Modification of ERα by UFM1 Increases Its Stability and Transactivity for Breast Cancer Development

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The post-translational modification (e.g., phosphorylation) of estrogen receptor α (ERα) plays a role in controlling the expression and subcellular localization of ERα as well as its sensitivity to hormone response. Here, we show that ERα is also modified by UFM1 and this modification (ufmylation) plays a crucial role in promoting the stability and transactivity of ERα, which in turn promotes breast cancer development. The elevation of ufmylation via the knockdown of UFSP2 (the UFM1-deconjugating enzyme in humans) dramatically increases ERα stability by inhibiting ubiquitination. In contrast, ERα stability is decreased by the prevention of ufmylation via the silencing of UBA5 (the UFM1-activating E1 enzyme). Lys171 and Lys180 of ERα were identified as the major UFM1 acceptor sites, and the replacement of both Lys residues by Arg (2KR mutation) markedly reduced ERα stability. Moreover, the 2KR mutation abrogated the 17β-estradiol-induced transactivity of ERα and the expression of its downstream target genes, including pS2, cyclin D1, and c-Myc: this indicates that ERα ufmylation is required for its transactivation function. In addition, the 2KR mutation prevented anchorage-independent colony formation by MCF7 cells. Most notably, the expression of UFM1 and its conjugating machinery (i.e., UBAS, UFC1, UFL1, and UFBP1) were dramatically upregulated in ERα-positive breast cancer cell lines and tissues. Collectively, these findings implicate a critical role attributed to ERα ufmylation in breast cancer development by ameliorating its stability and transactivity.

Keywords: breast cancer, estrogen receptor alpha, proteasome, transactivity, ubiquitin, ubiquitin-fold modifier 1

INTRODUCTION

Estrogen receptor α (ERα), a member of the nuclear receptor superfamily, is a ligand-regulated transcription factor (Mangelsdorf et al., 1995). Upon binding with estrogen (e.g., 17β-estradiol; henceforth referred to as estradiol), ERα forms a dimeric complex, translocates to the nucleus from the cytoplasm, and binds to the estrogen responsive elements (ERE) of target genes for transcriptional activation (Nilsson et al., 2001). Numerous types of post-translational modifications (PTM), such as phosphorylation, acetylation, sumoylation, and ubiquitination, participate in the control of ERα functions, such as the regulation of interactions with chromatin, coactivator recruitment, nuclear localization, receptor stability, and hormone responsiveness (Le Romancer et al., 2011). Interestingly, proteasome activity has been shown to be required not only for ERα degradation but also for ERα transactivity. Thus, the ubiquitination of ligand-bound ERα is thought to be critical for promoter clearance and the reinitiation of transcription (Preisler-Mashek et al., 2002; Reid et al., 2003).
Ubiquitin-fold modifier 1 (UFM1) is a newly discovered ubiquitin-like protein (Komatsu et al., 2004). Like ubiquitin and other ubiquitin-like proteins (Song et al., 2021), UFM1 is conjugated to target proteins by a three-step enzyme system: UBA5, a UFM1-activating E1 enzyme; UFL1, a UFM1-conjugating E2 enzymes; and UFL1, a UFM1 E3 ligases (Daniel and Liebau, 2014; Komatsu et al., 2004; Yoo et al., 2015). In addition, a UFM1-binding protein (UFBP1, also known as DDRGK1) is required for protein modification by UFM1 (Yoo et al., 2014). This modification process (ufmylation) can be reversed by UFM1-specific proteases (UFSPs) that comprise UFS1 and UFS2 (Kang et al., 2007). Unlike in murine systems, however, UFS1 in humans is inactive due to the lack of the Cys box, which is essential for catalytic activity (Yoo et al., 2015). Therefore, in humans, only UFS2 serves as a functional enzyme that deconjugates UFM1 from ufmylated proteins and generates matured UFM1 molecules from its precursor (Yoo et al., 2014:2015).

Recent studies have demonstrated that ufmylation plays important roles in controlling numerous cellular processes, such as erythroid differentiation (Tatsumi et al., 2012), endoplasmic reticulum stress response (Tatsumi et al., 2012), DNA damage response (Lemaire et al., 2011: Zhang et al., 2012), fatty acid metabolism (Gannavaram et al., 2012), and neuronal development (Colin et al., 2016; Hamilton et al., 2017). In addition, the aberrant expression of UFBP1 and UFL1 has been implicated in the development of gastric cancer (Hu et al., 2021; Lin et al., 2019). Furthermore, the ufmylation of ASC1 (a transcriptional coactivator of ERα) has been shown to promote breast cancer development (Yoo et al., 2014). Poly-UFM1 chains conjugated to ASC1 serve as molecular scaffolds that recruit other transcription coactivators (e.g., p300 and SRC1) as well as itself to ERα. This leads to the transcriptional activation and expression of ERα downstream target genes, such as pS2, c-Myc, and cyclin D, all of which promote mammary cell proliferation for breast cancer development.

To date, as many as nine different types of PTM of ERα, including phosphorylation, acetylation, nitrosylation, parmi- toxylation, glycosylation, ubiquitination, and sumoylation, have been identified (Le Romancer et al., 2011: Zhou et al., 2014), illustrating ERα as a protein with one of the most diverse sets of post-translational modification. In this regard, we questioned whether ERα could also be modified by UFM1. In the present study, we show that ERα indeed serves as a target for ufmylation. Like ubiquitination, the ufmylation of endogenous ERα occurred only when the cells are treated with estradiol. This ligand-inducible ERα ufmylation promoted the stability of ERα, the expression of its target genes, and anchorage-independent cell growth. Moreover, the expression of UFM1 and its conjugating machinery (including UFBP1) were dramatically upregulated in ERα-positive breast cancer cell lines and tissues. Taken together, our findings implicate a critical role attributed to ERα ufmylation in breast cancer development.

MATERIALS AND METHODS

Materials
ERα cDNA was cloned into pcDNA-HisMax and pCMV2-Flag. Antibodies against ERα (H-184), c-Myc (9E10), UFM1 (I-16), UFBP1 (G-17), and UFS2 (C-7) were purchased from Santa Cruz Biotechnology (USA). Anti-UBA5, anti-UFC1 (Abcam, UK), anti-UFL1 (Bethyl Laboratories, USA), anti-Xpress (Thermo Fisher Scientific, USA), anti-His (BD Biosciences, USA), and anti-Flag M2 (Sigma-Aldrich, USA) antibodies were also obtained. shRNAs were purchased from Open Biosystems. Target sequences used for shRNAs were: AACAGAAACTTTA-ACACGT for UBA5 and AATAACCTTGCAGGTCTTCAGC for UFS2 (all from 5’ to 3’), which are the same as that we had previously used (Yoo et al., 2014).

Cell culture and transfection
HEK293T and mammary cells were cultured at 37°C in DMEM containing 1 µg/ml of streptomycin, 100 units/ml of penicillin, and 10% fetal bovine serum (FBS). For treatment with estradiol, cells were grown in the same medium containing phenol red-free DMEM supplemented with 5% charcoal-filtered FBS (i.e., estradiol-stripped medium). All media were obtained from Hyclone Laboratories (USA). Plasmids were transfected to cells by using Lipofectamine with PLUS reagent (Thermo Fisher Scientific), NeonTM Transfection System (Thermo Fisher Scientific), Metafectene reagent (Bior- ontex Laboratories, Germany), or jetPEI™ DNA Transfection Reagent (Polyplus-transfection, France).

Ufmylation assay
Cells transfected with appropriate vectors were cultured for 36 h. They were lysed by boiling in 150 mM Tris-HCl (pH 8), 5% SDS, and 30% glycerol, and then diluted 20 fold with buffer-A consisting of 50 mM Tris-HCl (pH 8), 150 mM NaCl, 10 mM imidazole, 0.5% NP-40 or 1% Triton X-100, 2 mM NEM, and 1× protease inhibitor cocktail (Roche, Switzerland). They were incubated with Ni-NTA resins for 3 h at 4°C, washed with buffer-A containing 20 mM imidazole, boiled in SDS-sampling buffer, and centrifuged. Supernatants were subjected to immunoblot analysis.

Pull-down analysis and immunoprecipitation
For pull-down analysis, cell lysates were prepared and treated with NTA-agarose resins. Precipitates were washed three times with a wash buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 20 mM imidazole, and 0.5% NP-40). Bound proteins were eluted by boiling in 2× SDS-sampling buffer and subjected to immunoblot analysis. For immunoprecipitation, cells were lysed in buffer-A and their lysates were incubated with appropriate antibodies for 2 h at 4°C and then with 30 µl of 50% slurry of protein A-Sepharose (Sigma-Aldrich) for 1 h. The resins were spun and boiled in SDS-sampling buffer. The samples were then subjected to immunoblot analysis.

Luciferase assay
Cells were transfected with pcDNA-pβ-Gal and ERE-Luc. After incubation for 24 h, cells were treated with estradiol for 24 h, harvested, and assayed for luciferase. The enzyme activity was measured in a luminometer, and normalized by β-galactosidase expression with a luciferase system (Promega, USA).
Reverse transcription polymerase chain reaction (RT-PCR) and real-time quantitative PCR (qPCR)

Total RNAs were isolated from cells by using TRizol (Thermo Fisher Scientific). RT-PCR was performed using SuperScript III (Thermo Fisher Scientific), according to the manufacturer’s instructions. Messenger RNAs (mRNAs) were quantified by qPCR using ABI Prism 7700 sequence detection system (Applied Biosystems, USA). Primers used in qPCR were: GGTGC-CCTTGGAGCAGA and GGGCGAAGATCACCTTTGT for pS2; GCTGCTCTGTGAGAACAAGC and AAGGTGTTCAATGAAATC-GTGC for cyclin D1; TCCACAACATCGACAACTACG and CACTGTCACAATGACCTT for c-Myc (all from 5’ to 3’).

Immunocytochemistry

Mammary cells were grown on coverslips. After transfection, they were fixed by incubation with 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min. Cells were then washed three times with PBS containing 0.1% Triton X-100, and permeabilized with 0.5% Triton X-100 in PBS for 5 min. After blocking with 3% bovine serum albumin (BSA) in PBS for 30 min, cells were incubated overnight at 4°C with appropriate antibodies. After washing with PBS containing 0.1% Triton X-100, cells were incubated for 1 h with FITC- or TRITC-conjugated secondary antibody in PBS containing 3% BSA. Cells were then observed using a confocal laser scanning microscope (LSM 700; Carl Zeiss, Germany).

Immunohistochemistry

Tumor tissues were embedded in OCT compound. Cryo-sections (10 µm) were permeabilized and fixed with methanol. Sections were then incubated with 3% H2O2 in PBS for 10 min to quench endogenous peroxidase activity. After treatment with a blocking solution (5% horse serum, 3% BSA, and 0.1% Triton X-100 in PBS), they were incubated with primary antibodies in blocking solution overnight at 4°C. Sections were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. Signals were detected using 3,3'-diaminobenzidine as a substrate. They were then counterstained with hematoxylin.

RESULTS

ERα is a target substrate for ufmylation

To determine whether ERα serves as a target protein for ufmylation, we first examined its ability to interact with UFL1, the UFM1 E3 ligase. We also tested if UFBP1 interacts with ERα, since it is an essential component for the ufmylation of target substrates, such as ASC1 (Yoo et al., 2014). ERα was ectopically expressed with UFL1 or UFBP1 in HEK293T cells. Immunoprecipitation analysis revealed that ERα interacts with UFL1 (Fig. 1A) and UFBP1 (Fig. 1B). Moreover, endogenous ERα was capable of interacting with both UFL1 and UFBP1 (Fig. 1C) in MCF7 cells. To determine whether ERα can indeed be ufmylated, HEK293T cells were transfected with vectors overexpressing ERα, UFM1, UFC1 (E2), UFL1 (E3), and UFBP1. Figure 1D clearly shows that ERα was modified by UFM1. Henceforth, UFM1, UFC1, UFL1, and UFBP1 are referred as the UFM1-conjugating system. Note that UBA5 (E1) was not overexpressed as its basal level in cells is sufficient for the ufmylation of target proteins (Yoo et al., 2014). These results indicate that ERα is a new target for ufmylation.

UFSP2 is the major deufmylating enzyme in humans (Kang et al., 2007). To determine whether UFSP2 can deufmylate ERα, we first examined if they interact with each other. Supplementary Figure S1A shows that ERα interacts with UFSP2.

**Fig. 1.** ERα is a target substrate for ufmylation. (A) HisMax-ERα was expressed in HEK293T cells with or without Flag-UFL1. Cell lysates were subjected to immunoprecipitation with the anti-Flag antibody followed by immunoblotting with the anti-Flag and anti-Xpress antibodies. They were also directly probed with the same antibodies. (B) Experiments were performed as above but by expressing Flag-UFBP1 instead of Flag-UFL1. (C) Lysates of MCF7 cells were subjected to immunoprecipitation with the anti-IgG or anti-ERα antibodies followed by immunoblotting with the anti-UFL1, anti-UFBP1, and anti-ERα antibodies. (D) HisMax-ERα was expressed in HEK293T cells with or without Myc-UF1C1 (E2), Myc-UFL1 (E3), Myc-UFBP1, and Flag-UFM1 (i.e., the UFM1-conjugating system). Cell lysates were subjected to pull-down with NTA resins followed by immunoblotting with the anti-Flag antibody.

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as well as with its inactive variant (CS), of which the catalytic Cys residue was substituted with Ser by site-directed mutagenesis. We next examined the ability of UFSP2 to deufmylate ERα. Supplementary Figure S1B shows that UFSP2, but not CS, was able to completely reverse ERα ufmylation, indicating that ERα is a substrate of UFSP2. Taken together, these results indicate that ERα can be reversibly modified by UFSP1.

Ubiquitin has seven Lys residues, all of which are capable of forming isopeptide bonds to generate poly-ubiquitin chains on target proteins (Heride et al., 2014). UFSP2 has been shown to generate poly-UFM1 chains on ASC1 (Yoo et al., 2014). To determine whether ERα utilizes Lys69 to generate poly-UFM1 chains on target proteins (Supplementary Fig. S2). These results suggest that ERα can be reversibly modified by UFSP2.

Identification of UFM1 acceptor sites in ERα
To determine UFM1 acceptor sites in ERα, its deletion fragments (termed Δ1-Δ6) were generated as in Fig. 2A and subjected to a ufmylation assay. Δ1, Δ2, and Δ4 could be ufmylated whereas Δ3, Δ5, and Δ6 could not (Supplementary Figs. S3A and S3B); this indicates that ufmylation sites reside in Δ1 (i.e., amino acids 1-180). Since Δ1 contains four Lys residues (K32, K48, K171, and K180), each residue was substituted with Arg. The resulting variants of Δ1 were expressed in HEK293T cells and subjected to a ufmylation assay. Ufmylation of K171R and K180R was dramatically reduced compared to that of K32R and K48R (Supplementary Fig. S3C), suggesting that both K171 and K180 are the major ufmylation sites. To confirm this, K171R, K180R, and K171R/ K180R (2KR) mutations were generated in full-length ERα. Figure 2B clearly shows that 2KR double mutation completely abrogates ERα ufmylation. Taken together, these results indicate that both K171 and K180 serve as the major UFM1 acceptor sites in ERα.

Estradiol has two different types of receptors: ERα and ERβ (Jensen, 2012; Kuiper et al., 1996). Therefore, we examined whether ERβ could also be ufmylated. Figure 2C shows that ERβ is not a substrate for ufmylation. This finding raised a question of whether ERβ has UFM1 acceptor sites. A sequence comparison revealed that the amino acid residues of ERβ corresponding to the ufmylation sites (K171 and K180) of ERα are replaced by Pro and Arg, respectively (fig. 2D). These results indicate that ERβ lacks UFM1 acceptor sites, unlike ERα.

UFm1-stabilizes ERα by inhibiting ubiquitination
The ubiquitination and proteasome-mediated degradation of ERα is known to participate in the control of the transactivity and stability of ERα (Reid et al., 2003). To determine whether ufmylation influences the stability of ERα, MCF7 cells expressing ERα or the 2KR mutant were incubated with cycloheximide, a protein synthesis inhibitor. In the absence of estradiol, ERα remained stable for at least 12 h regardless of whether

![Fig. 2. Identification of UFM1 acceptor sites in ERα.](image)

(A) Fragments (Δ1-Δ6) of ERα were generated and tagged with HisMax to their N-termini. FL denotes full length; AF-1 denotes activation function-1; DBD denotes DNA binding domain; AF-2 denotes activation function-2; and LBD denotes ligand binding domain. (B) ERα (FL) and its variants carrying K171R, K180R, and K171R/K180R (2KR) mutations were expressed in HEK293T cells with the UFM1-conjugating system. The cell lysates were subjected to pull-down with NTA resins followed by immunoblotting with the anti-Flag antibody. (C) HisMax-tagged ERα and ERβ were expressed in HEK293T cells with the UFM1-conjugating system. The cell lysates were subjected to pull-down with NTA resins followed by immunoblotting with the anti-Flag antibody. (D) The amino acid sequence (161-192) of human ERα was compared to that of human ERβ as well as of ERα from other sources.
it is able to be ufmylated or not (Fig. 3A). Conversely, in the presence of estradiol, ERα was rapidly degraded with a half-life of about 3-6 h (Fig. 3B), as previously reported (Eckert et al., 1984). Interestingly, the 2KR mutation further accelerated the ligand-induced degradation (Fig. 3B), suggesting that ufmylation stabilizes ERα. Moreover, UBA5 knockdown (i.e., the prevention of ufmylation) led to a marked decrease in ERα stability, whereas UFSP2 knockdown (i.e., an increase in ufmylation) resulted in the complete stabilization of ERα (Fig. 3C). Note that both shUBA5 and shUFSP2 effectively silenced the expression of the corresponding proteins (data not shown). These results strongly suggest that ERα ufmylation prevents its proteasome-mediated degradation by inhibiting ubiquitination.

To determine whether the stability of endogenous ERα is indeed influenced by ERα ufmylation, MCF7 cells were incubated with estradiol and MG132 (a proteasome inhibitor) and then subjected to immunoprecipitation analysis. The level of ufmylated ERα dramatically increased at 3 h but then disappeared at 6 h; in contrast, the level of ubiquitinated ERα gradually increased up to 6 h (Fig. 3D, left panels). UBA5 knockdown abrogated ERα ufmylation as expected but led to a marked increase in ERα ubiquitination (Fig. 3D, middle panels). Conversely, UFSP2 knockdown, which caused a dramatic increase in ERα ufmylation, abolished ERα ubiquitination, (Fig. 3D, right panels). These results indicate that ufmylation stabilizes ERα by inhibiting ubiquitination.

**Ufmylation promotes ERα transactivity and cell growth**

We next examined the effect of ERα ufmylation on its transactivity by transfecting the reporter vector ERE-Luc to MCF7 cells. Ectopic expression of ERα in the presence of estradiol led to an increase in ERα transactivity, and this increase was further elevated by the coexpression of the UFM1-conjugating system (Fig. 4A). However, the 2KR mutation abrogated the stimulatory effect of the UFM1-conjugating system, suggesting that ERα ufmylation promotes ERα transactivity. To determine whether ERα ufmylation indeed leads to the promotion of its target gene expression, we measured the transcript levels of pS2, cyclin D1, and c-Myc in the presence and absence of estradiol. Ectopic expression of ERα resulted in a significant increase in the mRNA levels of genes, whereas this increase was markedly reduced by the 2KR mutation (Fig. 4B). These results indicate that ERα ufmylation ameliorates ERα transactivity.

More than 70% of breast cancer is ERα positive (Jozwik and Carroll, 2012). To determine whether a ufmylation-mediated increase in ERα transactivity could lead to the promotion

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**Fig. 3. Ufmylation stabilizes ERα by inhibiting ubiquitination.** (A) MCF7 cells expressing ERα or its variant carrying the 2KR mutation were treated with cycloheximide (200 µg/ml) and incubated for increasing periods. Cell lysates were prepared at each time point and subjected to immunoblotting with the anti-ERα and anti-actin antibodies (left panel). Band intensity was quantified by using the Image J program: the value was set as 1.0 at time 0, and relative values were used at other times (right panel). Data are mean ± SD (n = 3). Ed denotes estradiol. (B) Experiments were performed as in (A), except that the cells were also treated with 10 nM estradiol prior to incubation. (C) Experiments were carried out as in (B), except by using cells transfected with non-specific shRNA (shNS). UBA5-specific shRNA (shUBA5), or UFSP2-specific shRNA (shUFSP2). (D) Cells expressing shNS, shUBA5, or shUFSP2 were treated with 10 µM MG132 and incubated for increasing periods in the presence of estradiol (10 nM). At each time of incubation, cells lysates were prepared and subjected to immunoprecipitation with the anti-ERα antibody followed by immunoblotting with the anti-UFM1 and anti-ubiquitin antibodies.
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of cell growth and, in turn, to breast cancer development, we examined the effect of the overexpression of ERα and 2KR in MCF7 cells on anchorage-independent colony formation. As expected, the depletion of endogenous ERα through the use of ERα-specific shRNA (shERα) prevented colony formation (Fig. 4C). However, in cells where endogenous ERα had been depleted, the overexpression of ERα, but not 2KR, led to a dramatic increase in the number of colonies. These results indicate that ERα ufmylation plays a critical role in the growth of ERα-positive MCF7 cells.

Upregulation of the UFM1-conjugating system in breast cancer cell lines and tissues
To determine whether the ufmylation of ERα is indeed associated with breast cancer development, we compared the expression of the UFM1-conjugating system, including UBA5, in breast cancer cells to that in normal cells. Both the protein and mRNA levels of all components of the UFM1-conjugating system in ERα-positive MCF7 and BT-474 cells were substantially higher than those in normal MCF10A cells and ERα-negative MDA-MB-231 cells (Figs. 5A and 5B). Immunocytochemical analysis also revealed that the expression of the UFM1-conjugating system was upregulated in MCF7 cells as compared to that in MCF10A and MDA-MB-231 cells (Fig. 5C). These findings implicate a crucial role attributed to ufmylation in ERα-positive breast cancer development.

We next examined the expression pattern of the UFM1-conjugating system in human breast cancer tissues. Expression of the UFM1-conjugating system in ERα-positive human breast tumor tissues (Figs. 6A and 6B) was markedly increased, similar to that in the ERα-positive cancer cell lines that were tested (Figs. 5A and 5B). Immunohistochemical analyses also showed that the expression of the UFM1-conjugating system was dramatically elevated in ERα-positive tumors as compared to that in normal tissues (Fig. 6C). Collectively, these results strongly suggest that the upregulation of the UFM1-conjugating system, which increases the stability and transactivity of ERα, plays a crucial role in the development of ERα-positive breast cancer.

DISCUSSION
In the present study, we demonstrated that ERα ufmylation plays a critical role in the positive regulation of ERα stability and transactivity and, in turn, breast cancer development. This conclusion is based on our findings that the elevation

Fig. 4. Ufmylation of ERα promotes its transactivity and cell growth. (A) ERα or its variant carrying the 2KR mutation were expressed with or without the UFM1-conjugating system (marked as UFM1-S) in MCF7 cells. Cells were also transfected with ERE-Luc. After incubation for 24 h with or without 10 nM estradiol (Ed), cell lysates were assayed for luciferase activity. The activity seen in cells transfected with the empty vector was expressed as 1.0 and the other were as its relative values. (B) Cells were prepared as above but without transfection of the reporter vector. After incubation, the cells were subjected to qPCR analysis to determine the mRNA levels of pS2, cyclin D1, and c-Myc. The mRNA level observed in the absence of estrogen was expressed as 1.0 and the other were as its relative values. All data are mean ± SD (n = 3). (C) ERα-specific shRNA (shERα) was transfected to MCF7 cells with a pcDNA vector overexpressing shERα-insensitive ERα (iERα) or 2KR (i2KR). Cells were then grown on soft agar in the presence or absence of 10 nM estradiol. After incubation for 4 weeks, colonies were stained with crystal violet. Scale bar = 500 µm.
of ERα ufmylation ameliorates ERα stability and transactivity, whereas its reduction exhibits opposite effects (Fig. 3). Consistently, ERα ufmylation promoted the expression of its target genes, such as pS2, cyclin D1, and c-Myc, all of which are known to promote the proliferation of mammary cells (Fig. 4). Of particular interest was the finding that the expression...
of all components of the UFm1-conjugating machinery is dramatically increased in ERα-positive breast cancer cell lines and tissues compared to that in normal and ERα-negative cell lines and tissues (Figs. 5 and 6). Taken together, these findings indicate that ERα ufmylation is tightly associated with breast cancer development. In this regard, the components of the UFm1-conjugating system could be excellent therapeutic drug targets as well as diagnostic markers of breast cancer.

UFSP2 is a deufmylating enzyme, and therefore the elevation of its expression in cells would reverse ERα ufmylation. Noteworthy, however, is the observation that the expression of UFSP2 was up-regulated in ERα-positive MCF7 and BT-474 breast cancer cells, although to a lesser extent than the UFm1-conjugating system (Fig. 5A). Since UFSP2 plays an essential role in the generation of matured UFm1 from its precursor in addition to its role in the reversal of the ufmylation process, a moderate increase in UFSP2 levels may be required for efficient ERα ufmylation.

It is also of note that the protein level of UFm1 in ERα-positive breast cancer tissues was similar to that in normal or ERα-negative tissues (Fig. 6A), despite the finding that its mRNA level was dramatically upregulated in ERα-positive tissues (Fig. 6B). Immunohistochemical analysis also showed that the protein level of UFm1 in ERα-positive breast cancer tissues was substantially higher than that in normal tissues (Fig. 6C). Whereas UFm1 as observed in SDS-PAGE gels represents its monomeric form, UFm1 detected by immunohistochemical analysis (as well as by immunocytochemical analysis in Fig. 5C) should represent both the free and conjugated forms. Since the expression of all other components of the UFm1-conjugating system was upregulated in ERα-positive breast cancer tissues, it is likely that a large portion of free UFm1 has been conjugated to cellular proteins in addition to ERα.

It has been shown that proteasome inhibitors prevent estradiol-induced ERα transactivity, and conversely, transcription inhibitors prevent the proteasome-mediated breakdown of ERα (Presler-Mashek et al., 2002; Reid et al., 2003). Therefore, ERα transactivity and proteasome activity are thought to

**Fig. 6. Upregulation of the UFm1-conjugating system in ERα-positive breast tumors.** (A) Lysates of normal (N1-N7), ERα-positive (T1-T7), and ERα-negative (T8-T14) tumor tissues from breast cancer patients were prepared and subjected to immunoblot analysis. (B) Total RNA was also extracted from the tissues and subjected to qPCR. The mRNA levels observed in normal tissues were set as 1.0 (dotted line), and relative values were used for the other cases. Data are mean ± SD (n = 7). (C) Normal and ERα-positive tumor tissues were subjected to immunohistochemical analysis. The large boxes (scale bars = 30 µm) show the magnified view of the small boxes (scale bars = 20 µm).
be intrinsically linked to each other in the repeated termination-and-reinitiation of transcription. The evidence presented in this study indicate that the reversible modification of ERα by UFM1 is an essential process that links the estradiol-induced transactivation of ERα to the subsequent degradation of ERα by proteasome. First, ERα ufmylation increases the stability of ERα by inhibiting ubiquitination, thus maintaining the receptor level. Second, ERα ufmylation occurs prior to the modification of ERα by ubiquitin, providing a time gap for the transactivation of its target genes. Third, ERα is subsequently deufmylated, thus enabling the ubiquitination and proteasomal degradation of the receptor for promoter clearance. Finally, both the ufmylation and ubiquitination of ERα occur only when estradiol is present, indicating that the sequential modification of ERα is signal-specific. Therefore, we conclude that the estradiol-induced transactivation function of ERα is essentially regulated by three sequential reactions: ufmylation-deufmylation-ubiquitination. A critical unanswered question, however, is how the UFSP2-mediated deufmylation of ERα is timely regulated.

Based on our findings as well as of others, we propose a model for the role of the sequential modification of ERα by UFM1 and ubiquitin in the transactivation pathway (Fig. 7). First, the UFM1-conjugating system, including UBAS, generates poly-UFM1 chains on both ASC1 and estradiol-bound ERα dimers, although it is unclear whether ERα binds to ERE before or after modification by UFM1. Poly-UFM1 chains conjugated to ERα protect the receptor from ubiquitination, perhaps by blocking the interaction of ubiquitin E3 ligase(s). Considering the findings that claim poly-UFM1 chains ligated to ASC1 serve as scaffolds to recruit other transcriptional coactivators to ERα (Yoo et al., 2014), it appears possible that poly-UFM1 chains conjugated to ERα may also serve a function in the recruitment of ASC1 and other coactivators to form active transcriptional complexes with RNA polymerase II. Once transcription proceeds for the expression of ERα target genes, both ERα and ASC1 are deufmylated by UFSP2 and subsequently ubiquitinated by ubiquitin E3 ligase(s) for degradation by 26S proteasome. The resulting empty ERE can accommodate new estradiol-bound ERα for the next round of transcription.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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AUTHOR CONTRIBUTIONS
H.M.Y., J.Y.K., and J.H.P. performed experiments. All authors contributed data analysis and interpretation. H.M.Y. and C.H.C. conceived the project, wrote the manuscript, and secured the funding.

CONFLICT OF INTEREST
The authors have no potential conflicts of interest to disclose.
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