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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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**In estrogen target cells, estrogen receptor-$\alpha$ (ER$\alpha$) 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$\alpha$ is sequestered within a stable chaperone protein complex consisting of heat shock protein 90 (Hsp90) and cochaperones. However, the molecular mechanism(s) regulating ER$\alpha$ 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$\alpha$ protein degradation was investigated. In ER-negative HeLa cells transfected with ER$\alpha$ and CHIP, ER$\alpha$ proteasomal degradation increased, whereas ER$\alpha$-mediated gene transcription decreased. In contrast, CHIP depletion by small interference RNA resulted in increased ER$\alpha$ 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$\alpha$ down-regulation. In addition, coimmunoprecipitation assays demonstrated that ER$\alpha$ and CHIP associate through the CHIP TPR domain. In ER$\alpha$-positive breast cancer MCF7 cells, CHIP overexpression resulted in decreased levels of endogenous ER$\alpha$ protein and attenuation of ER$\alpha$-mediated gene expression. Furthermore, the ER$\alpha$-CHIP interaction was stimulated by the Hsp90 inhibitor geldanamycin (GA), resulting in enhanced ER$\alpha$ degradation; this GA effect was further augmented by CHIP overexpression but was abolished by CHIP depletion. Finally, ER$\alpha$ dissociation from CHIP by various ER$\alpha$ ligands, including 17$\beta$-estradiol, 4-hydroxytamoxifen, and ICI 182,780, interrupted CHIP-mediated ER$\alpha$ degradation. These results demonstrate a role for CHIP in both basal and GA-induced ER$\alpha$ degradation. Furthermore, based on our observations that CHIP promotes ER$\alpha$ degradation and attenuates receptor-mediated gene transcription, we suggest that CHIP, by modulating ER$\alpha$ stability, contributes to the regulation of functional receptor levels, and thus hormone responsiveness, in estrogen target cells. (*Molecular Endocrinology* 19: 2901–2914, 2005)**

THE PRIMARY MEDIATORS of 17$\beta$-estradiol (E2) action, the major female sex steroid hormone, are the estrogen receptors ER$\alpha$ and ER$\beta$. 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$\alpha$ and its various coactivators and corepressors (2, 3).

To strictly control cellular responses, the cellular synthesis and turnover of the ER$\alpha$ protein dynamically fluctuates with changing cellular environments (4). For example, in the absence of ligand, ER$\alpha$ is a short-lived protein (half-life of 4–5 h) and undergoes constant degradation (5). In the presence of ligand, by contrast, the turnover rate of ER$\alpha$ 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 (ICI), RU 58,668], heat shock protein (Hsp) 90 inhibitors [geldanamycin (GA) and radicicol], ATP depletion (oligomycin and hypoxia) and aryl hydrocarbon agonists; these all induce degradation and rapid down-regulation of ER$\alpha$

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Abbreviations: CHIP, Carboxyl terminus of Hsc70-interacting protein; CHIPi, CHIP-siRNA expression construct; CMV, cytomegalovirus promoter; DMSO, dimethylsulfoxide; E2, 17$\beta$-estradiol; ER$\alpha$, estrogen receptor-$\alpha$; ERE, estrogen response element; FBS, fetal bovine serum; GA, geldanamycin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HA, hemagglutinin; Hsp, heat shock protein; ICI, ICI 182,780; Luc, firefly luciferase; OHT, 4-hydroxytamoxifen; siRNA, small interference RNA; SV40, simian virus 40 promoter; TPR, tetratricopeptide repeat.

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levels (6–12). In contrast, the partial agonist/antagonist 4-hydoxytamoxifen (OHT), thyroid hormone, and protein kinase K activators (forskolin, 8-bromo-cAMP) all block receptor degradation, subsequently increasing ERα protein levels (13–15).

Although both basal and ligand-induced ERα 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 ERα 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 synthesis (17, 19, 22, 24); conversely, neither ERα transcriptional activity nor new protein synthesis are needed for ICI-induced ERα degradation (19, 20, 22). In addition, various stimuli induce distinct changes in the conformation and cellular compartmentalization of ERα (22, 25–27), and these may be associated with receptor ubiquitination.

Like other members of the steroid receptor superfamily, unliganded ERα, by associating with various Hsp90-based chaperone complexes, is maintained in a ligand-binding competent conformation (28). Although these associations do not influence ERα ligand-binding affinity, Hsp90 chaperone complexes appear to regulate ERα stability because Hsp90 disruption induces rapid ERα 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 (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 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 receptor, androgen receptor, Smad1/4, and ErbB2 (30–33), we investigated a regulatory role for CHIP in ERα stability. Our results demonstrate that CHIP, likely through a chaperone intermediate, associates with ERα and consequently facilitates both basal and GA-induced receptor degradation in human cancer cells.

## RESULTS

### CHIP Overexpression Decreases and CHIP Knockdown Increases ERα Protein Levels

To investigate the effect of CHIP overexpression on steady-state levels of ERα, ER-negative HeLa cells were cotransfected with constructs expressing CHIP (pcDNA-His6-CHIP) and ERα (pSG5-ERα). ERα protein levels were subsequently determined by immunoblot analysis. Overexpression of CHIP decreased ERα protein levels in a dose-dependent manner (Fig. 1A). To control for transfection efficiency, the green fluorescent protein (GFP) was also included in transfection. No effect of CHIP on GFP expression level was observed (Fig. 1A), demonstrating that CHIP-induced down-regulation of ERα was specific. Next, we examined whether CHIP-induced ERα down-regulation could be inhibited by CHIP-specific small interference RNA (siRNA). Compared with cells transfected with CHIP only, cotransfection of pBS/U6/CHIPi, a CHIP-siRNA expression construct (33), dramatically decreased the level of exogenous CHIP (Fig. 1B, *upper*

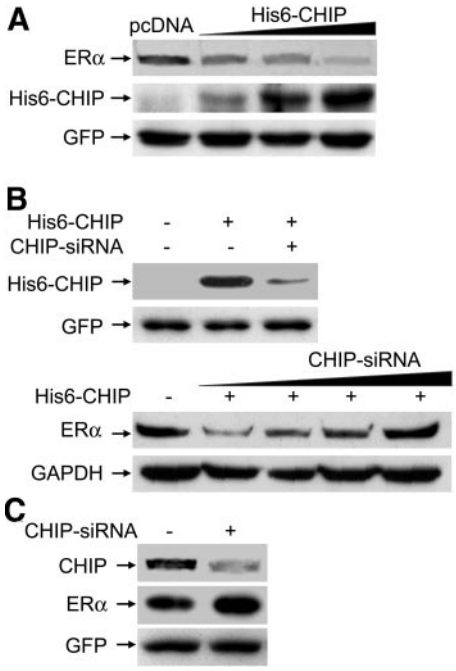


**Fig. 1.** CHIP Overexpression Decreases and CHIP Knockdown Increases ERα Protein Levels

A, Overexpression of CHIP down-regulates ERα protein levels. HeLa cells were transfected with 250 ng pSG5-ERα, 100 ng CMV-GFP, and various amounts (0, 50, 100, and 250 ng) of pcDNA-his6-CHIP. B, Expression of CHIP-siRNA attenuates CHIP-induced ERα down-regulation. In the *upper panel*, HeLa cells were transfected with 250 ng pcDNA-his6-CHIP, with or without 250 ng pBS/U6/CHIPi, as indicated. In the lower panel, HeLa cells were transfected with 250 ng pSG5-ERα, 250 ng pcDNA-his6-CHIP, and various doses (150, 300, 500, and 1000 ng) of pBS/U6/CHIPi. C, Knockdown of endogenous CHIP increases ERα level. HeLa cells were transfected with 250 ng pSG5-ERα and either 250 ng pcDNA-his6-CHIP or 250 ng pBS/U6/CHIPi, as indicated. For all experiments, $3 \times 10^5$ HeLa cells were plated in 60-mm dishes, cultured in hormone-free medium for 3 d, and then transfected with LipofectAMINE Plus Reagent. Cell lysates were prepared 24 h after transfection. Protein levels were determined by immunoblotting with specific antibodies. Exogenous His6-CHIP and endogenous CHIP were detected by anti-His6 and anti-CHIP, respectively. GFP and GAPDH were used as transfection control and SDS-PAGE loading controls, respectively. Representative results of two independent experiments, each performed in duplicate, are shown.

*panel*). However, pBS/U6/CHIPi had no effect on GFP level, confirming that the CHIP-siRNA specifically blocks CHIP expression (Fig. 1B). The effect of CHIP-siRNA on CHIP-induced ERα down-regulation was then examined. As shown in Fig. 1B (*lower panel*), cotransfection of CHIP-siRNA, in a dose-dependent fashion, attenuated ERα down-regulation induced by exogenous CHIP. Collectively, these results demonstrate that CHIP overexpression can down-regulate ERα protein level in HeLa cells.

To examine a role for endogenous CHIP in regulation of ERα protein levels, HeLa cells, which are known to express CHIP (30), were cotransfected with pBS/U6/CHIPi and ERα. Expression of CHIP-siRNA decreased the level of endogenous CHIP by 60%, and correspondingly increased ERα protein level by 1.6-fold (Fig. 1C), indicating that endogenous CHIP plays a role in controlling ERα level in HeLa cells.

### CHIP Down-Regulates ERα Levels through the Ubiquitin Proteasome Pathway

To determine whether proteasome activity is required for CHIP-induced ERα down-regulation, HeLa cells were cotransfected with pcDNA-His6-CHIP and pSG5-ERα, treated with the protease inhibitor MG132, and subjected to immunoblotting. As shown in Fig. 2A, a 6-h treatment with MG132 completely blocked CHIP-induced down-regulation of ERα. To examine whether polyubiquitination is required for CHIP-induced ERα degradation, a mutant ubiquitin, UbK0, with all lysines replaced by arginines (34), was used. Previously, we showed that the UbK0 protein could efficiently block E2-induced ERα degradation (35). Expression of UbK0, but not wild-type ubiquitin, restored ERα protein levels (Fig. 2B), demonstrating that CHIP stimulates ERα degradation through the ubiquitin and proteasome pathway.

### CHIP Targets Mature ERα 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 ERα down-regulation by CHIP was due to the selective ubiquitination of unfolded or misfolded receptor protein, we examined the effect of OHT, a selective ER modulator, on CHIP-mediated ERα degradation. It has been shown that OHT can dissociate ERα 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 ERα (with no functional OHT-binding pocket), then, in the presence of CHIP, OHT treatment should not restore ERα levels. On the other hand, if CHIP targets mature ERα, OHT treatment should rescue the receptor protein from CHIP-induced degradation. HeLa cells were thus cotransfected with pcDNA-His6-CHIP and pSG5-ERα and treated with OHT for 6 h before lysate preparation. OHT treatment completely abolished CHIP-

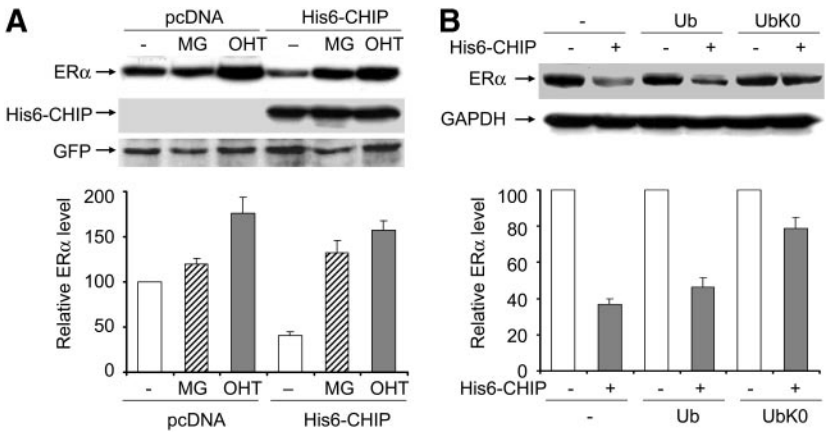


**Fig. 2.** The Proteasome Inhibitor MG132, Partial ERα-Antagonist OHT, and Ubiquitin Mutant UbK0, All Block CHIP-Induced ERα Degradation

A, The proteasome inhibitor MG132 and the partial ERα antagonist OHT block CHIP-induced ERα down-regulation. HeLa cells were transfected with 250 ng pSG5-ERα and 100 ng CMV-GFP, along with 250 ng pcDNA (vector control) or pcDNA-His6-CHIP, then treated with DMSO (vehicle), 10 μM MG312 or 1 μM OHT for 6 h before immunoblot analysis. Protein levels of ERα, CHIP and GFP were determined by immunoblotting with anti-ERα, 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 ERα down-regulation. HeLa cells were transfected with 250 ng pSG5-ERα, with or without 250 ng pcDNA-His6-CHIP, pcDNA-Ub, or pCS2-UbK0, as indicated. ERα protein levels were determined by immunoblotting with anti-ERα. GAPDH was used as a loading control for SDS-PAGE. For all experiments, $3 \times 10^5$ HeLa cells were plated in 60-mm dishes, cultured in hormone-free medium for 3 d, and then transfected with LipofectAMINE Plus Reagent. Cell lysates were prepared 24 h after transfection. The band density of exposed films was evaluated with ImageJ software. Relative ERα levels were presented as the mean ± SE of three independent experiments, each performed in duplicate.

induced ERα down-regulation (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 ERα.

### Both the TPR and U-Box Domains Are Essential for CHIP-Induced ERα Down-Regulation

To examine whether the ubiquitin ligase activity and chaperone interaction domain are required for CHIP-induced ERα degradation, two mutant CHIP constructs were used: 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 ERα 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 ERα protein.

### The TPR Domain of CHIP Is Required for the CHIP-ERα Interaction

As CHIP appears to be linked to ERα degradation, we investigated whether CHIP associates with the receptor. HeLa cells were cotransfected with ERα and CHIP, and coimmunoprecipitation analysis performed using an ERα-specific antibody. The results revealed a complex containing both CHIP and ERα (Fig. 3B). Because CHIP(K30A) exhibited no effect on ERα turnover (Fig. 3A), we examined whether the TPR domain is required for the CHIP-ERα interaction. In HeLa cells cotransfected with ERα and CHIP(K30A), the CHIP mutant was not detected in the precipitated ERα complex (Fig. 3B), demonstrating a requirement for the TPR domain in the CHIP-ERα 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 ERα degradation.

### CHIP Interacts with Endogenous ERα, in Breast Cancer Cells, to Induce Receptor Ubiquitination and Degradation

Having demonstrated a role for CHIP (possibly in association with chaperones) in degradation of exogenous ERα in HeLa cells, it was of interest to examine the effect of CHIP on stability and function of endogenous ERα in breast cancer cells. In human breast cancer MCF7 cells, overexpression of CHIP resulted in a dose-dependent ERα down-regulation (Fig. 4A). Coimmunoprecipitation analysis of MCF7 cells transfected with pcDNA-His6-CHIP revealed both CHIP and ERα in the immunocomplexes precipitated by either an ERα-specific or anti-His6 antibody (Fig. 4B), suggesting that CHIP associates with endogenous

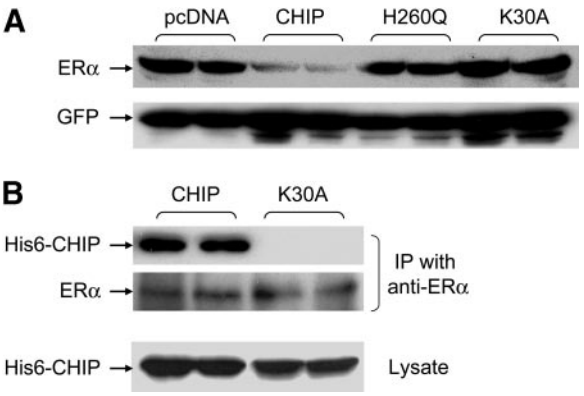


**Fig. 3.** Both the TPR and U-Box Domains Are Required for CHIP-Induced ERα Down-Regulation

A, Both the TRP and U-box domains are required for CHIP to down-regulate ERα. HeLa cells were transfected with 250 ng pSG5-ERα, 100 ng CMV-GFP, along with 250 pcDNA (control) or various CHIP constructs, as indicated. ERα and GFP protein levels were determined by immunoblotting with anti-ERα and anti-GFP, respectively. GFP was used as control for transfection efficiency and SDS-PAGE loading. B, The TRP domain is required for CHIP-ERα interaction. HeLa cells were transfected with 250 ng pSG5-ERα, along with 250 ng pcDNA-His6-CHIP or pcDNA-His6-CHIP(K30A). ERα protein in cell lysates was precipitated with anti-ERα. The presence of CHIP in the precipitated ERα complex was determined by immunoblotting with anti-His6. The same blot was reprobed with anti-ERα to assess the amount of ERα 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 $3 \times 10^5$ cells/dish, cultured in hormone-free medium for 3 d, and then transfected with LipofectAMINE Plus Reagent. Cell lysates were prepared 24 h after transfection. Representative results of two independent experiments, each performed in duplicate, are shown. IP, Immunoprecipitation.

ERα. In addition, both Hsc70 and Hsp90 were detected in the precipitated ERα complex (Fig. 4B). These results indicate that CHIP can associate with endogenous ERα-Hsp90/Hsc70 complexes to down-regulate ERα level in breast cancer cells.

To determine whether CHIP promotes polyubiquitination of endogenous ERα, we examined the ubiquitination status of ERα in MCF7 cells transfected with hemagglutinin-tagged ubiquitin (HA-Ub), plus a vector control (pcDNA) or a CHIP-expressing construct. To block proteasomal degradation of polyubiquitinated proteins, transfected cells were treated with MG132 for 6 h before lysate preparation. An ERα-specific antibody was then used for immunoprecipitation, and the presence of ubiquitinated ERα in the immunocomplex was 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 ERα exhibited a typical high-molecular-weight smear on the blot membrane, and overexpression of CHIP markedly increased smear intensity, suggesting elevated receptor

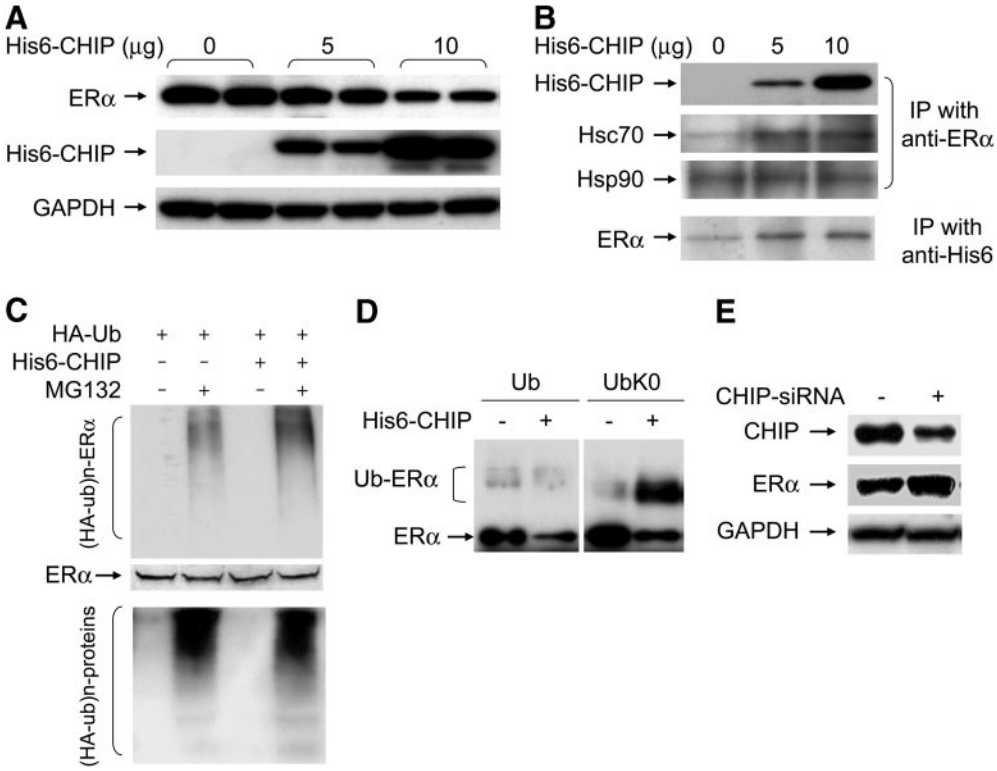


**Fig. 4.** CHIP Interacts with Endogenous ERα and Induces ERα Ubiquitination and Degradation in Breast Cancer MCF7 Cells

A, Overexpression of CHIP down-regulates endogenous ERα levels in MCF7 cells. MCF7 cells were plated in 100-mm dishes at a density of $1 \times 10^6$ cells/dish, cultured in hormone-free medium for 3 d, and transfected with various amounts (0, 5, or 10 μg) of pcDNA-His6-CHIP using FuGENE. Twenty-four hours after transfection, whole cell lysates were prepared, and protein levels of ERα and CHIP determined by immunoblotting with anti-ERα and anti-His6, respectively. GAPDH was used as an SDS-PAGE loading control. B, CHIP associates with ERα-Hsp complex in MCF7 cells. MCF7 cells were transfected as in panel A and subjected to coimmunoprecipitation analysis. ERα and CHIP were precipitated with anti-ERα and anti-His6, respectively. The presence of CHIP, Hsc70, Hsp90, or ERα in the precipitated complexes was determined by immunoblotting with anti-His6, anti-Hsc70, anti-Hsp90, or anti-ERα, respectively. C, Expression of CHIP enhances endogenous ERα polyubiquitination in MCF7 cells. MCF7 cells were plated in 60-mm dishes at a density of $5 \times 10^5$ cells/dish cultured in hormone-free medium for 3 d, 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 ERα protein was precipitated with anti-ERα. The presence of ubiquitin-conjugated ERα in the immunocomplex was detected by immunoblotting with anti-HA (*upper panel*). The same membrane was reprobed with anti-ERα to assess the amount of precipitated ERα (*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*). D, CHIP increases ERα ubiquitination in MCF7 cells expressing UbK0. MCF7 cells were plated as in panel C and transfected with 500 ng pcDNA-HA-Ub or 500 ng pCS2-UbK0, along with 250 ng pcDNA or pcDNA-His6-CHIP, as indicated. Twenty-four hours after transfection, whole cell lysates were prepared and ERα protein was detected by immunoblotting with anti-ERα. E, Knockdown of endogenous CHIP increases ERα level. MCF7 cells were plated as in panel C and transfected with 2 μg vector or pBS/U6/CHIPi using FuGENE. Forty-eight hours after transfection, whole cell lysates were prepared, and protein levels of CHIP and ERα were determined by immunoblotting with anti-CHIP and anti-ERα, respectively. GAPDH was used as an SDS-PAGE loading control. For all experiments, representative results of two independent experiments, each performed in duplicate, are shown. IP, Immunoprecipitation.

polyubiquitination (Fig. 4C, *upper panel*). In contrast, CHIP had no effect on overall protein ubiquitination (Fig. 4C, *lower panel*).

A possible limitation of *in vivo* ubiquitination assays is that the immunocomplex may contain multiple polyubquitinated species, not just the target protein of interest. To corroborate the observation that CHIP promotes ERα ubiquitination, we examined the effect of CHIP on ERα-ubiquitination in MCF7 cells transfected with UbK0. This mutant ubiquitin competes with endogenous ubiquitin and terminates ubiquitin chains, resulting in the accumulation of oligoubiquitin-ERα conjugates, which upon immunoblotting with ERα antibody can be detected as mobility-shifted bands. In MCF7 cells transfected with wild-type ubiquitin, overexpression of CHIP had no effect on the intensity of ERα-ubiquitination (Fig. 4D, *left panel*), presumably due to the rapid degradation of polyubiquitinated ERα. However, in cells transfected with UbK0, overexpression of CHIP remarkably increased the amount of oligoubiquitinated ERα (Fig. 4D, *right panel*), confirming that overexpression of CHIP promotes ERα ubiquiti-

nation. Together, these results suggest that CHIP, by facilitating receptor ubiquitination, targets endogenous ERα for proteasome-mediated degradation.

### Knockdown of Endogenous CHIP by siRNA Increases ERα Level in MCF7 Cells

The above experiments showed that overexpression of CHIP promotes ERα polyubiquitination and degradation in breast cancer cells. Conversely, we wanted to examine whether knockdown of endogenous CHIP protein by CHIP-siRNA could increase endogenous ERα level. Transfection of MCF7 cells with pBS/U6/CHIPi decreased the level of endogenous CHIP by 60% (Fig. 4E, *upper panel*) and increased the level of ERα level by 1.5-fold (Fig. 4E, *lower panel*), indicating that endogenous CHIP plays a role in basal turnover of ERα in breast cancer cells.

### CHIP Down-Regulates ERα-Mediated Gene Expression

Having established a role for CHIP in ERα ubiquitination and receptor turnover, we next examined the effect of CHIP on ERα-mediated gene transactivation. HeLa cells were transiently transfected with ERα and an estrogen-responsive reporter (ERE-pS2-Luc), plus various CHIP (CHIP, H260Q, K30A, CHIP-siRNA) or control (pcDNA) constructs. Twenty-four hours after transfection, cells were treated for 6 h with vehicle [dimethylsulfoxide (DMSO)] or E2 (10 nM) and luciferase activity then measured. In a parallel experiment, a constitutive reporter [simian virus 40 promoter-firefly luciferase (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-induced ERE-pS2-Luc expression, whereas the CHIP mutants had no effect on ERα-mediated gene transactivation (Fig. 5A). Conversely, depletion of endogenous CHIP by siRNA increased both basal and E2-induced ERE-pS2-Luc expression ($P < 0.05$, Fig. 5B). Similarly, in MCF7 cells, overexpression of CHIP, but not U-box or TPR mutant, attenuated ERα-mediated reporter gene expression (Fig. 6A), whereas knockdown of endogenous CHIP by siRNA augmented ERα-mediated reporter gene expression (Fig. 6B). To examine the effect of knocking down CHIP on the expression of an endogenous ERα target gene, MCF7 cells were transfected with CHIP-siRNA, and *pS2* mRNA levels were examined. As shown in Fig. 6C, both basal and E2-induced expression of *pS2* mRNA were significantly increased. Together, these results demonstrate that CHIP coordinately regulates ERα protein levels and ERα-mediated gene transactivation.

### GA Induces ERα Degradation through a CHIP-Dependent Mechanism

The Hsp90 inhibitor, GA, binds to the amino-terminal ATP/ADP-binding 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 ERα degradation through the ubiquitin-proteasome pathway (9, 29, 37). Whether CHIP plays a role in GA-induced ERα degradation has not been previously investigated. Thus, we examined the effects of CHIP overexpression and depletion on GA-induced ERα degradation. In HeLa cells transfected with ERα, GA treatment resulted in a time-dependent ERα down-regulation (Fig. 7A); this effect was enhanced by CHIP overexpression (Fig. 7A). Conversely, CHIP depletion by siRNA completely abolished GA-induced ERα down-regulation (Fig. 7A).

To investigate the effect of GA on the CHIP-ERα interaction, HeLa cells were transfected with ERα and

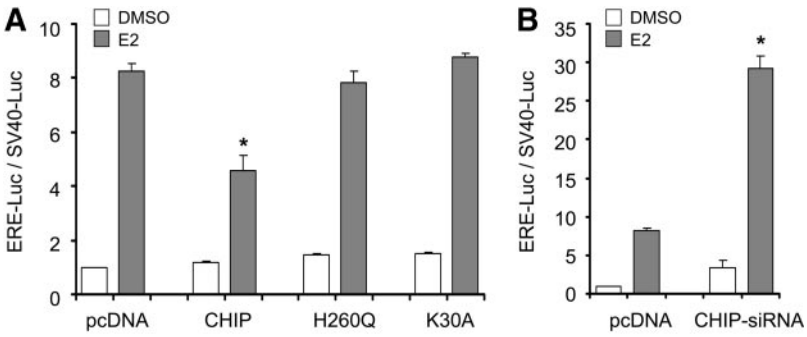


**Fig. 5.** CHIP Down-Regulates ERα-Mediated Reporter Gene Expression in HeLa Cells

HeLa cells were plated in 12-well dishes at a density of $1 \times 10^5$/well, grown in hormone-free medium for 3 d, and transfected with 10 ng pSG5-ERα, 250 ng ERE-pS2-Luc, 250 ng various CHIP constructs (A) or pBS/U6/CHIPi (B). Twenty-four hours after transfection, cells were treated for 6 h 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 ± SE from three independent experiments, with each performed in quadruplicate. *, $P < 0.05$ (Student's *t* test, *vs.* pcDNA treated with E2).

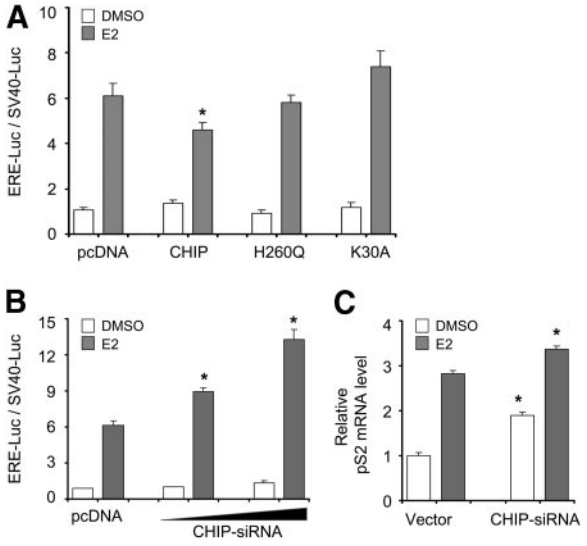


**Fig. 6.** CHIP Down-Regulates ERα-Mediated Gene Expression in MCF7 Cells

A, Overexpression of CHIP Inhibits ERα-Mediated Reporter Gene Expression. MCF7 cells were plated in 12-well dishes at a density of $1 \times 10^5$/well, grown in hormone-free medium for 3 d, and transfected with 250 ng ERE-pS2-Luc, along with 250 ng various CHIP constructs. Twenty-four hours after transfection, cells were treated with DMSO or 10 nM E2 for 6 h and then assayed for luciferase. The ERE-pS2-Luc activity was normalized to SV40-Luc activity (determined in a parallel experiment where ERE-pS2-Luc was replaced with SV40-Luc). Results are expressed as the mean ± SE from three independent experiments, each performed in quadruplicate. *, $P < 0.05$ (Student's *t* test, *vs.* pcDNA treated with E2). B, Knockdown of CHIP by siRNA increases ERα-mediated reporter gene expression. MCF7 cells were plated as in panel A and transfected with 250 ng ERE-pS2-Luc and various amounts (0, 250, and 500 ng) of pBS/U6/CHIPi. Twenty-four hours after transfection, cells were treated and subjected to luciferase analysis, as in panel A. C, Knockdown of CHIP by siRNA increases expression of pS2 mRNA. MCF7 cells were plated in 100 mm dishes at a density of $1 \times 10^6$/dish, grown in hormone-free medium for 3 d, and transfected with 5 μg vector or pBS/U6/CHIPi using FuGENE. Forty-eight hours after transfection, cells were treated for 6 h with DMSO or 10 nM E2. The mRNA level of pS2 was determined by real-time quantitative PCR. The relative pS2 mRNA levels were normalized with β-actin mRNA and expressed as mean ± SE from three independent experiments, each performed in duplicate. *, $P < 0.05$ (Student's *t* test, CHIP-siRNA *vs.* pcDNA).

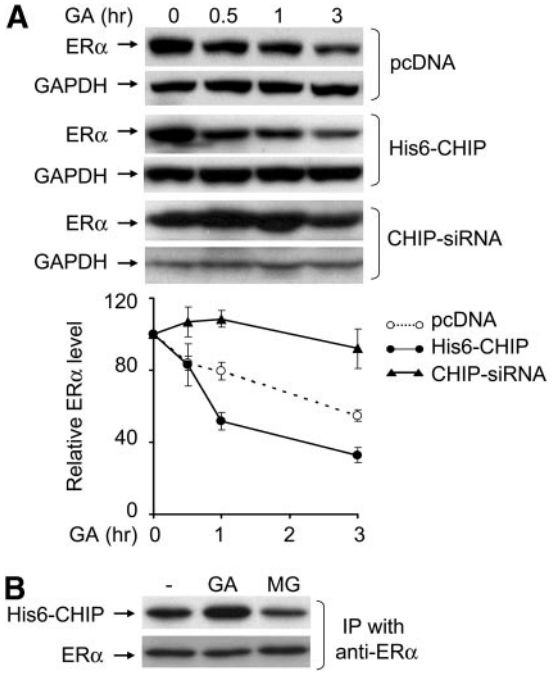


**Fig. 7.** Disruption of Hsp90 Function Induces ERα Degradation through a CHIP-Dependent Mechanism in HeLa Cells

A, CHIP overexpression augments and CHIP depletion by siRNA blocks GA-induced ERα degradation. HeLa cells were plated in 60-mm dishes at a density of $3 \times 10^5$ cells/dish, cultured in hormone-free medium for 3 d, and transfected with 250 ng pSG5-ERα, 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 μM GA for 0, 0.5, 1, and 3 h. Cell lysates were immunoblotted with anti-ERα. GAPDH was used as an SDS-PAGE loading control. The band density of exposed films was evaluated with ImageJ software. Relative ERα levels were presented as mean ± SE from three independent experiments. B, GA enhances CHIP-ERα interaction. HeLa cells were plated as in panel A and transfected with 250 ng pSG5-ERα and 250 ng pcDNA-His6-CHIP. Twenty-four hours after transfection, cells were untreated or treated with 1 μM GA or 10 mM MG132 for 1 h before lysate preparation. ERα protein was precipitated by anti-ERα and the presence of CHIP determined by immunoblotting with anti-His6. The same membrane was then reprobed with anti-ERα to assess the amount of precipitated ERα in the same complex. Representative results of three independent experiments, each performed in duplicate, are shown. IP, Immunoprecipitation.

CHIP, and coimmunoprecipitation was performed with an ERα-specific antibody. The amount of CHIP in the precipitated ERα complex increased after a 1-h GA treatment (Fig. 7B), suggesting that GA promotes ERα degradation by recruiting CHIP to the chaperone-ERα complex. Because CHIP can associate with ubiquitinated proteins through its U-box domain (31), ERα ubiquitination may play a role in the GA-induced ERα-CHIP interaction. We thus examined the interaction between ERα and CHIP in the presence of the proteasome inhibitor MG132. We reasoned that if CHIP preferentially interacts with ubiquitinated ERα, then MG132, by enhancing the accumulation of polyubiquitinated ERα, would increase the ERα-CHIP interaction. However, MG132 treatment did not increase the amount of CHIP precipitated with the ERα complex (Fig. 7B), suggesting that the GA-induced ERα-CHIP interaction occurs before ERα polyubiquitination.

To establish a role for CHIP in GA-induced ERα degradation under physiologically relevant conditions, the consequence of knocking down endogenous CHIP by siRNA on ERα degradation was examined in MCF7 cells. GA induced rapid ERα down-regulation in MCF7

cells transfected with a pcDNA control plasmid (Fig. 8A), consistent with previous reports (9, 29). However, expression of CHIP-siRNA significantly impaired GA-induced ERα down-regulation (Fig. 8A). In addition, we performed a coimmunoprecipitation analysis to examine the effect of GA treatment on the association between endogenous CHIP and ERα. As shown in Fig. 8B, GA treatment increased the amount of CHIP that coimmunoprecipitated with ERα. Based on these results, we suggest that GA induces ERα degradation by enhancing the recruitment of CHIP to ERα-chaperone complexes.

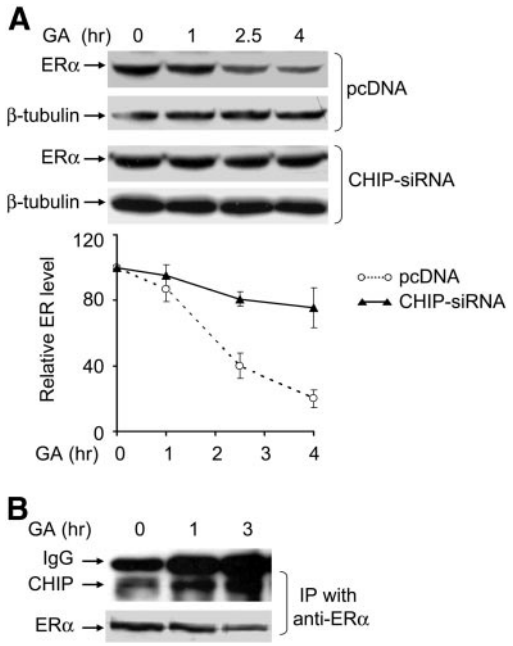


**Fig. 8.** CHIP Is Required for GA-Induced ERα Degradation in Breast Cancer MCF7 Cells

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

### Effects of Ligand Binding on GA-Induced ERα Degradation

Ligand binding results in disassembly of the ERα-Hsp90 chaperone complex, due to competition for overlapping binding sites and conformational changes within the ERα protein (28). Because GA stimulated the CHIP-ERα interaction (Figs. 7B and 8B), we investigated whether ligand binding, by interrupting the CHIP-ERα interaction, could interfere with GA-induced ERα degradation. Toward this, ERα protein levels were examined in MCF7 cells: 1) exposed to E2, 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-h treatment with E2, OHT, or ICI. As expected, E2, ICI and GA treatment, but not OHT, dramatically down-regulated ERα levels in MCF7 cells (Fig. 9A, *upper panel*). Exposure to E2 or OHT, either before (Fig. 9A, *middle panel*) or shortly after (Fig. 9A, *lower panel*) GA treatment, completely abolished GA-induced ERα degradation. In contrast to what was observed with E2 and OHT, ICI exposure, neither before (Fig. 9A, *middle panel*) nor shortly after (Fig. 9A, *lower panel*) GA treatment, failed to protect ERα against degradation.

To examine the effect of these ligands on the CHIP-ERα interaction, 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 ERα 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 and ERα. Because these ligands have dramatically different effects on ERα stability, our results indicate that after dissociation from the Hsp90 chaperone complex, distinct downstream pathways exist for ERα degradation. Because E2 alone can induce ERα degradation through a transcription coupled mechanism (17, 19, 22, 24), it was somewhat unexpected to observe that ERα was stable during the combined treatment of GA and E2 (Fig. 9A). One explanation is that Hsp90 activity (inhibited by GA) is required for transcription-coupled ERα degradation. The OHT-ERα 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 ERα degradation is likely due to disruption of the CHIP-ERα interaction (Fig. 9B). ICI also interrupts the GA-induced CHIP-ERα interaction (Fig. 9B) but fails to stabilize ERα (Fig. 9A), suggesting that the ERα-ICI complex is targeted for degradation through a CHIP-independent, GA-insensitive pathway.

### Effect of CHIP and GA on ER α Cellular Localization

CHIP and Hsp90 are located primarily in the cytoplasm (30), whereas ERα is primarily a nuclear-localized pro-

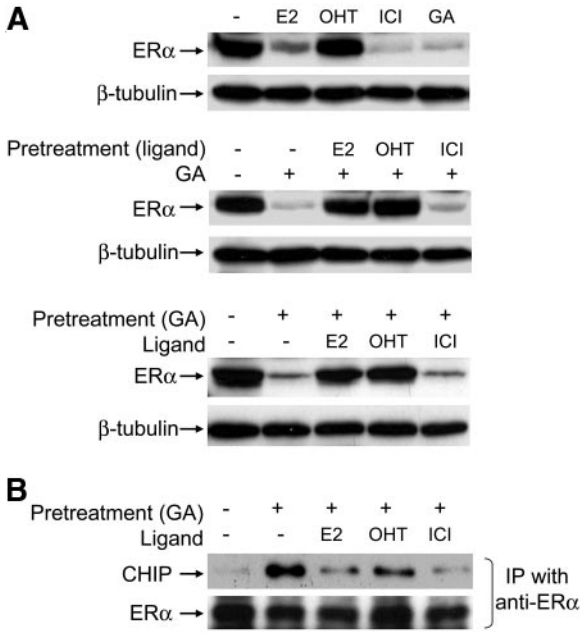


**Fig. 9.** Effect of Ligand Binding on Geldanamycin-Induced ERα Degradation

A, ERα 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 $3 \times 10^5$ cells/dish and cultured in hormone-free medium for 3 d. *Upper panel*, Cells were treated with vehicle, 10 nM E2, 1 μM OHT, 100 nM ICI or 1 μM GA for 6 h; *middle panel*, cells were exposed to indicated ligand for 30 min before a 6-h GA treatment; *lower panel*, 30 min after GA treatment, cells were exposed to indicated ligand for 5.5 h. For all experiments, ERα levels were determined by immunoblotting with anti-ERα. β-Tubulin was used as SDS-PAGE loading control. B, Effect of ligands on GA-induced CHIP-ERα interaction. MCF7 cells were plated in 100-mm dishes at a density of $1 \times 10^6$ cells/dish, cultured in hormone-free medium for 3 d, and then transfected with 5 μg pcDNA-His6-CHIP by using FuGENE. Twenty-four hours after transfection, the cells were treated with 1 μM GA for 30 min, followed by a 30-min treatment with indicated ligands (100 nM E2, 1 μM OHT, and 100 nM ICI). ERα protein from the cell lysates was precipitated using anti-ERα. CHIP presence in the precipitated ERα complex was determined by immunoblotting with anti-His6. The same membrane was reprobed with ERα antibody to assess the amount of precipitated ERα. Representative results of two independent experiments, each performed in duplicate, are shown. IP, Immunoprecipitation.

tein (39). To determine whether CHIP overexpression, or GA treatment, could affect the cellular distribution of ERα, HeLa cells were transfected with a GFP-ERα fusion protein (40) and the cellular distribution of green fluorescence was 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-ERα (Fig. 10A). In contrast, ICI treatment, either alone or in the presence of transfected CHIP, resulted in the appearance of green fluorescence in the cytoplasm (Fig. 10A, *bottom two panels*). This observation is consistent with a previous study by Dauvois *et al.* (7) showing that ICI induces cytoplasmic retention of ERα. In addition, in HeLa cells transfected with GFP-ERα 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*). These GFP foci were not observed in GA-treated cells cotransfected with CHIP (Fig. 10A, *right middle panel*). Although the identity of the GFP foci is unknown, one possibility is that these represent aggregated GFP-ERα, resulting from the combined effect of Hsp90 inhibition and high expression levels of GFP-ERα. CHIP overexpression may promote both basal and GA-induced ERα degradation, preventing GFP-ERα aggregate formation. Consistent with this interpretation, we found that expression of CHIP decreased the number of GFP-ERα-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-mediated ERα degradation occurs within the nucleus.

## DISCUSSION

The cellular level of ERα determines both estrogen sensitivity and responsiveness (2, 35, 42). Steady-state levels of ERα 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 ERα degradation is primarily mediated by the ubiquitin proteasome pathway, the molecular mechanism(s) by which cells regulate ERα 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 ERα turnover. Furthermore, CHIP-induced receptor degradation occurs through the ubiquitin proteasome pathway. Overexpression of CHIP promotes ERα degradation, accompanied by a decrease in ERα-mediated gene transactivation. Conversely, inhibition of CHIP by siRNA increases ERα levels and up-regulates ERα-mediated gene transactivation. Thus, this is the first report that CHIP, by modulating the cellular concentration of ERα, plays a role in regulating estrogen action.

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

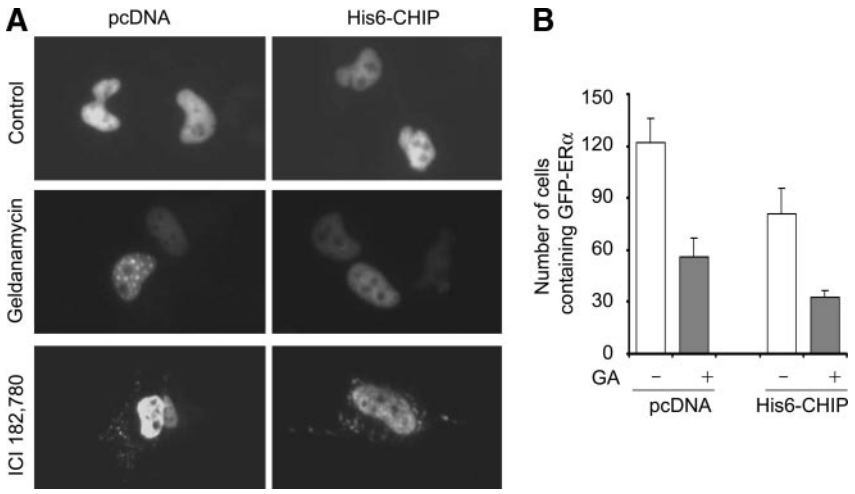


**Fig. 10.** Effect of CHIP and GA on ERα Cellular Localization

HeLa cells were plated in six-well dishes at a density of $1 \times 10^5$ cells/dish, cultured in hormone-free medium for 3 d, and transfected with 250 ng GFP-ERα 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 μM GA or 100 nM ICI for 6 h. The fluorescence of GFP-ERα was then examined using an inverted microscope (Axiovert 40 CFL) (A). The number of cells expressing GFP-ERα from 10 microscope fields is shown in the histogram (B). Representative results of two independent experiments, each performed in triplicate, are shown. *Open bar*, Vehicle-treated controls; *gray bar*, GA treatment (1 μM, 6 h).

hibitor-induced ERα degradation; and 2) CHIP targets functional ERα (correctly folded, ligand-binding competent receptor protein) for degradation.

Several lines of evidence from our study support the conclusion that CHIP targets functional ERα for degradation. First, OHT treatment completely blocked CHIP-induced ERα degradation, suggesting that ERα reaches a correctly folded conformation, competent for ligand binding, before CHIP-directed degradation. Secondly, CHIP overexpression down-regulated ERα levels and decreased ERα-mediated gene expression, whereas CHIP depletion by siRNA up-regulated ERα levels and increased ERα-mediated gene transcription. This coordinate regulation of ERα levels and activity suggests that CHIP targets functional ERα for degradation. Thirdly, CHIP plays a role in GA-induced ERα degradation by primarily targeting Hsp90-associated, transcriptionally competent ERα (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.* (43) observed that CHIP overexpression increased ERα 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 ERα 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 ERα (35). In the present study, we also used 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.* (43), pRSVβGal was used as an internal control. When we used a similar construct, CMVβGal, we found that overexpression of either wild-type CHIP or TPR mutant (K30A), but not U-box mutant (H260Q), dramatically decreased CMVβGal expression in a dose-dependent manner (data not shown). Based on these observations, we suggest that βGal is not a suitable control reporter for studying the effect of CHIP on gene transcription.

Our results, with data from Tateishi *et al.* (43), suggest a role for the Hsp90 chaperone complex in the regulation of cellular ERα levels. A summary of distinct ERα degradation pathways is depicted in Fig. 11. Nascent ERα is translocated into nucleus, and by associating with Hsp90, receptor protein is maintained in a ligand-binding competent conformation, ready for subsequent activation (28). In the absence of ligand or other activation signals, CHIP constantly targets chaperone-associated ERα for degradation, thereby limiting cellular concentrations of receptor protein. Ligand binding disassembles the ERα-Hsp90 complex and thus protects ERα from CHIP-mediated degradation. However, depending on the ligand, ERα stability can vary considerably, suggesting that different downstream destructive pathways exist. Furthermore, the ERα-ligand interaction could play a definitive role in pathway use. For example, when activated by E2, ERα is degraded through a transcription-coupled mechanism (17, 19, 22, 24). Pretreatment with GA, however, abolished E2-induced ERα degradation (Fig. 9A), suggesting that Hsp90 activity is required for transcrip-

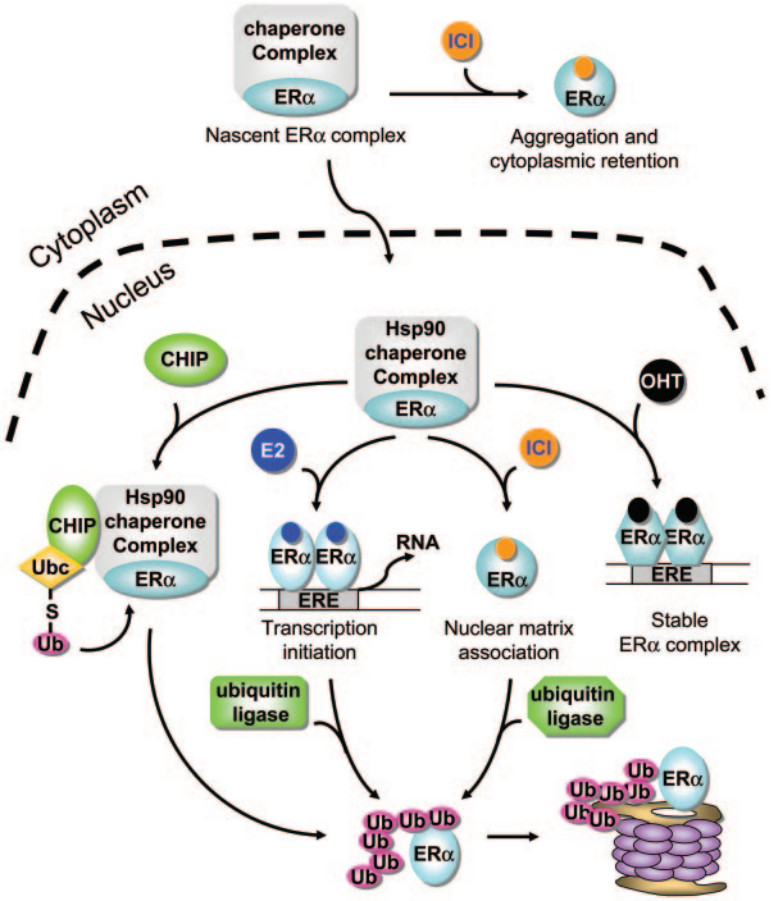


**Fig. 11.** Schematic Summary of Distinct ERα Degradation Pathways

Nascent ERα is translocated into nucleus. There, by associating with Hsp90, the receptor is maintained in a ligand-binding competent conformation, ready for subsequent activation. In the absence of ligand or other activation signals, CHIP constantly targets Hsp90-associated ERα for degradation. Ligand binding disassembles the ERα-Hsp90 complex and thus protects ERα from CHIP-mediated degradation. However, depending upon the ligand, distinct downstream destructive pathways are engaged in the degradation of liganded ERα. When activated by E2, ERα is degraded through a transcription-coupled mechanism. In response to ICI, nuclear ERα-ICI complex is immobilized to the nuclear matrix and undergoes rapid proteasomal degradation. In addition, ICI induces cytoplasmic retention and aggregation of nascent ERα OHT-ERα complexes are stable, likely due to the lack of transcriptional activity.

tion-coupled ERα degradation. In support of this possibility, the Hsp90-p23 complex has been shown to play a role in disassembling the nuclear 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 nuclear ERα-ICI complex is immobilized to the nuclear matrix and undergoes rapid degradation, in association with cytoplasmic retention of aggregated nascent ERα (7, 8, 22, 27, 40, 48). Although it is not clear how intracellular localization influences receptor degradation, the unique distribution pattern of ERα after treatment with ICI-182,780, together with the fact that ICI-induced receptor degradation is independent of ERα transcription activity, support the possibility that the pure antiestrogen and E2 use distinct degradation pathways for ERα. Taken together with our previous observation that an intact NEDD8 conjugation pathway is essential for ICI-induced ERα degradation in breast cancer cells (49), we suggest that destruction of the ICI-liganded receptor requires a cullin-based ubiquitin ligase.

Abnormal expression of ERα has long been associated with both the initiation and progression of breast cancer (50). An increase in the number of ERα-positive cells, as well as increased individual cell ERα content, have frequently been detected in malignant breast tumors (42). Furthermore, increased ERα 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 ERα degradation, and breast cancer progression (52). Collectively, these observations indicate that alterations in ERα degradation pathways may contribute to deregulation of ERα, perhaps leading to enhanced estrogen action in breast tumors. Based on our results, the chaperone/

CHIP pathway, by regulating ERα levels, likely contributes to the development/progression of that disease; and such a possible role merits further examination.

## MATERIALS AND METHODS

### Materials

The following antibodies and reagents were used in this study: anti-ERα (HC20) and anti-β-tubulin (SC9104) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-HA tag (3F10; Roche Molecular Biochemicals, Indianapolis, IN); anti-ERα (Ab-10) and anti-GFP (GFP01) (NeoMarkers, Inc., Fremont, CA); anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Chemicon International, Inc., Temecula, CA); anti-CHIP (PA1-015, Affinity Bioreagents, Golden, CO); anti-Hsp90 (SPA-830) and anti-Hsc70 (SPA-816) (Stressgene, Victoria, British Columbia, 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 Corp., San Diego, CA); LipofectAMINE Plus Reagent (Life Technologies, Inc., Logan, UT); FuGENE (Roche Molecular Biochemicals, Indianapolis, IN); 17β-estradiol, OHT, GA and MG132 (Sigma Chemical Co., St. Louis, MO); ICI (Tocris Cookson Ltd., Ellisville, MO); passive lysis buffer and luciferase assay system (Promega Corp., Madison, WI); fetal bovine serum (FBS) and dextran-coated charcoal-stripped FBS (Hyclone Laboratories, Inc., Logan, UT); cell culture supplementary reagents (Life Technologies, Inc., Rockville, MD).

### Plasmid Construction

The construction of pSG5-ERα(HEGO), ERE2-pS2-Luc, SV40-Luc, pcDNA-HA-Ub, pCS2-UbK0 and cytomegalovirus promoter (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 Drs. Neckers and Patterson (36), the pBS/U6/CHIPi construct by Dr. Chang (33), and the GFP-ERα construct by Dr. Stenoien (40).

### 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 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 μg/ml streptomycin, and 10% FBS. MCF7 cells were maintained in the same medium, with the addition of 6 ng/ml insulin. Before experiments, cells were cultured in hormone-free medium (phenol red-free MEM with 3% dextran-coated charcoal-stripped FBS) for 3 d. 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 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% glycerol, and protease inhibitor cocktail III]. After 15 min incubation on ice, extracts were sonicated (3 × 20 sec), insoluble material removed by centrifugation (15 min at 12,000 × *g*), and supernatant protein concentration determined using a Bio-Rad protein assay kit. Five percent β-mercaptoethanol was added to the protein extracts before heating at 90 C for 5 min. Protein extracts (50 μg per lane) were fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and 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 National Institutes of Health 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 determined using the Luciferase Assay System.

### CHIP siRNA Construct

The pBS/U6/CHIPi construct was kindly provided by Dr. Zhijie Chang (33). The siRNA expressed by the pBS/U6/CHIPi construct starts with GGG (position 233–251 bp relative to the ATG start site in the CHIP cDNA).

### Quantitative Real-Time PCR

Total RNA was prepared by a RNAeasy Mini Kit (QIAGEN, Valencia, CA), according to the manufacturer's protocol. RNA (2 μg) was reverse-transcribed in a total volume of 40 μl containing 400 U Moloney murine leukemia virus reverse transcriptase (New England Biolabs, Beverly, MA), 400 ng random hexamers (Promega), 80 U ribonuclease inhibitor and 1 mM deoxynucleotide triphosphates. The resulting cDNA was used in subsequent quantitative real-time PCRs, performed in 1× iQ SYBR Green Supermix (Bio-Rad) with 5 pmol forward and reverse primers as previously described (35).

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