Estrogen-Dependent Gene Transcription in Human Breast Cancer Cells Relies upon Proteasome-Dependent Monoubiquitination of Histone H2B

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Abstract

The estrogen receptor-α (ERα) determines the phenotype of breast cancers where it serves as a positive prognostic indicator. ERα is a well-established target for breast cancer therapy, but strategies to target its function remain of interest to address therapeutic resistance and further improve treatment. Recent findings indicate that proteasome inhibition can regulate estrogen-induced transcription, but how ERα function might be regulated was uncertain. In this study, we investigated the transcriptome-wide effects of the proteasome inhibitor bortezomib on estrogen-regulated transcription in MCF7 human breast cancer cells and showed that bortezomib caused a specific global decrease in estrogen-induced gene expression. This effect was specific because gene expression induced by the glucocorticoid receptor was unaffected by bortezomib. Surprisingly, we observed no changes in ERα recruitment or assembly of its transcriptional activation complex on ERα target genes. Instead, we found that proteasome inhibition caused a global decrease in histone H2B monoubiquitination (H2Bub1), leading to transcriptional elongation defects on estrogen target genes and to decreased chromatin dynamics overall. In confirming the functional significance of this link, we showed that RNA interference–mediated knockdown of the H2B ubiquitin ligase RNF40 decreased ERα-induced gene transcription. Surprisingly, RNF40 knockdown also supported estrogen-independent cell proliferation and activation of cell survival signaling pathways. Most importantly, we found that H2Bub1 levels decrease during tumor progression. H2Bub1 was abundant in normal mammary epithelium and benign breast tumors but absent in most malignant and metastatic breast cancers. Taken together, our findings show how ERα function might be regulated was uncertain. In this study, we investigated the transcriptome-wide effects of the proteasome inhibitor bortezomib on estrogen-regulated transcription in MCF7 human breast cancer cells and showed that bortezomib caused a specific global decrease in estrogen-induced gene expression. This effect was specific because gene expression induced by the glucocorticoid receptor was unaffected by bortezomib. Surprisingly, we observed no changes in ERα recruitment or assembly of its transcriptional activation complex on ERα target genes. Instead, we found that proteasome inhibition caused a global decrease in histone H2B monoubiquitination (H2Bub1), leading to transcriptional elongation defects on estrogen target genes and to decreased chromatin dynamics overall. In confirming the functional significance of this link, we showed that RNA interference–mediated knockdown of the H2B ubiquitin ligase RNF40 decreased ERα-induced gene transcription. Surprisingly, RNF40 knockdown also supported estrogen-independent cell proliferation and activation of cell survival signaling pathways. Most importantly, we found that H2Bub1 levels decrease during tumor progression. H2Bub1 was abundant in normal mammary epithelium and benign breast tumors but absent in most malignant and metastatic breast cancers. Taken together, our findings show how ERα function is blunted by bortezomib treatment as a result of reducing the downstream ubiquitin-dependent function of H2Bub1. In supporting a tumor suppressor role for H2Bub1 in breast cancer, our findings offer a rational basis to pursue H2Bub1-based therapies for future management of breast cancer. Cancer Res; 71(17); 1–15. ©2011 AACR.

Introduction

The estrogen receptor-α (ERα) is a ligand-activated transcription factor which plays an essential role in a number of physiologic processes, including development, fertility, cardiovascular function, and bone metabolism (1), by inducing rapid and dramatic changes in gene expression. Importantly, over two thirds of breast tumors express ERα and depend upon its activity for their growth (1, 2). As such, antiestrogen therapies such as tamoxifen, fulvestrant (Faslodex), and aromatase inhibitors have become standard treatments for ERα-positive breast cancer. Nevertheless, about one third of ERα-positive breast cancers become resistant to antiestrogen therapy and develop into more aggressive hormone-independent tumors (3). Thus, more effective therapies which act on additional aspects of ERα function may help to eliminate ERα-positive tumors at an earlier stage and increase overall patient survival (4).

One potential target of breast cancer therapy may be the ubiquitin–proteasome system (UPS; ref. 5). The conjugation of multiple ubiquitin moieties (polyubiquitination) linked to one another through lysine residue 48 (K48) of ubiquitin generally lead to the degradation of the target protein by the 26S proteasome (6). Ubiquitination is carried out by ubiquitin ligases which frequently contain either RING or HECT

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domains. Several ubiquitin ligases, such as RNF12 (7), have been shown to bind directly to ERα and regulate its activity. Furthermore, a number of studies have suggested that proteasome inhibition alters ERα-dependent gene transcription, although different effects and mechanisms have been suggested (8–12). Proposed mechanisms of action for proteasome inhibition included decreased ERα mobility within the nucleus (10, 11) and decreased ERα binding and recruitment of cofactors (10). In addition, changes in RNA polymerase (RNAP) II phosphorylation have been observed following proteasome inhibition (13). However, most of these studies utilized the laboratory proteasome inhibitor MG132, which is not suitable for clinical use, and were mostly based on the investigation of a limited number of target genes.

Recently, the proteasome inhibitor bortezomib (Velcade) was shown to be safe for clinical use (14) and was approved by the Food and Drug Administration for the treatment of multiple myeloma and mantle cell lymphoma (15). Although clinical trials for the treatment of metastatic breast cancer were initially disappointing when used as a single agent (14), a new study combining the pure antiestrogen fulvestrant with bortezomib suggests that the combination of antiestrogen therapies with proteasome inhibition may indeed increase treatment efficacy (16). In line with these results, clinical trials utilizing the combination of bortezomib and fulvestrant are currently underway for the treatment of ER-positive metastatic tumors in postmenopausal patients (NCT01142401). Therefore, a more complete understanding of the effects of proteasome inhibition on ERα activity is essential for assessing the molecular mechanisms involved and determining which types of tumors (i.e., nonmetastatic vs. metastatic; ERα-positive vs. ERα-negative) may respond better to proteasome inhibitors such as bortezomib.

In this study, we sought to determine the effects of the clinically safe proteasome inhibitor bortezomib on the rapid induction of estrogen-regulated gene transcription in MCF7 breast cancer cells. We provide evidence that bortezomib blocks estrogen-induced gene transcription by a unique posttranscriptional initiation mechanism involving a loss of histone H2B monoubiquitination (H2Bub1) and changes in chromatin dynamics. Moreover, we observe a dramatic loss of H2Bub1 during tumor progression which may lead to estrogen-independent cell proliferation and increased metastatic properties.

**Methods**

**Cell culture, transfections, and siRNAs**

MC7 cells were obtained from K. Pantel (University Hospital Hamburg-Eppendorf, Hamburg, Germany) and A549 cells were obtained from M. Dobbelstein (University of Göttingen). Both cell lines were grown in phenol red-free high-glucose Dulbecco’s Modified Eagle’s Media (DMEM; Invitrogen) supplemented with 10% bovine growth serum (Thermo Scientific). The identity of both the cell lines was routinely verified microscopically on the basis of cellular morphology and the expression of ERα and estrogen responsiveness (for MCF7 cells). Prior to hormone treatments, MCF7 and A549 cells were grown in DMEM containing 5% charcoal-dextran–treated FBS (CSS; HyClone) 1 to 2 days prior to treatment with 10 nmol/L 17β-estradiol (Sigma-Aldrich) or 100 nmol/L dexamethasone (Sigma-Aldrich), respectively, as indicated. For blocking proteasome activity, cells were pretreated for 15 minutes with 1 of the 3 following chemical proteasome inhibitors: 50 nmol/L bortezomib (LC Laboratories), 20 μmol/L MG132 (Biomol), or 1 μmol/L epoxomicin (Biomol). MCF10A cells were obtained from M. Oren (Weizmann Institute of Science, Rehoovot, Israel) and grown in DMEM/F-12 medium supplemented with 5% horse serum (Sigma), 20 ng/mL epidermal growth factor (EGF; Sigma), 0.5 μg/mL hydrocortisone (Sigma), 0.1 μg/mL cholera toxin (Sigma), and 10 μg/mL insulin (Sigma). Cellular identity was regularly verified microscopically on the basis of cell morphology. Transfections were done using Lipofectamine RNAiMAX (Invitrogen) for siRNAs and Lipofectamine 2000 (Invitrogen) for plasmid DNA according to the manufacturer’s instructions. siRNAs are listed in Supplementary Table S1.

**Chromatin fractionation, Western blot, and tissue microarray analysis**

Chromatin fractionation was done as described (17) and analyzed by Western blotting with antibodies and dilutions listed in Supplementary Table S6. Tissue microarray analyses were carried out essentially as described previously (7, 18) with the modifications listed in Supplementary Methods using the newly generated anti-H2Bub1 mouse monoclonal antibody 7B4 and the tissue microarrays BR8010 and BR951 (Biomax) containing normal mammary epithelial tissue samples, as well as benign, malignant, and metastatic samples.

**Cell migration, colony formation, and apoptosis assay**

Cell migration was assayed by seeding 100,000 MCF10A cells 48 hours after transfection with the respective siRNAs into 8.0-μm PET track-etch membrane cell culture inserts (BD Bioscience). Cells were grown for another 48 hours before fixation with methanol for 10 minutes. Migrated cells were visualized by crystal violet staining [0.1% (w/v) crystal violet, 10% (v/v) formaldehyde] for 10 minutes. For determining proliferative capacity, 20,000 MCF7 cells were plated in normal growth medium which was replaced 10 hours later by hormone-deprived medium. Cells were treated with 17β-estradiol for 7 days. Subsequently, colonies were fixed with 70% methanol for 30 minutes on ice before staining with crystal violet solution for 2 hours. Apoptosis was analyzed using the Guava Nexin Assay (Guava Technologies, Millipore) according to the manufacturer’s instructions.

**Fluorescence recovery after photobleaching**

For fluorescence recovery after photobleaching (FRAP) analyses, MCF7 cells were transiently transfected with a plasmid in which the open reading frame of ERα was cloned in frame into the pEGFP-C2 vector (Clontech Laboratories) and grown for 48 hours in estrogen-free medium before conducting analyses. Eight-bit time lapse images of cells were acquired at a frame rate of 3 frames per second with a confocal microscope setup and its software (TCS SP2 AOBS; Leica) with a 40× oil immersion objective (Type HCX PL Apo CS, NA 1.25;
The pinhole was set at 81.40 μm and the zoom factor was adjusted to obtain 62.5 × 62.5 μm² images with voxel size of 244.26 × 244.26 nm². After recording 10 images, the green fluorescent protein (GFP) signal in a 13.2 × 2.9 μm² region within a nucleus was bleached with an 488-nm laser beam and 120 postbleaching images were taken. To correct the intensity from imaging bleaching and background signal in the bleached region of the postbleaching series, the formula

\[ I_{\text{corr}}(t) = \frac{I_{\text{ori}}(t) - I_{\text{bg}}}{I_{\text{ori}}(0) - I_{\text{bg}}} \]

was used, where \( I_{\text{corr}}(t) \) represents the corrected intensity at a certain time point; \( I_{\text{ori}} \) represents the original intensity; \( I_{\text{bg}} \) is the average background signal in the series; and \( I_{\text{ori}} \) is the GFP signal from another nucleus in the same image field used for measuring the GFP bleaching from image acquisition. The fluorescence recovery of the bleached region was then fitted with a simple exponential function using the SigmaPlot (Systat Software) with 3 variables, \( I_{\text{thor}}(t) = y_0 - a \times e^{-bt} \), where \( y_0 \) is the theoretical maximum intensity and \( b \) is the time constant. The Welch's two-sample t test was carried out using the free statistical software R (19) for statistical computing and graphics to evaluate the significance of the data.

**Gene expression, chromatin immunoprecipitation, and quantitative real-time PCR**

RNA was isolated from cells with the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using random nonamer primers (Metabion). Chromatin immunoprecipitation (ChIP) and subsequent real-time PCR analyses were conducted essentially as previously described (7, 20) with modifications described in Supplementary Methods and the antibodies listed in Supplementary Table S6. ChIP and input samples were quantified using a standard curve made from all cDNA samples. Prior to statistical analysis, all quantitative real-time PCR (qRT-PCR) samples were normalized to an internal reference gene (28S ribosomal RNA or hnRNPK). The expression levels were determined relative to the vehicle-treated control sample and expressed as “relative mRNA expression.”

**Microarray analyses**

Total RNA for microarray experiments was isolated and transcriptome-wide gene expression analysis was conducted on samples treated for 24 hours using GeneChip Human Gene 1.0 ST Array (Affymetrix) carried out at the Transcriptome Analysis Laboratory, University of Göttingen. For mRNA expression profiling after 6-hour treatments, RNA samples were prepared in the same way and Illumina whole-genome gene expression analysis using a human HT-12 v4 beadchip was conducted by the Vancouver Prostate Centre Laboratory for Advanced Genome Analysis, Vancouver, Canada. Gene expression data were analyzed using log2 transformation and quantile normalization of expression levels (21). To determine significant differences of expression levels between the different groups, a moderated Student’s t test was computed on a gene-by-gene basis using the empirical Bayes statistics in the "LIMMA" package (22). To avoid a high number of false positives and to stay below a false discovery rate of 5%, P values were adjusted for multiple testing using the Benjamini–Hochberg method (23). All analyses were conducted using the free statistical software R (version 2.12.2; ref. 19). All gene expression data have been deposited into the Geo Expression Omnibus repository under the accession numbers GSE30931 and GSE31118.

**Chromosome conformation capture**

Chromosome conformation capture (3C) analysis was done as described (24) with the modifications described in the Supplementary Methods section. For comparison of interactions, DNA derived from bacterial artificial chromosome clones (BAC information in Supplementary Table S5) was isolated, digested, and ligated similarly to 3C samples and used for serial dilutions and quantitative analyses essentially as described (25). qRT-PCR analysis is described in Supplementary Methods. 3C values were normalized with values from an ampiclon located between restriction sites to control for equal amounts of input DNA. The normalized levels were graphed relative to the nontreated control sample (set to 1) and represented as “normalized relative interaction.”

**Results**

**Bortezomib blocks proteasome activity in MCF7 breast cancer cells**

To investigate the effects of a clinically utilized proteasome inhibitor on ERα activity, we validated the ability of bortezomib to block proteasome activity and determined the optimal concentration of bortezomib for blocking proteasome activity in ERα-positive MCF7 breast cancer cells. As shown in Supplementary Figure S1A, bortezomib effectively increased polyubiquitination of cellular proteins already at a concentration of 10 nmol/L and exhibited a similar efficiency to that of the laboratory proteasome inhibitors MG132 and epoxomicin (Supplementary Fig. S1B). Knockdown of the proteasome subunits PSMB3 or PSMB5 induced a similar accumulation of polyubiquitinated proteins compared with bortezomib treatment (Supplementary Fig. S1C). Surprisingly, in contrast to previous reports (16, 26), whereas bortezomib effectively decreased estrogen-induced proteasomal degradation of ERα (Supplementary Fig. S1D) in a manner similar to that reported for MG132 (9, 27), we observed little or no effect of bortezomib on fulvestrant-induced ERα downregulation (Supplementary Fig. S1E).

**Bortezomib does not affect RNAPII carboxy-terminal domain phosphorylation and only mildly impairs ERα nuclear mobility**

A previous study suggested that proteasome inhibition affected glucocorticoid receptor (GR)-regulated transcription by increasing the global phosphorylation of the RNAPII carboxy-terminal domain (CTD; ref. 13). Therefore, we tested whether bortezomib treatment also affects RNAPII phosphorylation in MCF7 cells. However, as shown in Figure 1A, no
major changes in the phosphorylation of any of the commonly phosphorylated residues (Ser2, Ser5, or Ser7) of the RNAPII CTD were observed following bortezomib treatment.

Decreased nuclear mobility of ERα following proteasome inhibition was also proposed as a mechanism by which MG132 treatment impairs ERα binding to target genes and estrogen-regulated transcription (10, 11). We substantiated these data using FRAP where we observed a dramatic decrease in ERα mobility following MG132 treatment with essentially no recovery observed up to 30 seconds after photobleaching (Fig. 1B). However, in comparison to MG132, bortezomib only mildly decreased ERα mobility (Fig. 1C), with effects more similar to those of estrogen.

In stark contrast to MG132, a nearly complete recovery of GFP-ERα signal was observed 30 seconds after photobleaching following bortezomib treatment.

**Neither short-term bortezomib treatment nor proteasome subunit knockdown induces apoptosis in MCF7 cells**

To test for secondary effects caused by the induction of apoptosis, we investigated the effects of 24- or 48-hour bortezomib treatment and transfection of siRNAs against the proteasome subunits PSMB3 or PSMB5. As shown in Supplementary Figure S2, neither 24-hour bortezomib treatment nor transfection of PSMB3 or PSMB5 siRNAs significantly induced...
apoptosis in MCF7 cells. Only after prolonged bortezomib treatment (48 hours) were apoptotic effects observed. Therefore, all subsequent experiments were carried out at time points of 24 hours or less.

**Bortezomib blocks estrogen-induced gene expression**

To determine the transcriptome-wide effects of bortezomib treatment on estrogen-regulated gene expression, we carried out microarray analyses using MCF7 cells treated with estrogen alone or a combination of bortezomib and estrogen for either 24 hours (Fig. 2A and B) or 6 hours (Supplementary Fig. S4). We also compared these effects with those of knocking down the proteasome subunits PSMB3 and PSMB5 (Fig. 2A–C).

Our microarray results for both 24 and 6 hours of estrogen or combined estrogen and bortezomib treatment show that proteasome inhibition dramatically decreases estrogen-induced gene expression (Fig. 2A and B; Supplementary Fig. S4). These effects were specific because an overwhelming majority of estrogen-induced genes (209 of 302) were decreased in their induction (Fig. 2B). In contrast, although bortezomib also had a significant effect on the expression of non–estrogen-regulated or estrogen-repressed genes, these conditions showed a similar number of genes whose expression was decreased or increased by proteasome inhibition. The effects of bortezomib on estrogen-induced gene transcription are specifically due to proteasome inhibition because siRNA-mediated knockdown of PSMB3 or PSMB5 resulted in similar effects on estrogen-induced gene expression (Fig. 2A and B) with a significant overlap in the changes observed (Fig. 2C).

To verify the results of these microarray studies, we also conducted qRT-PCR studies on several representative genes following 2, 6, or 24 hours of estrogen, bortezomib, or combined treatments. As shown in Figure 2D, the estrogen-induced expression of all genes tested (CXCL12, GREB1, TFF1, PGR, PKIB, and WISP2) was dramatically decreased by bortezomib treatment at each time point, except for TFF1 which showed decreased induction at both 2 and 6 hours but an increased induction at 24 hours (Fig. 2D). The effects observed on the TFF1 gene at 24 hours seem to be due to indirect effects most likely caused by changes in the stability or expression of a gene-specific cofactor. Consistent with the absence of an effect of bortezomib on the fulvestrant-induced decrease in ERα protein levels, we observed no effect of bortezomib on the fulvestrant-induced decreases in ERα-dependent gene transcription and ERα binding (Supplementary Fig. S3). Further microarray studies conducted after 6 hours of treatment (Supplementary Fig. S4) showed similar, albeit weaker, effects of bortezomib treatment on estrogen-induced transcription. Therefore, we conclude that proteasome inhibition most likely decreases ERα-dependent estrogen-induced gene transcription by a direct mechanism in a manner distinct from that of the antiestrogen fulvestrant.

**GR activity is not affected by bortezomib treatment**

The laboratory proteasome inhibitor MG132 has significant effects on GR-dependent gene expression (13). Thus, we also tested the specificity of proteasome inhibition toward ERα-dependent gene transcription by treating the glucocorticoid-responsive A549 lung cancer cell line with bortezomib and dexamethasone. In contrast to the effects observed with ERα, we observed no effects of bortezomib treatment on the rapid induction of the 4 glucocorticoid-responsive genes investigated (FKBP5, GILZ, SGK1, and SLC19A2; Fig. 3A). Furthermore, no effect of bortezomib on GR binding to these genes was observed (Fig. 3B).

**Bortezomib treatment does not affect ERα binding or cofactor recruitment to estrogen-induced genes**

On the basis of the results of a previous study investigating the effects of MG132 on the TFF1 gene (10), we utilized ChIP analyses to test whether proteasome inhibition by bortezomib affects ERα binding to endogenous estrogen-induced genes and the subsequent assembly of factors involved in transcriptional activation. However, no significant decrease in ERα binding to any of the 10 investigated binding sites was observed (Fig. 4A; Supplementary Figs. S5 and 6A). Given the importance of the p160 family of nuclear receptor coactivators in controlling estrogen-induced gene transcription and cellular proliferation (28), we also investigated whether the binding of GRIP1 (also called SRC2 and TIF2) was affected by bortezomib treatment. Like ERα, GRIP1 showed an increased recruitment to several ERα binding sites following estrogen treatment, and this recruitment was unaffected by bortezomib treatment (Fig. 4B; Supplementary Fig. S6B).

**The induction of long-range chromosomal interactions does not require proteasome activity**

Unbiased chromosome- and genome-wide studies showed that a large fraction of ERα binding sites are greater than 10 kb away from the genes that they regulate (29). Consistently, ERα frequently colocalizes with the cohesin component STAG1 (30) and thereby induces dramatic changes in higher order chromatin structure by inducing long-range chromosomal interactions between different ERα binding sites (25). Components of the mediator complex, which has been shown to play an essential role in regulating ERα function (31), also colocalize and cooperate with cohesin to regulate tissue-specific gene expression (32). Therefore, we also examined the recruitment of MED12 (Fig. 4C; Supplementary Fig. S6C) and STAG1 (Fig. 4D, Supplementary Fig. S6D) to ERα-binding sites which nucleate long-range chromosomal interactions (25) and were previously shown to colocalize with cohesin (30). These studies revealed that neither MED12 nor STAG1 recruitment was affected by bortezomib treatment. Consistent with the preserved recruitment of both MED12 and cohesin to ERα binding sites, we also observed no effect of bortezomib treatment on estrogen-induced changes in long-range chromosomal interactions as assessed by 3C analyses on the CXCL12, GREB1, and TFF1 genes (Fig. 4E; Supplementary Figs. S6E and F and S7).

**Proteasome inhibition does not affect transcriptional initiation steps but decreases efficient transcriptional elongation**

On the basis of the normal recruitment of ERα, transcriptional coactivators, and the induction of long-range chromo-
somal interactions, we next tested which subsequent transcriptional step may be affected by proteasome inhibition. Given the significant role of histone H3 acetylation (H3Ac) during transcriptional initiation, we investigated whether estrogen-induced changes in this epigenetic mark near the transcriptional start site (TSS) of ERα target genes may be affected by bortezomib treatment. However, no effect of bortezomib on H3Ac was observed on any of the investigated genes (Fig. 4F; Supplementary Fig. S8A). Despite the significant decrease observed in their mRNA levels (Fig. 5D2), the recruitment of RNAPII to the TSS was also unaffected on both the CXCL12 and GREB1 genes and only mildly affected on the TFF1 gene (Fig. 4G; Supplementary Fig. S8B). These results are supported by a previous study which concluded that many ERα target genes are regulated primarily at the level of transcriptional elongation (33). Therefore, we concluded that a posttranscriptional initiation step such as transcriptional elongation likely leads to the decrease in mRNA expression observed on most ERα target genes following bortezomib treatment.

Phosphorylation of RNAPII at serine 2 of its CTD by cyclin-dependent kinase-9 (CDK9) plays a critical role during transcriptional elongation by serving as a scaffold to promote the binding of transcriptional regulatory and chromatin-modifying proteins (34). Therefore, we investigated the effects of bortezomib treatment on CDK9 recruitment to the TSS of CXCL12, GREB1, and TFF1 following estrogen, bortezomib, or combined treatments in MCF7 cells. As shown in Figure 4H and Supplementary Figure S8C, bortezomib had little or no effect on CDK9 recruitment in the presence of estrogen. Thus, we next tested whether the ability of RNAPII to traverse ERα target genes was affected by proteasome inhibition. Indeed, as shown in Figure 4I and Supplementary Figure S8D, we observed a decrease in the elongating (Ser2 phosphorylated) form of RNAPII at the 3′ end of the investigated estrogen-regulated genes, whereas the total levels of Ser2 phosphorylation were unchanged (see Fig. 1A).

Proteasome inhibition decreases H2B monoubiquitination and affects histone exchange

On the basis of the normal recruitment of CDK9 but decreased ability of RNAPII to transcribe the entire length of the investigated ERα target genes, we hypothesized that bortezomib-induced changes in chromatin structure may prevent transcriptional elongation. One histone modification which is associated with the transcribed regions of active genes (35) is dependent upon Ser2 phosphorylation of the RNAPII CTD (20) and has been implicated in transcriptional elongation is histone H2Bub1 (36). Furthermore, proteasome inhibition causes a rapid decrease in H2Bub1 within minutes of treatment (35, 37). In contrast to the stabilizing effect of proteasome inhibition on many ubiquitinated proteins, the effect on H2Bub1 is related to the transient nature of H2Bub1 and is a consequence of a rapid depletion of the pool of free ubiquitin in the nucleus (37, 38). Consistent with the previously published results for other proteasome inhibitors (35, 37), we observed a significant decrease in H2Bub1 following bortezomib treatment (Fig. 5A). These effects were specific for H2Bub1 because other histone modifications associated with active genes and mark transcriptional initiation (H3 lysine 9/14 acetylation; H3Ac) and elongation (H3 lysine 36 trimethylation; H3K36me3) were unaffected by bortezomib treatment.

H2Bub1 functions during transcriptional elongation by cooperating with the facilitates chromatin transcription (FACT) histone chaperone complex to increase histone exchange and open chromatin structure to allow passage of RNAPII through chromatin during transcription (36). Importantly, H2Bub1 is localized exclusively in a chromatin-associated form (39) and seems to be a requisite for both transcription-associated (36) and DNA repair–associated histone exchange (39). Therefore, we tested whether the bortezomib-induced decrease in H2Bub1 levels also leads to decreased recruitment of the FACT complex to chromatin by separating the nucleoplasmic and chromatin-bound fractions from MCF7 cells treated with estrogen, bortezomib, or combined treatments. Indeed, as shown in Figure 5B and consistent with the global decrease in H2Bub1 levels, the recruitment of the FACT component SSR1P1 to chromatin was significantly impaired by bortezomib treatment.

The positive effect of histone chaperones such as FACT during transcriptional elongation and other DNA-associated processes is achieved by the removal and reinsertion of components of the core nucleosome. This effect can be observed by analyzing the soluble fraction (i.e., “non-chromatin bound fraction”) of core histones such as H3. A recent report showed that H2Bub1 is essential for DNA damage-induced changes in the pool of free H3 (39). Consistently, we also observed that the bortezomib-induced loss of total (Fig. 5A) and chromatin-bound H2Bub1 (Fig. 5B) resulted in decreased nucleoplasmic H3 (Fig. 5B). Thus, bortezomib seems to decrease histone exchange by blocking H2B mono-ubiquitination.

Figure 2. The bulk of estrogen-regulated genes is negatively influenced by proteasome inhibition using bortezomib. A, mRNA expression profiling of estrogen-regulated genes after 24-hour treatment. The heatmap shows log2 fold changes (FC) in experiments 17β-estradiol versus control (E2), bortezomib + 17β-estradiol versus 17β-estradiol (Bort + E2), and PSMB siRNA + 17β-estradiol versus control siRNA + 17β-estradiol (siRNA PSMB + E2; columns) for genes which are significantly (q < 0.05; p values adjusted to false discovery rate) regulated by estrogen [FC < log2 (1.5) or FC > log2 (1.5) rows]. The color key ranges from red marking downregulated to blue marking upregulated genes; mean values, n = 3. PSMB knockdown samples depict all genes which were either up- or downregulated in all 4 following comparisons: PSMB3 versus control 1, PSMB3 versus control 2, PSMB5 versus control 1, and PSMB5 versus control 2 siRNAs. B, effect of proteasome inhibition and knockdown on genes which are significantly influenced by estrogen. Numbers of genes depicted in the heatmap which are affected by estrogen, bortezomib, or the knockdown of 20S subunits. E2 genes: |q| < 0.05, FC < log2 (1.5); |q| > 0.05, FC > log2 (1.5); Bort + E2/PSMB + E2 genes: |q| < 0.1, negative FC; |q| > 0.1, positive FC. C, Venn diagram showing the overlap of genes that are significantly upregulated by estrogen [q < 0.05, FC > log2 (1.5)] and that are downregulated by bortezomib and/or knockdown of PSMB3 and PSMB5 (negative FC). D, total mRNA was extracted from MCF7 cells, pretreated for 15 minutes with 50 nmol/L bortezomib (Bort) or vehicle (ethanol, Cont) and incubated with 10 nmol/L 17β-estradiol (E2) for 2, 6, or 24 hours. The expression levels of estrogen target genes CXCL12, GREB1, TFF1, PGR, PKB, and WISP2 were normalized to 28S ribosomal RNA graphed relative to the control sample and expressed as "relative mRNA expression"; mean values ± SD, n = 2.
Depletion of the histone H2B ubiquitin ligase RNF40 specifically results in decreased estrogen-dependent gene transcription and enables estrogen-independent cell proliferation

Histone H2B monoubiquitination is carried out by a heterodimeric ubiquitin ligase complex containing RNF20 and RNF40 (36, 40) in which both proteins are essential for maintaining H2Bub1 levels (20). Given the association of H2Bub1 with active gene transcription and its connection to transcriptional elongation, we tested whether a loss of H2Bub1 following RNF40 knockdown in MCF7 cells impairs estrogen-regulated gene transcription in a manner similar to bortezomib treatment. Indeed, RNF40 knockdown significantly decreased the induction of CXCL12, GREB1, and
**Figure 4.** Bortezomib treatment has no effect on ERα, GRIP1, MED12, and STAG1 recruitment to target genes, does not influence estrogen-induced long-range interaction, shows no effect on the transcriptional initiation complex assembly but decreases RNAPII elongation on estrogen target genes. A–D and F–I, after a 15-minute pretreatment with 50 nmol/L bortezomib (Bort) or vehicle (ethanol, Cont), MCF7 cells were incubated with 10 nmol/L 17β-estradiol (E2) for 2 hours. ERα (A), GRIP1 (B), MED12 (C), STAG1 (D), H3Ac (F), RNAPII (G), CDK9 (H), and RNAPII p-Ser2 (I) binding to the indicated CXCL12 gene sites was analyzed via ChIP analysis using specific antibodies. ChIP analysis was conducted as in Figure 3B; mean values ± SD, n = 3. E, graphical scheme of the tested 3C sites on the CXCL12 locus. The interacting sites were chosen on the basis of their published ChIA-PET interactions by Fullwood and colleagues (25) and are labeled according to published ChIP-on-chip data by Carroll and colleagues (29). 3C assay was done as described in the Methods section. Purified 3C DNA samples were quantified by qPCR using a standard curve from BAC DNA containing the CXCL12 locus which was digested and ligated as for 3C samples. 3C values were normalized to values from an internal control site that lies between restriction enzyme sites, graphed relative to the control sample (set to 1), and represented as “normalized relative interaction”; mean values ± SD, n = 3.

**TFF1** expression following estrogen treatment (Fig. 5C; Supplementary Fig. S9A), without decreasing the protein levels of any of the investigated cofactors MED12, GRIP1, or CDK9 (Supplementary Fig. S9B). This effect was specific for ERα-regulated transcription, as the rapid induction of several GR target genes following dexamethasone treatment of A549 cells was unaffected by RNF40 knockdown (Fig. 5D; Supplementary Fig. S9C).

Our previous work indicated a potential tumor suppressor role for H2Bub1 (41). Therefore, we tested whether a loss of
Proteasome inhibition using bortezomib decreases histone H2B monoubiquitination. Knockdown of the H2B ubiquitin ligase RNF40 decreases estrogen-induced target gene expression but enhances estrogen-independent proliferative capacity of breast cancer cells. A, after growing MCF7 cells in hormone-deprived medium for 3 days, cells were pretreated with either vehicle (ethanol) or 50 nmol/L bortezomib (Bort) for 15 minutes before incubating with 10 nmol/L 17β-estradiol (E2) for 2 hours. Whole protein extracts were analyzed via Western blotting with the indicated antibodies. β-Actin is shown as loading control. B, MCF7 cells were kept in hormone-depleted medium for 2 days and then treated (as in A). Nuclear protein extracts were separated into soluble and insoluble (chromatin) fractions and analyzed by Western blotting for SSRP1, histone H3, H2Bub1, and H2B protein levels. MCF7 (C) and A549 (D) cells transfected with RNF40 siRNAs for 24 hours were grown in hormone-free medium for an additional 48 hours before stimulation with either 10 nmol/L 17β-estradiol (E2; C) or 100 nmol/L dexamethasone (Dex; D) for 2 hours. Hormone-induced gene expression was normalized to hnRNPK (C) or 28S ribosomal RNA (D) levels and graphically represented as in Figure 2D; mean values ± SD, n = 3. E and F, clonogenic assay after RNF40 knockdown. MCF7 cells were transfected with control or RNF40 siRNA. The next day, cells were replated into 6-well plates. After attachment, growth medium was changed to hormone-deprived 5% CSS growth medium and cells stimulated with 10 nmol/L 17β-estradiol (E2) for 7 days (E). Efficient knockdown of RNF40 was verified and ERα, p-AKT, AKT, p-ERK, ERK, and H2Bub1 protein levels were analyzed by Western blotting. H2B was used as loading control (F).
H2Bub1 mediated by knockdown of RNF40 might affect estrogen-dependent cellular proliferation. Interestingly, RNF40 knockdown led to estrogen-independent proliferation of MCF7 cells (Fig. 5E and F). This effect was not further potentiated by estrogen treatment. In contrast, control transfected cells depended upon estrogen treatment of proliferation (Fig. 5E, top).

ERα-independent proliferation is frequently associated with increased activation of the phosphoinositide-3-kinase (PI3K)/AKT and extracellular signal–regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling pathways (42). To determine whether a loss of RNF40 expression also leads to the activation of estrogen-independent cell proliferation signaling, we examined the activation of both AKT and ERK. Interestingly, RNF40 knockdown was sufficient to significantly activate both signaling pathways (Fig. 5F).

**H2Bub1 decreases during tumor progression**

Although our previous study indicated a potential tumor suppressor role for RNF20 (41), no study to date has investigated the presence of H2Bub1 in normal and cancerous tissues. Therefore, we developed a new H2Bub1-specific monoclonal antibody using a previously published branched-peptide antigen strategy (35) and carried out immunohistochemical analyses of tissue microarrays containing normal mammary epithelial tissue as well as benign, malignant, and metastatic breast cancer samples. These studies yielded a total of 109 interpretable results in which the nuclei of both normal mammary ductal epithelial cells (Fig. 6A) and all 18 benign tumor samples stained positive for H2Bub1 (Fig. 6B and C). In contrast, only 21 of 64 malignant breast cancer samples (32%) stained positive for H2Bub1 and only 3 of the 19 metastatic tumor samples stained positive for H2Bub1 (Fig. 6B and C; Table 1). As a control, we also stained normal and tumor samples for (total) H2B (Supplementary Fig. S10). As expected, all samples stained positive for H2B, confirming that the decreased H2Bub1 staining during breast cancer progression is due to decreased monoubiquitination of H2B and not a loss of nuclear histones. Thus, decreased H2B monoubiquitination correlates with breast cancer progression and metastasis in a small cohort of tumor samples. Furthermore, on the basis of these results and the importance of cell migration for metastasis, we also investigated the effects of RNF40 knockdown on the migratory potential of a normal mammary epithelial cell line. Consistent with a central role for H2Bub1 in suppressing metastasis, increased AKT and ERK activity following RNF40 knockdown, and with our previous study with RNF20 (41), siRNA-mediated knockdown of RNF40 resulted in increased migration of MCF10A cells (Fig. 6D).

**Discussion**

Inhibition of proteasome activity has been shown to be effective in treating some types of cancers including multiple myeloma and mantle cell lymphoma. However, its effectiveness in the treatment of breast cancer is not clear. Moreover, its utility for different stages and subtypes of breast cancer needs to be determined. On the basis of the proposed necessity of proteasome activity for ERα-induced gene transcription (9, 10), we sought to determine the effects of the clinical proteasome inhibitor bortezomib on estrogen-regulated gene transcription and investigated its mechanism of action. We provide the first evidence that bortezomib-mediated proteasome inhibition functions to limit transcriptional elongation of estrogen-induced genes by causing a global decrease in H2Bub1 levels. This in turn prevents histone chaperone recruitment to chromatin and increased nucleosome dynamics on ERα target genes.

This “nonproteolytic” effect of proteasome inhibition challenges the general assumption that proteasome inhibition functions primarily by preventing the degradation of specific proteins required for essential cellular processes (43, 44). Although these mechanisms likely influence the ultimate inhibition of cell-cycle progression and the induction of apoptosis after prolonged bortezomib treatment in MCF7 cells, they do not seem to be responsible for the rapid effects on estrogen-induced gene transcription that we have observed.

A recent study reported a combinatorial effect of fulvestrant and bortezomib on the induction of apoptosis and laid the groundwork for ongoing clinical trials combining these 2 treatments (16). In this study, we were unable to see any effects of bortezomib treatment on the rapid fulvestrant-induced ERα downregulation and the repression of ERα target gene transcription or ERα binding. Thus, as suggested by the reported induction of an unfolded protein response, the long-term effects of combined bortezomib and fulvestrant treatment are probably not due to changes in ERα-dependent gene transcription but rather a secondary effect induced by the accumulation of a highly polyubiquitinated protein. However, additional experiments are clearly needed to address whether and how these 2 therapies work synergistically, their mechanism of action, and the contribution of altering ERα transcriptional activity in eliciting their effects.

According to our data, we propose a new mechanism whereby bortezomib treatment decreases the ability of ERα to activate estrogen-responsive genes by leading to a rapid and global loss of H2Bub1. This effect is due to a rapid depletion of free ubiquitin in the nucleus (37, 38). On the basis of the very dynamic nature of H2B monoubiquitination (20), the depletion of nuclear ubiquitin prevents new ubiquitination of H2B and causes a rapid loss of H2Bub1 levels. This is in stark contrast to most previously proposed models which invoked the degradation of specific transcriptional complexes as being essential for ERα activity. Although a stabilization of some gene-specific coactivator proteins following bortezomib treatment likely influences the regulation of some estrogen-responsive genes at later time points (e.g., TFF1), our data suggest that H2Bub1-dependent recruitment of the FACT histone chaperone complex and the subsequent changes in nucleosome dynamics which allow passage of transcribing RNAPII through ERα target genes is a likely mechanism whereby bortezomib very rapidly decreases the majority of estrogen-induced gene transcription.
Figure 6. H2Bub1 expression in human normal breast tissue, tumors, and metastasis. A, representative immunohistochemical image displaying H2Bub1 protein expression in normal breast tissue; bar = 100 μm. B, bar graph representing the immunohistochemical analysis of the H2Bub1 staining in normal adjacent tissue, the indicated breast tumors, or metastasis. Shown are relative numbers in "% samples" as well as the absolute numbers of samples on the bottom of each bar. C, representative sample pictures of a tissue microarray analysis showing immunohistochemical H2Bub1 staining in benign, malignant, and metastatic breast tumor samples. Samples were categorized into 2 groups as either negative or positive for H2Bub1 (shown in left top corners of the images); bar = 100 μm. D, cell migration assay after RNF40 knockdown. Control or RNF40 depleted MCF10A cells were seeded onto cell culture inserts and allowed to migrate for 48 hours. Representative images from an experiment are shown, bar = 500 μm.
Consistent with the data presented here on estrogen-regulated genes, we and others have shown that H2Bub1 is probably most important for signal-specific induction of gene expression (36, 41). These effects are likely to be due to the need for major changes in chromatin structure caused by disruption of the 30-nm chromatin fiber by monoubiquitination of H2B (45). Genes which are already active or repressed may not require the presence or absence of H2Bub1 to remain in these states. In contrast, the activation of silent or lowly expressed genes which lie within a specific chromatin context is more likely to require the addition or loss of H2Bub1 because the activation of these genes will require dynamic alterations in the structure of the surrounding chromatin. Interestingly, these effects seem to be signal specific because the activation of gene transcription by GR did not require RNF40 expression.

A particularly striking and potentially therapy-relevant finding of this study is the correlation of a loss of H2Bub1 with tumor progression and the effects of RNF40 knockdown on both cellular proliferation and migration. Despite a significant decrease in the induction of estrogen-regulated gene transcription, we observed no significant effect of RNF40 knockdown on cellular proliferation in the presence of estrogen. Instead, RNF40 knockdown allowed for estrogen-independent proliferation of MCF7 cells. These paradoxical findings were initially very surprising. However, our findings that the AKT and ERK cell survival and proliferative signaling pathways are activated suggest that the effects of RNF40 knockdown may extend beyond its effects on chromatin structure. Consistent with this hypothesis, the obligate heterodimeric partner of RNF40, RNF20, has been shown to interact with both isoforms of EBP1 (46) which has been implicated in directly controlling the activity of nuclear AKT (47) and the EGF receptor (48). Although, it is likely that the increased activation of the ERK and AKT pathways may be related to this interaction, it is presently unknown how or if RNF40 affects the activity of EBP1. Thus, a loss of RNF40 or RNF20 activity during tumor progression may be an important step in the development of hormone-independent breast cancer by simultaneously decreasing ERα-dependent cell differentiation and activating cell survival and proliferative signaling.

We hypothesize that the loss of H2Bub1 during tumor progression and metastasis may lead to decreased cellular differentiation and reversion to a stem cell–like phenotype. In support of this, the mRNA levels of the H2B deubiquitinating enzyme USP22 correlate with an undifferentiated stem-like phenotype in multiple types of cancer (49), and its protein expression was shown to correlate with breast cancer progression and patient outcome (50). Thus, multiple mechanisms which diminish H2B monoubiquitination such as decreased RNF20 or RNF40 expression or increased USP22 expression may similarly lead to a loss of H2Bub1 and reversion to a stem cell–like phenotype.

In this study, we have uncovered a novel mechanism whereby proteasome inhibition blocks ERα-activated gene expression by interfering with histone H2B monoubiquitination and histone exchange. Moreover, we provide the first evidence that H2Bub1 decreases during tumorigenesis. Additional studies into the various mechanisms of proteasome inhibitor action need to be conducted to determine which tumors may respond best to bortezomib treatment. For example, more differentiated ERα/H2Bub1-positive tumors might be predicted to respond to H2Bub1-reducing therapies such as bortezomib which simultaneously decrease ERα-dependent gene transcription and induce apoptosis. In contrast, more advanced, less differentiated, ERα-dependent tumors may benefit from therapies which increase H2Bub1 levels and lead to a more differentiated phenotype. To address these aspects, additional immunohistochemical studies investigating the presence of H2Bub1 in a larger cohort of patients with more extensive immunohistologic characterization, patient follow-up information and hormone responsiveness as well as in vivo animal model studies should be conducted. We hypothesize that H2Bub1 may represent an important new diagnostic marker and potential therapeutic target in breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Table 1. Statistical analysis of tissue microarray analyses: Fisher’s exact test

<table>
<thead>
<tr>
<th>Normal adjacent tissue</th>
<th>Benign tumor</th>
<th>Malignant tumor</th>
<th>Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>8</td>
<td>18</td>
<td>21</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>0</td>
<td>43</td>
</tr>
</tbody>
</table>

P < 0.0001

P < 0.0001

P = 0.0004

P < 0.0001
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