Histone Deacetylase Inhibitor Trichostatin A Represses Estrogen Receptor α-Dependent Transcription and Promotes Proteasomal Degradation of Cyclin D1 in Human Breast Carcinoma Cell Lines

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ABSTRACT

Purpose: **Estrogen receptor α (ERα)-positive breast cancer cell lines are up to 10 times more sensitive than ERα-negative cell lines to the antiproliferative activity of the histone deacetylase inhibitor trichostatin A (TSA). The purpose of the study was to investigate the mechanisms underlying this differential response.**

Experimental Design and Results: **In the ERα-positive MCF-7 cell line, TSA repressed ERα and cyclin D1 transcription and induced ubiquitin dependent proteasomal degradation of cyclin D1, leading primarily to G_1-S-phase cell cycle arrest. By contrast, cyclin D1 degradation was enhanced but its transcription unaffected by TSA in the ERα-negative MDA-MB-231 cell line, which arrested in G_2-M phase. Cyclin D1 degradation involved Skp2/p45, a regulatory component of the Skp1/Cullin/F-box complex; silencing *SKP2* gene expression by RNA interference stabilized cyclin D1 and abrogated the cyclin D1 down-regulation response to TSA.**

Conclusions: **Tamoxifen has been shown to inhibit ERα-mediated cyclin D1 transcription, and acquired resistance to tamoxifen is associated with a shift to ERα-independent cyclin D1 up-regulation. Taken together, our data show that TSA effectively induces cyclin D1 down-regulation through both ERα-dependent and ERα-independent mechanisms, providing an important new strategy for combating resistance to antiestrogens.**

INTRODUCTION

Histone deacetylase (HDAC) inhibitors such as the natural antifungal antibiotic trichostatin A (TSA; refs. 1, 2) inhibit the proliferation of tumor cells in culture and *in vivo* by inducing cell cycle arrest, differentiation and/or apoptosis (3); reviewed in (4). In response to HDAC inhibition, accumulation of hyperacetylated core histones in chromatin leads to transcriptional activation of certain genes such as the *CDKN1A* gene, which encodes the $p21^{WAF1/CIP1}$ cyclin-dependent kinase inhibitor (5), but leads to transcriptional repression of other genes including the *CCND1* gene encoding cyclin D1 (6).

D-type cyclins collectively control progression through the cell cycle by activating their cyclin-dependent kinase partners CDK4 and CDK6, which leads to phosphorylation of the retinoblastoma protein and release of the E2F family of transcription factors, which, in turn, leads to advancing through G_1 into S phase of the cell cycle (7). Cyclin D1 is strongly implicated in mammary oncogenesis. Cyclin D1 accumulation is normally tightly regulated, but overexpression of the cyclin occurs in some 50% of human breast cancers (8, 9). Cyclin D1 overexpression is seen in all histologic types of breast cancer and at all stages from carcinoma *in situ* through metastatic disease but not in premalignant lesions (10, 11). Transgenic mice that overexpress cyclin D1 in mammary tissue develop breast cancers (12) and cyclin D1-deficient mice are resistant to breast cancers induced by *neu* and *ras* oncogenes (13).

Cyclin D1 may be overexpressed as a result of *CCND1* gene amplification or chromosomal translocation (14–16), stabilization of cyclin D1 mRNA (17), or defective degradation of cyclin D1 protein (18). D-type cyclins as well as cyclin E, p21, p27, and E2F-1 are ubiquitinated and targeted for degradation by the 26S proteasome. Phosphorylation of cyclin D1 on threonine 286 by glycogen synthase kinase 3β (GSK-3β) targets cyclin D1 for ubiquitination (19). Skp2, a regulatory component of the Skp1/Cullin/F-box complex, is implicated in the ubiquitination of cyclin D1; cyclin D1 levels are modestly elevated in Skp2−/− mouse embryo fibroblasts (20), expression of Skp2 antisense induces accumulation of cyclin D1 (20), and defective cyclin D1 degradation in the SK-UT-1B uterine tumor cell line can be rescued by stable transfection of Skp2 but not by a splice variant of Skp2 that does not bind Skp1 and remains in the cytoplasm (21). However, a direct association between Skp2 and cyclin D1 has yet to be confirmed.

Estrogen receptor α (ERα) is a member of the nuclear receptor superfamily of transcription factors that have highly

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conserved DNA and ligand-binding domains (LBDs) and regulate gene expression in a ligand-dependent fashion. ERα stimulates transcription on binding to *cis*-acting estrogen response elements (EREs) in the promoters of estrogen-regulated target genes (22–24). The proliferative response of mammary epithelial cells to estrogen is, in part, due to ERα-mediated transient activation of *CCND1* gene transcription (25). There is no classical ERE in the *CCND1* promoter (26). Instead, ERα upregulation of the *CCND1* promoter is mediated mainly by cyclic AMP response element (CRE) but also by AP-1 and Sp1 sites (27, 28). Reduction in cyclin D1 mRNA and protein expression is an early and critical event in antiestrogen action (29, 30), and inducible or constitutive cyclin D1 overexpression can confer resistance to antiestrogens (31, 32).

Cyclin D1 can activate ERα transcription by promoting the binding of both ligand-bound and unliganded ERα to ERE sequences in estrogen-regulated genes (33). Cyclin D1 can thereby enhance transcription of ERE-containing genes in both the presence and the absence of estrogen. This effect of cyclin D1 makes it possible for ERα-positive breast cancer cells to bypass the requirement for estrogen and provides a mechanism for estrogen-independent proliferation of cyclin D1-overexpressing breast cancer cells (8) that cannot be inhibited by antiestrogens (8).

We previously observed that ERα-positive breast cancer cell lines are highly sensitive to the antiproliferative activity of TSA compared with ERα-negative cell lines (34). Here we show that ERα and cyclin D1 down-regulation play a central role in this differential response. For the first time, we demonstrate that TSA not only inhibits ERα mediated cyclin D1 transcription in an ERα-positive breast cancer cell line but also induces ubiquitin-dependent proteasomal degradation of cyclin D1 in both ERα-positive and ERα-negative breast cancer cell lines. Silencing of *SKP2* gene expression by RNA interference stabilizes cyclin D1 and abrogates the cyclin D1 down-regulation response to TSA. Cyclin D1 degradation is, therefore, a critical event in the cytostatic action of TSA. Previous studies have shown that antiestrogens inhibit ERα mediated cyclin D1 transcription and acquired resistance to antiestrogens is associated with a shift toward ERα-independent cyclin D1 up-regulation. Taken together, our results demonstrate that HDAC inhibition can effectively inhibit cyclin D1 expression through both ERα-dependent and ERα-independent mechanisms, providing an important new strategy for overcoming endocrine resistance in breast cancer.

MATERIALS AND METHODS

Chemicals. Stock solutions of trichostatin A (TSA) and HC-toxin (Sigma-Aldrich, Dorset, United Kingdom) 2 mmol/L in dimethyl sulfoxide (DMSO), MG-132 (Merck Biosciences Ltd., Nottingham, United Kingdom) 10 mmol/L in DMSO, and leptomycin B 5 μg/ml in 70% (v/v) methanol (Sigma-Aldrich) were stored at −20°C until use. All other chemicals and biochemicals were the highest quality available from commercial sources.

Cell Lines. MCF-7 and MDA-MB-231 human breast carcinoma and SK-UT-1B uterine cancer cell lines (American Type Culture Collection, Rockville, MD) were cultured in DMEM containing 10% (v/v) fetal calf serum, 2 mmol/L L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. For estrogen depleted conditions, cells were grown in phenol red-free DMEM supplemented with 5% (v/v) dextran-coated charcoal-stripped fetal calf serum, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin as described previously (35). Cells were cultured at 37°C in 5% CO_2 humidified atmosphere.

Cell Proliferation Assay. Breast cancer cell lines were counted in a hemocytometer after detachment with 0.25% (w/v) trypsin in Dulbecco's PBS without Ca^{2+} or Mg^{2+} (DPBS, Sigma-Aldrich) containing 0.02% (w/v) EDTA. Viability was determined by trypan blue exclusion. For each cell line, cells were seeded in 96-well microtiter plates at optimal densities determined in prior experiments to ensure exponential growth for the duration of the assay. After a 24-h incubation, growth medium was replaced with experimental medium. For determining the concentration of TSA that inhibited cell proliferation by 50% (IC_{50}), the experimental medium contained TSA at final concentrations ranging from 10^{-10} mol/L to 10^{-5} mol/L in log dilutions and 0.1% (v/v) DMSO or growth medium containing 0.1% (v/v) DMSO as a vehicle control. To determine the concentration of 17β-estradiol that stimulated MCF-7 cell proliferation by 50% (EC_{50}), experimental medium contained 17β-estradiol at final concentrations ranging from 10^{-14} mol/L to 10^{-8} mol/L in log dilutions and 0.1% (v/v) EtOH or growth medium containing 0.1% (v/v) EtOH as a vehicle control. Cell proliferation at various time points was determined with the sulforhodamine B colorimetric assay (36), and the results expressed as the mean for six replicates as a percentage of vehicle control (taken as 100%).

Flow Cytometry. MCF-7 and MDA-MB-231 cells were treated as indicated. Floating and adherent cells were collected by centrifugation (500 × *g* for 5 minutes at 4°C) and washed twice with PBS. Cells were then fixed in 90% EtOH and stored at 4°C. For analysis, the samples were washed once in PBS and stained by resuspension in PBS containing propidium iodide (PI; 50 μg/mL) and RNase A (2 μg/mL) for 30 minutes at 4°C. Single cell suspensions were analyzed on a FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA) with CellQuest (BD Biosciences) acquisition software. PI fluorescence was measured through a 585/42 nm band pass filter, and list mode data were acquired on a minimum of 12,000 single cells defined by a dot plot of PI width *versus* PI area. Data analysis was done in ModFit LT (Verity Software House, Topsham, ME) with PI width *versus* PI area to exclude cell aggregates.

Immunoprecipitation and Immunoblot Analysis. Cells treated as indicated were harvested in 5 mL of medium, pelleted by centrifugation (1,000 × *g* for 5 minutes at 4°C), washed twice with ice-cold PBS and lysed in ice-cold HEPES lysis buffer [50 mmol/L HEPES (pH 7.5), 10 mmol/L NaCl, 5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100 and a cocktail of protease inhibitors] on ice for 30 minutes. Lysates were clarified by centrifugation (15,000 × *g* for 10 minutes at 4°C). The supernatants were then separated and either analyzed immediately or stored at −80°C. Equivalent amounts of protein (20–50 μg) from total cell lysates were resolved on precast 4–12% Bis-Tris gradient gels (Invitrogen

Ltd., Paisley, United Kingdom) and transferred onto polyvinylidene difluoride membranes (Hybond P; Amersham Biosciences United Kingdom Limited, Little Chalfont, United Kingdom) with a Novex XCell system (Invitrogen). Membranes were blocked overnight at 4°C in blocking buffer [5% (w/v) nonfat dried milk, 150 mmol/L NaCl, 10 mmol/L Tris (pH 8.0) and 0.05% (v/v) Tween 20]. Proteins were detected by incubation with primary antibodies diluted in blocking buffer at room temperature for 1 hour. Rabbit polyclonal anti-HDAC1 (ab7028; Abcam Ltd., Cambridge, United Kingdom), mouse monoclonal anti-ERα (NCL-L-ER-6F11/2, Novocastra Ltd., Newcastle upon Tyne, United Kingdom), rabbit polyclonal anti-CDK2 (sc-163, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal anti-CDK4 (sc-260, Santa Cruz Biotechnology), rabbit polyclonal anti-CDK6 (sc-177, Santa Cruz Biotechnology), mouse monoclonal anti-cyclin D1 (sc-20044, Santa Cruz Biotechnology), rabbit polyclonal anti-Skp2/p45 (sc-7164, Santa Cruz Biotechnology), mouse monoclonal anti-CUL-1 (sc-12761, Santa Cruz Biotechnology), rabbit polyclonal anti-α tubulin (sc-5546, Santa Cruz Biotechnology), goat polyclonal anti-actin (sc-1615, Santa Cruz Biotechnology), mouse monoclonal anti-p21 and mouse monoclonal anti-cyclin E antibodies (both kind gifts from Dr. E. W-F. Lam, Imperial College London, London, United Kingdom) were used. Blots were then incubated at room temperature with horseradish peroxidase-conjugated secondary antibody. Bands were visualized by enhanced chemiluminescence (Supersignal West Pico, Perbio Science, United Kingdom Ltd., Cheshire, United Kingdom) followed by exposure to autoradiography film (Kodak BioMax ML-light or MR-1).

For immunoprecipitations, rabbit polyclonal anti-green fluorescent protein (anti-GFP) antibody (sc-8334, Santa Cruz Biotechnology) was added to lysate at a concentration of 2 μg/mL. After incubation for 2 hours at 4°C, 50 μL of Protein A/G agarose (Santa Cruz Biotechnology) 50% (v/v) slurry in lysis buffer was added, and the incubation continued for an additional 60 minutes. After washing four times with ice-cold HEPES lysis buffer, immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by immunoblotting with mouse monoclonal anti-GFP (sc-9996, Santa Cruz Biotechnology) and anti-ubiquitin antibodies (sc-8017, Santa Cruz Biotechnology).

Reverse Transcription-Polymerase Chain Reaction. Total RNA was prepared from breast cancer cell lines (~2×10^7 cells) by guanidine isothiocyanate lysis followed by silica gel membrane purification (RNeasy mini kit; Qiagen Ltd., West Sussex, United Kingdom). The concentration and purity of RNA were determined by measuring the spectrophotometric absorption at 260 nm to 280 nm. Integrity of the RNA was confirmed by 1.2% denaturing formaldehyde agarose gel electrophoresis with ethidium bromide staining. Reverse transcription (RT)-PCR reactions were done with a OneStep RT-PCR kit according to the manufacturer's instructions (Qiagen). Primer pairs used for ERα (*ESR1*) RT-PCR were forward, 5′-CAGATGGCCACAGTTTCC-3′, and reverse, 5′-CCAAGAGCAAGTTAGGAGCAAACAG-3′, and for cyclin D1 (*CCND1*) forward, 5′-AACAGAAGTGCGAGGAGGAG-3′, and reverse, 5′-CTGGCATTTTGGAGAGGAAG-3′.

Expression Plasmids. *CCND1* was amplified from human breast carcinoma cDNA with the primers forward, 5′-GGAATTCCCCAGCCATGGAA-3′, and reverse, 5′-CGGGATCCCGCCCTCAGAT-3′. PCR products were digested and cloned into the *Eco*RI and *Bam*HI sites of the pEGFP-C1 mammalian expression vector (BD Biosciences).

Transfections. Small interfering RNA (siRNA) technology was used to silence expression of specific genes. Synthetic 21-nt RNA duplexes (Dharmacon Inc., Lafayette, CO) were transfected into breast cancer cell lines with Oligofectamine reagent according to the manufacturer's instructions (Invitrogen). Control scrambled siRNA, *ESR1* siRNA, *CCND1* siRNA or *SKP2* siRNA duplexes were used. For plasmids, Fugene 6 transfection reagent (Roche Diagnostics Ltd., East Sussex, United Kingdom) was used according to the manufacturer's protocol.

Fluorescence Microscopy. MCF-7 and SK-UT-1B cells stably transfected to express GFP-tagged cyclin D1 were grown on coverslips and treated as indicated. Coverslips were washed gently with PBS and fixed in methanol at −20°C for 10 minutes. The cells were subsequently mounted in Vectashield mounting medium containing 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI; Vector Laboratories Ltd., Peterborough, United Kingdom) to counterstain DNA and were observed with an Olympus BX60 fluorescent microscope equipped with the Micrometastatic Detection System (Applied Imaging International Ltd., Newcastle upon Tyne, United Kingdom) for analysis of fluorescent images.

Statistical Analysis. The concentration of TSA that inhibited cell proliferation by 50% (IC_{50}) or 90% (IC_{90}) and the concentration of 17β-estradiol that effectively stimulated cell proliferation by 50% (EC_{50}) was determined graphically in each case with nonlinear regression analysis to fit data to the appropriate dose-response curve (GraphPad Prism version 4.0; GraphPad Software Inc., San Diego, CA). The extra sum of squares F test was used for comparative statistical analysis of best fit logIC_{50} values. Cell proliferation responses to siRNA treatments at various time points were compared with two-way ANOVA with Bonferroni after tests for multiple comparisons. Statistical significance was set at the 5% level ($\alpha = 0.05$).

RESULTS

Differential Antiproliferative Potency of TSA in ERα-Positive and ERα-Negative Breast Cancer Cell Lines. TSA was a potent inhibitor of breast cancer cell proliferation. The ERα-positive MCF-7 cell line was highly sensitive to this action of TSA (IC_{50}, 36 nmol/L; 95% confidence interval, 20–65 nmol/L) compared with the ERα-negative MDA-MB-231 cell line (IC_{50}, 242 nmol/L; 95% confidence interval, 182–320 nmol/L; $P = 0.0002$, $F = 178.9$; Fig. 1*A*). ERα mRNA expression was confirmed by RT-PCR and ERα protein expression by immunocytochemistry and immunoblot experiments. The dose-dependent proliferative response of MCF-7 cells to the ERα ligand 17β-estradiol (EC_{50} 11 pmol/L; 95% confidence interval, 6–21 pmol/L) indicated that the expressed ERα was functionally active (Fig. 1*B*).

Cell Cycle Response of MCF-7 and MDA-MB-231 Cells to TSA. For these experiments, we initially used TSA at a concentration (1 μmol/L) determined in prior experiments to induce hyperacetylation of histone H3 and H4 and to inhibit proliferation of breast cancer cell lines by 90% (IC_{90}) at 48

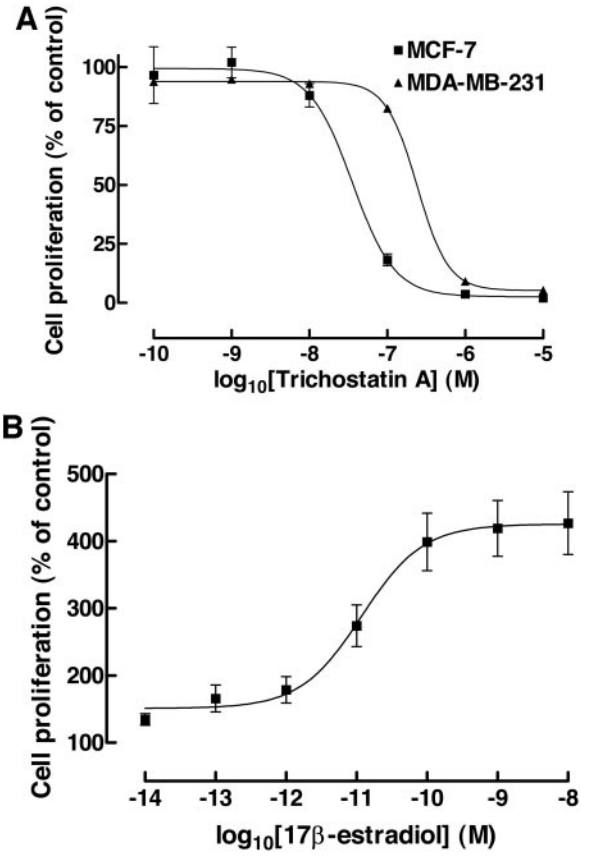


Fig. 1 *A*, effects of TSA on proliferation of breast cancer cell lines. ERα-positive MCF-7 (■) and ERα-negative MDA-MB-231 (▲) breast cancer cell lines were treated with the indicated concentrations of TSA for 48 hours. Cell proliferation was then quantified with the sulforhodamine B colorimetric assay as described in Materials and Methods. Results are the mean ± SD of six replicates expressed as a percentage of the control value in vehicle treated cells. *B*, proliferative response of MCF-7 cells to ERα ligand. Cells were treated with 17β-estradiol at a range of concentrations as indicated for 96 hours. Cell proliferation was then measured as described in *A*.

hours. There were striking differences in the cell cycle distribution of MCF-7 and MDA-MB-231 cells treated with 1 μmol/L TSA for 24 hours (Fig. 2). MCF-7 cells arrested in the G_1-S phase and G_2-M phases whereas MDA-MB-231 cells arrested primarily in G_2-M phase of the cell cycle. In both cell lines, a small population of apoptotic or necrotic cells accumulated in sub-G_1. The observed effects of TSA on the cell cycle were concentration dependent; 1 nmol/L or 10 nmol/L TSA for 24 hours did not affect the cell cycle distribution of MCF-7 or MDA-MB-231 cells, 100 nmol/L TSA arrested MCF-7 cells in G_1-S phase but had little effect on the cell cycle of MDA-MB-231 cells, and at 1 μmol/L, TSA MCF-7 cells arrested in both G_1-S phase and G_2-M, whereas MDA-MB-231 cells arrested mainly in G_2-M (Fig. 2). Consistent differences in the cell cycle distribution of MCF-7 and MDA-MB-231 cells exposed to 1 μmol/L TSA prompted us to use this concentration of TSA in subsequent experiments.

TSA Depleted ERα and Cyclin D1 in MCF-7 Cells. Immunoblot analysis of MCF-7 and MDA-MB-231 cells showed that TSA treatment (1 μmol/L for 24 hours) resulted in a marked down-regulation of cyclin D1 levels (Fig. 3). The effects of 1 μmol/L HC-toxin, a cyclic tetrapeptide HDAC inhibitor used as a positive control, were indistinguishable from TSA. Cellular levels of CDK4 and CDK6 fell sharply in MDA-MB-231 cells but were either unchanged or only marginally reduced in MCF-7 cells, although p21 was up-regulated in both cell types. It is notable that the cyclin E level was substantially induced by TSA or HC-toxin in both cell lines, but the basal level of the cyclin was lower in MDA-MB-231 cells. ERα was strikingly down-regulated in MCF-7 cells treated with TSA or HC-toxin.

TSA Lowered ERα Expression and Promoted Proteasomal Degradation of Cyclin D1 in MCF-7 Cells. ERα and cyclin D1 are important regulators of MCF-7 cell proliferation and we, therefore, sought to investigate further the effect of TSA on stability of these proteins. ERα and cyclin D1 were partially down-regulated in response to 10 nmol/L TSA but exposure to 1 μmol/L TSA induced complete degradation of both proteins (Fig. 4*A*). p21 up-regulation (Fig. 4*A*) and G_1-S-phase cell cycle arrest (Fig. 2) both occurred at 100 nmol/L TSA. In MCF-7 cells cultured for 24 hours in estrogen-depleted medium, 1 μmol/L TSA induced a rapid reduction in ERα and cyclin D1 protein levels within 2 to 4 hours, followed by a progressive decline to almost undetectable levels by 12 hours, which persisted for at least up to 24 hours (Fig. 4*B*). ERα down-regulation was accelerated in response to TSA treatment of MCF-7 cells cultured in estrogen-containing complete medium (Fig. 4*C*), an effect that may be attributable to concomitant ligand-induced proteasomal degradation of the receptor (37). Interestingly, p21 was consistently down-regulated at 2 to 6 hours, with subsequent stabilization and up-regulation occurring between 6 and 24 hours (Fig. 4*B* and *C*). The rapid fall in cyclin D1 protein was accompanied by only partial down-regulation of cyclin D1 mRNA levels, which suggested that the initial effects of TSA on the stability of this protein occur mainly at the posttranslational level (Fig. 4*C*). In contrast, there was a steady accumulation of p21 mRNA during the first 6 hours of TSA treatment. p21 protein levels fell during the first 6 hours of TSA treatment (Fig. 4*C*) but then increased progressively during the subsequent 18-hour period (Fig. 4*B*). Treatment of MCF-7 cells with the proteasome inhibitor MG132 (50 μmol/L for 4 hours) resulted in elevated p21 and cyclin D1 levels, confirming the rapid turnover of these proteins via the ubiquitin-dependent degradation pathway (Fig. 4*D*; refs. 18, 38). Importantly, cotreatment with MG132 completely abolished TSA-induced p21 and cyclin D1 degradation, indicating that TSA promotes proteasomal degradation of these proteins in MCF-7 cells. Both TSA and MG132 down-regulated ERα protein levels, and cotreatment had an additive effect (Fig. 4*D*). In contrast to p21 and cyclin D1, TSA-induced down-regulation of ERα does not, therefore, occur via ubiquitin-dependent proteasomal degradation. Leptomycin B (LMB; 10 ng/mL for 6 hours), a potent inhibitor of CRM1-dependent nuclear export (39), also induced elevated p21 and cyclin D1 levels in MCF-7 cells (Fig. 4*E*). This observation is in keeping with previous reports linking cyclin D1 degradation and nuclear export (40, 41). In contrast to MG132, however, LMB only

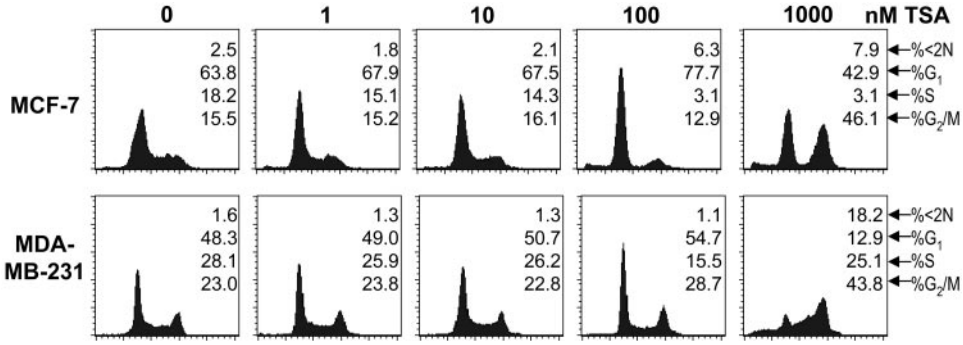


Fig. 2 Effects of TSA on cell cycle progression of breast cancer cell lines. MCF-7 and MDA-MB-231 breast carcinoma cell lines were treated with TSA at various concentrations as specified for 24 hours. Cells were fixed, permeablized, and stained with propidium iodide to measure DNA content by fluorescence-activated cell sorting (FACS) analysis. The percentages of cells (*Y*-axis) in each phase of the cell cycle determined by their DNA contents (*X*-axis) are indicated. (<2N, hypodiploid.)

partially inhibited TSA-induced cyclin D1 degradation (Fig. 4*E*). Nuclear export is, therefore, not a strict requirement for TSA-induced cyclin D1 proteasomal degradation.

TSA-Induced Skp2/p45 Up-Regulation. The Skp2/p45 F-box protein component of the Skp/Cullin/F-box (SCF) complex has been linked to the ubiquitin-dependent proteolysis of p21 and to the ubiquitination, nuclear export, and subsequent proteasomal degradation of cyclin D1 (20, 41). This suggested to us that TSA might induce ubiquitin-dependent degradation of p21 and cyclin D1 by up-regulation of Skp2/p45. Skp2/p45 did indeed accumulate in MCF-7 cells after 2 to 6 hours of exposure to 1 μmol/L TSA, and there was a concomitant increase in Cullin 1 (Cul1) protein (Fig. 4*B* and *C*). Up-regulation of Skp2/p45 correlated with the observed initial reduction in p21 protein levels (Fig. 4*C*). The Skp2/p45 up-regulation response to TSA was inhibited by cycloheximide indicating a requirement for protein synthesis (Fig. 5*A*). Silencing endogenous *SKP2* gene expression by RNA interference not only stabilized p21 and cyclin D1 but also inhibited TSA-induced down-regulation of these proteins in MCF-7 cells (Fig. 5*B*).

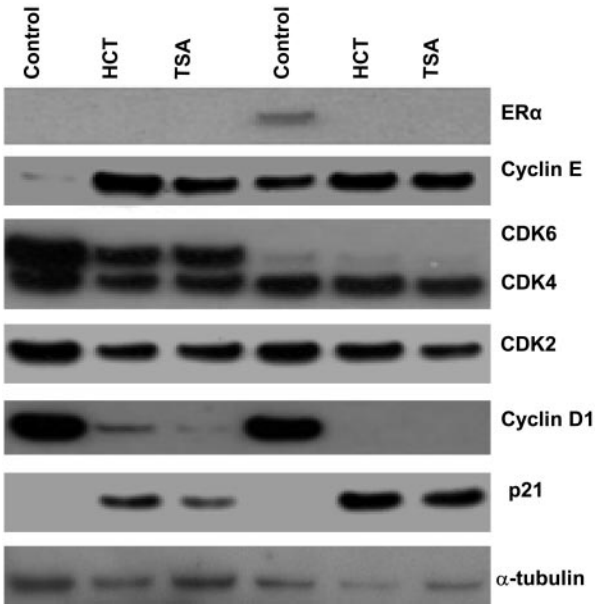


Fig. 3 Effects of histone deacetylase inhibitors on expression levels of ERα and cell cycle regulatory proteins in breast cancer cell lines. MDA-MB-231 cells (*Lanes 1–3*) and MCF-7 cells (*Lanes 4–6*) were treated with 1 μmol/L TSA, 1 μmol/L HC-toxin (*HCT*) as a positive control, or vehicle control for 24 hours. Cell lysates were prepared and the expression levels of ERα and cell cycle regulatory proteins were analyzed by Western blotting as described in the Materials and Methods.

Next we used the SK-UT-1B uterine tumor cell line to further investigate the role of Skp2/p45 in the cyclin D1 down-regulation response to TSA. SK-UT-1B cells express a splice variant of Skp2/p45 that does not migrate into the nucleus, and cellular extracts fail to promote the formation of polyubiquitinated cyclin D1 species *in vitro* (21). As a consequence, the half-life of cyclin D1 is prolonged in SK-UT-1B cells, which results in elevated levels of the cyclin (42). In contrast to its effect on cyclin D1 levels in MCF-7 cells, TSA treatment (1 μmol/L for 6 hours) of SK-UT-1B cells resulted in only a partial reduction in cyclin D1 protein levels and residual protein was still detectable at 24 hours (Fig. 5*C*). MG132 alone (50 μmol/L for 6 hours) also reduced cyclin D1 protein levels. Furthermore, MG132 failed to inhibit TSA-induced cyclin D1 down-regulation in SK-UT-1B cells. In fact, cotreatment with TSA and MG132 had at least an additive effect on the down-regulation of cyclin D1 levels in SK-UT-1B cells (Fig. 5*C*) that was reminiscent of the effect of these compounds on ERα protein levels in MCF-7 cells (Fig. 4*D*). In contrast, p21 protein levels were elevated in response to both TSA and MG132. TSA, therefore, fails to promote the rapid proteasomal degradation of cyclin D1 in SK-UT-1B cells.

We generated a recombinant GFP-tagged cyclin D1 (GFP-cyclin D1) vector under the control of a cytomegalovirus (CMV) promoter to further compare the effects of TSA on cyclin D1 in MCF-7 and SK-UT-1B cells. Vector integrity was confirmed by restriction enzyme digestion and sequencing. In general, the cellular localization and response to MG132 or LMB of the recombinant protein was similar to that of endogenous cyclin D1 protein in MCF-7 cells (Fig. 5*D* and not shown). In contrast to its effect on endogenous cyclin D1,

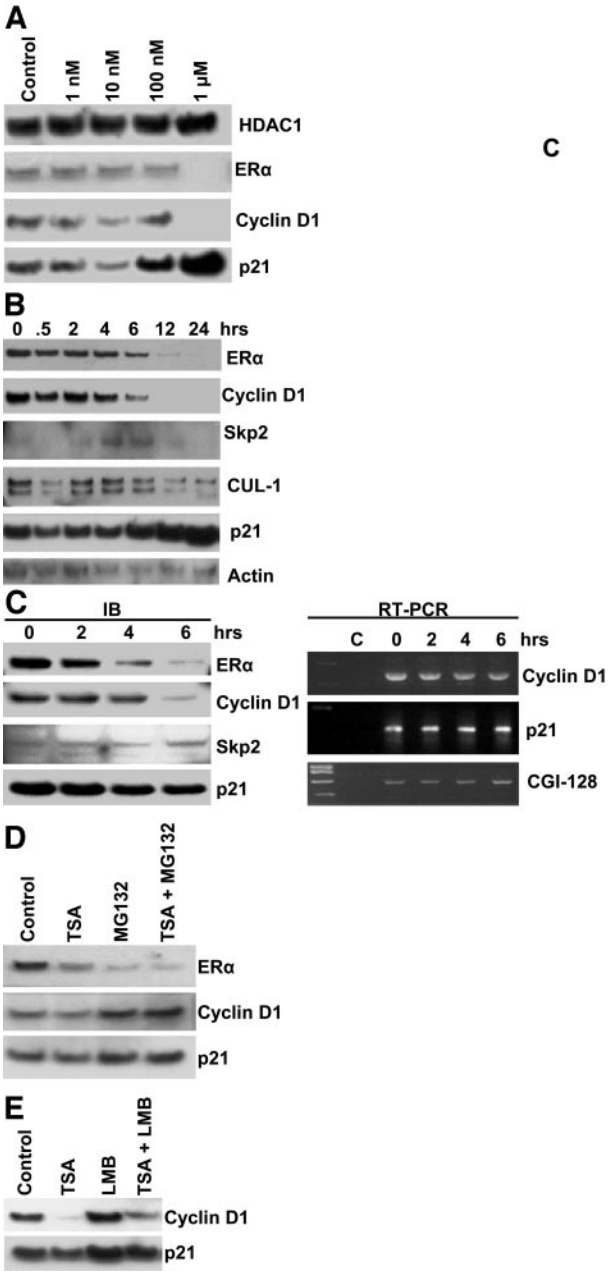
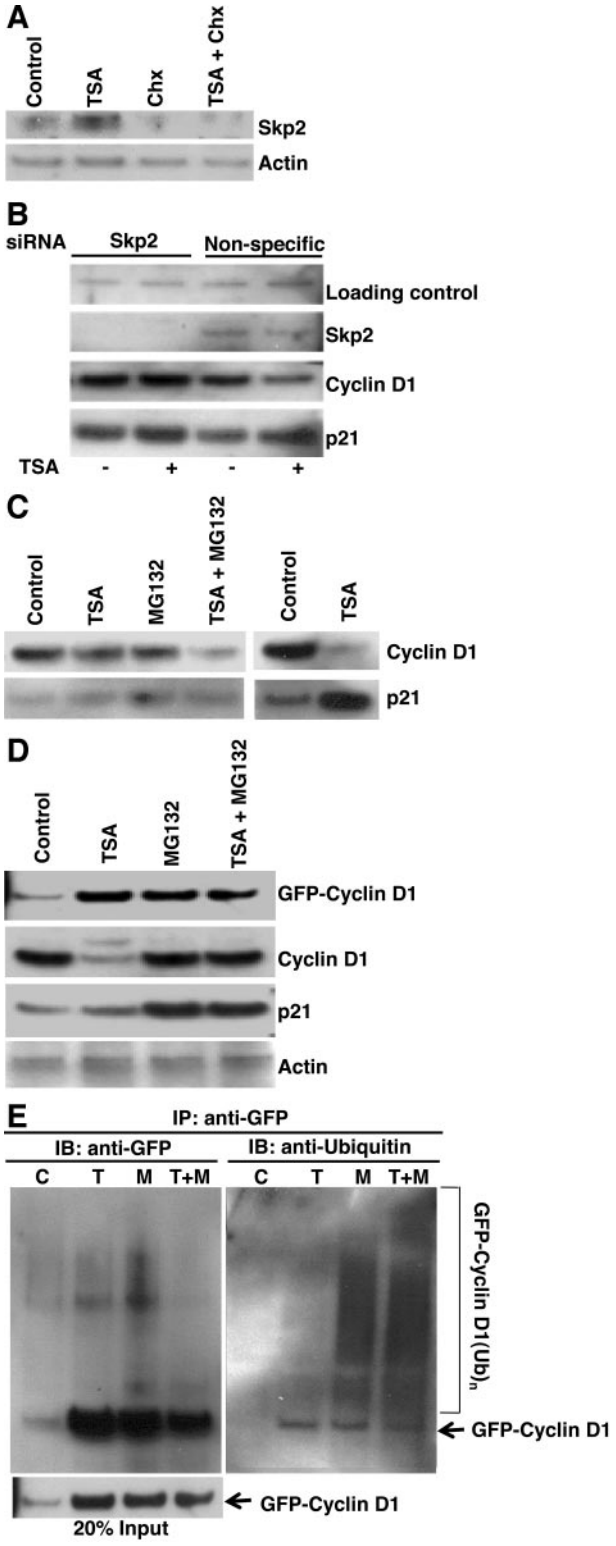


Fig. 4 *A*, concentration-dependent effects of TSA on expression levels of ERα and cell cycle regulatory proteins in MCF-7 cells. Cells were treated with TSA at the indicated concentrations or vehicle for 24 hours, cell lysates were prepared, and expression levels of ERα, cyclin D1 and p21 were analyzed by Western blot as described in the Materials and Methods. HDAC1 was used as a loading control. *B*, Skp2/p45 accumulates in MCF-7 cells after exposure to TSA. Cells were treated with 1 μmol/L TSA for the indicated times [hours (*hrs*)] and expression levels of ERα, cyclin D1, Skp2/p45, CUL-1, and p21 were analyzed by Western blot as described in the Materials and Methods. *C*, immunoblot (*IB*) analysis of ERα, cyclin D1, Skp2/p45, and p21 protein levels following 1 μmol/L TSA treatment of MCF-7 cells for the indicated time periods [hours, (*hrs*); *left panel*]. A representative agarose gel analyzing RT-PCR products for cyclin D1 and p21 is shown with a quantitative loading control (CGI-128; *right panel*). *D*, the proteasome inhibitor MG132 abrogates TSA-induced degradation of p21 and cyclin D1 but not ERα in MCF-7 cells. Immunoblot analysis of ERα, cyclin D1, and p21 protein levels in MCF-7 cells treated with 1 μmol/L TSA, 50 μmol/L MG132 (*MG132*), or the combination (*TSA + MG132*), for 4 hours. *E*, LMB, an inhibitor of CRM1-dependent nuclear export, partially inhibits the cyclin D1 degradation response to TSA in MCF-7 cells. Cyclin D1 and p21 protein levels were analyzed by Western blotting in MCF-7 cells treated with 1 μmol/L TSA, 10 ng/mL LMB, or the combination (*TSA + LMB*), for 6 hours.

however, TSA induced up-regulation of the GFP-cyclin D1 protein in MCF-7 cells (Fig. 5*D*). In contrast to endogenous cyclin D1, GFP- cyclin D1 expression is controlled by a CMV D1, and p21 protein levels in MCF-7 cells treated with 1 μmol/L TSA, promoter, which may account for the up-regulation response to TSA. Subsequent immunoprecipitation with a polyclonal anti-GFP antibody, followed by sequential immunoblotting with monoclonal anti-GFP and anti-ubiquitin antibodies, revealed that TSA and MG132 additively promote the accumulation of higher molecular weight, polyubiquitinated GFP-cyclin D1 species in MCF-7 cells (Fig. 5*E*).

Next, we used fluorescence microscopy to compare the effects of TSA on GFP-cyclin D1 levels and subcellular localization in MCF-7 and SK-UT-1B cells. Treatment with TSA (1 μmol/L for 6 hours) or MG132 (50 μmol/L for 6 hours) resulted in the accumulation of GFP-cyclin D1 within the nucleus and cytoplasm of both cell lines (Fig. 6). MG132-induced stabilization of GFP-cyclin D1 was far more pronounced in MCF-7 cells than in SK-UT-1B cells. This is consistent with the notion that SK-UT-1B cells, lacking functional Skp2/p45, have an impaired ability to target cyclin D1 for ubiquitin-dependent proteasomal degradation. In MCF-7 cells, cotreatment with TSA and MG132 resulted in relocalization of GFP-cyclin D1 to the cytoplasm (Fig. 6), in keeping with our observation that LMB partially inhibits TSA-induced cyclin D1 degradation (Fig. 4*E*). In contrast, GFP-cyclin D1 remained nuclear in response to cotreatment with TSA and MG132 of SK-UT-1B cells, which presumably fail to ubiquitinate GFP-cyclin D1 (Fig. 6). Taken together, these findings strongly suggest that the early and rapid down-regulation of p21 and cyclin D1 in response to TSA treatment of MCF-7 cells is dependent on Skp2/p45 up-regulation. TSA-induced Skp2/p45 up-regulation, in turn, may result in increased cyclin D1 polyubiquitination, nuclear export, and degradation within both the nucleus and the cytoplasm of MCF-7 cells.

TSA Repressed ERα and Cyclin D1 Transcription in MCF-7 Cells. We then wished to examine the effects of more prolonged exposure to TSA on ERα and cyclin D1 transcription in breast carcinoma cell lines. Reverse transcription-PCR analysis showed that TSA (1 μmol/L for 12 hours) repressed transcription of both ERα and cyclin D1 in MCF-7 cells, whereas the 17β-estradiol (100 nmol/L for 12 hours) control did not affect ERα transcription and the up-regulated cyclin D1 mRNA levels (Fig. 7*A* and *B*). In MDA-MB-231 cells, however, cyclin D1 mRNA expression was only slightly reduced after TSA treatment, whereas estradiol, as expected, had no effect (Fig. 7*C*). TSA-induced cyclin D1 down-regulation was only partially inhibited by 50 μmol/L cycloheximide, which suggested an indirect mechanism of transcriptional repression (Fig. 7*B*). Cycloheximide did not affect the transcriptional repression of ERα by TSA, which indicated that this was a direct effect (Fig. 7*B*).



Silencing *ESR1* And *CCND1* Gene Expression Inhibited MCF-7 Cell Proliferation. To investigate the mechanism whereby TSA repressed transcription of cyclin D1 in MCF-7 cells and to further characterize the role of ERα in this action, we transfected subconfluent MCF-7 cells with pooled *ESR1* siRNA duplexes to silence ERα gene (*ESR1*) expression through RNA interference. The resultant effect on expression of cyclin D1 was examined by immunoblot analysis after 96 hours (Fig. 8*A*). ERα protein expression was effectively abolished, leading to reduction in cyclin D1 levels at 96 hours and a parallel, down-regulation of p21 expression. We, therefore, conclude that in MCF-7 cells, TSA-induced transcriptional repression of cyclin D1 results in part from the effect of TSA on ERα expression.

Next we examined the functional effects of silencing *ESR1* and *CCND1* gene expression on MCF-7 cell proliferation. ERα- or cyclin D1-specific siRNA pools or scrambled siRNA control pool duplexes were transfected into MCF-7 cells, and cell proliferation was then quantified by sulforhodamine B assay at various time points for up to 7 days. Immunoblot analysis confirmed that *CCND1* gene expression had been successfully silenced in cells transfected with cyclin D1-specific siRNA; cyclin D1 protein levels could not be detected at the time of seeding and remained undetectable for 7 days, whereas cyclin D1 levels were unaffected in control siRNA-transfected cells (Fig. 8*A* and data not shown). Specific *ESR1* gene expression was successfully silenced in parallel experiments (Fig. 8*A* and data not shown). Specific silencing of either *ESR1* or *CCND1* gene expression had a profound inhibitory effect on the proliferation of MCF-7 cells (Fig. 8*B*). Although the mock and nonspecific siRNA control cells proliferated rapidly, the ERα- or cyclin D1-siRNA–transfected MCF-7 cells remained almost quiescent throughout the 7-day period of analysis (Fig. 8*B*).

Fig. 5 *A*, immunoblot analysis of Skp2/p45 protein levels in MCF-7 cells treated with 1 μmol/L TSA, 50 μmol/L cycloheximide (*Chx*), or the combination (*TSA + Chx*) for 12 hours. Actin was used as a quantitative loading control. *B*, effects of silencing *SKP2* gene expression on Skp2, cyclin D1, and p21 protein levels. MCF-7 cells treated with 1 μmol/L TSA (+) or vehicle control (−) for 2 hours were transfected with *SKP2* siRNA duplex (*Skp2*) or a scrambled siRNA control (nonspecific). Expression levels of Skp2, cyclin D1, and p21 protein were then analyzed by Western blotting as described in the Materials and Methods. *C*, immunoblot analysis of cyclin D1 and p21 protein levels in SK-UT-1B cells treated with 1 μmol/L TSA, 50 μmol/L MG132 (*MG132*), or the combination (*TSA + MG132*) for 6 hours (*left panel*) or 24 hours (*right panel*). *D*, MCF-7 cells that had been stably transfected to express GFP-tagged cyclin D1 were treated with 1 μmol/L TSA, 50 μmol/L MG132 (*MG132*), or the combination (*TSA + MG132*), for 6 hours. Cellular levels of recombinant GFP-tagged cyclin D1 (*GFP-Cyclin D1*), endogenous cyclin D1, and p21 proteins were compared by immunoblot analysis along with actin as a quantitative loading control. *E*, TSA and the proteasome inhibitor MG132 additively promote the accumulation of polyubiquitinated GFP-cyclin D1 [*GFP-cyclin D1(Ub)$_n$*] species in MCF-7 cells. MCF-7 cells were stably transfected with a plasmid expressing GFP-tagged cyclin D1. Cells were treated with 1 μmol/L TSA (*T*), 50 μmol/L MG132 (*M*), the combination (*T+M*), or control (*C*) for 6 hours. Proteins were then extracted, and recombinant GFP-cyclin D1 was immunoprecipitated with a polyclonal anti-GFP antibody (*IP: anti-GFP*) and analyzed by immunoblotting with monoclonal anti-GFP (*IB: anti-GFP*) and anti-ubiquitin (*IB: anti-*Ubiquitin) antibodies.

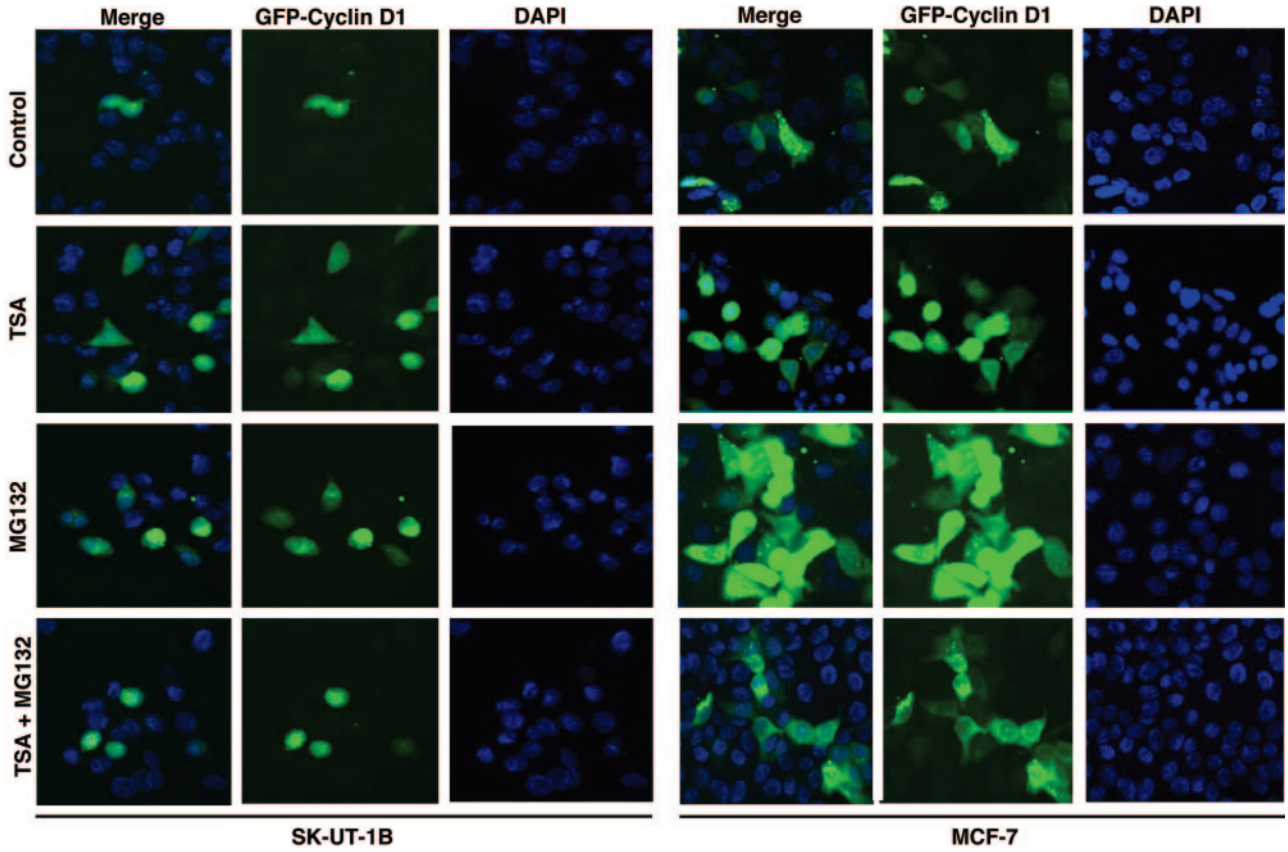


Fig. 6 Subcellular localization of GFP-tagged cyclin D1 in MCF-7 and SK-UT-1B cells treated with 1 μmol/L TSA, 50 μmol/L MG132, or the combination (*TSA + MG132*), for 6 hours. Cells were transfected with plasmid expressing GFP-cyclin D1 before being subjected to fluorescence microscopy as described in the Materials and Methods (*DAPI*, 4′,6′-diamidino-2-phenylindole dihydrochloride).

Thus, down-regulating ERα or cyclin D1 by silencing gene expression, powerfully inhibited the proliferation of ERα-positive MCF-7 cells. We conclude that the combined down-regulation of both ERα and cyclin D1 may, therefore, account for the increased sensitivity of proliferating MCF-7 breast cancer cells to TSA (Fig. 9).

DISCUSSION

The observation that ERα-positive breast cancer cell lines were up to 10 times more sensitive than ERα-negative cell lines to growth inhibition by TSA (43) has been confirmed by another group (44), but the mechanism has remained elusive. Differential sensitivity of ERα-positive breast cancer cell lines has also been reported to occur with other HDAC inhibitors from different structural classes such as the short-chain fatty acid sodium butyrate (45) and the cyclic tetrapeptide HC-toxin (44). Unlike the depsipeptide HDAC inhibitor FR901228 (46), TSA is not a substrate for p-glycoprotein, and efflux of TSA is unaltered in MCF-7 cells expressing a multidrug resistance phenotype (44). Differential sensitivity to TSA is also not associated with differences in the global activities of histone acetyltransferase or HDAC enzymes (43, 44). It has been suggested that $p21^{WAF1/CIP1}$ may be expressed at higher levels and that the *CDKN1A* gene may be more sensitive to the effects of TSA in ERα-positive compared with ERα-negative breast cancer cells (44). However, in the present study, basal levels and up-regulation of $p21^{WAF1/CIP1}$ in response to TSA were similar in ERα-positive and ERα-negative breast cancer cell lines. The *CDKN1A* gene and $p21^{WAF1/CIP1}$ are, therefore, unlikely to be the primary molecular targets of TSA. Furthermore, altered expression levels of $p21^{WAF1/CIP1}$ cannot account for the differential sensitivity of ERα-positive compared with ERα-negative breast cancer cell lines observed in our study.

In the present study, TSA effectively inhibited cyclin D1 up-regulation in breast cancer cell lines through both ERα-dependent and ERα-independent mechanisms. Furthermore, there were fundamental differences in the cell cycle response of ERα-positive and ERα-negative breast cancer cell lines to TSA, which could account for differential sensitivity to the antiproliferative effects of this HDAC inhibitor. In the ERα-positive MCF-7 breast cancer cell line, TSA not only repressed ERα and cyclin D1 transcription but also induced ubiquitin-dependent proteasomal degradation of cyclin D1, leading to cell cycle arrest in G_1-S phase. In contrast, TSA enhanced cyclin D1 degradation but only slightly affected its transcription in the ERα-negative MDA-MB-231 breast cancer cell line, which arrested in G_2-M phase. We were able to show that transcriptional down-regulation of cyclin D1 by TSA was an indirect effect of ERα transcriptional repression. Silencing *ESR1* gene expression by RNA interference down-regulated cyclin

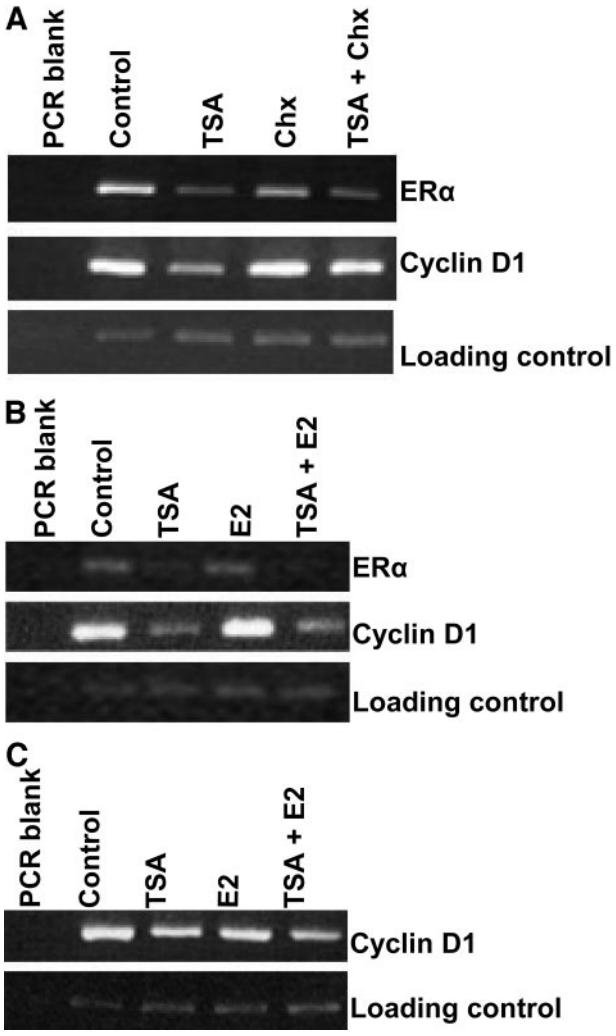


Fig. 7 *A* and *B*, RT-PCR analysis of ERα and cyclin D1 mRNA expression in MCF-7 cells. Cells were treated with 1 μmol/L TSA, 50 μmol/L cycloheximide (*Chx*), 100 nmol/L 17β-estradiol (*E2*), or the indicated combinations (*TSA + Chx*, *TSA + E2*), for 12 hours. Representative agarose gels, analyzing RT-PCR products for ERα and cyclin D1, are shown with a quantitative loading control (CGI-128). *C*, RT-PCR analysis of cyclin D1 mRNA expression levels in MDA-MB-231 cells. Cells were treated with 1 μmol/L TSA, 100 nmol/L 17β-estradiol (*E2*), or the combination (*TSA + E2*), for 12 hours and were analyzed by RT-PCR as described in *A* and *B*.

D1 expression. Furthermore, silencing of *ESR1* or *CCND1* gene expression comparably inhibited MCF-7 cell proliferation. We conclude that the sensitivity of ERα-positive breast cancer cells to growth inhibition by TSA may, therefore be, due to the combined effects of ERα transcriptional repression and enhanced cyclin D1 degradation.

The emergence of endocrine resistance in previously sensitive ERα-positive breast cancers is a major limitation in the treatment of breast cancer with anti-estrogens or estrogen withdrawal therapy. Early studies reported that ectopic expression of cyclin D1 was sufficient to stimulate proliferation of ERα-positive breast cancer cell lines in culture (31) and neutralizing antibodies to cyclin D1-arrested estrogen-treated MCF-7 cells in G_1-S phase (47). Anti-estrogens, including tamoxifen and the pure ERα antagonist/down-regulator fulvestrant (formerly known as ICI 182,780), inhibit ERα-dependent cyclin D1 up-regulation, abrogating the proliferative response of ERα-positive breast cancer cells to estrogenic stimulation and arresting cells in G_1-S phase of the cell cycle (25, 30). In one study, constitutive expression of cyclin D1 was associated with tamoxifen resistance in an MCF-7-derived cell line (48). Other investigators found that inducible overexpression of cyclin D1 could confer endocrine resistance and overcome the growth-inhibitory effects of tamoxifen and fulvestrant in ERα-positive breast cancer cell lines (32). In this context, elevated cyclin D1 mRNA levels in human breast carcinomas have been associated with a

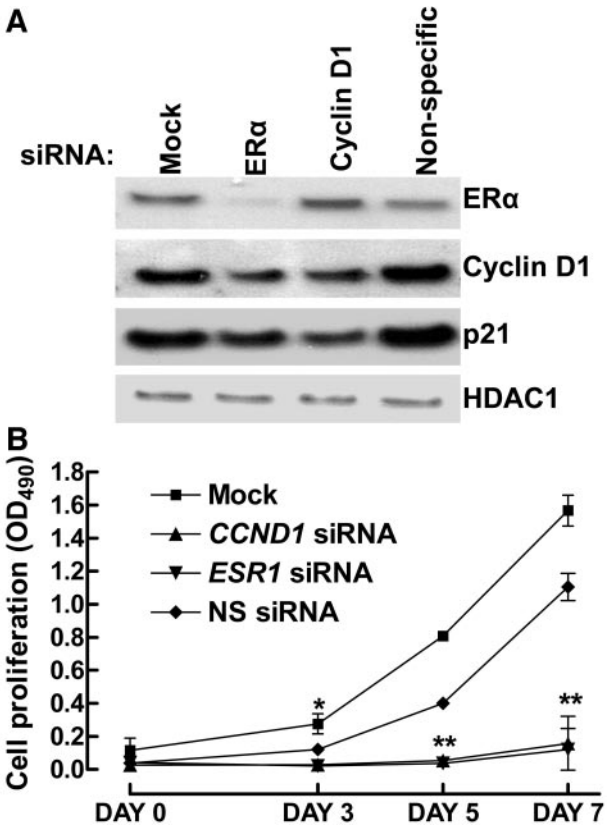


Fig. 8 *A*, effects of silencing *ESR1* or *CCND1* gene expression on ERα, cyclin D1, and p21 protein levels. MCF-7 cells were mock transfected or transfected with ERα-specific, cyclin D1-specific, or scrambled (nonspecific) siRNA pools. Cells were then cultured for 96 hours, cell lysates were prepared, and protein levels of ERα, cyclin D1, and p21 were determined by immunoblot analysis as described in the Materials and Methods. HDAC1 was used as a loading control. *B*, effects of silencing *CCND1* or *ESR1* gene expression on MCF-7 cell proliferation. Cells were mock transfected (■) or transfected with ERα-specific (*ESR1*; ▼), or cyclin D1 (*CCND1*; ▲)-specific siRNA pools, or scrambled nonspecific (*NS*; ◆) siRNA control pools. Cell proliferation at various time points up to 7 days was quantified by sulforhodamine B assay as described in the Materials and Methods. Mean and SD values are shown. *ESR1* and *CCND1* siRNA-transfected MCF-7 cell proliferation differed significantly from mock-transfected controls at day 3 (*, $P < 0.01$) and from both mock and nonspecific siRNA-transfected controls at days 5 and 7 (**, $P < 0.001$).

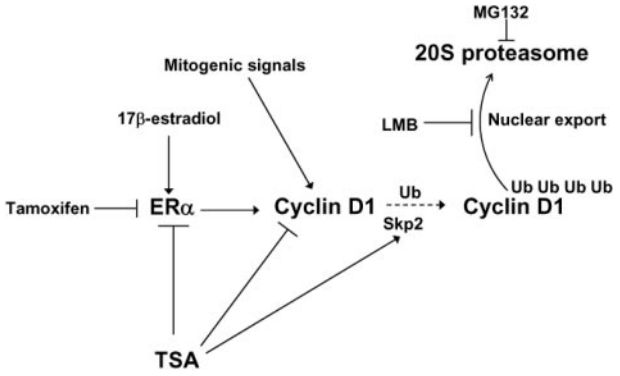


Fig. 9 Mechanisms of TSA action in MCF-7 cells. TSA repressed ERα and cyclin D1 transcription and induced ubiquitin (*Ub*)-dependent proteasomal degradation of cyclin D1. Cyclin D1 degradation involved Skp2/p45, a regulatory component of the Skp1/Cullin/F-box complex. Cyclin D1 degradation in response to TSA was abolished by cotreatment with the proteasome inhibitor MG132 but was only partially inhibited by LMB. Nuclear export is, therefore, not a strict requirement for TSA-induced cyclin D1 proteasomal degradation.

shorter duration of clinical response to tamoxifen (49). Overexpression of cyclin D1 renders breast cancer cells less dependent on mitogenic stimuli for proliferation and a shift toward ERα-independent cyclin D1 up-regulation has been implicated in the development of endocrine resistance (50). Cyclin D1 potentiates the transcription of ERα-responsive genes by enhancing the binding of the receptor to ERE sequences in these target genes (33). Cyclin D1 can bind directly to the LBD of the ERα and activate ERα transcription by protein–protein interactions (33, 51). Activation of ERα by cyclin D1 is independent of CDK binding, occurs in the presence or absence of estrogen, and cannot be inhibited by antiestrogens (33). This action of cyclin D1 can enable ERα-positive breast cancer cells to escape from their requirement for estrogen, providing a mechanism for estrogen-independent proliferation of cyclin D1-overexpressing breast cancer cells that is resistant to antiestrogens. The ability of TSA to inhibit both ERα-dependent and ERα-independent cyclin D1 up-regulation may, therefore, be an effective means of preventing or overcoming endocrine resistance in breast cancer.

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