

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

Hee Min Yoo^{1,2,3,*}, Jong Ho Park¹, Jae Yeon Kim¹, and Chin Ha Chung^{1,4,*}

¹School of Biological Sciences, Seoul National University, Seoul 08826, Korea, ²Biometrology Group, Korea Research Institute of Standards and Science (KRISS), Daejeon 34113, Korea, ³Department of Precision Measurement, University of Science & Technology (UST), Daejeon 34113, Korea, ⁴The Second Division of Natural Sciences, Korea National Academy of Sciences, Seoul 06579, Korea

*Correspondence: hmy@kriss.re.kr (HMY); chchung@snu.ac.kr (CHC)

<https://doi.org/10.14348/molcells.2022.0029>

www.molcells.org

The post-translational modification (e.g., phosphorylation) of estrogen receptor α (ER α) plays a role in controlling the expression and subcellular localization of ER α as well as its sensitivity to hormone response. Here, we show that ER α is also modified by UFM1 and this modification (ufmylation) plays a crucial role in promoting the stability and transactivity of ER α , which in turn promotes breast cancer development. The elevation of ufmylation via the knockdown of UFSP2 (the UFM1-deconjugating enzyme in humans) dramatically increases ER α stability by inhibiting ubiquitination. In contrast, ER α stability is decreased by the prevention of ufmylation via the silencing of UBA5 (the UFM1-activating E1 enzyme). Lys171 and Lys180 of ER α were identified as the major UFM1 acceptor sites, and the replacement of both Lys residues by Arg (2KR mutation) markedly reduced ER α stability. Moreover, the 2KR mutation abrogated the 17 β -estradiol-induced transactivity of ER α and the expression of its downstream target genes, including pS2, cyclin D1, and c-Myc; this indicates that ER α 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., UBA5, UFC1, UFL1, and UFBP1) were dramatically upregulated in ER α -positive breast cancer cell lines and tissues. Collectively, these findings implicate a critical role attributed to ER α 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 (ER α), a member of the nuclear receptor superfamily, is a ligand-regulated transcription factor (Mangelsdorf et al., 1995). Upon binding with estrogen (e.g., 17 β -estradiol; henceforth referred to as estradiol), ER α 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 ER α 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 ER α degradation but also for ER α transactivity. Thus, the ubiquitination of ligand-bound ER α is thought to be critical for promoter clearance and the reinitiation of transcription (Preisler-Mashek et al., 2002; Reid et al., 2003).

Received 22 February, 2022; revised 14 March, 2022; accepted 14 March, 2022; published online 26 May, 2022

eISSN: 0219-1032

©The Korean Society for Molecular and Cellular Biology.

©This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-sa/3.0/>.

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; UFC1, 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 UFSP1 and UFSP2 (Kang et al., 2007). Unlike in murine systems, however, UFSP1 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 UFSP2 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 (Lemaire et al., 2011; Zhang et al., 2012), DNA damage response (Liu et al., 2020; Qin et al., 2019; Wang et al., 2019), 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 ER α) has been shown to promote breast cancer development (Yoo et al., 2014). Poly-UFM1 chains conjugated to ACS1 serve as molecular scaffolds that recruit other transcription coactivators (e.g., p300 and SRC1) as well as itself to ER α . This leads to the transcriptional activation and expression of ER α 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 ER α , including phosphorylation, acetylation, nitrosylation, palmitoylation, glycosylation, ubiquitination, and sumoylation, have been identified (Le Romancer et al., 2011; Zhou et al., 2014), illustrating ER α as a protein with one of the most diverse sets of post-translation modification. In this regard, we questioned whether ER α could also be modified by UFM1. In the present study, we show that ER α indeed serves as a target for ufmylation. Like ubiquitination, the ufmylation of endogenous ER α occurred only when the cells are treated with estradiol. This ligand-inducible ER α ufmylation promoted the stability of ER α , 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 ER α -positive breast cancer cell lines and tissues. Taken together, our findings implicate a critical role attributed to ER α ufmylation in breast cancer development.

MATERIALS AND METHODS

Materials

ER α cDNA was cloned into pcDNA-HisMax and pCMV2-Flag.

Antibodies against ER α (H-184), c-Myc (9E10), UFM1 (I-16), UFBP1 (G-17), and UFSP2 (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: AACAGAACTTTA-ACACGT for UBA5 and AATAACTGCAGGCTTCAGC for UFSP2 (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 μ g/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 (Biontex 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 \times 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 \times 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 μ l 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- β -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: GGTCG-CCTTGGAGCAGA and GGGCGAAGATCACCTTGTT for pS2; GCTGCTCCTGGTGAACAAGC and AAGTGTTCATGAAATC-GTGCG for cyclin D1; TCCACACATCAGCACAACACTACG and CACTGTCCAACCTGACCCTCTTG 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 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 (LSM700; Carl Zeiss, Germany).

Immunohistochemistry

Tumor tissues were embedded in optimal cutting temperature (OCT) compound. Cryo-sections (10 μ m) 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

ER α is a target substrate for ufmylation

To determine whether ER α 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 ER α , since it is an essential component for the ufmylation of target substrates, such as ASC1 (Yoo et al., 2014). ER α was ectopically expressed with UFL1 or UFBP1 in HEK293T cells. Immunoprecipitation analysis revealed that ER α interacts with UFL1 (Fig. 1A) and UFBP1 (Fig. 1B). Moreover, endogenous ER α was capable of interacting with both UFL1 and UFBP1 (Fig. 1C) in MCF7 cells. To determine whether ER α can indeed be ufmylated, HEK293T cells were transfected with vectors overexpressing ER α , UFM1, UFC1 (E2), UFL1 (E3), and UFBP1. Figure 1D clearly shows that ER α 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 ER α is a new target for ufmylation.

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

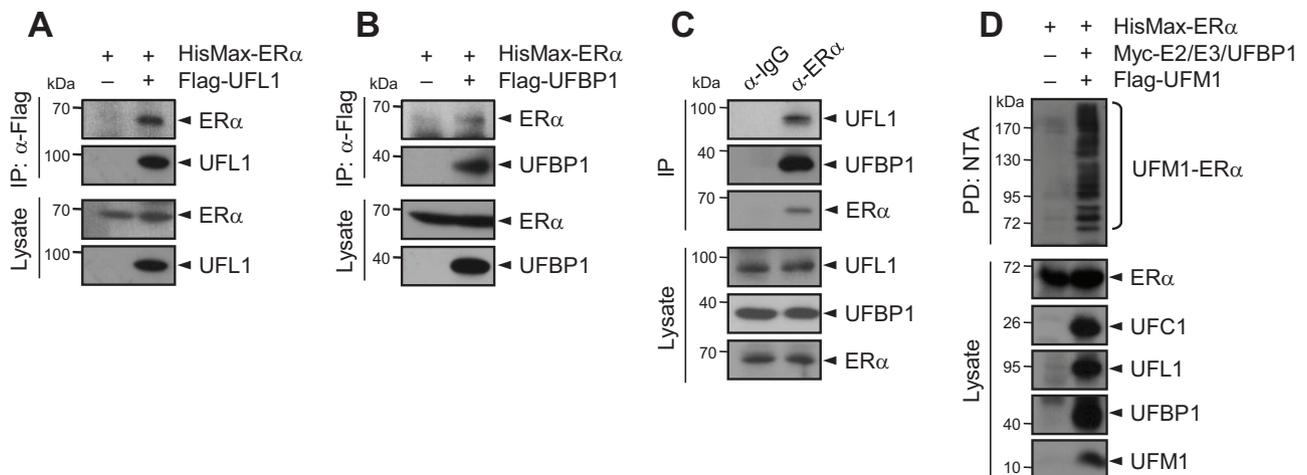


Fig. 1. ER α is a target substrate for ufmylation. (A) HisMax-ER α was expressed in HEK293T cells with or without Flag-UFL1. Cell lysates were subjected to immunoprecipitation with the anti-Flag antibody followed by immunoblotting with the anti-Flag and anti-Xpress antibodies. They were also directly 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-ER α antibodies followed by immunoblotting with the anti-UFL1, anti-UFBP1, and anti-ER α antibodies. (D) HisMax-ER α was expressed in HEK293T cells with or without Myc-UFC1 (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.

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 ER α . **Supplementary Figure S1B** shows that UFSP2, but not CS, was able to completely reverse ER α ufmylation, indicating that ER α is a substrate of UFSP2. Taken together, these results indicate that ER α can be reversibly modified by UFM1.

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). UFM1 has six Lys residues, but only Lys69 has been shown to generate poly-UFM1 chains on ASC1 (Yoo et al., 2014). To determine whether UFM1 also utilizes Lys69 to form poly-UFM1 chains on ER α , we produced various UFM1 variants as follows: Lys69 substituted with Arg (K69R); all Lys residues, except Lys69, substituted with Arg (K69); all six Lys residues substituted with Arg (K0). Through a ufmylation assay with the variants, it was revealed that only K69 could generate poly-UFM1 chains on ER α (**Supplementary Fig. S2**). These results suggest that UFM1 utilizes only Lys69 to generate poly-UFM1 chains on most, if not all, target proteins.

Identification of UFM1 acceptor sites in ER α

To determine UFM1 acceptor sites in ER α , its deletion fragments (termed Δ 1- Δ 6) were generated as in **Fig. 2A** and subjected to a ufmylation assay. Δ 1, Δ 2, and Δ 4 could be ufmylated whereas Δ 3, Δ 5, and Δ 6 could not (**Supplementary Figs. S3A and S3B**); this indicates that ufmylation sites reside in Δ 1 (i.e., amino acids 1-180). Since Δ 1 contains four Lys residues (K32, K48, K171, and K180), each residue

was substituted with Arg. The resulting variants of Δ 1 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 ER α . **Figure 2B** clearly shows that 2KR double mutation completely abrogates ER α ufmylation. Taken together, these results indicate that both K171 and K180 serve as the major UFM1 acceptor sites in ER α .

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

Ufmylation stabilizes ER α by inhibiting ubiquitination

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

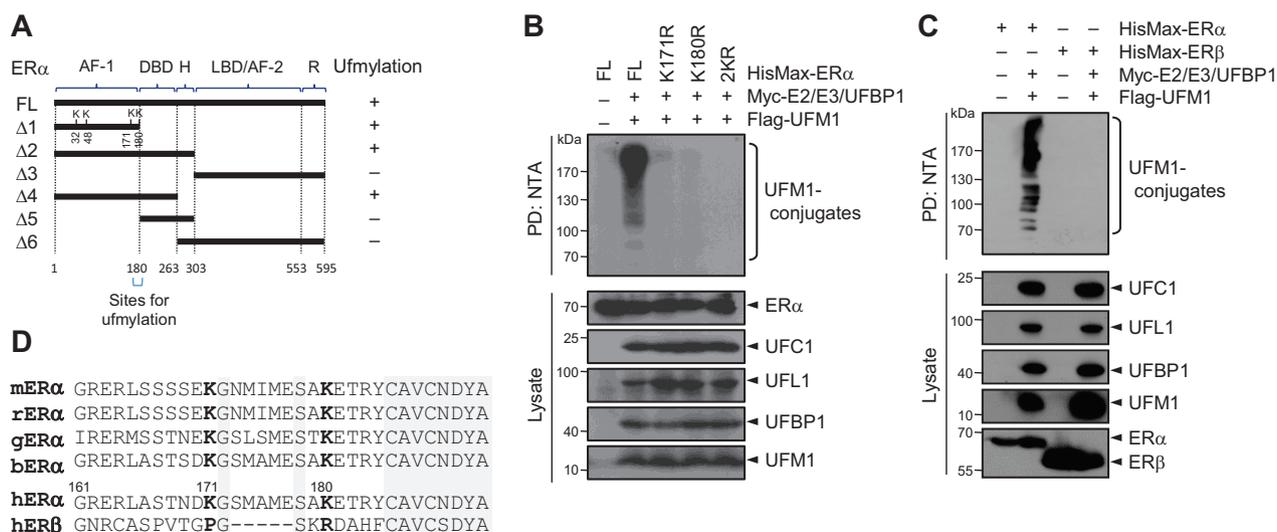


Fig. 2. Identification of UFM1 acceptor sites in ER α . (A) Fragments (Δ 1- Δ 6) of ER α were generated and tagged with HisMax to their N-termini. FL denotes full length; AF-1 denotes activation function-1; DBD denotes DNA binding domain; AF-2 denotes activation function-2; and LBD denotes ligand binding domain. (B) ER α (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 ER α and ER β 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 ER α was compared to that of human ER β as well as of ER α from other sources.

it is able to be ufmylated or not (Fig. 3A). Conversely, in the presence of estradiol, ER α 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 ER α . Moreover, UBA5 knockdown (i.e., the prevention of ufmylation) led to a marked decrease in ER α stability, whereas UFSP2 knockdown (i.e., an increase in ufmylation) resulted in the complete stabilization of ER α (Fig. 3C). Note that both shUBA5 and shUFSP2 effectively silenced the expression of the corresponding proteins (data not shown). These results strongly suggest that ER α ufmylation prevents its proteasome-mediated degradation by inhibiting ubiquitination.

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

increase in ER α ufmylation, abolished ER α ubiquitination, (Fig. 3D, right panels). These results indicate that ufmylation stabilizes ER α by inhibiting ubiquitination.

Ufmylation promotes ER α transactivity and cell growth

We next examined the effect of ER α ufmylation on its transactivity by transfecting the reporter vector *ERE-Luc* to MCF7 cells. Ectopic expression of ER α in the presence of estradiol led to an increase in ER α 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 ER α ufmylation promotes ER α transactivity. To determine whether ER α 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 ER α 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 ER α ufmylation ameliorates ER α transactivity.

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

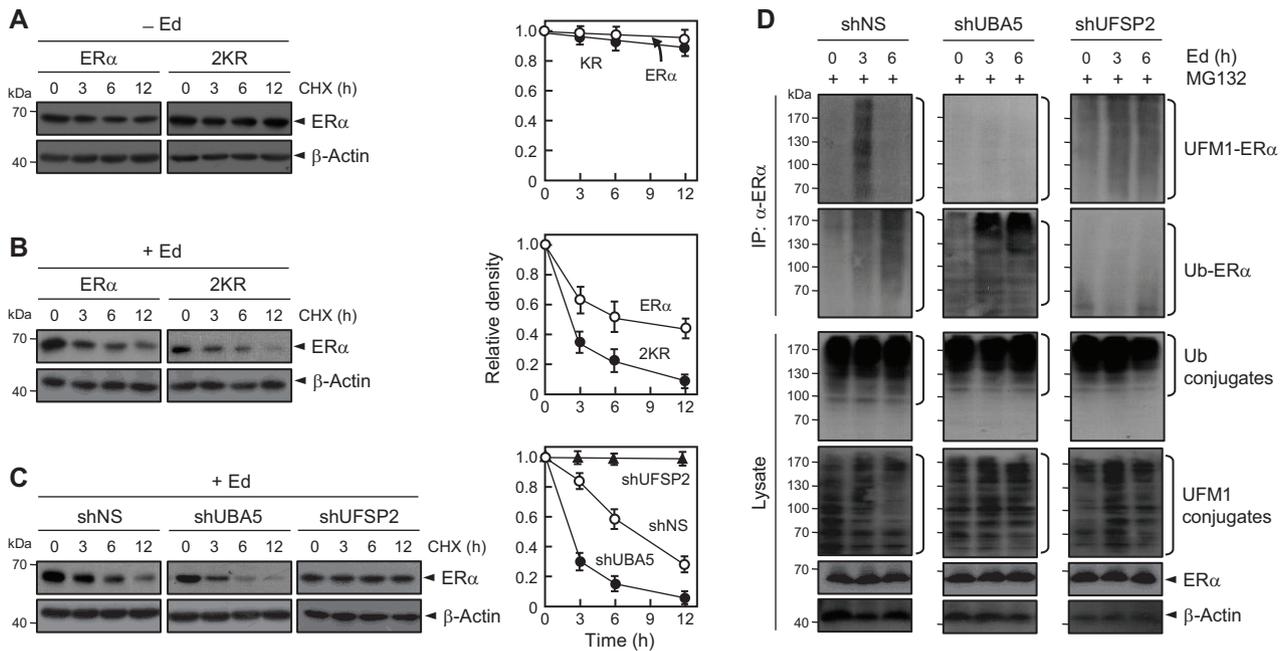


Fig. 3. Ufmylation stabilizes ER α by inhibiting ubiquitination. (A) MCF7 cells expressing ER α or its variant carrying the 2KR mutation were treated with cycloheximide (200 μ g/ml) and incubated for increasing periods. Cell lysates were prepared at each time point and subjected to immunoblotting with the anti-ER α 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 \pm 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 μ M 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-ER α antibody followed by immunoblotting with the anti-UFM1 and anti-ubiquitin antibodies.

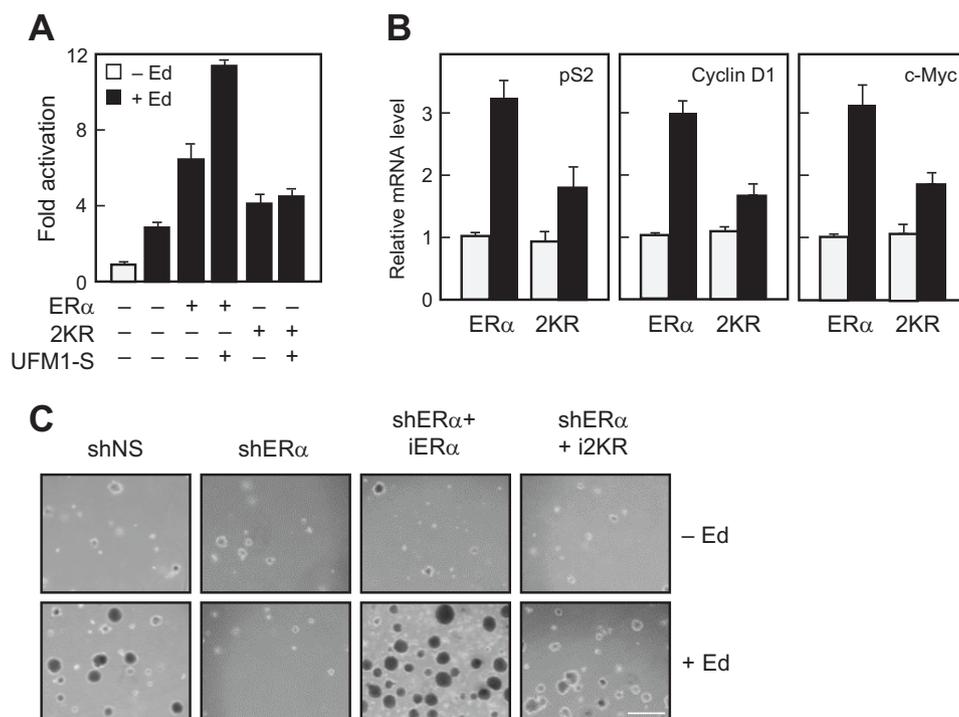


Fig. 4. Ufmylation of ER α promotes its transactivity and cell growth. (A) ER α or its variant carrying the 2KR mutation were expressed with or without the UFM1-conjugating system (marked as UFM1-S) in MCF7 cells. Cells were also transfected with *ERE-Luc*. After incubation for 24 h with or without 10 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 \pm SD (n = 3). (C) ER α -specific shRNA (shER α) was transfected to MCF7 cells with a pcDNA vector overexpressing shER α -insensitive ER α (iER α) 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 μ m.

of cell growth and, in turn, to breast cancer development, we examined the effect of the overexpression of ER α and 2KR in MCF7 cells on anchorage-independent colony formation. As expected, the depletion of endogenous ER α through the use of ER α -specific shRNA (shER α) prevented colony formation (Fig. 4C). However, in cells where endogenous ER α had been depleted, the overexpression of ER α , but not 2KR, led to a dramatic increase in the number of colonies. These results indicate that ER α ufmylation plays a critical role in the growth of ER α -positive MCF7 cells.

Upregulation of the UFM1-conjugating system in breast cancer cell lines and tissues

To determine whether the ufmylation of ER α 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 ER α -positive MCF7 and BT-474 cells were substantially higher than those in normal MCF10A cells and ER α -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 ER α -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 ER α -positive human breast tumor tissues (Figs. 6A and 6B) was markedly increased, similar to that in the ER α -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 ER α -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 ER α , plays a crucial role in the development of ER α -positive breast cancer.

DISCUSSION

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

of ER α ufmylation ameliorates ER α stability and transactivity, whereas its reduction exhibits opposite effects (Fig. 3). Consistently, ER α ufmylation promoted the expression of its tar-

get 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

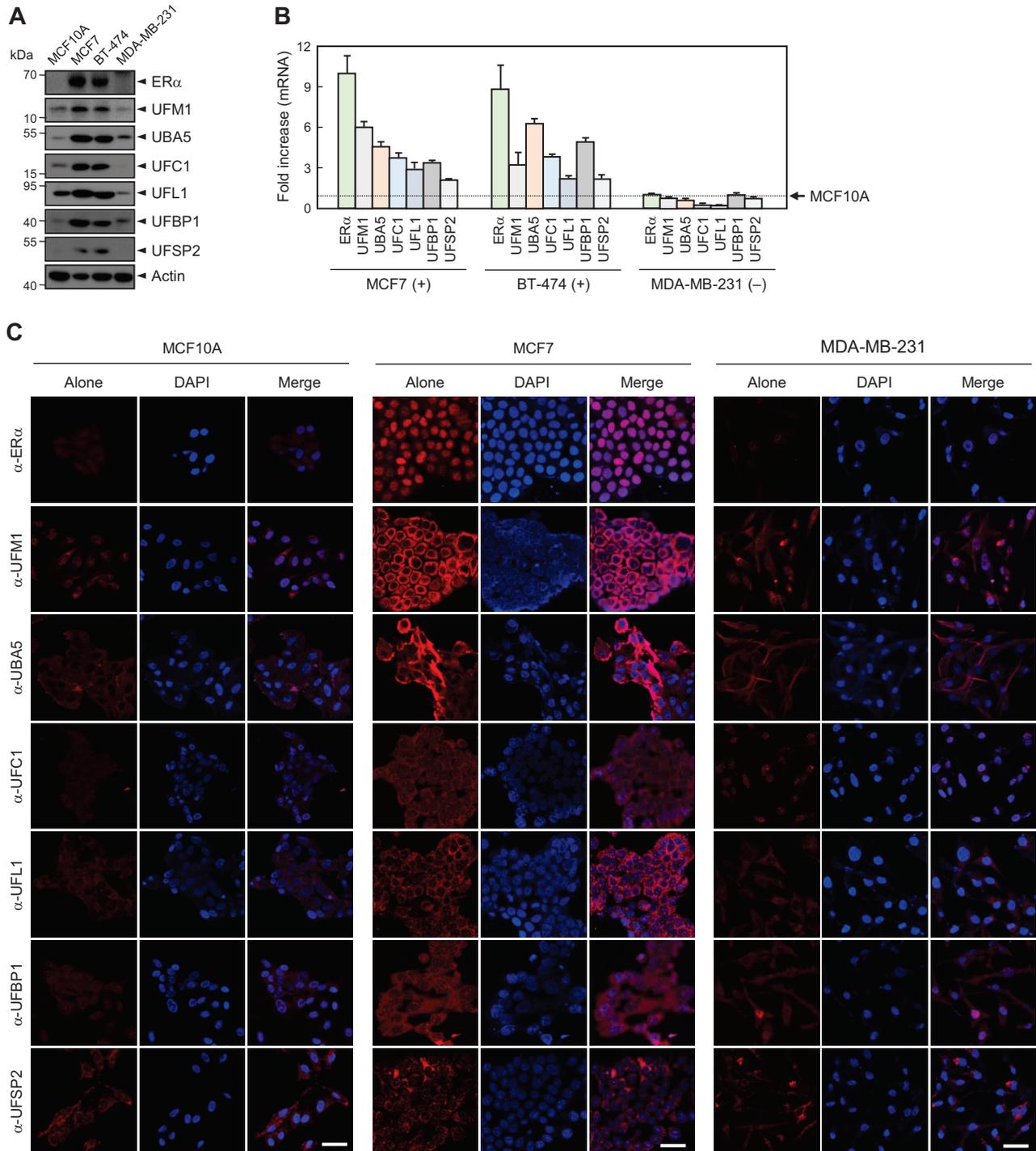


Fig. 5. Upregulation of the UFM1-conjugating system in ER α -positive breast cancer cells. (A) Lysates of normal (MCF10A), ER α -positive (MCF7 and BT-474), and ER α -negative (MDA-MB-231) cells were prepared and subjected to immunoblot analysis. (B) Total RNA was also extracted from the cells and subjected to qPCR. The mRNA levels observed in MCF10A cells were set as 1.0 (dotted line), and relative values were used for the other cases. Data are mean \pm SD (n = 3). (C) Indicated cells were subjected to immunocytochemistry. Scale bars = 50 μ m.

of all components of the UFM1-conjugating machinery is dramatically increased in ER α -positive breast cancer cell lines and tissues compared to that in normal and ER α -negative cell lines and tissues (Figs. 5 and 6). Taken together, these findings indicate that ER α 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 ER α ufmylation. Noteworthy, however, is the observation that the expression of UFSP2 was up-regulated in ER α -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 ER α ufmylation.

It is also of note that the protein level of UFM1 in

ER α -positive breast cancer tissues was similar to that in normal or ER α -negative tissues (Fig. 6A), despite the finding that its mRNA level was dramatically upregulated in ER α -positive tissues (Fig. 6B). Immunohistochemical analysis also showed that the protein level of UFM1 in ER α -positive breast cancer tissues was substantially higher than that in normal tissues (Fig. 6C). Whereas UFM1 as observed in SDS-PAGE gels represent 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 ER α -positive breast cancer tissues, it is likely that a large portion of free UFM1 has been conjugated to cellular proteins in addition to ER α .

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

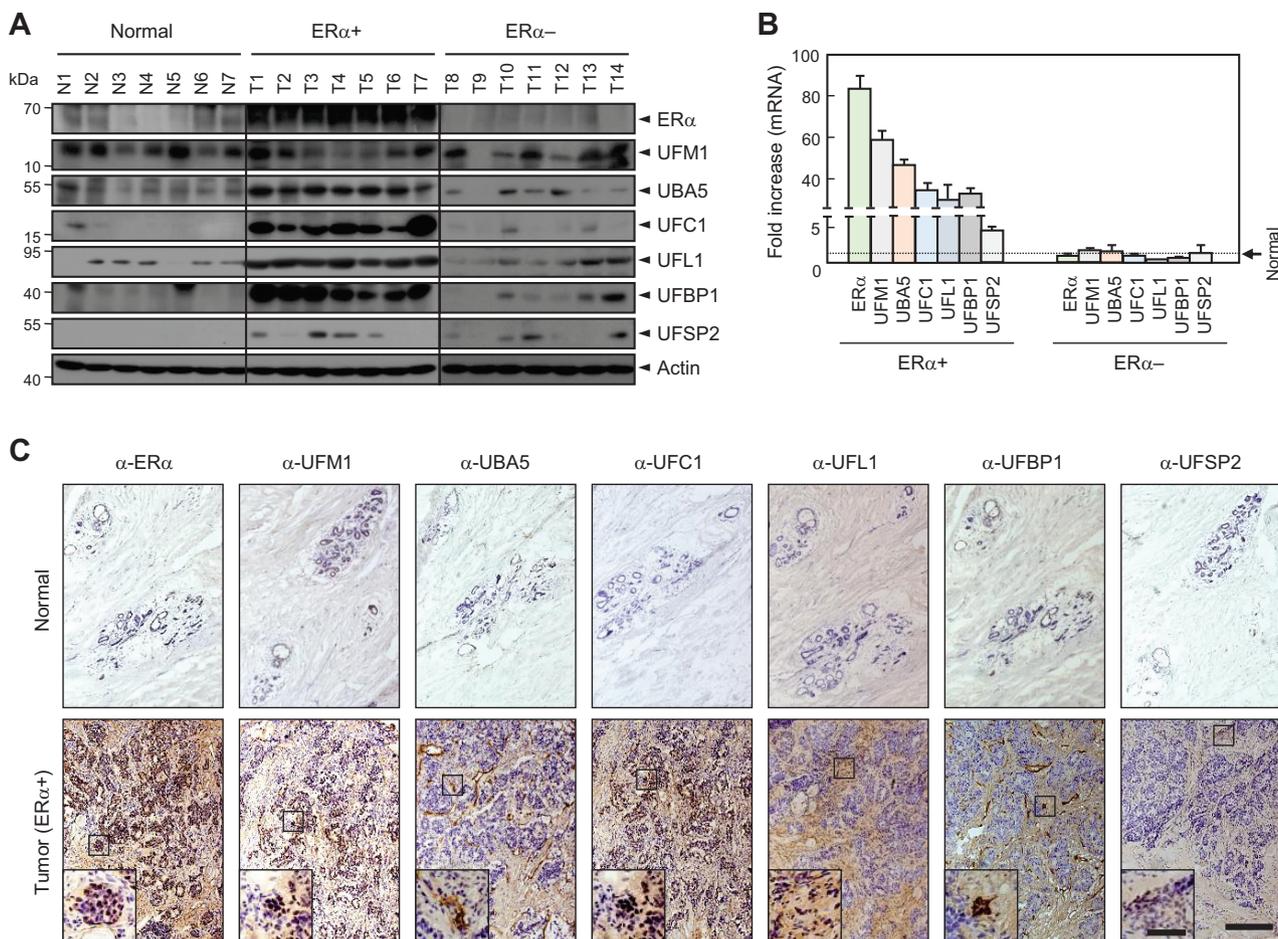


Fig. 6. Upregulation of the UFM1-conjugating system in ER α -positive breast tumors. (A) Lysates of normal (N1-N7), ER α -positive (T1-T7), and ER α -negative (T8-T14) tumor tissues from breast cancer patients were prepared and subjected to immunoblot analysis. (B) Total RNA was also extracted from the 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 \pm SD ($n = 7$). (C) Normal and ER α -positive tumor tissues were subjected to immunohistochemical analysis. The large boxes (scale bars = 30 μ m) show the magnified view of the small boxes (scale bars = 20 μ m).

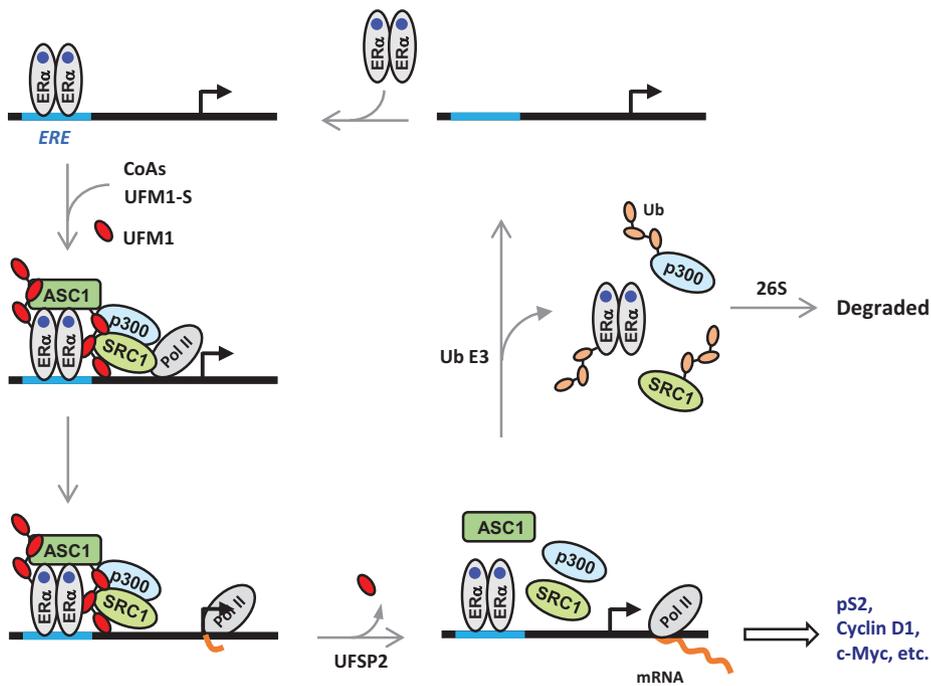


Fig. 7. A model for the role of the sequential modification of ER α by UFM1 and ubiquitin in the transactivation pathway.

The UFM1-conjugating system (UFM1-S) generates poly-UFM1 chains on both ASC1 and ER α . Poly-UFM1 chains ligated to ER α blocks ubiquitination, whereas poly-UFM1 chains on ASC1 recruit other transcriptional coactivators (CoAs) to form transcriptional complexes with RNA polymerase II (Pol II). Once transcription proceeds, both ER α and ASC1 are deufmylated by UFSP2 and then ubiquitinated by ubiquitin E3 ligases for degradation by proteasome (26S). The resulting empty ERE can accommodate new estradiol-bound ER α for the next round of transcription. The blue circles in the ER α dimer indicate estradiol.

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 ER α by UFM1 is an essential process that links the estradiol-induced transactivation of ER α to the subsequent degradation of ER α by proteasome. First, ER α ufmylation increases the stability of ER α by inhibiting ubiquitination, thus maintaining the receptor level. Second, ER α ufmylation occurs prior to the modification of ER α by ubiquitin, providing a time gap for the transactivation of its target genes. Third, ER α is subsequently deufmylated, thus enabling the ubiquitination and proteasomal degradation of the receptor for promoter clearance. Finally, both the ufmylation and ubiquitination of ER α occur only when estradiol is present, indicating that the sequential modification of ER α is signal-specific. Therefore, we conclude that the estradiol-induced transactivation function of ER α is essentially regulated by three sequential reactions: ufmylation-deufmylation-ubiquitination. A critical unanswered question, however, is how the UFSP2-mediated deufmylation of ER α is timely regulated.

Based on our findings as well as of others, we propose a model for the role of the sequential modification of ER α by UFM1 and ubiquitin in the cyclic transactivation pathway (Fig. 7). First, the UFM1-conjugating system, including UBA5, generates poly-UFM1 chains on both ASC1 and estradiol-bound ER α dimers, although it is unclear whether ER α binds to ERE before or after modification by UFM1. Poly-UFM1 chains conjugated to ER α 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 ER α (Yoo et al., 2014), it appears possible that poly-UFM1 chains conjugated to ER α 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 ER α target genes, both ER α 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 ER α to initiate the next round of transcription.

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

ACKNOWLEDGMENTS

We thank Dong-Young Noh for providing human breast cancer tissues. This work was supported by National Academy of Sciences, ROK and National Research Foundation of Korea (NRF-2005-084-C00025) to C.H.C. and by Korea Research Institute of Standards and Science (“Establishment of measurement standards for Chemistry and Radiation,” KRIS-2022-GP2022-0003), the Ministry of Science and ICT (MSIT), National Research Foundation of Korea (NRF-2016M3A7B6908929, NRF-2019M3A9F3065868, and NRF-2021M3C1C3097638) to H.M.Y. This study was approved by the Seoul National University Hospital (SNUH) Institutional Review Board (IRB) (approval No. 1207-119-420).

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.

ORCID

Hee Min Yoo <https://orcid.org/0000-0002-5951-2137>
Jong Ho Park <https://orcid.org/0000-0002-0488-7791>
Jae Yeon Kim <https://orcid.org/0000-0002-0542-9071>
Chin Ha Chung <https://orcid.org/0000-0003-0788-9343>

REFERENCES

- Colin, E., Daniel, J., Ziegler, A., Wakim, J., Scrivo, A., Haak, T.B., Khiati, S., Denomme, A., Amati-Bonneau, P., Charif, M., et al. (2016). Biallelic variants in UBA5 reveal that disruption of the UFM1 cascade can result in early-onset encephalopathy. *Am. J. Hum. Genet.* *99*, 695-703.
- Daniel, J. and Liebau, E. (2014). The ufm1 cascade. *Cells* *3*, 627-638.
- Eckert, R.L., Mullick, A., Rorke, E.A., and Katzenellenbogen, B.S. (1984). Estrogen receptor synthesis and turnover in MCF-7 breast cancer cells measured by a density shift technique. *Endocrinology* *114*, 629-637.
- Gannavaram, S., Connelly, P.S., Daniels, M.P., Duncan, R., Salotra, P., and Nakhasi, H.L. (2012). Deletion of mitochondrial associated ubiquitin fold modifier protein Ufm1 in *Leishmania donovani* results in loss of beta-oxidation of fatty acid and blocks cell division in the amastigote stage. *Mol. Microbiol.* *86*, 187-198.
- Hamilton, E.M.C., Bertini, E., Kalaydjieva, L., Morar, B., Dojckakova, D., Liu, J., Vandeever, A., Curiel, J., Persoon, C.M., Diadato, D., et al. (2017). UFM1 founder mutation in the Roma population causes recessive variant of H-ABC. *Neurology* *89*, 1821-1828.
- Heride, C., Urbé, S., and Clague, M.J. (2014). Ubiquitin code assembly and disassembly. *Curr. Biol.* *24*, R215-R220.
- Hu, Z., Wang, X., Li, D., Cao, L., Cui, H., and Xu, G. (2021). UFBP1, a key component in ufmylation, enhances drug sensitivity by promoting proteasomal degradation of oxidative stress-response transcription factor Nrf2. *Oncogene* *40*, 647-662.
- Jensen, E. (2012). A conversation with Elwood Jensen. Interview by David D. Moore. *Annu. Rev. Physiol.* *74*, 1-11.
- Jozwik, K.M. and Carroll, J.S. (2012). Pioneer factors in hormone-dependent cancers. *Nat. Rev. Cancer* *12*, 381-385.
- Kang, S.H., Kim, G.R., Seong, M., Baek, S.H., Seol, J.H., Bang, O.S., Ovaa, H., Tatsumi, K., Komatsu, M., Tanaka, K., et al. (2007). Two novel ubiquitin-fold modifier 1 (Ufm1)-specific proteases, UfSP1 and UfSP2. *J. Biol. Chem.* *282*, 5256-5262.
- Komatsu, M., Chiba, T., Tatsumi, K., Iemura, S., Tanida, I., Okazaki, N., Ueno, T., Kominami, E., Natsume, T., and Tanaka, K. (2004). A novel protein-conjugating system for Ufm1, a ubiquitin-fold modifier. *EMBO J.* *23*, 1977-1986.
- Kuiper, G.G., Enmark, E., Peltö-Huikko, M., Nilsson, S., and Gustafsson, J.A. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. *Proc. Natl. Acad. Sci. U. S. A.* *93*, 5925-5930.
- Le Romancer, M., Poulard, C., Cohen, P., Sentis, S., Renoir, J., and Corbo, L. (2011). Cracking the estrogen receptor's posttranslational code in breast tumors. *Endocr. Rev.* *32*, 597-622.
- Lemaire, K., Moura, R.F., Granvik, M., Igoillo-Estève, M., Hohmeier, H.E., Hendrickx, N., Newgard, C.B., Waelkens, E., Cnop, M., and Schuit, F. (2011). Ubiquitin fold modifier 1 (UFM1) and its target UFBP1 protect pancreatic beta cells from ER stress-induced apoptosis. *PLoS One* *6*, e18517.
- Lin, J., Xie, X., Weng, X., Qiu, S., Yoon, C., Lian, N., Xie, J., Wang, J., Lu, J., Chen, Q., et al. (2019). UFM1 suppressed invasive activities of gastric cancer cells by attenuating the expression of PDK1 through PI3K/AKT signaling. *J. Exp. Clin. Cancer Res.* *38*, 410.
- Liu, J., Guan, D., Dong, M., Yang, J., Wei, H., Liang, Q., Song, J., Xu, L., Bai, J., Liu, C., et al. (2020). UFMylation maintains tumor suppressor p53 stability by antagonizing its ubiquitination. *Nat. Cell Biol.* *22*, 1056-1063.
- Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., et al. (1995). The nuclear receptor superfamily: the second decade. *Cell* *83*, 835-839.
- Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., Enmark, E., Pettersson, K., Warner, M., and Gustafsson, J.A. (2001). Mechanisms of estrogen action. *Physiol. Rev.* *81*, 1535-1565.
- Preisler-Mashek, M.T., Solodin, N., Stark, B.L., Tyrivier, M.K., and Alarid, E.T. (2002). Ligand-specific regulation of proteasome-mediated proteolysis of estrogen receptor- α . *Am. J. Physiol. Endocrinol. Metab.* *282*, E891-E898.
- Qin, B., Yu, J., Nowsheen, S., Wang, M., Tu, X., Liu, T., Li, H., Wang, L., and Lou, Z. (2019). UFL1 promotes histone H4 ufmylation and ATM activation. *Nat. Commun.* *10*, 1242.
- Reid, G., Hubner, M.R., Metivier, R., Brand, H., Denger, S., Manu, D., Beaudouin, J., Ellenberg, J., and Gannon, F. (2003). Cyclic, proteasome-mediated turnover of unliganded and liganded ER α on responsive promoters is an integral feature of estrogen signaling. *Mol. Cell* *11*, 695-707.
- Song, A., Wang, Y., Jiang, F., Yan, E., Zhou, J., Ye, J., Zhang, H., Ding, X., Li, G., Wu, Y., et al. (2021). Ubiquitin D promotes progression of oral squamous cell carcinoma via NF- κ B signaling. *Mol. Cells* *44*, 468-480.
- Tatsumi, K., Yamamoto-Mukai, H., Shimizu, R., Waguri, S., Sou, Y.S., Sakamoto, A., Taya, C., Shitara, H., Hara, T., Chung, C.H., et al. (2011). The Ufm1-activating enzyme Uba5 is indispensable for erythroid differentiation in mice. *Nat. Commun.* *2*, 181.
- Wang, Z., Gong, Y., Peng, B., Shi, R., Fan, D., Zhao, H., Zhu, M., Zhang, H., Lou, Z., Zhou, J., et al. (2019). MRE11 ufmylation promotes ATP activation. *Nucleic Acids Res.* *47*, 4124-4135.
- Yoo, H.M., Kang, S.H., Kim, J.Y., Lee, J.E., Seong, M.W., Lee, S.W., Ka, S.H., Sou, Y., Komatsu, M., Tanaka, K., et al. (2014). Modification of ASC1 by UFM1 is crucial for ER α transactivation and breast cancer development. *Mol. Cell* *56*, 261-274.
- Yoo, H.M., Park, J.H., Jeon, Y.J., and Chung, C.H. (2015). Ubiquitin-fold modifier 1 acts as a positive regulator of breast cancer. *Front. Endocrinol. (Lausanne)* *6*, 36.
- Zhang, Y., Zhang, M., Wu, J., Lei, G., and Li, H. (2012). Transcriptional regulation of the Ufm1 conjugation system in response to disturbance of the endoplasmic reticulum homeostasis and inhibition of vesicle trafficking. *PLoS One* *7*, e48587.
- Zhou, W. and Slingerland, J.M. (2014). Links between oestrogen receptor activation and proteolysis: relevance to hormone-regulated cancer therapy. *Nat. Rev. Cancer* *14*, 26-38.