGCTGGATGG-3' (start position 3997, within the intron); reverse primer, 5'-ACCA-CAATTCTGTCTTCACGG-3' (start position 4126, within the second exon); for PR: forward primer, 5'-TCAGTGGGCA-

GATGC TGTATTT-3'; and reverse primer, 5'-GCCACATGG-TAAGGCATAATGA-3' (70); for cathepsin D: forward primer, 5'-GTACATGATCCCCTGTGAGAGGT-3'; reverse primer, 5'-GGGACAGCTTGTAGCCTTTGC-3' (71); and for β -actin: forward primer, 5'-TGCCTGACATTAAGGAGAAG-3'; and reverse primer, 5'-GCTCGTAGCT CTTCTCCA-3'. Q-PCR was performed in 96-well optical plates (Bio-Rad) using an iCycler system (Bio-Rad) for 40 cycles (94 C for 10 sec, 60 C for 40 sec), after an initial 3-min denaturation at 94 C. The relative concentration of RNA was calculated using the $\Delta\Delta$ Ct method according to Relative Quantitation of Gene Expression (Applied Biosystems User Bulletin) with β -actin mRNA as an internal control. Results were expressed as relative RNA levels standardized such that values obtained in cells treated with vehicle (DMSO) only were set to 1.

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CHIP (Carboxyl Terminus of Hsc70-Interacting Protein) Promotes Basal and Geldanamycin-Induced Degradation of Estrogen Receptor-alpha

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Abbreviations: CHIP, carboxyl terminus of Hsc70-interacting protein; csFBS, dextran-coated charcoal-stripped fetal bovine serum; DMSO, Methyl sulfoxide; E2, 17 β -estradiol; ER α , estrogen receptor-alpha; ERE, estrogen response element; GA, geldanamycin; GFP, green fluorescence protein; HA, hemagglutinin; Hsp, heat shock protein; ICI, ICI 182,780; Luc, firefly luciferase; OHT, 4-hydroxytamoxifen; siRNA, small interference RNA; TPR, tetratricopeptide repeat.

Key Words: estrogen receptor, CHIP, geldanamycin, ubiquitin, Hsp90

ABSTRACT

20 In estrogen target cells, estrogen receptor-alpha (ER α) protein levels are strictly regulated. Although receptor turnover is a continuous process, dynamic fluctuations in receptor levels, mediated primarily by the ubiquitin-proteasome pathway, occur in response to changing cellular conditions. In the absence of ligand, ER α is sequestered within a stable chaperone protein complex consisting of heat shock protein 90 (Hsp90) and co-chaperones. However, the
25 molecular mechanism(s) regulating ER α stability and turnover remain undefined. One potential mechanism involves CHIP, the carboxyl terminus of Hsc70-interacting protein, previously shown to target Hsp90 interacting proteins for ubiquitination and proteasomal degradation. In the present study, a role for CHIP in ER α protein degradation was investigated. In ER-negative HeLa cells transfected with ER α and CHIP, ER α proteasomal degradation increased, while
30 ER α -mediated gene transcription decreased. In contrast, CHIP depletion by siRNA resulted in increased ER α accumulation and reporter gene transactivation. Transfection of mutant CHIP constructs demonstrated that both the U-box (containing ubiquitin ligase activity) and the tetratricopeptide repeat (TPR, essential for chaperone binding) domains within CHIP are required for CHIP-mediated ER α downregulation. In addition, coimmunoprecipitation assays
35 demonstrated that ER α and CHIP associate through the CHIP TPR domain. In ER α -positive breast cancer MCF7 cells, CHIP overexpression resulted in decreased levels of endogenous ER α protein and attenuation of ER α -mediated gene expression. Furthermore, ER α -CHIP interaction was stimulated by the Hsp90 inhibitor geldanamycin (GA), resulting in enhanced ER α degradation; this GA effect was further augmented by CHIP overexpression, but was abolished
40 by CHIP depletion. Finally, ER α dissociation from CHIP by various ER α ligands, including E2,

OHT, and ICI, interrupted CHIP-mediated ER α degradation. These results demonstrate a role for CHIP in both basal and GA-induced ER α degradation. Furthermore, based on our observations that CHIP promotes ER α degradation and attenuates receptor-mediated gene transcription, we suggest that CHIP, by modulating ER α stability, contributes to the regulation
45 of functional receptor levels, and thus hormone responsiveness, in estrogen target cells.

INTRODUCTION

The primary mediators of 17 β -estradiol (E2) action, the major female sex steroid hormone, are the estrogen receptors ER α and ER β . These receptors function as ligand-activated transcription factors, regulating expression of genes coordinating most physiological and many pathophysiological processes in estrogen target tissues (1). Tissue sensitivity, and the overall magnitude of response to E2 and other estrogens, is strongly influenced by a combination of factors, including cellular levels of ER α and its various coactivators and corepressors (2, 3).

To strictly control cellular responses, the cellular synthesis and turnover of the ER α protein dynamically fluctuates with changing cellular environments (4). For example, in the absence of ligand, ER α is a short-lived protein (half-life of 4-5 hours) and undergoes constant degradation (5). In the presence of ligand, by contrast, the turnover rate of ER α can be increased or decreased, depending upon the ligand, thus modulating receptor protein levels. Turnover-inducing factors and conditions include the cognate ligand E2, pure antiestrogens (ICI 164,384, ICI 182,780, RU 58,668), Hsp90 inhibitors (geldanamycin and radicicol), ATP depletion (oligomycin and hypoxia) and aryl hydrocarbon agonists; these all induce degradation and rapid downregulation of ER α levels (6-12). In contrast, the partial agonist/antagonist 4-hydroxytamoxifen (OHT), thyroid hormone, and protein kinase K activators (Forskolin, 8-bromo-cAMP) all block receptor degradation, subsequently increasing ER α protein levels (13-15).

Although both basal and ligand-induced ER α degradation are mediated by the ubiquitin-proteasome pathway (12, 13, 16-21), regulation of this pathway, at the molecular level, remains unclear. Emerging evidence suggests that multiple ER α degradation pathways exist, and the engagement of one pathway over another depends on the nature of the stimulus (19, 21-23). For example, E2-induced receptor degradation is coupled with transcription and requires new protein

70 synthesis (17, 19, 22, 24); conversely, neither ER α transcriptional activity nor new protein synthesis are needed for ICI 182,780-induced ER α degradation (19, 20, 22). In addition, various stimuli induce distinct changes in the conformation and cellular compartmentalization of ER α , these likely are associated with receptor ubiquitination (22, 25-27).

Like other members of the steroid receptor superfamily, unliganded ER α , by associating
75 with various Hsp90-based chaperone complexes, is maintained in a ligand-binding competent conformation (28). Although these associations do not influence ER α ligand-binding affinity, Hsp90 chaperone complexes appear to regulate ER α stability, as Hsp90 disruption induces rapid ER α degradation through the ubiquitin proteasome pathway (9, 28, 29). For regulation of such complexes, recent studies have identified the carboxyl terminus of Hsc70-interacting protein
80 (CHIP) as a ubiquitin ligase that directs chaperone substrates for ubiquitination and proteasomal degradation (30, 31). CHIP interacts with Hsp/Hsc70 and Hsp90 through an amino-terminal tetratricopeptide repeat (TPR) domain and catalyzes ubiquitin conjugation through a carboxyl-terminal U-box domain (30). As recent observations demonstrate that CHIP targets a number of Hsp70/90-associated proteins for ubiquitination and degradation, including the glucocorticoid
85 receptor, androgen receptor, Smad1/4, and ErbB2 (30-33), we investigated a regulatory role for CHIP in ER α stability. Our results demonstrate that CHIP, likely through a chaperone intermediate, associates with ER α and consequently facilitates both basal and geldanamycin-induced receptor degradation in human cancer cells.

90 RESULTS

Overexpression of CHIP downregulates ER α protein levels

To investigate the effect of CHIP overexpression on steady state levels of ER α , ER-negative HeLa cells were cotransfected with constructs expressing CHIP (pcDNA-His6-CHIP) and ER α (pSG5-ER α) and ER α protein levels subsequently determined by immunoblot analysis.

95 Overexpression of CHIP decreased ER α protein levels in a dose-dependent manner (Fig. 1A). To control for transfection efficiency, the green fluorescence protein (GFP) was also included in transfection. No effects of CHIP on GFP expression levels were observed (Fig. 1A), demonstrating that CHIP-induced downregulation of ER α was specific. To examine the effect of endogenous CHIP on ER α levels, we transfected HeLa cells with a CHIP-siRNA expressing
100 construct, pBS/U6/CHIPi (33). In HeLa cells transfected with CHIP, expression of CHIP-siRNA dramatically decreased CHIP expression, but showed no effect on GFP level (Fig. 1B, *upper panel*), confirming that CHIP-siRNA specifically blocks CHIP expression. In cells transfected with ER α , expression of CHIP-siRNA elevated ER α protein level (Fig. 1B, *middle panel*). In addition, expression of CHIP-siRNA attenuated CHIP-induced ER α downregulation
105 in a dose-dependent fashion (Fig. 1B, *lower panel*). Together, these results demonstrate that expression of CHIP protein alone can induce degradation of ER α and also indicate that ER α is continuously targeted for degradation by endogenous CHIP.

CHIP down-regulates ER α levels through the ubiquitin proteasome pathway

110 To determine whether proteasome activity is required for CHIP-induced ER α downregulation, HeLa cells were cotransfected with pcDNA-His6-CHIP and pSG5-ER α , treated with the proteasome inhibitor MG132, and subjected to immunoblotting. As shown in Fig. 2A,

6-hr treatment with MG132 completely blocked CHIP-induced downregulation of ER α . To examine whether polyubiquitination is required for CHIP-induced ER α degradation, a mutant ubiquitin, UbK0, with all lysines replaced by arginines (34), was utilized. Previously, we showed that the UbK0 protein could efficiently block E2-induced ER α degradation (35). Expression of UbK0, but not wild-type ubiquitin, restored ER α protein levels (Fig. 2B), demonstrating that CHIP stimulates ER α degradation through the ubiquitin and proteasome pathway.

120

CHIP targets mature ER α for degradation

It has been proposed that CHIP functions as a general ubiquitin ligase, responsible for ubiquitinating unfolded or misfolded proteins in a chaperone-dependent process (31). To examine whether ER α downregulation by CHIP was due to the selective ubiquitination of unfolded or misfolded receptor protein, we examined the effect of OHT, a selective estrogen receptor modulator, on CHIP-mediated ER α degradation. It has been shown that OHT can dissociate ER α from its chaperone complex and protect the receptor from both basal turnover and degradation induced by Hsp90-binding agents (8, 13, 21). We reasoned that if CHIP selectively targets immature or misfolded ER α (with no functional OHT-binding pocket), then, in the presence of CHIP, OHT treatment should not restore ER α levels. On the other hand, if CHIP targets mature ER α , OHT treatment should rescue the receptor protein from CHIP-induced degradation. HeLa cells were thus cotransfected with pcDNA-His6-CHIP and pSG5-ER α and treated with OHT for 6 h prior to lysate preparation. OHT treatment completely abolished CHIP-induced ER α downregulation (Fig. 2A), but had no effect on protein levels of CHIP and GFP, excluding the possibility that OHT treatment affects protein degradation in general. These

results demonstrate that CHIP induces degradation of correctly folded, ligand-binding competent ER α .

Both the TPR and U-box domains are essential for CHIP-induced ER α downregulation

140 To examine whether the ubiquitin ligase activity and chaperone interaction domain are required for CHIP-induced ER α degradation, two mutant CHIP constructs were utilized: 1) CHIP(K30A), a TPR domain mutant unable to interact with Hsp/Hsc70 or Hsp90; and 2) CHIP(H260Q), a U-box domain mutant unable to catalyze protein ubiquitin conjugation (36). In contrast to wild-type CHIP, neither CHIP(K30A) nor CHIP(H260Q) overexpression decreased
145 ER α protein levels (Fig. 3A). These results establish that both the chaperone interaction and ubiquitin ligase activity of CHIP are required for CHIP-targeted degradation of ER α protein.

The TPR domain of CHIP is required for the CHIP-ER α interaction

As CHIP appears to be linked to ER α degradation, we investigated whether CHIP
150 associates with the receptor. HeLa cells were cotransfected with ER α and CHIP, and coimmunoprecipitation analysis performed using an ER α -specific antibody. The results revealed a complex containing both CHIP and ER α (Fig. 3B). Because CHIP(K30A) exhibited no effect on ER α turnover (Fig. 3A), we examined whether the TPR domain is required for the CHIP-ER α interaction. In HeLa cells cotransfected with ER α and CHIP(K30A), the CHIP mutant was
155 not detected in the precipitated ER α complex (Fig. 3B), demonstrating a requirement for the TPR domain in the CHIP-ER α interaction. Because it is known that CHIP interacts with Hsp90 or Hsc/Hsp70 through the TPR domain (30), our results suggest that a chaperone intermediate is involved in CHIP-induced ER α degradation.

**CHIP interacts with endogenous ER α , in breast cancer cells, to induce receptor
160 ubiquitination and degradation**

Having demonstrated a role for CHIP (possibly in association with chaperones) in degradation of exogenous ER α in HeLa cells, we next examined the effect of CHIP on endogenous ER α levels. In human breast cancer MCF7 cells, overexpression of CHIP resulted in a dose-dependent ER α downregulation (Fig. 4A). Coimmunoprecipitation analysis of MCF7
165 cells transfected with pcDNA-His6-CHIP revealed both CHIP and ER α in the immunocomplexes precipitated by either an ER α -specific or anti-His6 antibody (Fig. 4B), suggesting that CHIP associates with endogenous ER α . Based on previous observations that CHIP can bind either Hsp/Hsc70 or Hsp90 (31), and that the TPR domain is essential for CHIP-ER α interaction (Fig. 3), we investigated the possibility that CHIP targets ER α for degradation by
170 associating with a chaperone-receptor complex. The association of Hsc70 and Hsp90 with ER α , in the presence or absence of overexpressed CHIP, was thus examined in MCF7 cells. Both Hsc70 and Hsp90 were detected in the precipitated ER α complex (Fig. 4B). However, CHIP overexpression slightly (but reproducibly) increased the level of Hsc70, but not Hsp90, in the ER α complex. These results indicate that CHIP may act to remodel the ER α chaperone
175 complex to favor receptor degradation.

To determine whether CHIP promotes polyubiquitination of endogenous ER α , we examined the ubiquitination status of ER α in MCF7 cells transfected with hemagglutinin-tagged ubiquitin (HA-Ub), plus a vector control (pcDNA) or a CHIP expression construct. To block proteasomal degradation of polyubiquitinated proteins, transfected cells were treated with
180 MG132 for 6 h prior to lysate preparation. An ER α -specific antibody was then used for immunoprecipitation, and the presence of ubiquitinated ER α in the immunocomplex detected by

immunoblotting with an HA antibody. To assess overall levels of protein ubiquitination, whole cell lysates were immunoblotted using an HA antibody. The polyubiquitinated ER α exhibited a typical high molecular weight smear on the blot membrane, and overexpression of CHIP
185 markedly increased smear intensity, suggesting elevated receptor polyubiquitination (Fig. 4C, *upper panel*). In contrast, CHIP had no effect on overall protein ubiquitination (Fig. 4C, *lower panel*). These results confirm that CHIP, by facilitating receptor polyubiquitination, targets endogenous ER α for proteasome-mediated degradation.

190 **CHIP downregulates ER α -mediated reporter gene expression**

Having established a role for CHIP in ER α ubiquitination and receptor turnover, we next examined the effect of CHIP on ER α -mediated gene transactivation. HeLa cells were transiently transfected with ER α and an estrogen responsive reporter (ERE-pS2-Luc), plus various CHIP (CHIP, H260Q, K30A, CHIPsiRNA) or control (pcDNA) constructs. Twenty-four hours after
195 transfection, cells were treated for 6 h with vehicle (DMSO) or E2 (10 nM) and luciferase activity then measured. In a parallel experiment, a constitutive reporter (SV40-Luc) was used to monitor transcription efficiency, as well as any general effects of the various CHIP constructs might have on luciferase expression. The ERE-pS2-Luc activities were then normalized to the corresponding SV40-Luc activities. Expression of wild type CHIP decreased (P<0.05) E2-
200 induced ERE-pS2-Luc expression, while the CHIP mutants had no effect on ER α -mediated gene transactivation (Fig. 5A). Conversely, CHIP depletion by siRNA increased both basal and E2-induced ERE-pS2-Luc expression ((P<0.01, Fig. 5B). Similarly, in MCF7 cells, overexpression of CHIP, but not U-box or TPR mutant, attenuated ER α -mediated reporter gene expression (Fig. 6A), while CHIP depletion by siRNA augmented ER α -mediated gene expression (Fig. 6B).

205 Together, these results demonstrate that CHIP targets functional ER α for degradation and thereby coordinately regulates ER α protein levels and ER α -mediated gene transactivation

Geldanamycin induces ER α degradation through a CHIP-dependent mechanism

The Hsp90 inhibitor, geldanamycin (GA), binds to the amino-terminal ATP/ADP-binding
210 domain of Hsp90, "locking" this chaperone protein in its ADP-bound conformation (9, 29, 37). CHIP has been reported to play a role in GA-induced degradation of ErbB2, a Hsp90 client protein (36, 38), and recent studies have shown that GA stimulates ER α degradation through the ubiquitin-proteasome pathway (9, 29, 37). Whether CHIP plays a role in GA-induced ER α degradation has not been previously investigated. Thus, we examined the effects of CHIP
215 overexpression and depletion on GA-induced ER α degradation. In HeLa cells transfected with ER α , GA treatment resulted in a time-dependent ER α downregulation (Fig. 7A); this effect was enhanced by CHIP overexpression (Fig. 7A). Conversely, CHIP depletion by siRNA completely abolished GA-induced ER α downregulation (Fig. 7A).

To investigate the effect of GA on CHIP-ER α interaction, HeLa cells were transfected
220 with ER α and CHIP, and coimmunoprecipitation performed with an ER α -specific antibody. The amount of CHIP in the precipitated ER α complex increased following a 1-hr GA treatment (Fig. 7B), suggesting that GA promotes ER α degradation by recruiting CHIP to the chaperone-ER α complex. Because CHIP can associate with ubiquitinated proteins through its U-box domain (31), ER α ubiquitination may play a role in the GA-induced ER α -CHIP interaction. We thus
225 examined the interaction between ER α and CHIP in the presence of the proteasome inhibitor MG132. We reasoned that, if CHIP preferentially interacts with ubiquitinated ER α , MG132, by

enhancing the accumulation of polyubiquitinated ER α , would increase the ER α -CHIP interaction. However, MG132 treatment did not increase the amount of CHIP precipitated with the ER α complex (Fig. 7B), suggesting that the GA-induced ER α -CHIP interaction occurs prior
230 to ER α polyubiquitination.

To establish a role for CHIP in GA-induced ER α degradation under physiologically relevant conditions, the consequence of CHIP depletion by siRNA on ER α degradation was examined in GA-treated MCF7 cells. In MCF7 cells transfected with a pcDNA control plasmid, GA induced rapid ER α downregulation (Fig. 8A), consistent with previous reports (9, 29).
235 However, transfection of CHIP-siRNA significantly impaired GA-induced ER α downregulation (Fig. 8A). In addition, enhanced association between endogenous CHIP and ER α was revealed by coimmunoprecipitation in MCF7 cells treated with GA (Fig. 8B). Based on these results, we suggest that CHIP is required for GA-induced ER α degradation.

240 **Effects of ligand binding on geldanamycin-induced ER α degradation**

Ligand binding results in disassembly of the ER α -Hsp90 chaperone complex, due to competition for overlapping binding sites and conformational changes within the ER α protein (28). Since GA stimulated the CHIP-ER α interaction (Fig. 7B and 8B), we investigated whether ligand binding, by interrupting the CHIP-ER α interaction, could interfere with GA-induced ER α
245 degradation by examining ER α protein levels in MCF7 cells: 1) exposed to E2, ICI 182,780 (ICI) or GA alone; 2) pretreated with vehicle, E2, OHT or ICI for 30 min, followed by a 6-h treatment with GA; and 3) pretreated with vehicle or GA for 30 min, followed by a 5.5-hr treatment with E2, OHT or ICI. As expected, E2, ICI and GA treatment, but not OHT,

dramatically downregulated ER α levels in MCF7 cells (Fig. 9A, *upper panel*). Exposure to E2
250 or OHT, either prior to (Fig. 9A, *middle panel*) or short after (Fig. 9A, *lower panel*) GA
treatment, completely abolished GA-induced ER α degradation. In contrast to what observed
with E2 and OHT, ICI exposure, neither prior to (Fig. 9A, *middle panel*) nor short after (Fig. 9A,
lower panel) GA treatment, failed to protect ER α against degradation.

To examine the effect of these ligands on the CHIP-ER α interaction,
255 coimmunoprecipitation analysis was performed on MCF7 cells transfected with CHIP. Cells
were pretreated with GA for 30 min, followed by a 30-min treatment with E2, OHT, or ICI. GA
treatment alone increased the amount of CHIP detected in the precipitated ER α complex;
however, this amount was substantially reduced by treatment with E2, OHT, or ICI (Fig. 9B).
These results demonstrate that all three ligands can interfere with the interaction between CHIP
260 and ER α . Because these ligands have dramatically different effects on ER α stability, our results
indicate that after dissociation from the Hsp90 chaperone complex, distinct downstream
pathways exist for ER α degradation. Because E2 alone can induce ER α degradation through a
transcription coupled mechanism (17, 19, 22, 24), it was somewhat unexpected to observe that
ER α was stable during the combined treatment of GA and E2 (Fig. 9A). One explanation is that
265 Hsp90 activity (inhibited by GA) is required for transcription-coupled ER α degradation. The
OHT-ER α complex lacks transcriptional activity in MCF7 cells and thus is not a substrate for the
transcription-coupled degradation pathway. Consequently, the ability of OHT to block GA-
induced ER α degradation was likely due to disruption of the CHIP-ER α interaction (Fig. 9B).
ICI also interrupted the GA-induced CHIP-ER α interaction (Fig. 9B) but failed to stabilize ER α
270 (Fig. 9A), suggesting that the ER α -ICI complex was targeted for degradation through a CHIP-
independent, GA-insensitive pathway.

Effect of CHIP and geldanamycin on ER α cellular localization

CHIP and Hsp90 are located primarily in the cytoplasm (30), while ER α is primarily a
275 nuclear-localized protein (39). To determine whether CHIP overexpression, or GA treatment,
could affect the cellular distribution of ER α . HeLa cells were transfected with a GFP-ER α
fusion protein (40) and the cellular distribution of green fluorescence examined. In control cells,
fluorescence was restricted to the nuclei (Fig. 10A, *top left panel*). CHIP coexpression or GA
treatment did not affect the nuclear localization of GFP-ER α (Fig. 10A). In contrast, ICI
280 treatment, either alone or with transfected CHIP, resulted in the presence of green fluorescence
in the cytoplasm (Fig. 10A, *bottom two panels*). This observation is consistent with a previous
study by Dauvois *et al.* showing that ICI induces cytoplasmic retention of ER α (7). In addition,
in HeLa cells transfected with GFP-ER α only, treatment with GA resulted in the appearance of
GFP foci in the nuclei of approximately 20% of transfected cells (Fig. 10A, *left middle panel*).
285 These GFP foci were not observed in GA-treated cells cotransfected with CHIP (Fig. 10A, *right
middle panel*). While the identity of the GFP foci is unknown, one possibility is that these
represent aggregated GFP-ER α , resulting from the combined effect of Hsp90 inhibition and high
expression levels of GFP-ER α . CHIP overexpression may promote both basal and GA-induced
ER α degradation, preventing GFP-ER α aggregate formation. Consistent with this interpretation,
290 we found that expression of CHIP decreased the number of GFP-ER α -expressing cells (Fig.
10B). Based on our results, and a recent finding that a small fraction of nuclear-localized CHIP
can promote nuclear protein degradation (41), we suggest that CHIP and GA-induced ER α
degradation both occur within the nucleus.

295 DISCUSSION

The cellular level of ER α determines both estrogen sensitivity and responsiveness (2, 35, 42). Steady state levels of ER α protein are tightly regulated through a rapid balance between receptor synthesis and turnover, according to changing cellular conditions (4). Although it has been well documented that ER α degradation is primarily mediated by the ubiquitin proteasome pathway, the molecular mechanism(s) by which cells regulate ER α stability are largely unknown. Here we report that the Hsc70/Hsp90-interacting protein CHIP plays a key role in both basal and Hsp90 inhibitor-induced ER α turnover. Furthermore, CHIP-induced receptor degradation occurs through the ubiquitin proteasome pathway. Overexpression of CHIP promotes ER α degradation, accompanied by a decrease in ER α -mediated gene transactivation. Conversely, inhibition of CHIP by siRNA increases ER α levels and upregulates ER α -mediated gene transactivation. Thus, this is the first report that CHIP, by modulating the cellular concentration of ER α , directly coordinates estrogen action.

During the preparation of this report, Tateishi and colleagues reported a similar finding, that CHIP plays a role in basal ER α turnover (43). Our findings agree with several conclusions from that study, including: 1) CHIP, through its TPR domain, associates with ER α -chaperone complexes; 2) CHIP promotes, through its TPR and U-box domains, both polyubiquitination and proteasomal degradation of unliganded ER α ; 3) CHIP-mediated ER α degradation occurs in the nucleus; and 4) ligand binding blocks CHIP-mediated ER α degradation by disrupting CHIP-ER α interaction. Here, we further extend the study of Tateishi *et al.* (43) in two significant aspects: 1) CHIP is required for Hsp90 inhibitor-induced ER α degradation; and 2) CHIP targets functional ER α for degradation (43).

Several lines of evidence from our study support the conclusion that CHIP targets functional ER α for degradation. First, OHT treatment completely blocked CHIP-induced ER α degradation, suggesting that ER α reaches a correctly folded conformation, competent for ligand binding, prior to CHIP-directed degradation. Secondly, CHIP overexpression downregulated ER α levels and decreased ER α -mediated gene expression, while CHIP depletion by siRNA upregulated ER α levels and increased ER α -mediated gene transcription. This coordinate regulation of ER α levels and activity suggests that CHIP targets functional ER α for degradation. Thirdly, CHIP plays a role in GA-induced ER α degradation by primarily targeting Hsp90-associated, transcriptionally competent ER α (29). Although originally believed to function as a general ubiquitin ligase, responsible for ubiquitinating unfolded or misfolded proteins in a chaperone-dependent process (31), more recent studies have demonstrated that CHIP also targets mature Hsp90 client proteins for degradation (33, 36).

Tateishi *et al.* (44) observed that CHIP overexpression increased ER α transcriptional activity. Although this was not observed in our study, the use of different estrogen-response element (ERE) and control reporter constructs for the functional analyses of ER α could account for this discrepancy. In the present study, an estrogen-responsive reporter construct (ERE-pS2-Luc), possessing two ERE copies within the pS2 promoter (44), was used. Our previous study demonstrated a close correlation between ERE-pS2-Luc expression and cellular concentration of ER α (35). In the present study, we also utilized a constitutively active construct, SV40-Luc, to monitor and normalize the effects of both CHIP and CHIP-siRNA on transfection efficiency and luciferase expression. In the study by Tateishi *et al.*, pRSV β Gal was used as an internal control (44). When we used a similar construct, CMV β Gal, we found that overexpression of either wild-type CHIP or TPR mutant (K30A), but not U-box mutant (H260Q), dramatically decreased

340 CMV β Gal expression in a dose-dependent manner (data not shown). Based on these observations, we suggest that β Gal is not a suitable control reporter for studying the effect of CHIP on gene transcription.

Our results, with data from Tateishi *et al.* (44), suggest a role for the Hsp90 chaperone complex in the regulation of cellular ER α levels. A summary of distinct ER α degradation pathways is depicted in Fig. 11. In the absence of activation signals, CHIP constantly targets 345 chaperone-associated ER α for degradation, thereby limiting cellular concentrations of receptor protein. Ligand binding disassembles the ER α -Hsp90 complex and thus protects ER α from CHIP-mediated degradation. However, depending on the ligand, ER α stability can vary considerably, suggesting that different downstream destructive pathways exist. Furthermore, the ER α -ligand interaction could play a definitive role in pathway utilization. For example, when 350 ER α -ligand interaction could play a definitive role in pathway utilization. For example, when activated by E2, ER α is degraded through a transcription-coupled mechanism (17, 19, 22, 24). Pretreatment with GA, however, abolished E2-induced ER α degradation (Fig. 9A), suggesting that Hsp90 activity is required for transcription-coupled ER α degradation. In support of this possibility, the Hsp90-p23 complex has been shown to play a role in disassembling the nuclear 355 receptor transcriptional complex from chromatin, a process believed to be a prerequisite for degradation of activated transcription factors (45-47). Conversely, through an unknown mechanism, the ER α -ICI complex is targeted for rapid degradation in MCF7 cells (48), in association with a cellular redistribution and aggregation of ER α (7, 8, 22, 27). Together, with our previous observation that an intact NEDD8 conjugation pathway is essential for ICI-induced 360 ER α degradation in breast cancer cells (49), we suggest that destruction of the ICI-liganded receptor requires a cullin-based ubiquitin ligase.

Abnormal expression of ER α has long been associated with both the initiation and progression of breast cancer (50). An increase in the number of ER α -positive cells, as well as increased individual cell ER α content, have frequently been detected in malignant breast tumors (42). Furthermore, increased ER α content has been shown to augment the magnitude of estrogen-stimulated gene expression, providing a growth advantage to breast cancer cells (2, 35, 49, 51). A recent study demonstrated a correlation between the loss of ERK7, a regulator of estrogen-induced ER α degradation, and breast cancer progression (52). Collectively, these observations indicate that alterations in ER α degradation pathways may contribute to deregulation of ER α , perhaps leading to enhanced estrogen action in breast tumors. Based on our results, the chaperone/CHIP pathway, by regulating ER α levels, likely contributes to the development/progression of that disease; and such a possible role merits further examination.

MATERIALS AND METHODS

Materials

375 The following antibodies and reagents were used in this study: anti-ER α (HC20) and anti- β -tubulin (SC9104) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-HA tag (3F10; Roche Molecular Biochemicals, Indianapolis, IN); anti-ER α (Ab-10) and anti-GFP (GFP01) (NeoMarkers, Inc., Fremont, CA); anti-GAPDH (Chemicon International, Inc., Temecula, CA); anti-CHIP (PA1-015, Affinity Bioreagents, Golden, CO); anti-Hsp90 (SPA-830) and anti-Hsc70
380 (SPA-816) (Stressgene, Victoria, BC, Canada); anti-His6 (8906-1, BD Biosciences, Palo, Alto, CA); protein G-agarose beads (Oncogene Research Products, San Diego, CA); horseradish peroxidase-conjugated second antibodies and SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL); protein assay kit (Bio-Rad laboratories Inc., Hercules, CA); protease inhibitor cocktail set III (Calbiochem-Novabiochem Corporation, San Diego, CA);
385 LipofectAMINE Plus Reagent (Life Technologies, Inc., Logan, UT); FuGENE (Roche Molecular Biochemicals, Indianapolis, IN); 17 β -estradiol, 4-hydroxytamoxifen, geldanamycin and MG132 (Sigma Chemical Co., St. Louis, MO); ICI 182,780 (Tocris Cookson Ltd., Ellisville, MO); Passive lysis buffer and luciferase assay system (Promega Corp., Medison, WI); fetal bovine serum (FBS) and dextran-coated charcoal-stripped FBS (csFBS) (Hyclone
390 laboratories, Inc., Logan, Utah); cell culture supplementary reagents (Life Technologies, Inc.; Rockville, MD).

Plasmid Construction

The construction of pSG5-ER α (HEGO), ERE2-pS2-Luc, SV40-Luc, pcDNA-HA-Ub, pCS2-
395 UbK0 and CMV-GFP have all been described previously (35). The pcDNA-His6-CHIP,

pcDNA-His6-CHIP(K30A), and pcDNA-CHIP(H260Q) constructs were kindly provided by Dr. Neckers and Cam Patterson (36), the pBS/U6/CHIPi construct by Dr. Chang (33), and the GFP-ER α construct by Dr. Stenoien (40).

400 **Cell Lines and Transient Transfection**

The human cervical carcinoma cell line HeLa and the breast cancer cell line MCF-7 were purchased from ATCC (Manassas, VA). HeLa cells were maintained in minimum essential medium (MEM) with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 50 units/ml penicillin, 50 μ g/ml streptomycin, and 10%
405 FBS. MCF7 cells were maintained in the same medium, with the addition of 6 ng/ml insulin. Prior to experiments, cells were cultured in hormone-free medium (phenol red free MEM with 3% dextran-coated charcoal-stripped FBS (csFBS)) for 3 days. For transfection, cells (80% confluence) were transfected with an equal amount of total plasmid DNA (adjusted with the corresponding empty vectors) by using LipofectAMINE Plus Reagent or FuGENE according to
410 the manufacturer's guidelines.

Immunoblotting, Immunoprecipitation and Luciferase Assay

For immunoblot analysis, whole cell extracts were prepared by suspending cells ($\sim 2 \times 10^6$) in 0.1 ml SDS lysis buffer (62 mM Tris, pH 6.8, 2% SDS, 10% Triton X-100, and protease inhibitor
415 cocktail III). After 15 min incubation on ice, extracts were sonicated (3 x 20 sec), insoluble material removed by centrifugation (15 min at 12,000 x g), and supernatant protein concentration determined using a BioRad protein assay kit. Five percent β -mercaptoethanol was added to the protein extracts before heating at 90 $^{\circ}$ C for 5 min. Protein extracts (50 μ g per lane) were

fractionated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and
420 probed with antibodies. Primary antibody was detected by horseradish peroxidase-conjugated
second antibody and visualized using an enhanced SuperSignal West Pico Chemiluminescent
Substrate. The band density of exposed films was evaluated with ImageJ software
(<http://rsb.info.nih.gov/ij/>). Immunoprecipitation was performed as described previously (49).
For luciferase assays, cell lysates were prepared with passive lysis buffer and luciferase activity
425 determined using the Luciferase Assay System.

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FIGURE LEGENDS

575 **Fig. 1.** Overexpression of CHIP downregulates ER α protein levels.

A. Overexpression of CHIP downregulates ER α protein levels. HeLa cells were transfected with 250 ng pSG5-ER α , 100 ng CMV-GFP, and various amounts (0, 50, 100 and 250 ng) of pcDNA-his6-CHIP. Protein levels of ER α , CHIP and GFP were determined by immunoblotting with anti-ER α , anti-His6 and anti-GFP, respectively. GFP expression was used as a control for
580 transfection efficiency and SDS-PAGE loading. *B. Expression of CHIP-siRNA attenuates CHIP-induced ER α downregulation.* HeLa cells were transfected with various constructs (250 ng for each) as indicated, and protein levels were determined by immunoblot analysis. GAPDH was used as an SDS-PAGE loading control. For all experiments, 3×10^5 HeLa cells were plated in 60-mm dishes, cultured in hormone-free medium for 3 days, and then transfected with
585 LipofectAMINE Plus Reagent. Cell lysates were prepared 24 hr after transfection. Representative results of two independent experiments, each performed in duplicate, are shown.

Fig. 2. The proteasome inhibitor MG132, partial ER α -antagonist OHT, and ubiquitin mutant
590 UbK0, all block CHIP-induced ER α degradation.

A. The proteasome inhibitor MG132 and the partial ER α antagonist OHT block CHIP-induced ER α downregulation. HeLa cells were transfected with 250 ng pSG5-ER α and 100 ng CMV-GFP, along with 250 ng pcDNA (vector control) or pcDNA-His6-CHIP, then treated with DMSO (vehicle), 10 μ M MG312 or 1 μ M OHT for 6 hr prior to immunoblot analysis. Protein
595 levels of ER α , CHIP and GFP were determined by immunoblotting with anti-ER α , anti-His6 and anti-GFP, respectively. GFP was used as a control for transfection efficiency and SDS-PAGE

loading. *B. Expression of the ubiquitin mutant UbK0 blocks CHIP-induced ER α downregulation.* HeLa cells were transfected with 250 ng pSG5-ER α , with or without 250 ng pcDNA-His6-CHIP, pcDNA-Ub or pCS2-UbK0, as indicated. ER α protein levels were
600 determined by immunoblotting with anti-ER α . GAPDH was used as an SDS-PAGE loading control. For all experiments, 3×10^5 HeLa cells were plated in 60-mm dishes, cultured in hormone-free medium for 3 days, and then transfected with LipofectAMINE Plus Reagent. Cell lysates were prepared 24 hr after transfection. The band density of exposed films was evaluated with ImageJ software. Relative ER α levels were presented as the mean \pm SE of three
605 independent experiments, each performed in duplicate.

Fig. 3. Both the TRP and U-box domains are required for CHIP-induced ER α downregulation.

A. Both the TRP and U-box domains are required for CHIP to downregulate ER α . HeLa cells were transfected with 250 ng pSG5-ER α , 100 ng CMV-GFP, along with 250 pcDNA (control)
610 or various CHIP constructs as indicated. ER α and GFP protein levels were determined by immunoblotting with anti-ER α and anti-GFP, respectively. GFP was used as control for transfection efficiency and SDS-PAGE loading. *B. The TRP domain is required for CHIP-ER α interaction.* HeLa cells were transfected with 250 ng pSG5-ER α , along with 250 ng pcDNA-His6-CHIP or pcDNA-His6-CHIP(K30A). ER α protein in cell lysates was precipitated with
615 anti-ER α . The presence of CHIP in the precipitated ER α complex was determined by immunoblotting with anti-His6. The same blot was re-probed with anti-ER α to assess the amount of ER α in the precipitated immunocomplex. The expression levels of CHIP or CHIP(K30A) in whole cell lysates was determined by immunoblotting with anti-His6 (lower

panel). For all experiments, HeLa cells were plated in 60-mm dishes at a density of 3×10^5 cells/dish, cultured in hormone-free medium for 3 days, and then transfected with LipofectAMINE Plus Reagent. Cell lysates were prepared 24 hr after transfection. Representative results of two independent experiments, each performed in duplicate, are shown.

Fig. 4. CHIP interacts with endogenous ER α and induces ER α ubiquitination and degradation in breast cancer MCF7 cells.

A. Overexpression of CHIP downregulates endogenous ER α levels in MCF7 cells. MCF7 cells were plated in 100-mm dishes at a density of 1×10^6 cells/dish, cultured in hormone-free medium for 3 days, and transfected with various amounts (0, 5 or 10 μ g) of pcDNA-His6-CHIP using FuGENE. Twenty four hours after transfection, whole cell lysates were prepared, and protein levels of ER α and CHIP determined by immunoblotting with anti-ER α and anti-His6, respectively. GAPDH was used as an SDS-PAGE loading control. *B. CHIP associates with ER α -Hsp complex in MCF7 cells.* MCF7 cells were transfected as in *A* and subjected to coimmunoprecipitation analysis. ER α and CHIP were precipitated with anti-ER α and anti-His6, respectively. The presence of CHIP, Hsc70, Hsp90 or ER α in the precipitated complexes was determined by immunoblotting with anti-His6, anti-Hsc70, anti-Hsp90, or anti-ER α , respectively. *C. Expression of CHIP enhances endogenous ER α polyubiquitination in MCF7 cells.* MCF7 cells were plated in 60-mm dishes at a density of 5×10^5 cells/dish cultured in hormone-free medium for 3 days, and transfected with 250 ng pcDNA-HA-Ub and 250 ng pcDNA or pcDNA-His6-CHIP. Twenty four hours after transfection, whole cell lysates were prepared and ER α protein was precipitated with anti-ER α . The presence of ubiquitin-conjugated

ER α in the immunocomplex was detected by immunoblotting with anti-HA (upper panel). The same membrane was reprobbed with anti-ER α to assess the amount of precipitated ER α (middle panel). Whole cell lysates were separated by SDS-PAGE and probed with HA antibody to determine the amount of total ubiquitinated proteins (lower panel). Representative results of two
645 independent experiments, each performed in duplicate, are shown.

Fig. 5. CHIP downregulates ER α -mediated reporter gene expression in HeLa cells.

HeLa cells were plated in 12-well dishes at a density of 1×10^5 /well, grown in hormone-free medium for 3 days, and transfected with 10 ng pSG5-ER α , 250 ng ERE-pS2-Luc, 250 ng
650 various CHIP constructs (A) or pBS/U6/CHIPi (B). Twenty four hours after transfection, cells were treated for 6 hr with DMSO or 10 nM E2 and then assayed for luciferase activity. The ERE-pS2-Luc activity was normalized to SV40-Luc activity, which was determined in a parallel experiment where ERE-pS2-Luc was replaced with SV40-Luc. The results are expressed as means \pm SE from three independent experiments, with each performed in quadruplicate. *
655 $p < 0.05$ (student's t-test, vs pcDNA treated with E2).

Fig. 6. CHIP downregulates ER α -mediated reporter gene expression in MCF7 cells.

MCF7 cells were plated in 12-well dishes at a density of 1×10^5 /well, grown in hormone-free medium for 3 days, and transfected with 250 ng ERE-pS2-Luc, along with 250 ng various CHIP
660 constructs (A), or various amounts (250, and 500 ng) of pBS/U6/CHIPi (B). Twenty four hours after transfection, cells were treated for 6 hr with DMSO or 10 nM E2 and assayed for luciferase. The ERE-pS2-Luc activity was normalized to SV40-Luc activity, which was determined in a parallel experiment where ERE-pS2-Luc was replaced with SV40-Luc. The results were

expressed as mean \pm SE from three independent experiments, each performed in quadruplicate. *

665 $p < 0.05$ (student's t-test, vs pcDNA treated with E2).

Fig. 7. Disruption of Hsp90 function induces ER α degradation through a CHIP-dependent mechanism in HeLa cells.

A. CHIP overexpression augments, while CHIP depletion by siRNA blocks, GA-induced ER α

670 *degradation.* HeLa cells were plated in 60-mm dishes at a density of 3×10^5 cells/dish, cultured in hormone-free medium for 3 days, and transfected with 250 ng pSG5-ER α , along with 250 ng pcDNA, pcDNA-His6-CHIP or pBS/U6/CHIPi by using LipofectAMINE Plus Reagent. Twenty four hours after transfection, the cells were treated with 1 μ M GA for 0, 0.5, 1 and 3 hr. Cell lysates were immunoblotted with anti-ER α . GAPDH was used as an SDS-PAGE loading
675 control. The band density of exposed films was evaluated with ImageJ software. Relative ER α

levels were presented as mean \pm SE from three independent experiments. *B. GA enhances*

CHIP-ER α interaction. HeLa cells were plated as in *A* and transfected with 250 ng pSG5-ER α

and 250 ng pcDNA-His6-CHIP. Twenty four hours after transfection, cells were untreated or treated with 1 μ M GA or 10 mM MG132 for 1 hr prior to lysate preparation. ER α protein was

680 precipitated by anti-ER α and the presence of CHIP determined by immunoblotting with anti-His6. The same membrane was then re-probed with anti-ER α to assess the amount of precipitated ER α in the same complex. Representative results of three independent experiments, each performed in duplicate, are shown.

685 **Fig. 8.** CHIP is required for geldanamycin-induced ER α degradation in breast cancer MCF7 cells.

A. CHIP depletion by CHIP-siRNA eliminates GA-induced ER α degradation. MCF7 cells were plated in 60-mm dishes at a density of 3×10^5 cells/dish, cultured in hormone-free medium for 3 days, and transfected with 500 ng pcDNA (control) or pBS/U6/CHIPi. Twenty four hours after
690 transfection, cells were treated with 1 μ M GA for 0, 1, 2.5 and 4 hr, and subjected to immunoblotting with anti-ER α . β -tubulin was used as SDS-PAGE loading control. The band density of exposed films was evaluated with ImageJ software. Relative ER α levels are presented as mean \pm SE from three independent experiments (lower panel). *B. GA stimulates CHIP-ER α interaction.* MCF7 cells were plated at 1×10^6 cells in 100-mm dishes, cultured in hormone-free
695 medium for 3 days, and treated with 1 μ M GA for 0, 1 and 3 hr prior to lysate preparation. ER α protein was precipitated by anti-ER α and the presence of CHIP examined by immunoblotting with anti-CHIP. The same membrane was then reprobbed with ER α antibody to assess the amount of precipitated ER α in the same complex. Representative results of two independent experiments, each performed in duplicate, are shown.

700

Fig. 9. Effect of ligand binding on geldanamycin-induced ER α degradation

A. ER α protein levels in MCF7 cells treated with GA before or after ligand exposure. MCF7 cells were plated in 60-mm dishes at a density of 3×10^5 cells/dish and cultured in hormone-free medium for 3 days. *Upper panel*, cells were treated with vehicle, 10 nM E2, 1 μ M OHT, 100
705 nM ICI or 1 μ M GA for 6 hr; *Middle panel*, cells were exposed to indicated ligand for 30 min prior to a 6-hr GA treatment; *Lower panel*, 30 min after GA treatment, cells were exposed to

indicated ligand for 5.5 hr. For all experiments, ER α levels were determined by immunoblotting with anti-ER α . β -tubulin was used as SDS-PAGE loading control. B. *Effect of ligands on GA-induced CHIP-ER α interaction.* MCF7 cells were plated in 100-mm dishes at a density of 1×10^6 cells/dish, cultured in hormone-free medium for 3 days, and then transfected with 5 μ g pcDNA-His6-CHIP by using FuGENE. Twenty four hours after transfection, the cells were treated with 1 μ M GA for 30 min, followed by a 30-min treatment with indicated ligands (100 nM E2, 1 μ M OHT and 100 nM ICI). ER α protein from the cell lysates was precipitated using anti-ER α . CHIP presence in the precipitated ER α complex was determined by immunoblotting with anti-His6. The same membrane was re-probed with ER α antibody to assess the amount of precipitated ER α . Representative results of two independent experiments, each performed in duplicate, are shown.

Fig. 10. Effect of CHIP and geldanamycin on ER α cellular localization.

HeLa cells were plated in 6-well dishes at a density of 1×10^5 cells/dish, cultured in hormone-free medium for 3 days, and transfected with 250 ng GFP-ER α and 250 ng pcDNA or pcDNA-His6-CHIP by using LipofectAMINE Plus Reagent. Twenty four hours after transfection, the transfected cells were treated with 1 μ M GA or 100 nM ICI for 6 hr. The fluorescence of GFP-ER α was then examined using an inverted microscope (Axiovert 40 CFL) (A). The number of cells expressing GFP-ER α from 10 microscope fields was present in the histogram (B). Representative results of two independent experiments, each performed in triplicate, are shown.

Fig. 11. Schematic summary of distinct ER α degradation pathways.

In the absence of activation signals, CHIP constantly targets Hsp90-associated ER α for
730 degradation. Ligand binding disassembles the ER α -Hsp90 complex and thus protects ER α from
CHIP-mediated degradation. However, depending upon the ligand, distinct downstream
destructive pathways are engaged in the degradation of liganded ER α . When activated by E2,
ER α is degraded through a transcription-coupled mechanism. The ER α -ICI complex is targeted
for a rapid degradation through a mechanism associated with cellular redistribution and
735 aggregation of ER α . The OHT-ER α complexes are stable, likely due to the lack of
transcriptional activity.

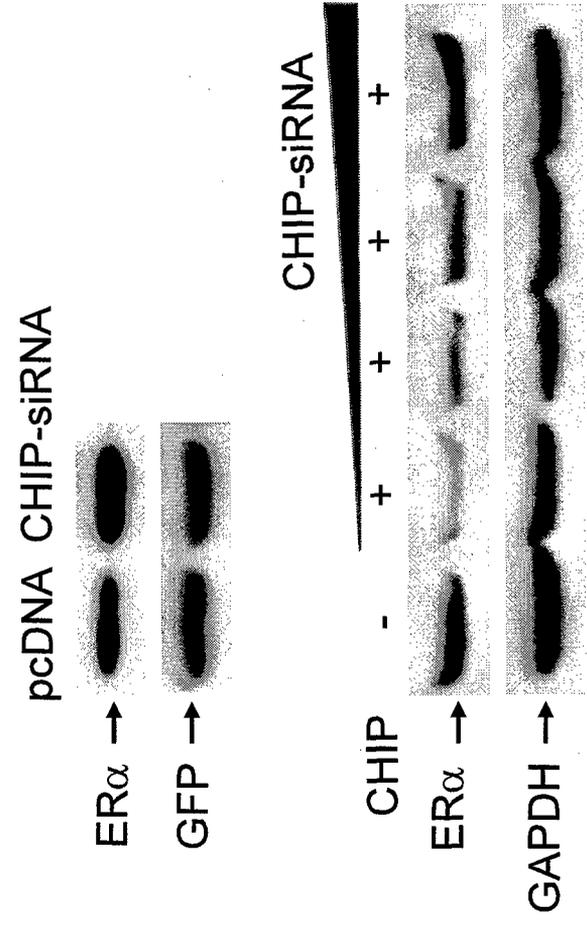
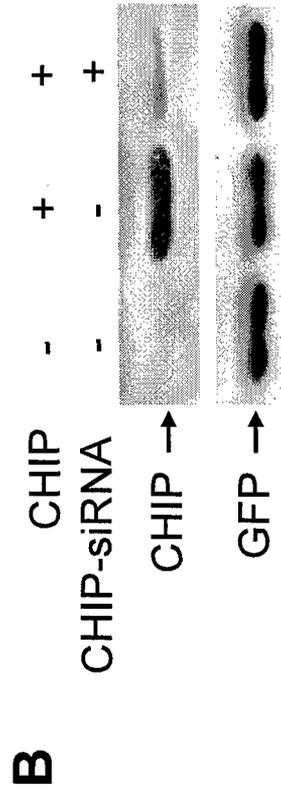
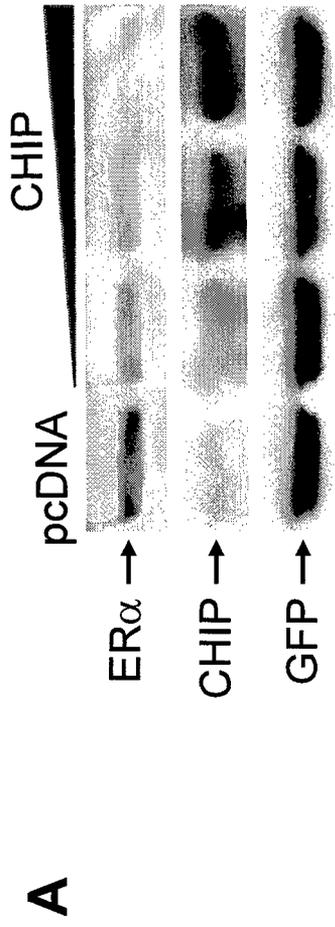


Fig. 1

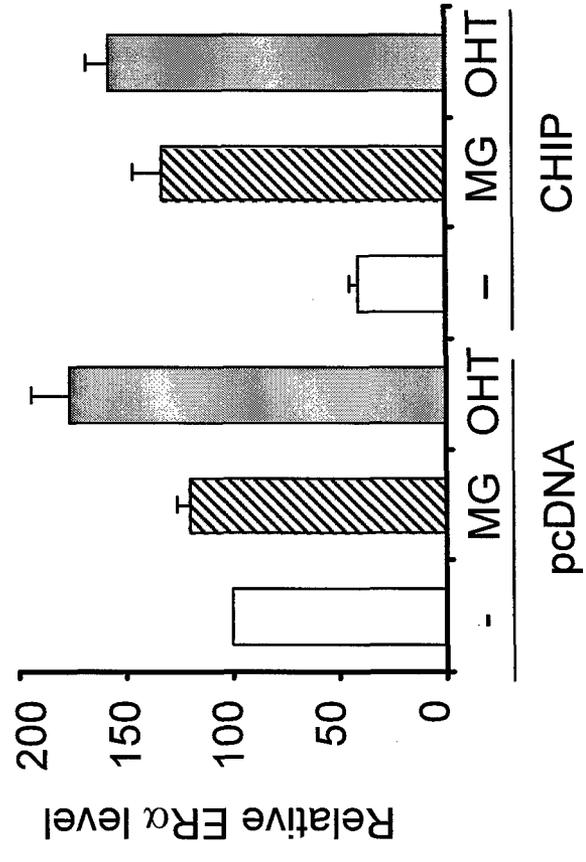
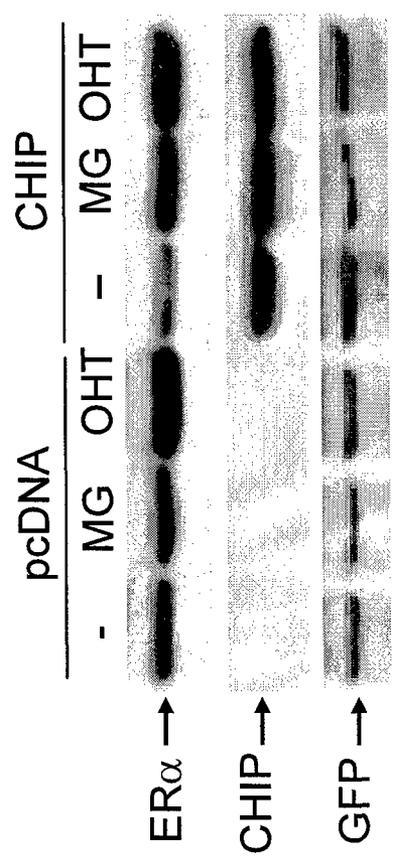
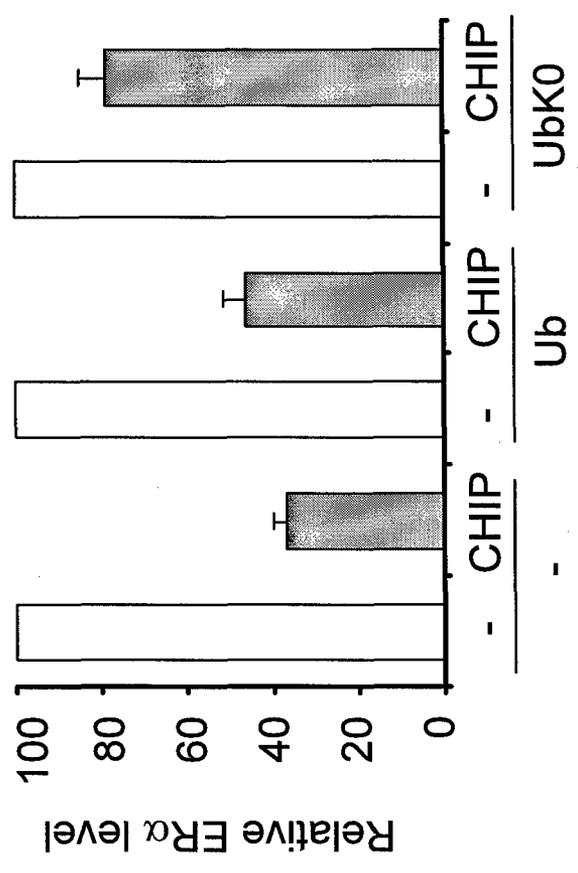
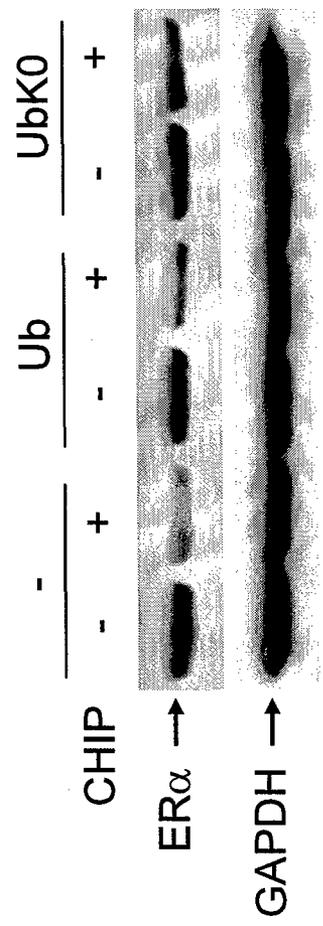
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Fig. 2

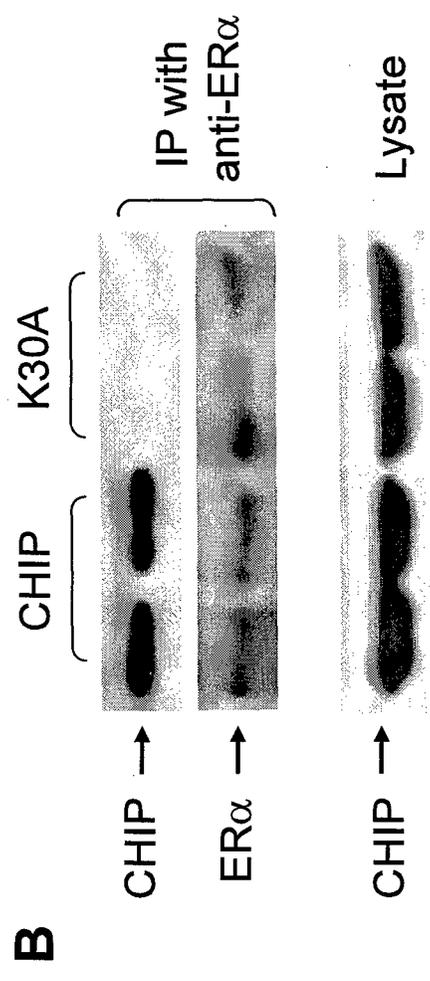
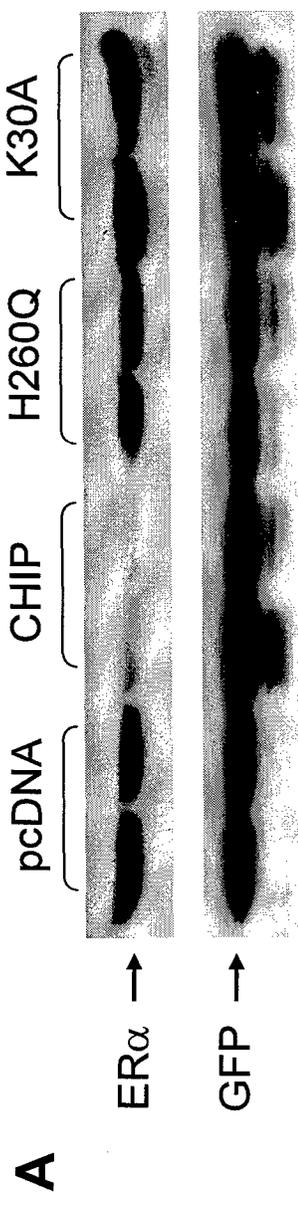


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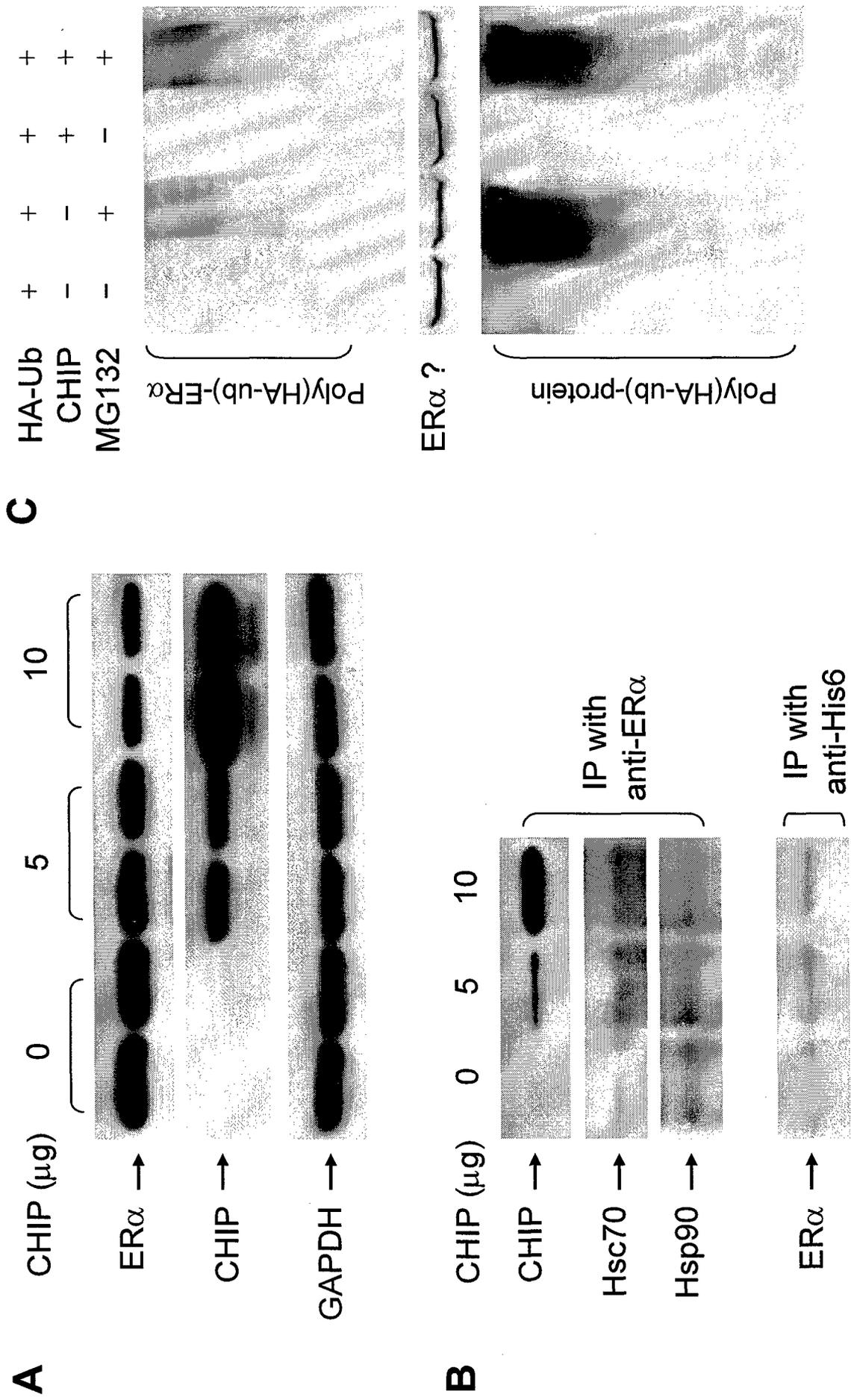


Fig. 4

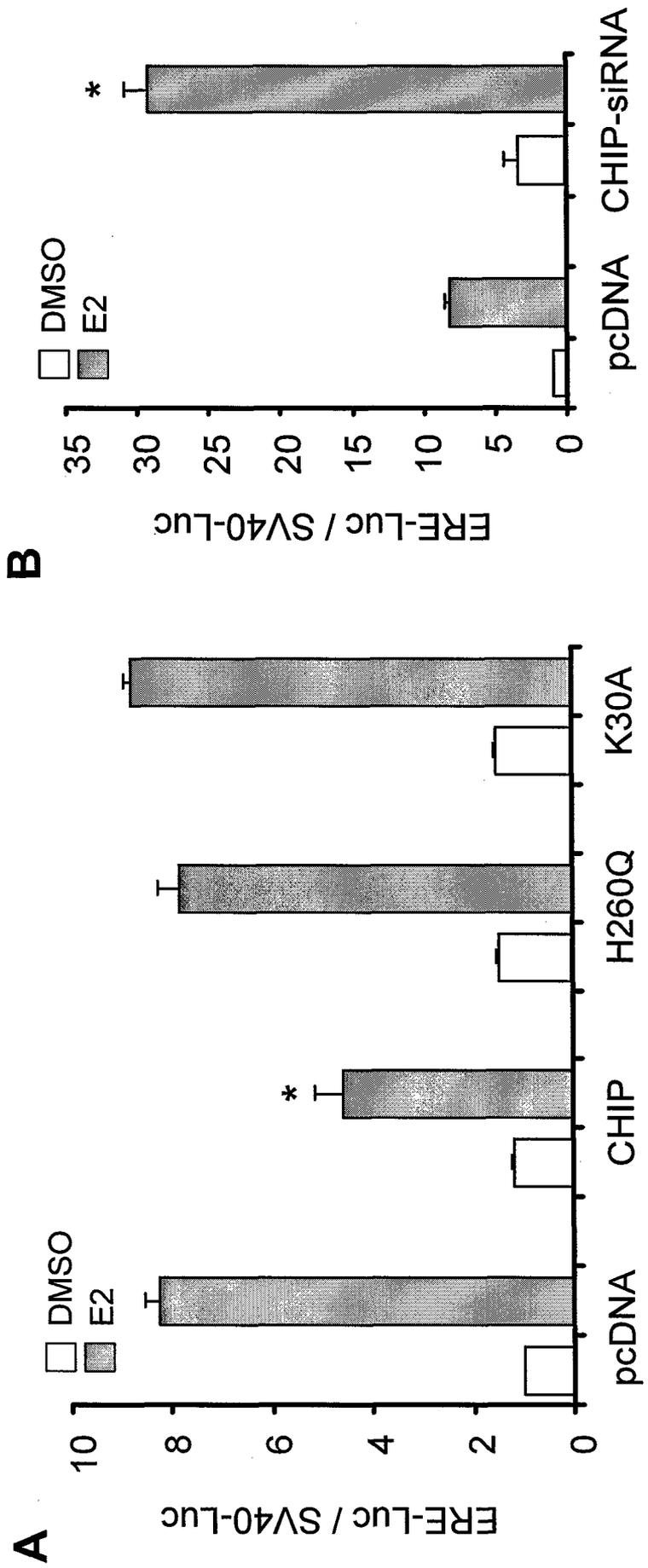


Fig. 5

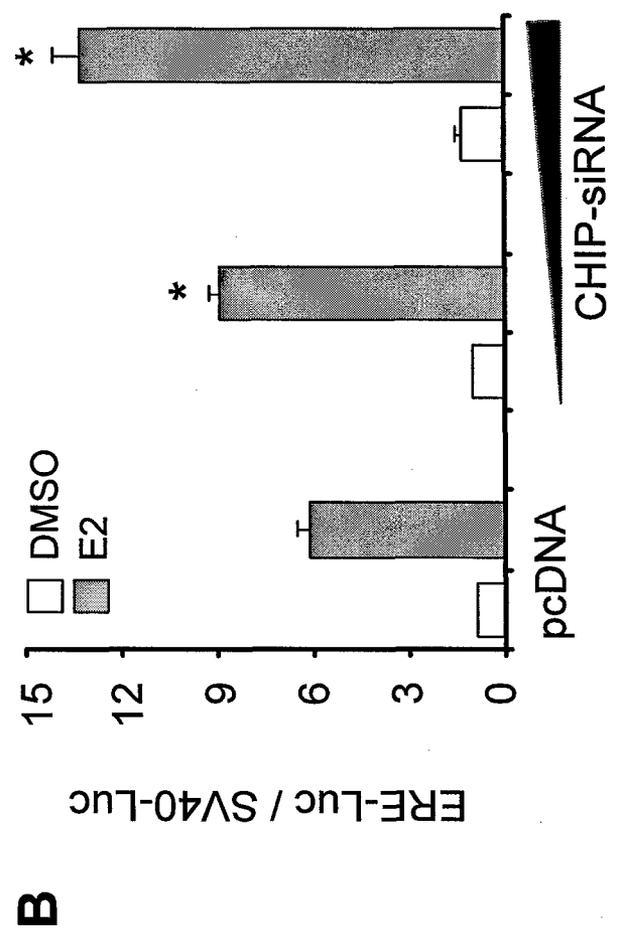
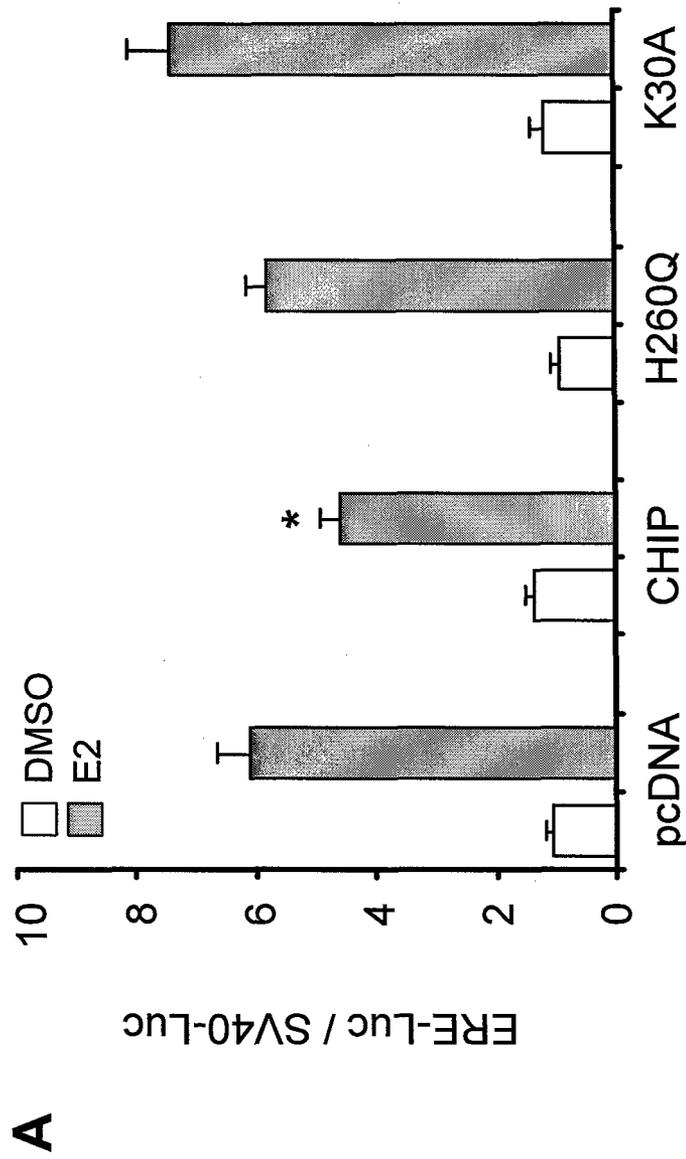


Fig. 6

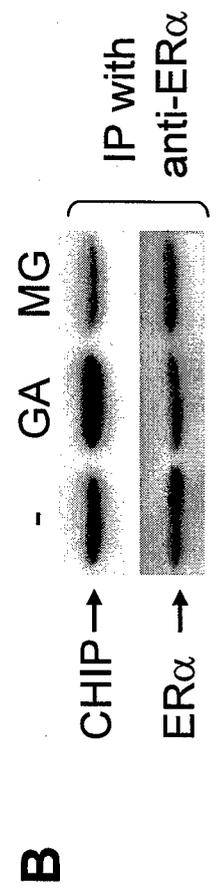
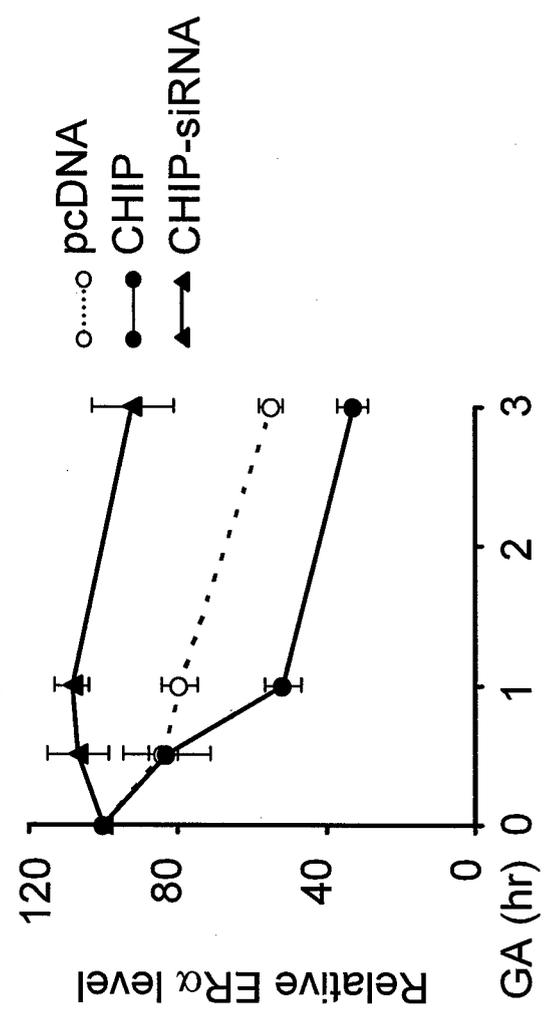
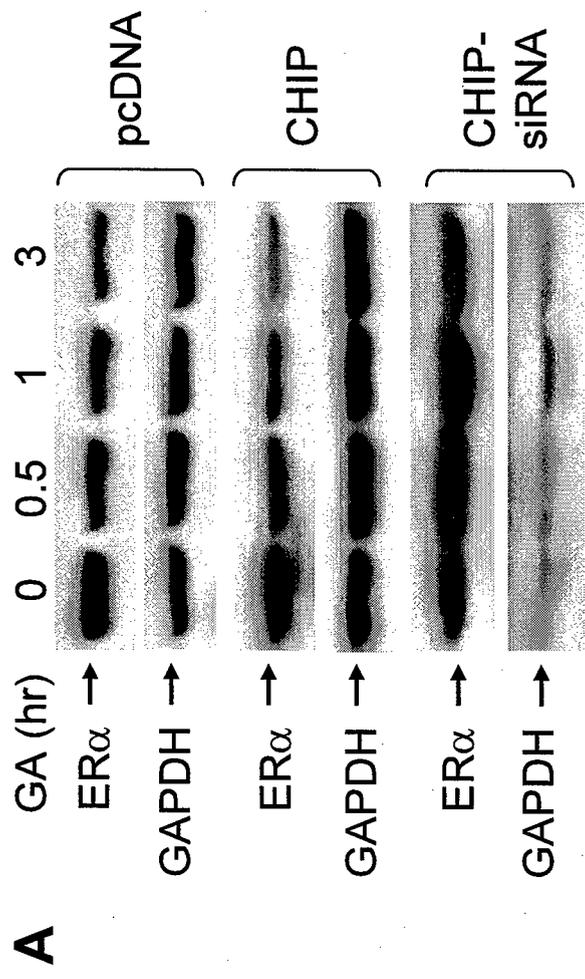


Fig. 7

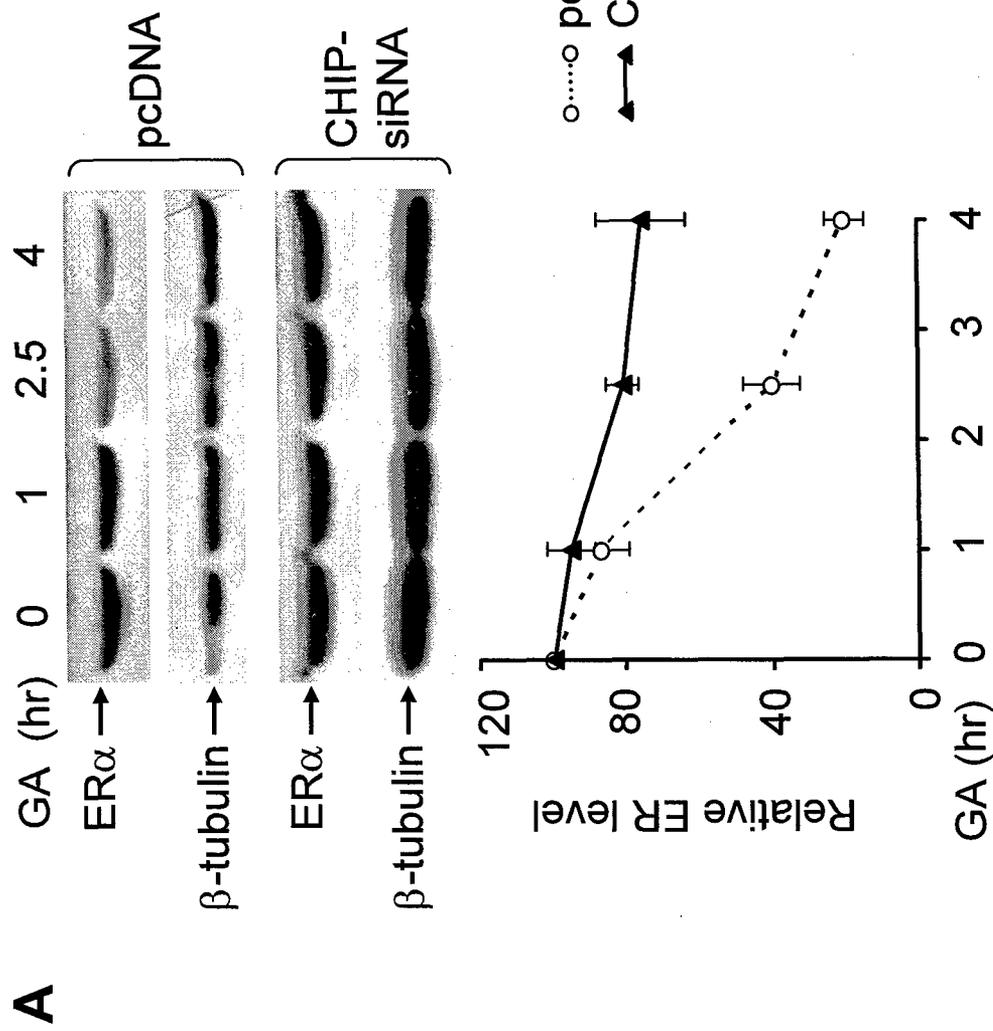


Fig. 8

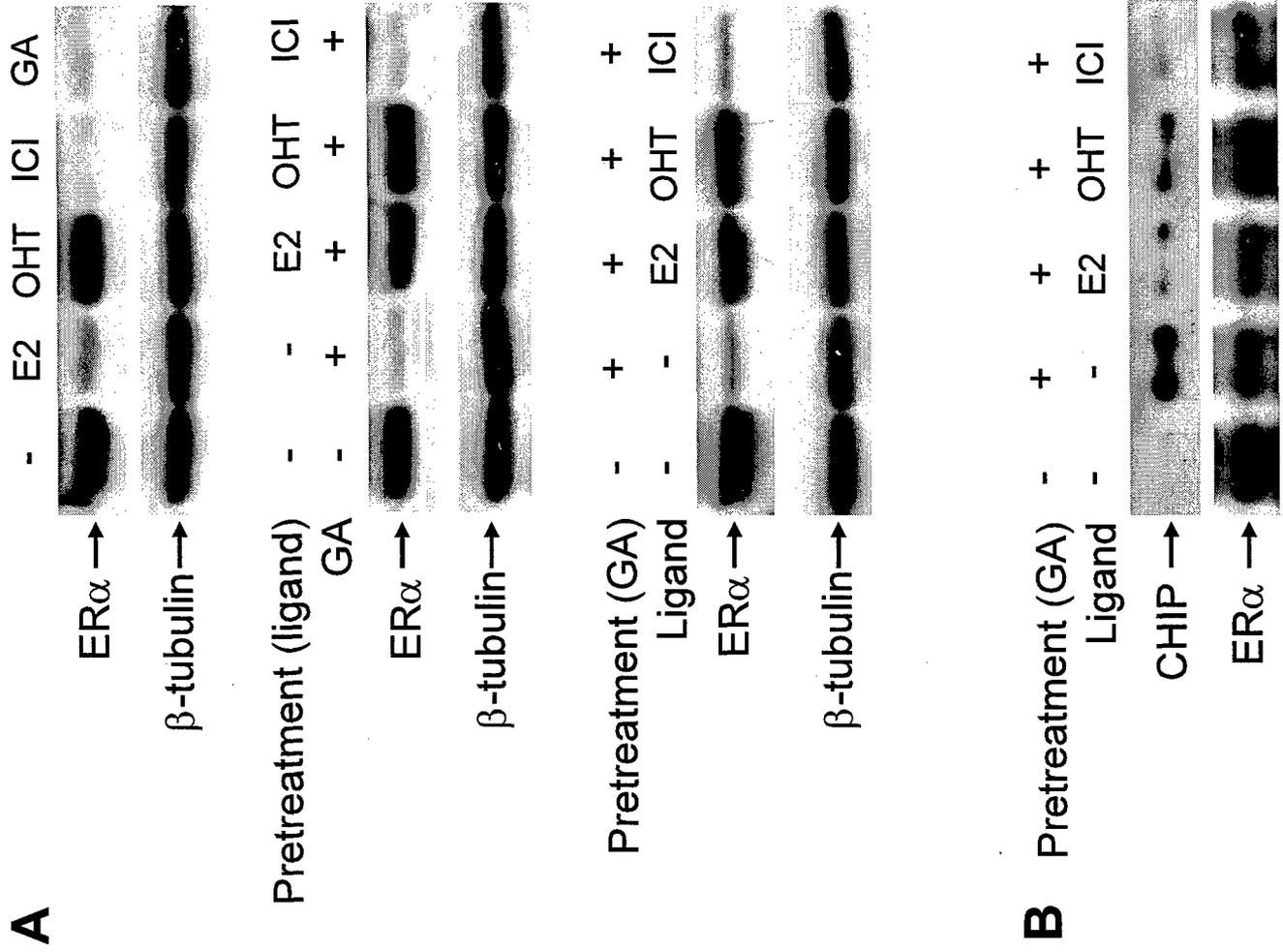


Fig. 9

B

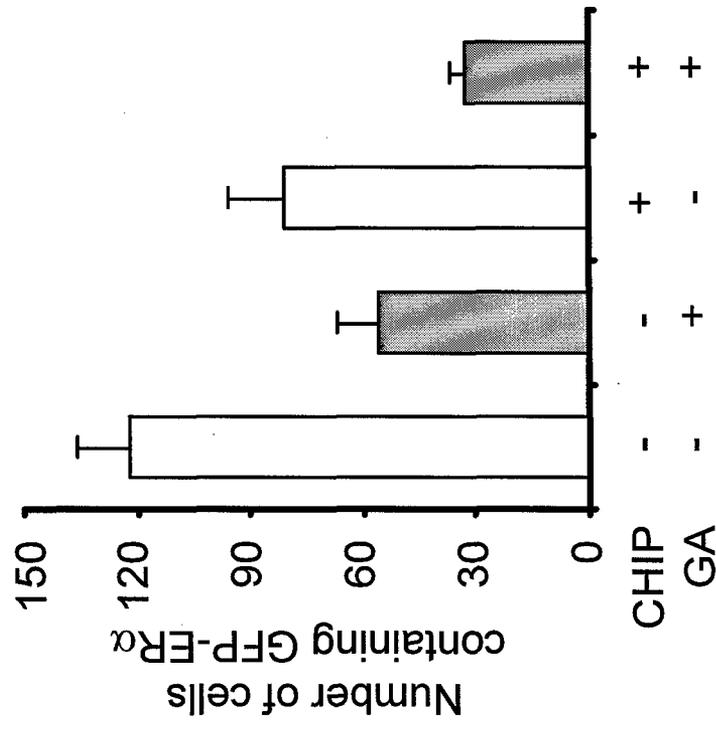
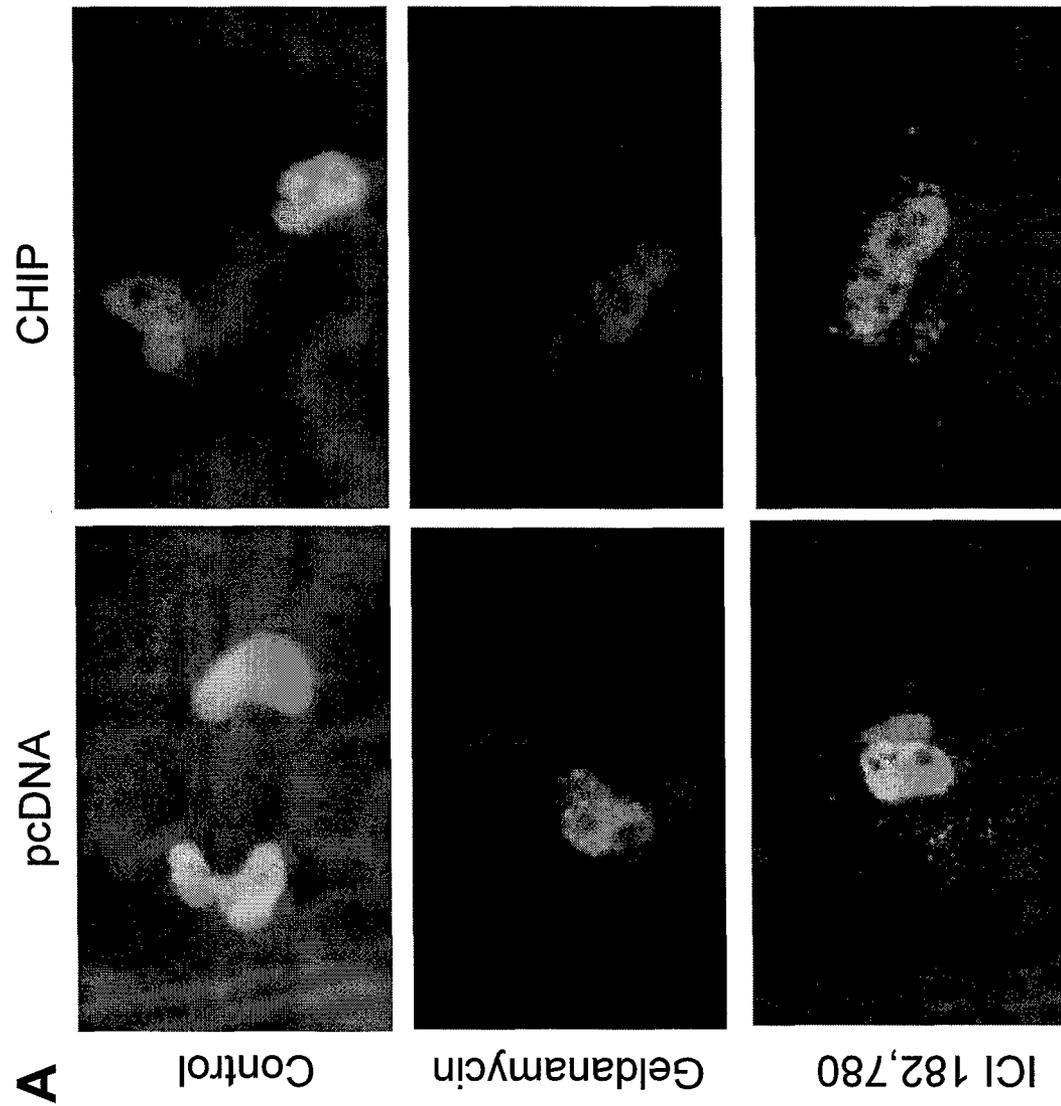


Fig. 10

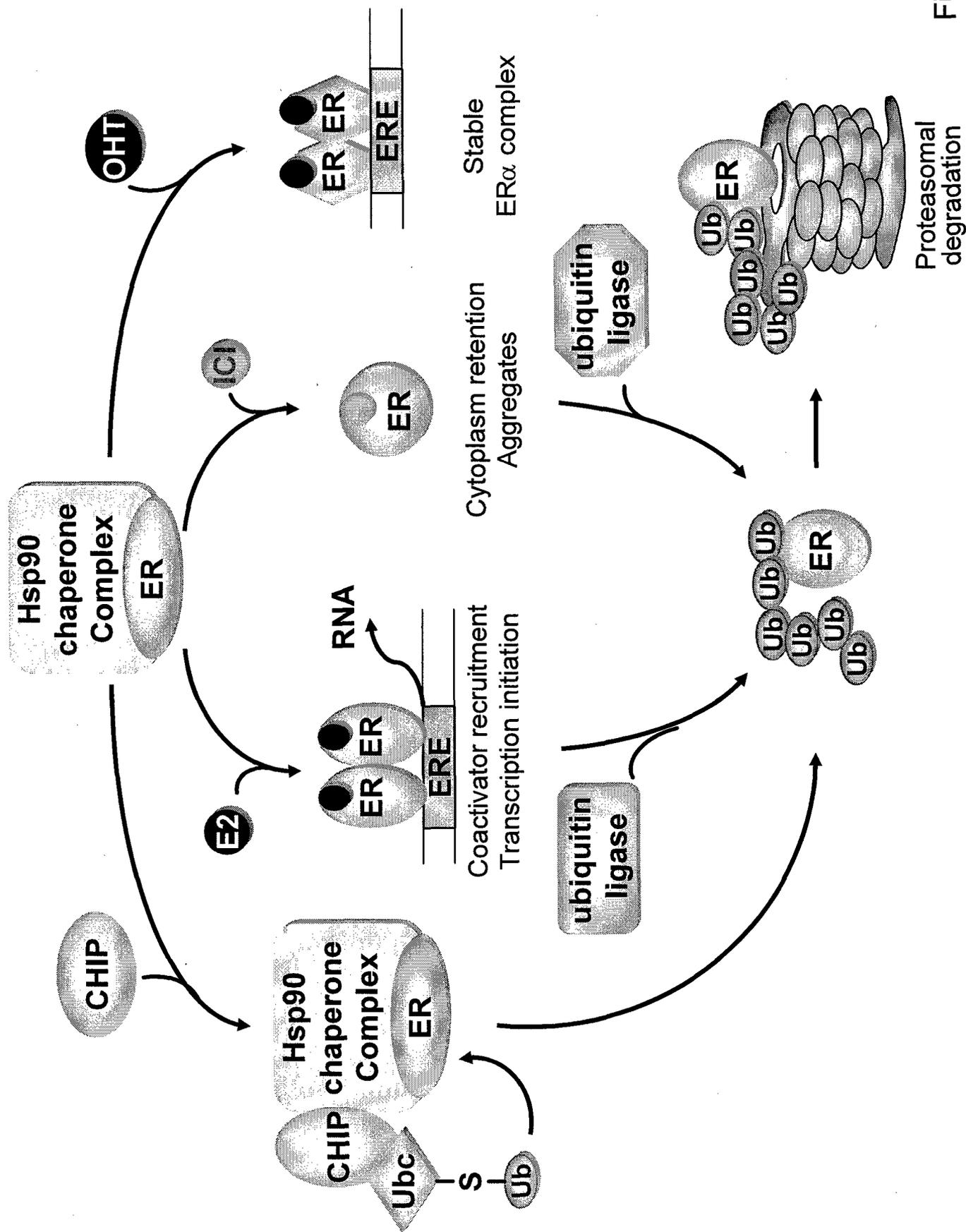


Fig. 11

Loss of Estrogen Receptor Signaling Triggers Epigenetic Silencing of Downstream Targets in Breast Cancer

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ABSTRACT

Alterations in histones, chromatin-related proteins, and DNA methylation contribute to transcriptional silencing in cancer, but the sequence of these molecular events is not well understood. Here we demonstrate that on disruption of estrogen receptor (ER) α signaling by small interfering RNA, polycomb repressors and histone deacetylases are recruited to initiate stable repression of the *progesterone receptor (PR)* gene, a known ER α target, in breast cancer cells. The event is accompanied by acquired DNA methylation of the *PR* promoter, leaving a stable mark that can be inherited by cancer cell progeny. Reestablishing ER α signaling alone was not sufficient to reactivate the *PR* gene; reactivation of the *PR* gene also requires DNA demethylation. Methylation microarray analysis further showed that progressive DNA methylation occurs in multiple ER α targets in breast cancer genomes. The results imply, for the first time, the significance of epigenetic regulation on ER α target genes, providing new direction for research in this classical signaling pathway.

INTRODUCTION

The steroid hormone estrogen is important for normal breast development, but it is also important for growth and progression of breast cancer. The molecular actions of estrogen are mediated by estrogen receptors (ERs), ER α and ER β . On ligand binding, ER α functions as a transcription factor by either binding to DNA targets or tethering to other transcription factors, such as AP-1 and SP-1 (1). These molecular interactions have been shown to positively or negatively modulate the activity of ER α downstream genes important to breast epithelial development.

It is known that estrogen signaling regulates the growth of some breast tumors, and antiestrogen therapies can effectively block this growth signaling, resulting in tumor suppression (2). However, most tumors eventually develop resistance to this endocrine therapy, and antiestrogens are mostly ineffective in patients with advanced disease (2). Mechanisms underlying this hormonal resistance are complex, involving intricate interactions between ER α and kinase networks (1, 2). In addition, epigenetic silencing of ER α is known to contribute to the antiestrogen resistance (1, 2). An emerging theme not yet inves-

tigated in this field is the subsequent influence on the expression of ER α downstream target genes.

Epigenetics can be defined as the study of heritable changes that modulate chromatin organization without altering the corresponding DNA sequence. DNA methylation, the addition of a methyl group to the fifth carbon position of a cytosine residue, occurs in CpG dinucleotides (3) and is a key epigenetic feature of the human genome. These dinucleotides are usually aggregated in stretches of 1- to 2-kb GC-rich DNA, called CpG islands, located in the promoter and first exon of ~60% of human genes (3, 4). Promoter methylation is known to participate in reorganizing chromatin structure and also plays a role in transcriptional inactivation (3, 5). Studies have suggested that the CpG island in an active promoter is usually unmethylated, with the surrounding chromatin displaying an "open" configuration, allowing for the access of transcription factors and other coactivators to initiate gene expression (6-8). Furthermore, transcription factor occupancy may make the promoter inaccessible to repressors or other chromatin-remodeling proteins. In contrast, the CpG island in an inactive promoter may become methylated, with the associated chromatin exhibiting a "closed" configuration. As a result, the methylated area is no longer accessible to transcription factors, disabling the functional activity of the promoter (7, 9, 10).

Recent studies have shown that establishing transcriptional silencing of a gene involves a close interplay between DNA methylation and histone modifications (7, 11). This process may be achieved by recruiting histone-modifying enzymes, such as histone deacetylases, which mediate posttranslational modification at the NH₂ terminus ends of histones (7, 11). As a result, chromatin modifications form distinct patterns, known as the "histone code," that may dictate gene expression (12-14).

Two models have been offered to describe the molecular sequence leading to the establishment of epigenetic gene silencing. One model suggests that histone modifications are the primary initiating event in transient repression (15, 16). DNA methylation subsequently accumulates in the targeted CpG island, creating a heterochromatin environment to establish a heritable, long-term state of transcriptional silencing. However, a second model is that DNA methylation can actually specify unique histone codes for maintaining the silenced state of a gene (17-20). In this case, DNA methylation may precede histone modifications. Clearly, this epigenetic process is complex, and multiple systems may be implemented for genes participating in different signaling pathways.

In this study, we investigated whether the removal of ER α signaling triggers changes in DNA methylation and chromatin structure of ER α target promoters. By using RNA interference (RNAi) to transiently disable ER α in breast cancer cells, we show, for the first time, that polycomb repressors and histone deacetylases assemble on the promoters of interrogated ER α target genes to participate in long-term transcriptional silencing. These events are later accompanied by a progressive accumulation of DNA methylation in the promoter re-

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Note: Supplementary data for this article can be found at Cancer Research Online (<http://cancerres.aacrjournals.org>). T. Huang is a consultant to Epigenomics, Inc., Berlin, Germany.

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gions of the now silent targets, leaving a heritable "mark" that may be stably transmitted to cell progeny.

MATERIALS AND METHODS

Cell Lines and Clinical Samples. The breast cancer cell line MCF-7 and its derived subline, C4-12, were routinely maintained in our laboratories. For the demethylating treatment, cells were plated at a density of 2×10^6 cells per 10-cm dish and pretreated with 2 or 5 $\mu\text{mol/L}$ 5-aza-2'-deoxycytidine (5-Aza-dC; Sigma, St. Louis, MO) for 5 days before treatment with 17β -estradiol (E_2 ; 10 nmol/L, 24 hours). Thirty-two invasive ductal carcinomas were obtained from patients undergoing breast surgery at the Ellis Fischel Cancer Center (Columbia, MO), in compliance with the institutional review board. Seven tumor-free breast parenchymas were used as controls. The ER status of tumor tissue was determined by immunohistochemical staining (21).

Transfection of Estrogen Receptor α Small Interfering RNAs. MCF-7 cells (60% confluent in a 3.5-cm-diameter culture dish) were starved in serum-free medium (minimal essential medium only) for 72 hours, followed by the addition of 10 nmol/L E_2 (E2758; Sigma) for 24 hours. The cells were then transfected with small interfering RNAs (siRNAs) for 4 to 5 hours with DMR1E-C reagent (Invitrogen, Carlsbad, CA). Double-stranded siRNA was generated using the *Silencer* siRNA Construction Kit (Ambion, Austin, TX). The siRNA oligonucleotides designed according to the ER α mRNA sequence (GenBank accession numbers AF_258449, 258450, and 258451) are as follows: (a) target sequence 1 (5'-AACCTCGGGCTGTGCTCTTTT), sense strand siRNA primer 5'-CCTCGGGCTGTGCTCTTTTCTGTCTC and antisense strand siRNA primer AAAAGAGCACAGCCCGAGGTTCTGTCTC; and (b) target sequence 17 (5'-AAACAGGAGGAAGAGCTGCCA), sense strand siRNA primer 5'-ACAGGAGGAAGAGCTGCCATTCTCTGTCTC and antisense strand siRNA primer 5'-TGGCAGCTCTTCTCTCTCTTCTGTCTC.

Media were changed after transfection. The cells were then harvested for total RNA (RNeasy Kit; Qiagen, Valencia, CA) and genomic DNA (QIAamp; Qiagen) isolation at various time periods after siRNA treatment.

Transfection of Estrogen Receptor α Expression Vector. C4-12 cells were transfected with pcDNA-ER α (C4-12/ER) or empty vector (C4-12/vec) using LipofectAMINE Plus Reagent (Life Technologies, Inc., Carlsbad, CA) and then exposed to an antibiotic (G418; 0.5 mg/mL) for 3 weeks. Expression of ER α in G418-resistant colonies was detected by immunoblotting with an anti-ER antibody (Chemicon, Temecula, CA).

Real-Time Reverse Transcription-Polymerase Chain Reaction. Total RNA (2 μg) was treated with DNase I to remove potential DNA contamination and then reverse transcribed using the SuperScript II reverse transcriptase (Invitrogen). Real-time polymerase chain reactions (PCRs) were then performed using puReTaq Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, NJ) and monitored by SYBR Green I (BioWhittaker, Walkersville, MD) using a Smart Cycler Real-Time PCR instrument (Cepheid, Sunnyvale, CA) for 42 cycles. PCR products of the expected size were also visualized on agarose gels stained with ethidium bromide. Alternatively, the reverse transcription-PCR (RT-PCR) reaction was conducted using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) in an iCycler system (Bio-Rad) for PR transcripts (22). The relative mRNA level of a given locus was calculated by Relative Quantitation of Gene Expression (Applied Biosystems, Foster City, CA) with *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) or β -actin mRNA as an internal control. The primers used for RT-PCR reactions are listed in Supplementary Table S1.

Immunofluorescence and Western Blot Analysis. MCF-7 cells (2×10^5) treated with or without ER α siRNAs were permeabilized with 0.5% Nonidet P-40/PBS and blocked with a 1:100 dilution of horse serum before incubation with primary anti-ER α antibody (1:1,000; mouse monoclonal antibody D-12; Santa Cruz Biotechnology, Santa Cruz, CA). Sample slides were washed with PBS and incubated in the dark with secondary antibody (1:500) conjugated with Texas Red (fluorescent antimouse IgG kit; Vector Laboratories, Burlingame, CA) for 1 hour. The slides were then mounted with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories) and observed under a fluorescence microscope (Zeiss Axioskop 40; Zeiss, Thornwood, NY). Images were captured by the AxioCam HRC camera and analyzed by AxioVision 5.05 software.

Small interfering RNA-treated cells and control cells were lysed in the presence of proteinase inhibitors. One hundred micrograms of protein were subjected to 7% SDS-PAGE and transferred to immunoblot membranes. The membranes were then incubated with mouse anti-ER α (MAB463; Chemicon) and labeled secondary antibody. GAPDH was used a loading control.

Chromatin Immunoprecipitation-Polymerase Chain Reaction. Cultured cells (2×10^6) were cross-linked with 1% formaldehyde and then washed with PBS in the presence of protease inhibitors. The cells were resuspended in lysis buffer, homogenized using a tissue grind pestle to release nuclei, and then pelleted by centrifugation. SDS-lysis buffer from a chromatin immunoprecipitation (ChIP) assay kit (Upstate Biotechnology, Lake Placid, NY) was used to resuspend the nuclei. The lysate was sonicated to shear chromatin DNAs and then centrifuged to remove cell debris. The supernatants were transferred to new tubes and incubated overnight with an antibody against ER α , YY-1, or EZH2 (Santa Cruz Biotechnology); HDAC1, MBD2, or MeCP2 (Upstate Biotechnology); and DNMT1, DNMT3a, or DNMT3b (Imgenex, San Diego, CA). Agarose slurry was then added to the mixture, and the chromatin-bound agarose was centrifuged. The supernatant was collected and used for total input (it serves as a positive control) in the ChIP-PCR assay. After elution, proteins were digested from the bound DNA with proteinase K. Phenol/chloroform-purified DNA was then precipitated and used in ChIP-PCR assays for a *progesterone receptor* (*PR*) promoter region. The primer sequences were 5'-GGCTTTGGGCGGGCCTCCCTA (sense strand) and 5'-TCTGCTGGCTCCGCTACTGCGG (antisense strand). After amplification, ^{32}P -incorporated PCR products were separated on 8% polyacrylamide gels and subjected to autoradiography using a Storm PhosphorImager (Amersham Biosciences).

Methylation-Specific Polymerase Chain Reaction. Genomic DNA (1 μg) from each sample was bisulfite-converted using the EZ DNA Methylation Kit (Zymo Research Corp., Orange, CA), according to the manufacturer's protocol. The converted DNA was eluted with 40 μL of elution buffer and then diluted 50 times for methylation-specific PCR (MSP). The primer sets designed for amplifying the methylated or unmethylated allele of the PR locus are listed in Supplementary Table S2. All PCR reactions were performed in PTC-100 thermocyclers (MJ Research, Watertown, MA) using AmpliTaq Gold DNA polymerase (Applied Biosystems). ^{32}P -incorporated amplified products were separated on 8% polyacrylamide gels and subjected to autoradiography using a Storm PhosphorImager (Amersham Biosciences).

Combined Bisulfite Restriction Analysis. Combined bisulfite restriction analysis (COBRA) was carried out essentially as described previously (23). Bisulfite-modified DNA (~ 10 ng) was used as a template for PCR with specific primers flanking the interrogated sites (*TaqI* or *BstUI*) of an ER α downstream target. Primer sequences used for amplification are listed in Supplementary Table S3. After amplification, radiolabeled PCR products were digested with *TaqI* or *BstUI*, which restrict unconverted DNA containing methylated sites. The undigested control and digested DNA samples were run in parallel on polyacrylamide gels and subjected to autoradiography. The percentage of methylation was determined as the intensity of methylated fragments relative to the combined intensity of unmethylated and methylated fragments.

Chromatin Immunoprecipitation on Chip. MCF-7 cells (2×10^7) were used to conduct ChIP with an antibody specific for ER α following the protocol described (see Chromatin Immunoprecipitation-Polymerase Chain Reaction). After chromatin coimmunoprecipitation, DNA was labeled with Cy5 fluorescence dye and hybridized to a genomic microarray panel containing $\sim 9,000$ CpG islands (24). Microarray hybridization and posthybridization washes have been described previously (25). The washed slides were scanned by a Gene Pix 4000A scanner (Axon, Union City, CA), and the acquired microarray images were analyzed with GenePix Pro 4.0 software. This ChIP-on-chip experiment was conducted twice.

Positive CpG island clones were sequenced, and the derived sequences were used to identify putative transcription start sites by Blastn⁵ or Blat.⁶ Both Genomatix⁷ and TFSEARCH⁸ programs were then used to localize the consensus sequences of the estrogen response elements (EREs) and other

⁵ <http://www.ncbi.nlm.nih.gov/BLAST/>.

⁶ <http://genome.cse.ucsc.edu/cgi-bin/hgBlat>.

⁷ http://www.genomatix.de/site_map/index.html.

⁸ <http://www.cbrc.jp/research/db/TFSEARCH.html>.

related transcription factor binding sites (AP-1, SP-1, cAMP-responsive element binding protein, and CEBP).

Differential Methylation Hybridization. Differential methylation hybridization (DMH) was performed essentially as described previously (25, 26). Briefly, 2 μ g of genomic DNA were digested by the 4-base frequent cutter *MseI*, which restricts bulk DNA into small fragments but retains GC-rich CpG island fragments (24). H-24/H-12 PCR linkers (5'-AGGCAACTGTGCTATC-CGAGGGAT-3' and 5'-TAATCCCTCGGA-3') were then ligated to the digested DNA fragments. The DNA samples were further digested with two methylation-sensitive endonucleases, *HpaII* and *BstUI*, and amplified by PCR reaction using H-24 as a primer. After amplification, test DNA from siRNA-treated cell lines or clinical samples was labeled with Cy5 (red) dye, whereas control DNA from the mock-transfected cell lines or normal female blood samples was coupled with Cy3 (green) dye. Equal amounts of test and control DNAs were cohybridized to a microarray slide containing 70 ER α promoter targets (average, 500 bp) identified from the ChIP-on-chip results. Posthybridization washing and slide scanning are described above. Normalized Cy5/Cy3 ratios of these loci were calculated by GenePix Pro 4.0.

Shrunken Centroids Analysis. DMH microarray data were analyzed by the procedure described online.⁹ This program incorporates graphic methods for automatic threshold choice and centroid classification.

Statistical Analyses. Differences of methylation or mRNA levels in experimental studies were analyzed by a paired *t* test. Methylation differences between two tumor groups were determined with a Pearson's χ^2 test. *P* < 0.05 was considered statistically significant.

RESULTS

RNA Interference Transiently Knocks Down Estrogen Receptor α Expression in Breast Cancer Cells. Although several *in vitro* systems and mouse models are available for analysis of estrogen signaling, to our knowledge, the recently described RNAi (27) has not been actively used in this area of research. We therefore used this technology to specifically repress ER α gene expression via targeted RNA degradation (28, 29). Six different ER α siRNAs, two of which have sequences homologous to a splice variant, were synthesized (Fig. 1A). These siRNAs (40 nmol/L) were individually transfected into MCF-7, an ER α -positive human breast cancer cell line. MCF-7 cells were cultured in the presence of E₂. Quantitative RT-PCR analysis showed that, 24 hours after transfection, two siRNAs, siRNAs 1 and 17, were capable of repressing ER α transcripts (Fig. 1B). Specifically for siRNA 1, we observed a >93% decrease of ER α mRNA. Immunofluorescence (Fig. 1C) and Western blot (Fig. 1D) analyses confirmed that this RNAi also dramatically reduced ER α protein synthesis. This inhibitory effect appeared to be transient, and the expression of ER α protein reappeared in cultured cells 4 weeks after RNAi withdrawal (Fig. 1D).

Epigenetic Silencing of the PR Gene Is Triggered by Estrogen Signal Disruption. We hypothesized that disruption of ER α signaling by siRNA may lead to the silencing of some positively regulated ER α targets governed by epigenetic mechanisms. To this end, a known ER α downstream target, the PR gene, was investigated in detail. In Fig. 2A and B, quantitative RT-PCR analysis showed that by 36 hours after treatment of MCF-7 cells with siRNA 1, the level of PR transcripts (PR-A and PR-B) was reduced by >95% (paired *t* test, *P* < 0.0001). Next, ChIP-PCR was performed to determine the status of chromatin remodeling at the 5'-end of the PR gene. The protein-DNA complexes were immunoprecipitated with antibodies to ER α or to specific modified histones (acetyl-H3, acetyl-H3-K9, and methyl-H3-K4) known to specify active transcription (7, 30). As shown in Fig. 2C, the presence of these active chromatin components was diminished over a period of 36 hours, coinciding with decreased ER α binding to the PR promoter region.

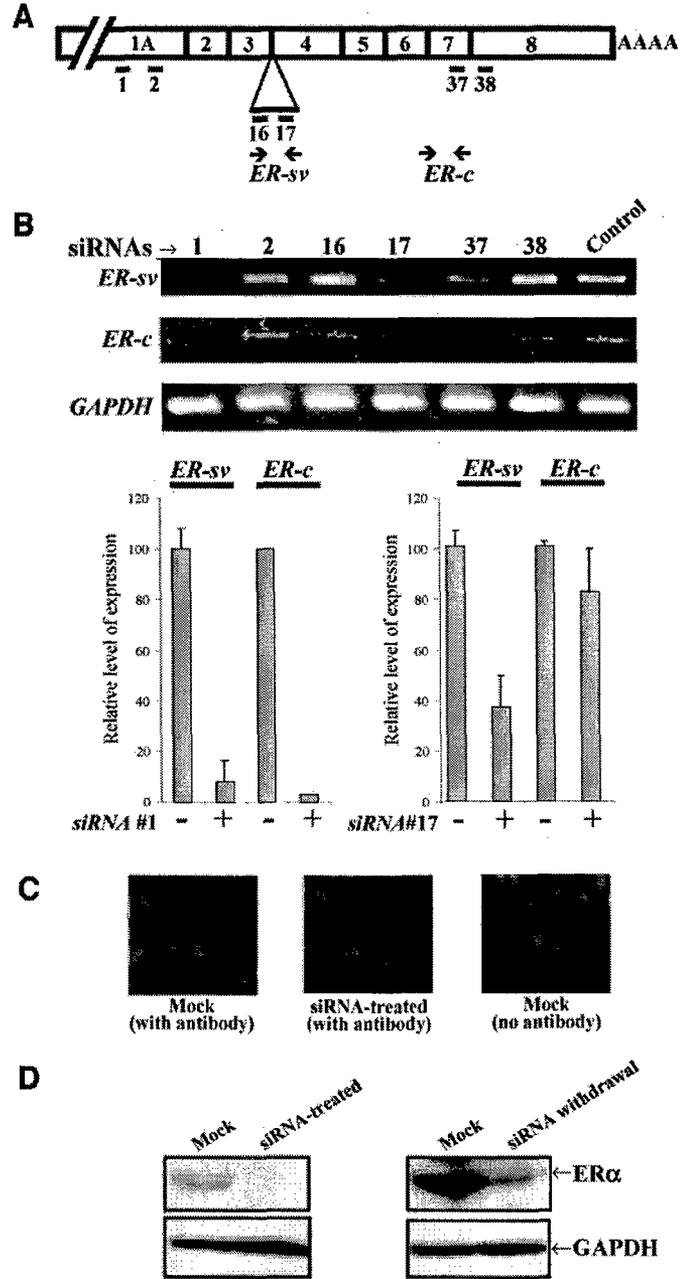
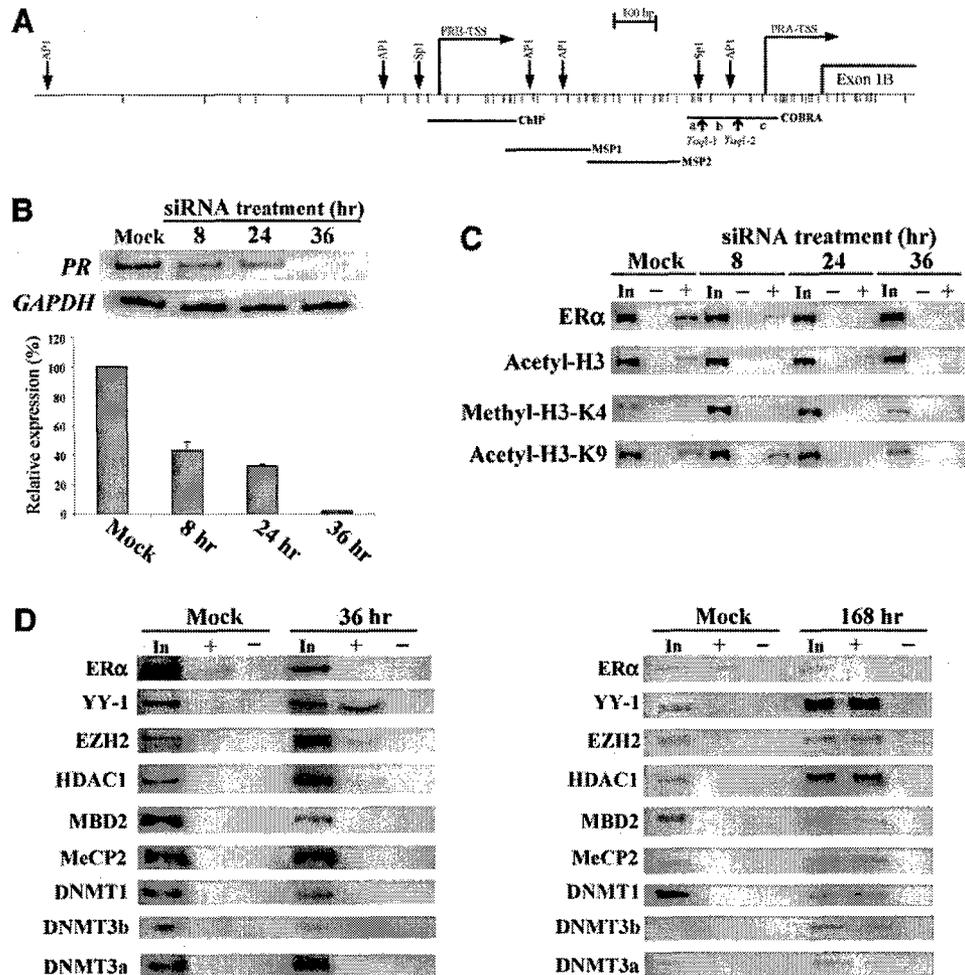


Fig. 1. Down-regulation of ER α by siRNAs. MCF-7 ER α -positive human breast cancer cells were transfected with six different double-stranded siRNAs for 4 hours. Cells were left untreated for an additional 24 hours, and total RNA and proteins were then harvested for analysis. Additionally, cells were left untreated for a prolonged period of 3 to 4 weeks (see RNAi withdrawal in D), and proteins were harvested for analysis. Mock-treated cells were transfected with vehicle only. A, map of ER α cDNA. The ER α mRNAs were transcribed from several unique first exons and seven other common exons. An additional splice variant (between exons 3 and 4) is also shown. Approximate locations of siRNAs (1, 2, 16, 17, 37, and 38) are indicated by bars. B, RT-PCR of ER α . PCR primers specifically for the splice variant (ER-sv) and the common transcripts (ER-c) are indicated by arrows in A. The GAPDH transcript was used as a control. The expression levels of ER-sv or ER-c were normalized against GAPDH (bottom panel) by real-time PCR. C, immunofluorescence analysis of ER α expression. Small interfering RNA 1- or mock-treated MCF-7 cells were cultured on microscope coverslips and incubated with an ER α antibody. Positive nuclei staining (Texas Red) was seen only in mock-treated cells (with antibody), but not in siRNA 1-treated (with antibody) or mock-treated (without antibody) cells. 4',6-Diamidino-2-phenylindole was used to counterstain nuclei (blue). D, Western blot analysis of ER α expression. Cellular protein products from various treatments were harvested and separated by SDS-PAGE for immunoblot analysis with ER α and GAPDH (loading control) antibodies, respectively. Results are representative of at least three independent experiments.

⁹ <http://www-stat.stanford.edu/~tibs/PAM>.

Fig. 2. Loss of estrogen signaling leads to epigenetic silencing of the *PR* gene. **A**, genomic map of the *PR* promoter CpG island. The positions of CpG sites in the genomic sequence are indicated by thin vertical lines. The positions of two alternative transcription start sites, *PRA* and *PRB*, respectively, are indicated by bent arrows. Potential transcription factor binding sites (AP-1 and Sp1) resulting from a TFSEARCH query (www.cbrc.jp/htbin/nph-tfsearch) are marked by vertical arrows. The location of the *PR* promoter fragment used for the ChIP assay and the two regions (MSP1 and MSP2) used for MSP are indicated by horizontal lines (see Fig. 3A). The region amplified for COBRA is underlined, and the two vertical arrows indicate the interrogating *TaqI* restriction (TCGA) sites (see Fig. 3C). **B**, time course inhibition of *PR* transcripts by siRNA treatment. MCF-7 cells were transfected with ER α siRNA 1 for the indicated time periods (8, 24, and 36 hours) and harvested for real-time RT-PCR. Mock-transfected cells were harvested at the 36 hour time point. Primers were designed to amplify a common region of the two known *PR* transcripts. Relative levels of *PR* transcripts were normalized against that of the *GAPDH* loading control. Results from three independent experiments are shown as means \pm SE. **C**, ChIP-PCR assay for activating chromatin modifications on the *PR* CpG promoter island. Chromatin DNA was immunoprecipitated with antibodies specific for ER α , acetylated histone H3 (acetyl-H3 and/or acetyl-H3-K9), or dimethyl-H3-K4. DNA fragments were amplified with a primer pair located in a *PR* CpG island region (indicated in A). The final radiolabeled products were separated on 6% polyacrylamide gels and subjected to autoradiography. *In*, total input; -, without antibody; +, with antibody. **D**, ChIP-PCR assays for repressive chromatin modifications on the *PR* promoter CpG island region at 36 and 168 hours after ER α siRNA treatment. Antibodies against polycomb repressors (YY-1 and EZH2), histone deacetylase (HDAC1), methyl-CpG binding proteins (MBD2 and MeCP2), and DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b) were used in ChIP-PCR assays. *In*, total input; -, without antibody; +, with antibody.



We speculated that this initial transcriptional inactivation might trigger further recruitment of repressor molecules to the *PR* promoter CpG island to subsequently establish a long-term silencing state. ChIP-PCR assays were conducted with a panel of antibodies raised for the polycomb repressors YY-1 and EZH2, histone deacetylase HDAC1, methyl-CpG-binding proteins MBD2 and MeCP2, and DNA methyltransferases DNMT1, DNMT3a, and DNMT3b. At 36 hours after siRNA treatment, YY-1 and EZH2 were bound to the promoter region (Fig. 2D). These polycomb proteins have previously been shown to target the regulatory regions of *homeobox* genes, the resulting repression of which can be tissue specific and important for early embryonic development (31, 32). Here we demonstrate for the first time that these proteins have an additional role in repressing an ER α target gene. Furthermore, the *PR* promoter was seen, at 36 hours, to recruit HDAC1 (Fig. 2D), a protein known to deacetylate histone protein tails, creating a repressive heterochromatin environment in the targeted promoter area (7, 9, 10). However, at this early time point (36 hours), ChIP-PCR analysis did not detect the presence of the DNA methyltransferase DNMT1 in the *PR* promoter CpG island area (Fig. 2D), nor did we observe the presence of MBD2 or MeCP2, which are known to bind methylated CpG sites. Only a faint band corresponding to DNMT3b was detected in the *PR* promoter area by 36 hours after ER α siRNA treatment of MCF-7 cells. Except for DNMT3a, the recruitment of these repressive proteins to the *PR* promoter CpG island was evident by 168 hours after siRNA treatment.

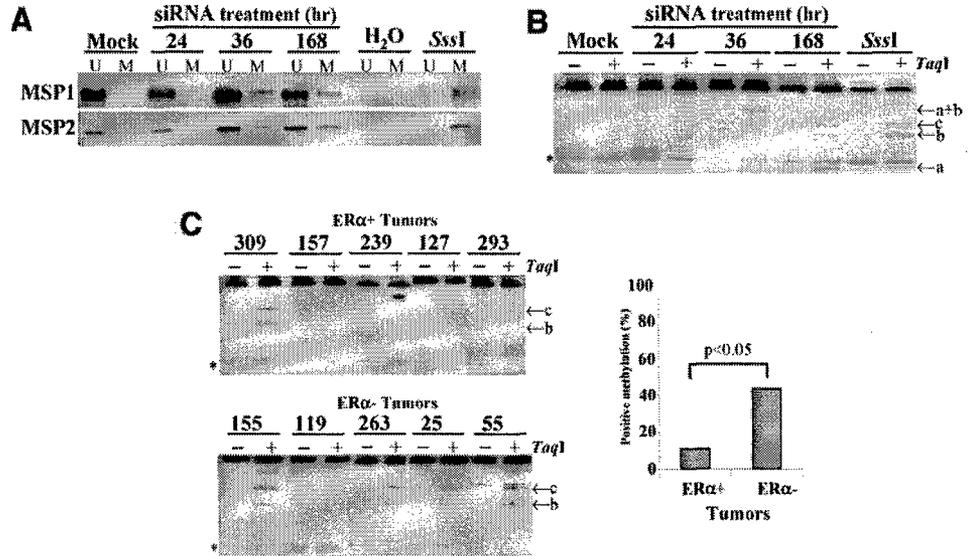
To further determine whether the recruitment of these epigenetic components could trigger *de novo* DNA methylation, MSP assays (33)

were conducted to survey two 5'-end regions of *PR* at different time periods after siRNA treatment. As shown in Fig. 3A, *PR* methylation was detected in amplified bisulfite-treated DNA only at 36 hours after treatment. This observation was independently confirmed by conducting semiquantitative COBRA (23). In the assay, ~10% of MCF-7 cells showed methylation in one (Fig. 2A, *TaqI*-2) of the two *PR* *TaqI* sites analyzed 36 hours after siRNA treatment (Fig. 3B). Both of these sites became methylated at a later time point (168 hours) of treatment (Fig. 3B). This study implies that acquired DNA methylation is a late event and that the density of DNA methylation may gradually accumulate at the 5'-end of *PR* after disrupting ER α signaling by siRNA.

Next, we determined whether this acquired promoter methylation could be observed in ER α -negative breast tumors. COBRA was therefore conducted in 32 primary tumors (16 ER α -negative and 16 ER α -positive tumors) and 7 normal controls (see representative examples in Fig. 3C). Consistent with the *in vitro* findings, *PR* promoter hypermethylation occurred more frequently in ER α -negative tumors (45%) than in ER α -positive tumors (10%) (χ^2 test, $P < 0.05$).

Reexpression of *PR* Requires Both Estrogen Signal Restoration and DNA Demethylation. The *in vitro* experimental results described above are based on transient siRNA treatment. To determine whether this signal disruption has a lasting impact on *PR* expression, we took advantage of an ER α -negative cell subline, C4-12, derived from ER α -positive MCF-7 cells by long-term hormonal depletion (34). A recent study has indicated that *PR* gene expression is absent in this cell line (35). We therefore examined whether stably reexpressing ER α could restore *PR* gene activity in several established C4-12

Fig. 3. DNA methylation analysis of the *PR* promoter by MSP and COBRA. A, bisulfite-treated DNA samples from siRNA- and mock-treated cells were used for amplification with specific primers for MSP1 and MSP2 (see Fig. 2A). Radiolabeled PCR products for unmethylated (Lanes U) and methylated (Lanes M) DNA strands were separated on 6% polyacrylamide gels. B, For COBRA, bisulfite DNA samples from siRNA- and mock-treated cells were amplified and digested with *TaqI* enzyme and then separated on polyacrylamide gels. The digested DNA fragments (a, b, and c) indicated by the arrows reflect methylation of *TaqI* restriction sites within the *PR* promoter CpG island (see Fig. 2A). An asterisk indicates PCR artifact or primer-dimer. C, COBRA of *PR* promoter in ER α -positive and -negative breast tumors. The percentage of positive methylation was calculated as the intensity of methylated fragments relative to the combined intensity of unmethylated and methylated fragments (right panel). All DNA methylation data shown here are representative of at least three independent experiments.



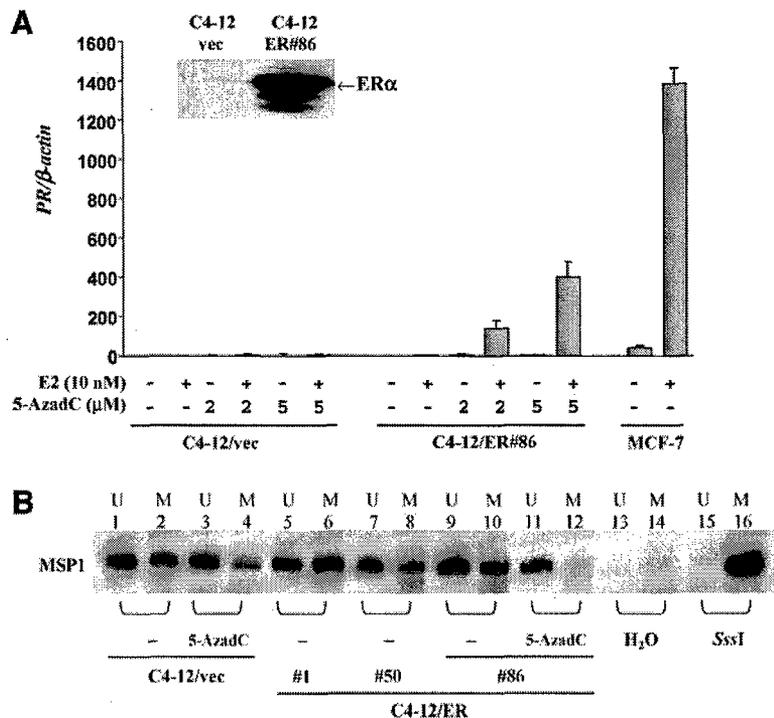
subclones (C4-12/vec, C4-12/ER#1, C4-12/ER#50, and C4-12/ER#86; see examples in Fig. 4A, inset).

Treatment of these subclones (e.g., C4-12/ER#86 in Fig. 4A) with E₂, however, failed to induce *PR* mRNA expression, demonstrating that reintroduction of ER α alone was insufficient to reactivate expression of a silent *PR* gene. To determine whether loss of *PR* expression was due to DNA methylation, C4-12/vec (i.e., cells stably transfected with empty vector) and C4-12/ER#86 cells were pretreated with 5-AzadC, a DNA demethylating agent, before E₂ treatment. As shown in Fig. 4A, sequential treatment with 5-AzadC followed by E₂ resulted in reexpression of *PR* mRNA in C4-12/ER#86 cells, but not in C4-12/vec cells, demonstrating that both ER α expression and DNA demethylation are required to restore *PR* expression. To further confirm that reactivation of the *PR* gene was due to DNA demethylation, the methylation status of the *PR* promoter CpG island region was examined by MSP (Fig. 4B). In contrast to MCF-7 cells in which the

PR promoter CpG island was unmethylated (Fig. 3A), methylation was observed in both C4-12/vec and C4-12/ER cells (Fig. 4B, Lanes 1, 2, and 5–10). However, after treatment with 5-AzadC, *PR* promoter methylation was partially reversed in C4-12/vec cells (Fig. 4B, Lanes 3 and 4) and completely removed in C4-12/ER#86 cells (Fig. 4B, Lanes 11 and 12). Together, these results demonstrate that the silencing of *PR* is maintained, in part, by DNA methylation in the ER α -negative C4-12 cells and that reactivation of the *PR* promoter requires both the presence of ER α and DNA demethylation.

DNA Methylation of Multiple Estrogen Receptor α Downstream Targets Is Triggered by Disrupting Receptor Signaling. To determine whether this epigenetically mediated silencing is a generalized event, we used ChIP-on-chip, a novel microarray-based method developed in our laboratory (36, 37), for a genome-wide screening of ER α downstream targets. In this case, we probed a panel of ~9,000 arrayed CpG island fragments with anti-ER α -coimmuno-

Fig. 4. Treatment of C4-12/ER cells with 5-AzadC restores *PR* mRNA expression. A, Expression levels of *PR* mRNA in C4-12/Vec and C4-12/ER cells were determined after treatment with the indicated doses of 5-AzadC for 5 days, followed by 10 nmol/L E₂ for 24 hours. *PR* mRNA levels were measured by quantitative real-time RT-PCR and normalized using β -actin mRNA levels. The results (X \pm SE) are shown from two independent experiments, each in triplicate. ER α expression in C4-12 cells #86 (A, inset) was detected by immunoblotting with an anti-ER α antibody. B, analysis of *PR* promoter by MSP assay. Bisulfite-treated DNAs from cells treated with or without 5-AzadC were amplified with specific primers for the *PR* promoter CpG island (i.e., the MSP1 region in Fig. 2A). Radiolabeled PCR products for unmethylated (Lanes U) and methylated (Lanes M) DNA strands were separated on 6% polyacrylamide gels. MSP data shown here are representative of three independent experiments.

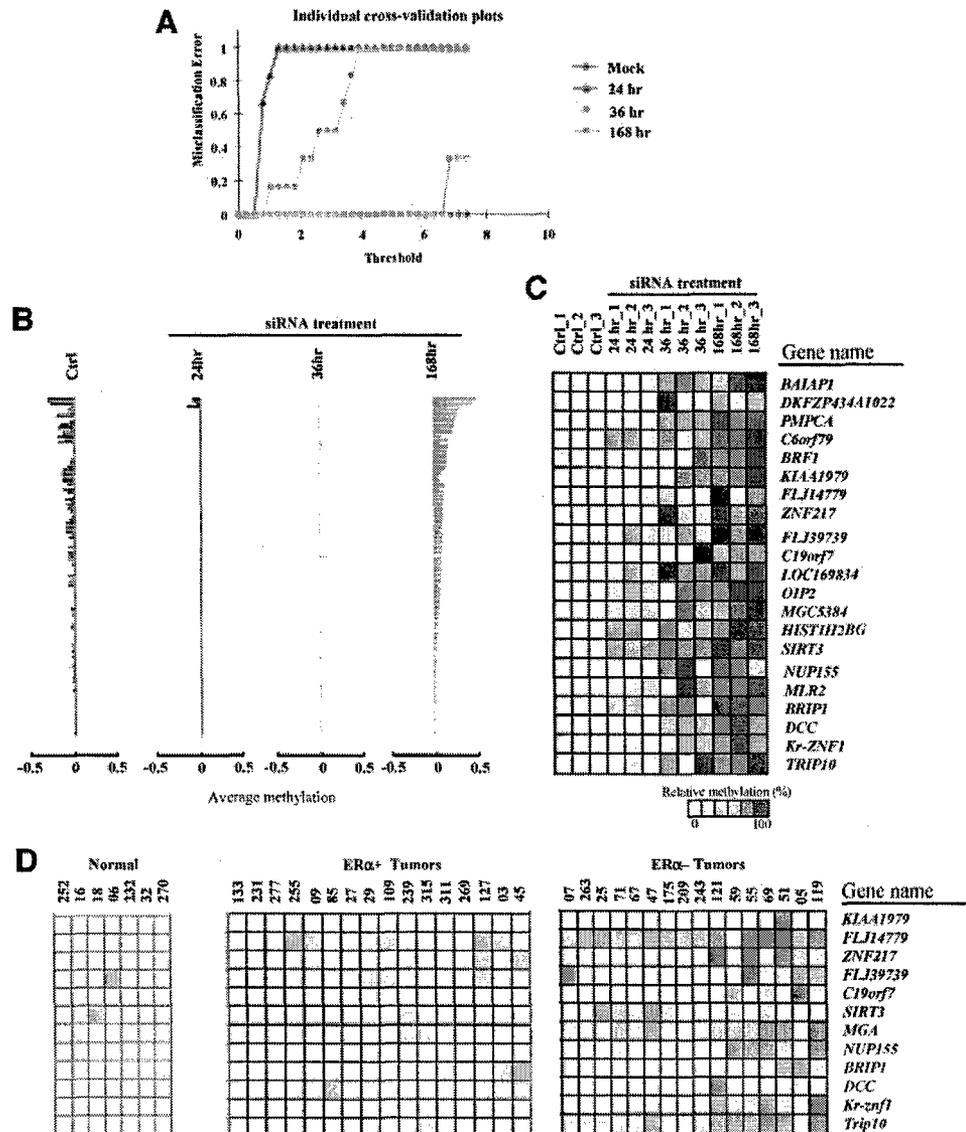


precipitated chromatin. Putative target sequences were used to search for the presence of ER α binding motifs, EREs, and other related binding sites (e.g., AP-1, SP-1, cAMP-responsive element binding protein, and CEBP) by using the Genomatrix⁷ and TFSEARCH⁸ programs. These computational algorithms identified a total of 70 unique ER α promoter targets, which were used to construct a sub-panel genomic microarray (see a partial list of the genes in Supplementary Table S4). The previously described DMH method (25, 26) was then used to determine the DNA methylation status of these ER α targets in siRNA-treated *versus* mock-treated MCF-7 cells. Amplicons representing genomic pools of methylated DNAs were prepared from these treated cells using our established protocols (25, 26). Cy5 (red dye)- and Cy3 (green dye)-labeled DNAs were prepared from siRNA- and mock-treated cells, respectively, and cohybridized to microscope slides containing the arrayed 70 unique ER α targets. ER α target loci methylated in siRNA-treated cells, but not in mock-treated cells, were expected to show greater Cy5/Cy3 hybridization signals. This is because methylated CpG sites are protected from methylation-sensitive restriction (*i.e.*, *HpaII* and *BstUI*) and could thus be amplified by a linker-PCR approach during amplicon preparation. In contrast, unmethylated CpG sites were restricted by the methylation-

sensitive enzymes, could not be amplified by PCR, and were thus devoid of hybridization signals.

To analyze our microarray data, we adapted the "shrunken centroids method" (38) to define the threshold setting for class prediction of methylated ER α target loci. This approach can be used to uniquely define the threshold level that statistically discriminates ER α loci commonly methylated in siRNA-treated cells from the same loci in mock-treated cells. After initial evaluation of the microarray data, we chose the threshold value 2.0 that generates less error (≤ 0.3) for cross-validation (data not shown). When the cross-validation variances from individual samples were plotted (Fig. 5A), many ER α target loci could be used to discriminate between siRNA-treated cells and mock-treated counterparts (manifested as having many loci with no misclassification error) at the 168 hour time point. However, this threshold level was not sufficiently stringent to discriminate between the mock- and siRNA-treated cell samples at 24 or 36 hours (manifested as having very few loci with low misclassification error). In Fig. 5B, the actual methylation status of individual loci, in comparison with the predicted centroids, is plotted to present an overall change of DNA methylation at different time periods of siRNA treatment. Relative to the overall predicted centroids, a positive value of a locus

Fig. 5. Acquired DNA methylation in multiple ER α downstream targets after estrogen signal disruption. Seventy ER α downstream targets were analyzed by DMH, as described in the text. Fluorescence-labeled methylation amplicons were prepared from siRNA-treated (24, 36, and 168 hours) and mock-treated (168 hours) MCF-7 cells, respectively, and cohybridized to ER α microarray slides. The hybridization output is the measured relative intensity of fluorescence reporter molecules. A, test error for different values of shrinkage. Shrunken centroids analysis was conducted using methylation microarray datasets (see detailed description in the text). Tenfold cross-validation was used to estimate the error rate, when a different degree of shrinkage was used to generate the centroids. B, Predicted centroids, shown as horizontal units, represent log ratios of DNA methylation. The order of the 70 ER α target loci is arbitrary. Methylation changes were seen only in a few loci at 24 or 36 hours after siRNA treatment; however, a significant methylation change was seen at 168 hours (7 days; $P < 0.05$), displaying positively shrunken values for these 70 loci. C, methylation heat map of the 21 selected ER α loci at different time periods after siRNA treatment. These loci were selected because a threshold (threshold = 2; error rate ≤ 0.3) from cross-validation showed fewer errors in methylation microarray experiments. As shown, DNA methylation of these loci accumulates progressively over time (168 hours) after the siRNA treatment. Data shown here represent three independent microarray experiments. D, methylation heat map of the top 12 methylated ER α loci in ER α -negative tumors. Microarray-based DMH was conducted in these clinical samples as described in the text. The derived microarray data were analyzed by the shrunken centroids method.



indicates more methylation during the treatment, whereas a negative value indicates less methylation. This shrunken centroid map revealed that *de novo* DNA methylation can be detected in a subset of ER α targets 168 hours after siRNA treatment, but not in cells treated for only 24 or 36 hours after treatment.

To validate the findings of the shrunken centroid analysis, unsupervised cluster analysis was performed on the microarray data, using the top 21 methylated loci selected by machine training ("heat map" shown in Fig. 5C). The result reaffirms the shrunken centroid data in that replicates of each treatment type are clustered together and that the level of methylation increased with the extent of siRNA treatment. A paired *t* test revealed that the methylation status of these 21 loci was significantly different ($P < 0.05$) between the mock-treated (ER α -positive) and siRNA-treated (ER α -negative) cells.

This microarray observation was independently validated by conducting expression and DNA methylation analyses on three newly identified ER α downstream targets, *TRIP10*, *Kr-Znf1*, and *DCC*. In general, the decreased levels of these mRNAs preceded the emergence of DNA methylation at their respective promoter CpG islands (Fig. 6A and B). This epigenetically mediated silencing also indirectly influenced the expression of *MTA3*, a gene known to be regulated via a downstream ER α target and to participate in Mi-2/NuRD nucleosome remodeling (Fig. 6B; ref. 39).

DNA Methylation of ER α Downstream Targets is Preferentially Observed in ER α -Negative Tumors. We next determined whether this *in vitro* finding could be seen *in vivo*. DMH was therefore conducted using the aforementioned 32 primary breast tumors and 7 normal controls. The derived microarray data were then analyzed by the shrunken centroid method. Although the methylation results of these 70 ER α target loci did not clearly segregate tumor samples into subclasses, we observed a general trend that methylated loci appear more frequently in ER α -negative tumors than in ER α -positive tumors ($P < 0.05$). Fig. 5D presents a heat map of the 12 most methylated loci in the studied breast tumors. As shown, we observed higher overall methylation in the ER α -negative tumors (6 of 16 tumors had $>40\%$ methylation in the loci analyzed) than in the ER α -positive tumors (only 1 of 16 tumors achieved the same level of methylation). Also, the total number of loci showing DNA methylation was greater in ER α -negative tumors, when compared with ER α -positive tumors. Only four loci showed a low level of methylation in normal breast samples. Methylation analysis by MSP was further conducted for *TRIP10* in these breast samples (Fig. 6C). Consistent with the microarray finding, *TRIP10* promoter hypermethylation was detected in 50% (8 of 16) of ER α -negative tumors but in none of the 16 ER α -positive tumors analyzed (χ^2 test, $P < 0.005$).

DISCUSSION

Understanding the sequence of how complex epigenetic events are established can provide important insights into the molecular mechanisms underlying gene silencing in cancer. However, the "chicken and egg" issue of which comes first, DNA methylation, histone modification, or others, is an ongoing debate in the epigenetic research community. Many early studies of this issue come from non-mammalian systems. Mutations in a histone methyltransferase specific for H3-K9 resulted in loss of DNA methylation in *Neurospora crassa* (15, 16), suggesting that histone methylation can initiate DNA methylation. In *Arabidopsis*, it has been shown that CpNpG methylation depends on a histone H3 methyltransferase (40), also indicating that histone methylation can direct DNA methylation. New evidence suggests that the reverse scenario can occur in heterochromatin (41). In this case, a self-reinforcing system is implemented, allowing for feedback from DNA methylation to histone methylation for the long-

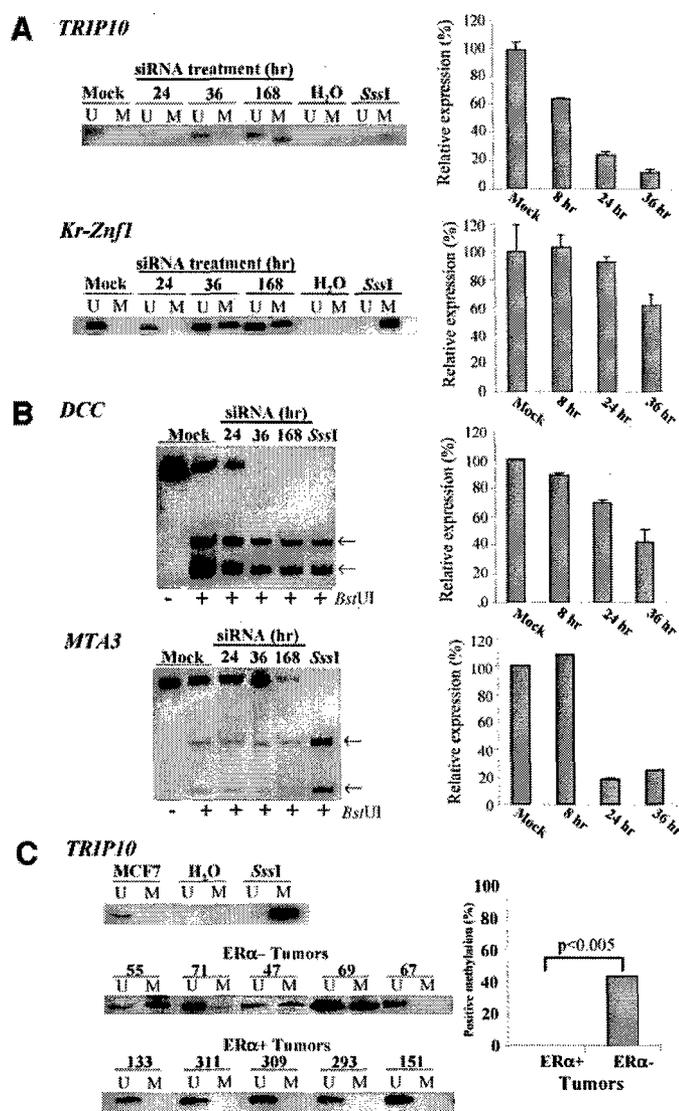


Fig. 6. Methylation and expression analysis of ER α target genes. **A**. For methylation-specific PCR assays, bisulfite-treated DNA samples from siRNA- or mock-treated cells were used for amplification with specific primers for *TRIP10* and *Kr-Znf1*, respectively. 32 P-labeled PCR products for unmethylated (Lanes U) and methylated (Lanes M) DNA strands were separated and displayed on 6% polyacrylamide gels (left panels). Messenger RNA levels of these genes were measured by quantitative real-time RT-PCR (right panels) and normalized using *GAPDH* mRNA expression levels, as described in the text. Results from three independent experiments are shown as means \pm SE. The expression level of these genes was significantly reduced by the siRNA treatment (paired *t* test, $P < 0.017$ for *TRIP10* and $P < 0.048$ for *Kr-Znf1*). **B**. For *DCC* and *MTA3* genes, COBRA was used to measure DNA methylation. Bisulfite DNA samples were amplified and digested with *Bst*UI enzyme and separated on polyacrylamide gels. The digested DNA fragments shown by arrows reflect methylation at the *Bst*UI restriction sites within the *DCC* promoter CpG island (left panels). Messenger RNA levels of these genes were measured by quantitative real-time RT-PCR (right panels) and normalized using *GAPDH* mRNA expression levels, as described in the text. Results from three independent experiments are shown as means \pm SE. The expression level of these genes was significantly reduced by siRNA treatment (paired *t* test, $P < 0.03$ for *DCC* and $P < 0.014$ for *MTA3*). **C**. MSP analysis of the *TRIP10* promoters in 16 ER α -positive and 16 ER α -negative breast tumors. Only representative results are shown.

term maintenance of a heterochromatin state in a gene (41). However, this epigenetic paradigm remains to be explored in mammalian systems. Earlier studies have shown that *in vitro* methylated transgenes can be targets for methyl-CpG-binding proteins, which in turn recruit repressor complexes containing histone deacetylases (17, 18). Fahrner *et al.* (19) suggested that DNA methylation of *hMLH1* can specify unique histone codes for the maintenance of a silenced state. They detected methyl histone 3-lysine 9 in the DNA methylated, transcrip-

tionally silenced promoter CpG island of *hMLH1* in a cancer cell line. Treatment with the DNA demethylating agent 5-AzacC alone, but with not the histone deacetylase inhibitor trichostatin A, resulted in reversal of this repressive histone modification. Taken together, these reports, as well as other studies, imply that in contrast to other organisms, histone modifications may be secondary to DNA methylation in initiating gene silencing in mammalian cells (17, 18, 20, 42).

A study by Bachman *et al.* (43), however, presents a different view with respect to the silencing of the *p16* gene in an experimental system using somatic knockout cells. These authors suggest that chromatin modifications are not totally dependent on prior DNA methylation to initiate gene silencing. In support of this observation, Mutskov and Felsenfeld (44) have recently demonstrated that histone modifications are the primary event associated with the silencing of a transgene, *ILR2*. In this case, a gradual increase in DNA methylation density in and around the *ILR2* promoter was observed after transfection. In contrast to previous observations, these two recent studies therefore suggest that DNA methylation sets up an epigenetic "mark" for the maintenance of long-term silencing, rather than initiating it. Clearly, this epigenetic process is complex and multifaceted, and it is possible that the sequence of epigenetic events for establishing and maintaining the silenced state of a gene can be locus or pathway specific.

The present study suggests that gene inactivation and histone modifications occur before DNA methylation at some ER α target loci. Depicted in Fig. 7 is a hypothetical gene containing an ERE site within the promoter area, the active transcription of which is directly dependent on estrogen signaling. On the removal of this signaling, down-regulation of this gene occurs immediately. Transcriptional repressors (*e.g.*, polycomb proteins) and histone deacetylases are then assembled to its promoter to initiate long-term transcriptional repression. Subsequent recruitment of DNA methyltransferases to the repressor complex methylates CpG sites in the adjacent area. This process may be gradual, with methylation density increasing over time in the targeted area (see the heat map in Fig. 5C). The buildup of DNA

methylation could set up a heritable mark that may eventually replace some of the original repressors to establish a heterochromatin state of long-term silencing. In this case, reactivation of ER α target genes could no longer be achieved by reestablishing estrogen signaling alone (see the example of *PR* in Fig. 4A); it also requires DNA demethylation. In addition to the *PR* gene, we suggest that establishment of epigenetic memory may occur in other critical ER α downstream loci in some breast cancer cells.

The occurrence of DNA methylation in a pathway-specific manner also has a new implication. Altered DNA methylation was originally thought to be a generalized phenomenon arising from a stochastic process in earlier studies (45, 46). This random methylation in tumor suppressor genes at their promoter CpG islands, thus silencing their transcripts, would provide tumor cells with a growth advantage. The specific epigenetic patterns observed in particular cancer types would therefore be derived from clonal selection of the proliferating cells. Some studies (26, 47, 48), however, have indicated that this epigenetic event is not random and that remodeling of the local chromatin structure of a gene may influence its susceptibility to specific DNA methylation. The present study provides some answers to this conundrum. Here we show that dysregulation of normal signaling in cancer cells may result in stable silencing of downstream targets, maintained by epigenetic machinery. This implies that the altered epigenetic condition is pathway specific, rather than a stochastic process in the ER α signaling pathway.

In conclusion, the present study implicates, for the first time, epigenetic influence (*i.e.*, chromatin remodeling and DNA methylation) on transcription of ER α downstream target genes and thus provides a new direction for research in this classical signaling pathway. Unlike irreversible genetic damage, epigenetic alterations are potentially reversible, providing an opportunity for therapeutic intervention in breast cancer. Histone deacetylase inhibitors, alone or together with DNA demethylating agents, may represent novel treatment approaches that could be combined with currently available chemotherapies. Our experimental evidence therefore provides a rationale for such treatment strategies designed to alter aberrant epigenetic processes in hormone-insensitive but receptor-positive breast tumors.

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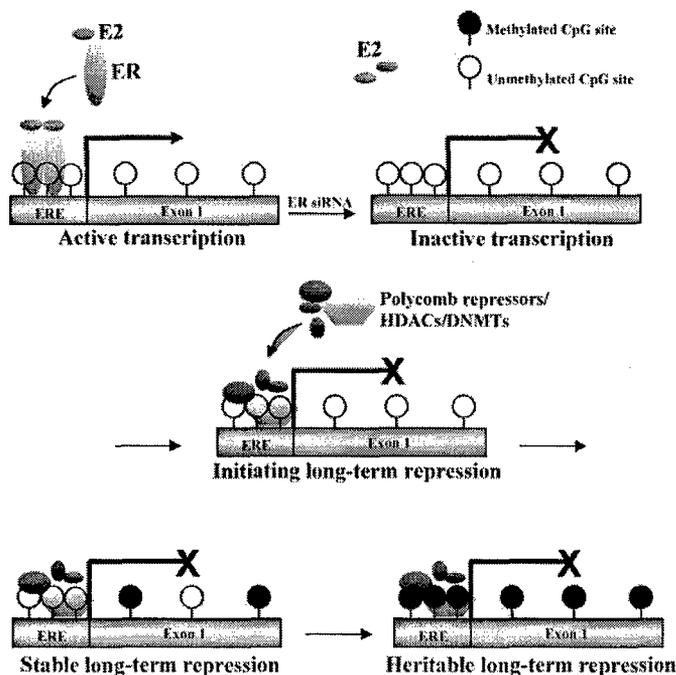


Fig. 7. A proposed model for the epigenetic hierarchy of long-term silencing in ER α downstream targets (see further explanation in the text). HDACs, histone deacetylases; DNMTs, DNA methyltransferases.

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