

Intrinsic ubiquitin E3 ligase activity of histone acetyltransferase Hbo1 for estrogen receptor α

By Masayoshi IIZUKA,^{*1,†} Takao SUSA,^{*1} Mimi TAMAMORI-ADACHI,^{*1}
Hiroko OKINAGA^{*2} and Tomoki OKAZAKI^{*1}

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Abstract: Estrogen receptors (ER) are important transcription factors to relay signals from estrogen and to regulate proliferation of some of breast cancers. The cycling of estrogen-induced DNA binding and ubiquitin-linked proteolysis of ER potentiates ER-mediated transcription. Indeed, several transcriptional coactivators for ER-dependent transcription ubiquitinate ER. Histone acetyltransferase (HAT) Hbo1/KAT7/MYST2, involved in global histone acetylation, DNA replication, transcription, and cellular proliferation, promotes proteasome-dependent degradation of ER α through ubiquitination. However, molecular mechanism for ubiquitination of ER α by Hbo1 is unknown. Here we report the intrinsic ubiquitin E3 ligase activity of Hbo1 toward the ER α . The ligand, estradiol-17 β , inhibited E3 ligase activity of Hbo1 for ER α *in vitro*, whereas hyperactive ER α mutants from metastatic breast cancers resistant to hormonal therapy, were better substrates for ER α ubiquitination by Hbo1. Hbo1 knock-down caused increase in ER α expression. Hbo1 is another ER α coactivator that ubiquitinates ER α .

Keywords: estrogen receptor, ubiquitination, histone acetyltransferase, Hbo1

Introduction

Steroid hormone estrogen has numerous physiological functions, including reproduction, bone homeostasis, cardiovascular health, behavior, cognition, and cellular proliferation. Estrogen transmits its signal into the cell through association with estrogen receptors (ER α and ER β). The ligand-ER α complex directly or indirectly binds to DNA, recruits co-regulatory protein complexes, and activates or inactivates transcription.^{1)–4)} In addition to genomic action, estrogen exerts its function through binding to DNA-binding transcription factors or through

membrane-bound ER α . Moreover, ER α action is modulated by co-regulators,^{5),6)} splicing,⁷⁾ miRNAs,⁸⁾ long non-coding RNAs,⁹⁾ and posttranslational modifications.¹⁰⁾ Clinically important, two-thirds of breast cancers are positive for ER α and respond to hormonal therapy.¹¹⁾

The ubiquitin-proteasome pathway¹²⁾ is responsible for basal and ligand-induced ER α degradation.^{13)–15)} Counterintuitively, this ligand-induced ER α degradation, potentiates transcriptional activation by ER α through cycling of DNA binding and ubiquitin-dependent proteolysis of ER α . ER α co-activators that ubiquitinate E3 include E6-AP, Mouse double minute 2 homologue (Mdm2), the breast cancer susceptibility gene 1/Brcal-associated RING domain protein (BRCA1/BARD1) estrogen-responsive finger protein (EFP), and SKP1–CUL1–F-box protein complex (SCF). In addition, growth-related signal pathways, such as mitogen-activated kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways, stimulate ubiquitination of ER α through ER α phosphorylation.¹⁶⁾

Hbo1, a histone acetyltransferase binding to origin recognition complex (ORC1),¹⁷⁾ regulates histone acetylation,^{18)–20)} replicational licensing,^{21),22)}

^{*1} Department of Biochemistry, Teikyo University School of Medicine, Tokyo, Japan.

^{*2} Department of Internal Medicine, Teikyo University School of Medicine, Tokyo, Japan.

[†] Correspondence should be addressed: M. Iizuka, Department of Biochemistry, Teikyo University School of Medicine, 2-11-1, Kaga, Itabashi-ku, Tokyo 173-8605, Japan (e-mail: miizuka@med.teikyo-u.ac.jp).

Abbreviations: ER: estrogen receptor; HAT: histone acetyltransferase; LBD: ligand-binding domain; MYST: MOZ, Ybf1/Sas3, Sas2 and Tip60; Hbo1: a histone acetyltransferase binding to origin recognition complex 1 (ORC1); KAT7: lysine acetyltransferase 7.

origin activation,²³⁾ adipogenesis,²⁴⁾ pluripotency and self-renewal of embryonic stem cells,²⁵⁾ and chromatin assembly of centromeres.²⁶⁾ Hbo1 is linked to cellular proliferation^{18),27)} and subject to functional modulation upon DNA damage.^{28),29)} Hbo1 belongs to MYST family of histone acetyltransferases.³⁰⁾ We have found that Hbo1 promotes ubiquitin-mediated degradation of ER α and modulates ER α -mediated transcription and proliferation.³¹⁾ Hbo1 appears to be a coactivator that ubiquitinates ER α . However, it is unclear whether acetylation of ER α by Hbo1 facilitates ER α ubiquitination or Hbo1 directly ubiquitinates ER α .

Here we investigated molecular mechanism of the promoted ubiquitination of ER α by Hbo1. The ligand-binding domain (LBD) of ER α was ubiquitinated by Hbo1. Hbo1 is an atypical ubiquitin E3 ligase for ER α . The self-ubiquitination activity of Hbo1 was mapped to the MYST domain. The ligand, estradiol-17 β , inhibited E3 ligase activity of Hbo1 toward ER α . In contrast, hyperactive ER α mutants found in metastatic breast cancers resistant to hormonal therapy, were better substrates for ubiquitination by Hbo1. Irrespective of the presence and absence of the ligand, knocking down Hbo1 expression caused increase in ER α expression. These results suggest that Hbo1 is a novel E3 ubiquitin ligase that activates ER α -dependent transcription.

Materials and methods

Chemicals and antibodies. 17 β -estradiol was purchased from Sigma-Aldrich (St. Louis, MO, USA), MG-132 from Enzo Life Sciences (Farmingdale, NY, USA), Cy5-conjugated Streptavidin from Jackson ImmunoResearch Laboratories (West Grove, PA, USA), and Coomassie Stain Solution from Bio-Rad (Hercules, CA, USA). The recombinant KAT7 (Hbo1 purified from Sf9 cells), MurF1, GST-tagged p53, and ubiquitin proteins were purchased from Active Motif (Carlsbad, CA, USA), R&D systems (Minneapolis, MN, USA), EMD Millipore (Temecula, CA, USA) and UBPBio (Aurora, CO, USA), respectively. Anti-Hbo1 antibody was described.¹⁷⁾ The following commercial antibodies were purchased: anti-FLAG monoclonal and anti- α -tubulin monoclonal from Sigma-Aldrich (St. Louis, MO, USA), anti-HA monoclonal from Roche (Basel, Switzerland), anti-ER α polyclonal from Santa Cruz (Dallas, TX, USA), anti-ubiquitin polyclonal from Cell Signaling Technology (Danvers, MA, USA), and anti-GST polyclonal and anti-His-tag monoclonal from Medical and Biological Laboratories (Nagoya, Japan).

Mutations in the ER α gene and purification of recombinant proteins. Deletion mutants of the human ER α gene³¹⁾ were made by amplifying corresponding DNA fragments using PCR. Base substitutions were made using PrimeStar Mutagenesis Basal kit (TaKaRa Biotechnology, Shiga, Japan) and the mutations were verified by sequencing. Recombinant proteins were expressed in bacteria BL21(DE3)-pLysS. GST-tagged ER α LBD (amino acids: 304–554) and GST-tagged human Mdm2 (a gift from Dr. Koji Okamoto, National Cancer Center Research Institute, Tokyo, Japan) were purified with Glutathione Sepharose.³²⁾ Throughout the GST-ER α LBD purification, estradiol-17 β was not included in the growth media or buffers for stabilization of ER α LBD,³³⁾ to assay the effect of ligand during the ubiquitination reaction. The His-tagged full-length human Hbo1 (amino acids: 1–611) protein was purified on Heparin Sepharose (GE Healthcare, Little Chalfont, UK), whereas His-Hbo1 (amino acids: 225–611) and His-Hbo1 (amino acids: 311–611) proteins²⁸⁾ were purified on Ni-NTA Agarose (Qiagen, Hilden, Germany).

Ubiquitination assay. *In vivo* ubiquitination assay was performed as previously.³¹⁾ For *in vitro* ubiquitination assay, reactions were carried out using a ubiquitinylation kit purchased from Enzo Life Sciences (Farmingdale, NY, USA) containing 2.5 μ M biotinylated or non-tagged ubiquitin, 100 nM His-tagged E1, approximately 1–2.5 μ M His-tagged E2, and 5 mM Mg-ATP, and 20 U/mL yeast inorganic pyrophosphatase (New England Biolabs, Ipswich, Massachusetts, USA). Detection was made by immunoblotting with Cy5-conjugated streptavidin (Cell Signaling Technology) or anti-ubiquitin antibody. His-tagged UbCH5c was used for Figs. 2A and 2E, whereas non-tagged UbCH5c (R&D Systems, Minneapolis, MN, USA) was used for all the other ubiquitination reactions. To affinity-purify His-tagged and GST-tagged proteins, stringent washing conditions were completed using 0.3 M NaCl and 0.1% Triton-X for Figs. 3D and 3E, and 0.5 M NaCl plus 0.1% Triton-X for Figs. 4, 5A, and 5D, respectively.

siRNA knock-down and quantitative reverse transcription (qRT)-PCR. siRNA and qRT-PCR experiments were as described previously.³⁴⁾ siRNAs for Hbo1 targeted nucleotide 92 of the coding sequences of Hbo1.³¹⁾ For qRT-PCR analysis, data was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Values were expressed as mean \pm SD ($n = 4$, unless otherwise

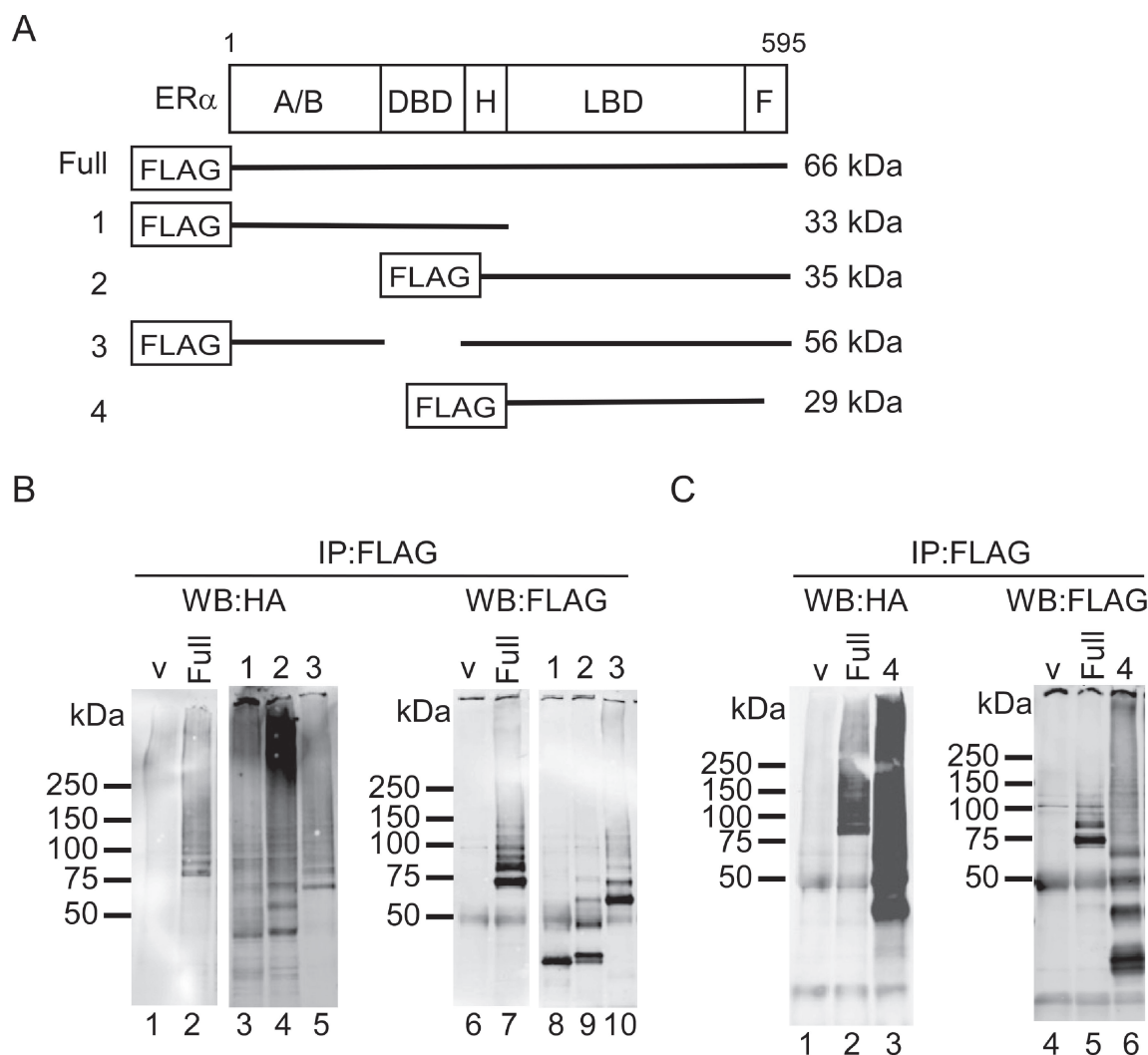


Fig. 1. Ubiquitination of ER α LBD stimulated by Hbo1. (A) Domain structure of the ER α protein. DBD: DNA-binding domain; LBD: ligand-binding domain; H: hinge region. Full and mutants 1 through 4, cover amino acids 1–595, 1–298, 282–595, 1–179 plus 269–595, and 304–554 of the ER α protein, respectively. Expected molecular weights are shown. (B–C) FLAG-tagged ER α mutants, Hbo1, and HA-ubiquitin were transfected into 293T cells. The cells were treated with MG-132 for 6 hours, lysed under denaturing conditions, and immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were blotted with anti-HA or anti-FLAG antibodies. v: empty FLAG vector; Full: FLAG-tagged full-length ER α .

stated). The PCR primer sets for Hbo1 and GAPDH were described.³¹⁾

Results

ER α LBD is preferentially ubiquitinated by Hbo1. To map sites of ER α ubiquitination promoted by Hbo1, deletion mutants of ER α were constructed (Fig. 1). FLAG-tagged ER α mutants, Hbo1, and HA-ubiquitin were transfected into 293T cells, followed by immunoprecipitation with anti-FLAG antibody under denaturing conditions, and immunoblotted with anti-HA and anti-FLAG anti-

bodies (Fig. 1B). High-molecular-weight ubiquitinated proteins were detected in mutants 1, 2, and 3 (lanes 3–5 and 8–10), comparable to the full-length ER α (lanes 2 and 7), suggestive of extensive ubiquitination in ER α . The ubiquitinated signals of mutants 2 and 3 were stronger than that of mutant 1 (lanes 4 and 5 compared to lane 3; lanes 9 and 10 compared to lane 8). This result indicated that the hinge region (H) and the ligand-binding domain (LBD), shared by mutants 2 and 3 of ER α , were preferentially ubiquitinated by Hbo1. As lysines 302 and 303 in the hinge region protect unliganded ER α

turnover,³⁵⁾ we predicted that the removal of the hinge region would boost ubiquitination of ER α . Deletion mutant 4 (amino acids: 304–554) was constructed, mostly composed of LBD, and assayed for ubiquitination by Hbo1 (Fig. 1C). Mutant 4 gave a stronger poly-ubiquitination signal than the full-length ER α (lanes 3 and 6). These results suggested that Hbo1 promotes a robust ubiquitination of the ER α LBD. From this point forward, we focused on the analysis of ER α LBD.

Self-ubiquitination of Hbo1 *in vitro*. To test whether Hbo1 alone has ubiquitin E3 ligase activity, an array of E2 ubiquitin conjugating enzymes for self-ubiquitination of commercial Hbo1 protein were screened by mixing commercial recombinant Hbo1 protein with E1, E2, ATP, and biotinylated ubiquitin and probing with Cy5-conjugated streptavidin (Fig. 2A). High-molecular-weight ubiquitination bands greater than 250 kDa (Ub_n-E3) were detected by using UbcH5b and UbcH5c as an E2 (lanes 12 and lane 14), similar to the control MurF1, a known E3 ligase³⁶⁾ (lane 2), suggesting self-ubiquitination of Hbo1. The lack of signal by immunoblotting of Hbo1 protein with anti-ubiquitin antibody (Fig. 2B, lanes 4 and 5) ruled out a possibility that the original preparation of Hbo1 protein was ubiquitinated. To confirm the E2 screen result by a different assay, we incubated the recombinant Hbo1 protein with E1, E2 (UbcH5c), ATP, and non-tagged ubiquitin and immunoblotted the reaction mixture with anti-ubiquitin antibody (Fig. 2C). High molecular-weight poly-ubiquitination signals were detected when Hbo1 was used as an E3 enzyme (lane 3), comparable to the positive control, MurF1 (lane 2), indicating a self-ubiquitination activity of Hbo1. Equal loading of E1 was confirmed by blotting with anti-His antibody (bottom panel, Fig. 2C). When any one component of the ubiquitin reaction was omitted (Fig. 2D), high molecular weight poly-ubiquitination was abolished. Hbo1 signal was diminished only when all the necessary components were included (bottom panel, lane 5). These results demonstrated dependency of the reaction on E1, E2, and ubiquitin. Similar experiment was done by incubation of biotinylated ubiquitin, His-tagged E1, His-tagged E2 (UbcH5c), and commercial Hbo1, followed by removal of E1 and E2 by mixing with Ni-NTA Agarose. The supernatant was probed for ubiquitin by immunoblotting with Cy5-streptavidin (Fig. 2E). High-molecular-weight ubiquitinated signals were detected only when all four components were included (lane 2). All these results suggested E3 ligase activity of Hbo1.

E3 ligase activity of Hbo1 mapped to the MYST domain. Although Hbo1 lacks RING domains, HECT domains, and RBR domains, which are characteristics of E3 ligases,³⁷⁾ it possesses two zinc-finger like motifs: one within the MYST domain and the other outside the MYST domain (Fig. 3A) like other atypical ubiquitin E3 ligases.^{38)–40)} To map the E3 ligase activity of Hbo1, deletion mutants of Hbo1 were bacterially purified (Fig. 3B). No signal by immunoblotting with anti-ubiquitin antibody against the purified Hbo1 deletion mutants (lanes 2 and 3, Fig. 3C) demonstrated lack of ubiquitin modification on those Hbo1 deletion mutants. We incubated His-tagged Hbo1 mutants with His-tagged E1, non-tagged E2, biotin-ubiquitin, and ATP, followed by affinity purification of His-tagged Hbo1 and E1 proteins, with Ni-NTA Agarose under stringent condition. The purified proteins, were probed with Cy5-streptavidin and anti-His antibody (Figs. 3D and 3E). Immunoblotting with anti-His antibody detected E1 in all lanes except for lane 2, where no E1 was included (bottom panels, Figs. 3D and 3E). On the other hand, Cy5-streptavidin blot revealed that the ubiquitinated E1 (approximately 120 kDa band) was detected in lanes 1, 3, and 5 (Ub-E1), where E1 and ubiquitin were included (top panels, Figs. 3D and 3E). As expected, the ubiquitination of E1 was dependent on ubiquitin and E1. In Fig. 3D, in Cy5-strepavidin blotting, bands were detected for His-Hbo1 (225–611), predominantly around 80 kDa in size, migrating slower than the non-modified His-tagged Hbo1 (225–611) solely in lane 5 where all the necessary components were included (bracketed). Those bands reacted with anti-His antibody (lane 10, arrowhead), suggesting that Hbo1 (225–611) has E3 ligase activity. In contrast, Hbo1 (311–611) produced an approximately 40 kDa band when all reagents were included (lanes 5 and 10, Fig. 3E), detected by both ubiquitin and His blots (arrowheads, Fig. 3E), suggestive of mono-ubiquitination. These results suggest that the MYST domain of Hbo1 has E3 ligase activity. The ubiquitin E3 ligase activity detected in bacterially purified Hbo1 protein eliminated a possibility that contaminants in commercial Hbo1 from Sf9 cells had ubiquitin E3 activity.

E3 ligase activity of Hbo1 for ER α . Prior to examining whether Hbo1 ubiquitinates ER α LBD, to confirm that our assay system for ubiquitination is appropriate, we checked whether Mdm2 ubiquitinates p53 *in vitro*⁴¹⁾ by our assay. We incubated His-E1, E2 (UbcH5c), GST-Mdm2 protein, biotinylated

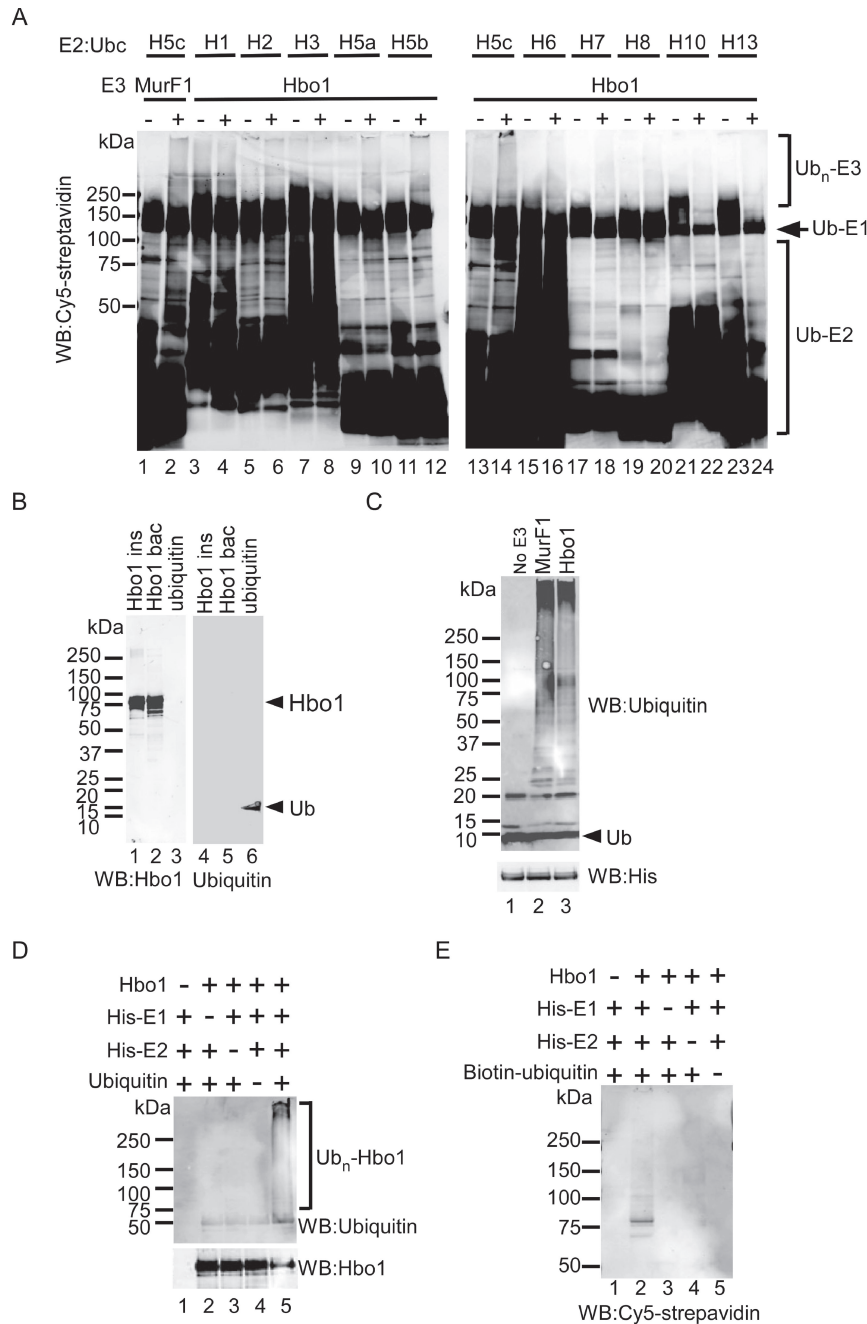


Fig. 2. Self-ubiquitination of Hbo1. (A) E2 screen for Hbo1 E3 ligase assay. Ten different His-tagged E2s were incubated with (+) or without (-) commercial recombinant Hbo1 in the presence of His-tagged E1, ATP, and biotinylated ubiquitin. The reaction mixture was run on SDS-PAGE, transferred onto the membrane, and probed with Cy5-conjugated streptavidin to detect ubiquitin. Recombinant MurF1 protein (1.25 µg) served as a positive control for E3 ligase activity (lanes 1 and 2). (B) Recombinant Hbo1 protein from insect cells (lanes 1 and 4) and from bacteria (lanes 2 and 5), and recombinant ubiquitin (lanes 3 and 6), were blotted with anti-Hbo1 or anti-ubiquitin antibodies. Ub: ubiquitin. (C) Commercial recombinant Hbo1 protein was incubated with His-tagged E1, E2 (UbcH5c), ATP, and non-tagged ubiquitin. The reaction mixture was run on SDS-PAGE, transferred onto the membrane, and probed with anti-ubiquitin or anti-His antibodies. Recombinant MurF1 protein (1.25 µg) served as a positive control (lane 2). Ub: ubiquitin. (D) Ubiquitination reaction mixture performed in the listed combination using commercial Hbo1 protein and non-tagged ubiquitin, was blotted with anti-ubiquitin or anti-Hbo1 antibodies. (E) Hbo1 protein from Sf9 cells (1.2 µg) was incubated in the listed combinations for 3 hours. After removal of His-tagged E1 and E2 by mixing with Ni-NTA Agarose, the reaction mixture was blotted with Cy5-conjugated streptavidin to detect ubiquitin.

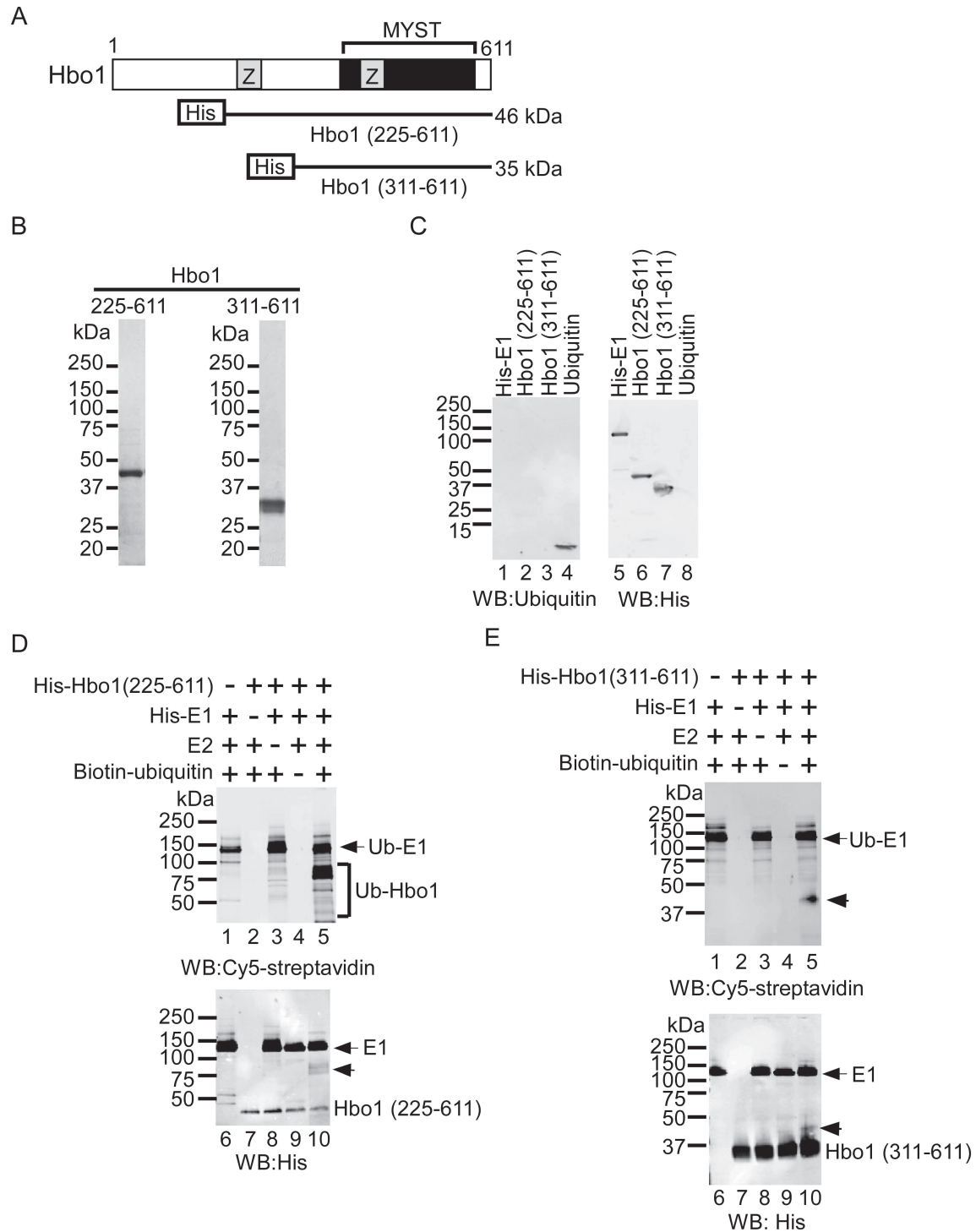


Fig. 3. MYST domain of Hbo1 has ubiquitin E3 ligase activity. (A) Schematic representation of Hbo1 domains and deletion mutants. Z: zinc finger; MYST: MYST domain. Expected molecular weights are shown. (B) Coomassie staining of His-tagged Hbo1 (amino acids: 225–611) and His-Hbo1 (amino acids: 311–611) proteins. (C) His-tagged E1, His-Hbo1 (225–611), His-Hbo1 (311–611), and non-tagged ubiquitin proteins were immunoblotted with anti-ubiquitin or anti-His antibodies. (D, E) Following ubiquitination reaction in the listed combination, His-tagged E1 and Hbo1 proteins (Hbo1 (225–611) for (D) and Hbo1 (311–611) for (E)) were affinity-purified with Ni-NTA Agarose under stringent condition, run on SDS-PAGE, and blotted with Cy5-conjugated streptavidin or anti-His antibody. Arrowheads: ubiquitinated Hbo1 deletion mutants.

ubiquitin, ATP, and GST-tagged p53, followed by affinity-purification of GST-tagged proteins under stringent condition. The GST-tagged Mdm2 and p53 proteins were analyzed by immunoblotting with Cy5-streptavidin and anti-GST antibody (Fig. 4A). No poly-ubiquitination of GST-p53 was observed without Mdm2 (lanes 1 and 4). On the other hand, high-molecular-weight poly-ubiquitination signal (lanes 3 and 6) confirmed that Mdm2 has intrinsic E3 ligase activity. When GST-p53 was incubated with GST-Mdm2, in addition to high-molecular-weight poly-ubiquitination signal, smeared bands ranging between 75 kDa and 100 kDa (bracketed) was detected (lanes 2 and 5), suggestive of ubiquitinated GST-p53. Thus, our assay could detect ubiquitinated substrates.

A test was performed to determine whether Hbo1 ubiquitinates ER α LBD *in vitro*. We incubated His-E1, E2 (UbcH5c), the commercial Hbo1 protein, biotinylated ubiquitin, ATP, and GST-tagged ER α LBD, followed by affinity-purification of GST-tagged ER α LBD protein under stringent condition. The GST-ER α LBD proteins were analyzed by immunoblotting with Cy5-streptavidin and anti-GST antibody (Fig. 4B). High-molecular-weight ubiquitinated proteins were only observed when all the necessary components were included (lanes 6 and 12). This result indicated that Hbo1 *per se* could ubiquitinate ER α *in vitro*. Next the mutant Hbo1 (311–611) was examined to determine whether it could ubiquitinate ER α LBD (Fig. 4C). Use of Hbo1 (311–611) protein as an E3 enzyme lacked a high-molecular-weight ubiquitination (lanes 2, 3, 6, and 7), suggesting that the MYST domain was not sufficient for ER α LBD ubiquitination by Hbo1. These results indicated that the N-terminal portion of Hbo1 was required for E3 ligase activity toward ER α LBD.

Estradiol-17 β inhibited ER α ubiquitination by Hbo1 *in vitro*. The ligand destabilizes ER α protein in a proteasome-dependent manner.^{14),15),42)} The association of ER α LBD with the ligand, estradiol-17 β , involves multiple amino acids via hydrogen bonds and hydrophobic contacts.⁴³⁾ To examine the effect of estradiol-17 β on ER α ubiquitination by Hbo1, estradiol-17 β was included in the ubiquitination reaction, followed by affinity-purification of GST-ER α LBD, and assayed for ubiquitin by immunoblotting (Fig. 5A). To negate an effect of ligand carryover from ER α LBD, estradiol-17 β was not used to stabilize ER α LBD throughout the purification procedures of GST-ER α LBD. With the increase of estradiol-17 β , high-molecular-weight

ubiquitination signal of ER α was reduced in a dose-dependent manner (lanes 3 and 4 compared to lane 2), suggesting that estradiol-17 β inhibited ER α ubiquitination by Hbo1 *in vitro*. As the ER α LBD bound tightly to ubiquitin,⁴⁴⁾ ubiquitinated E1 (about 120 kDa) was pulled down concomitantly with GST-ER α LBD, even under stringent conditions (arrowhead).

Suppression ER α expression by Hbo1 irrespective of the presence and absence of the ligand. Based on ligand-inducible degradation of ER α ,^{14),15),42)} degradation of ER α by Hbo1,³¹⁾ and inhibition of E3 ligase activity of Hbo1 for ER α by the ligand (Fig. 5A), we examined whether Hbo1 was involved in basal or estrogen-induced turnover of ER α . We knocked down expression of Hbo1 in a breast cancer cell line, MCF-7 cells, cultured the cells with or without estradiol-17 β , and examined expression of Hbo1 by qRT-PCR analysis (Fig. 5B) and of ER α by western blotting (Fig. 5C). A 72% reduction of Hbo1 mRNA expression by siRNA knock-down was achieved (Fig. 5B). In accord with the qRT-PCR result, Hbo1 protein decreased upon siRNA knock-down of Hbo1 (bottom panel, Fig. 5C). An equivalent loading of proteins onto each lane was judged by probing with anti- α -tubulin antibody (second panel, Fig. 5C). ER α expression (upper panel, Fig. 5C) was decreased upon estrogen treatment (lane 1 compared to lane 2) due to ligand-mediated turnover of ER α as previously reported.^{14),15)} With no ligand, depletion of Hbo1 caused an increase in ER α protein (lane 1 compared to 3), consistent with the previous study.³¹⁾ When treated with the ligand, ER α expression increased in Hbo1 siRNA cells compared to that of the siRNA control (lanes 2 and 4). These results suggest that Hbo1 regulates the stability of ER α , irrespective of the presence and absence of the ligand.

Enhanced ubiquitination of hyperactive ER α mutants by Hbo1. ER α somatic mutations in LBD, ER α -Y537S and ER α -D538G, found in ER-positive metastatic breast cancers, activate transcription even in the absence of ligand, providing a mechanism for resistance to hormonal therapy.^{45)–49)} Due to the linkage of ER α ubiquitination to transcriptional activation,¹⁶⁾ constitutive transcriptional activation by the hyperactive ER α mutants might be enhanced by ER α ubiquitination. To determine whether these hyperactive ER α mutants were good substrates of *in vitro* ubiquitination by Hbo1, we introduced the mutations by site-directed mutagenesis. The recombinant GST-tagged ER α LBD

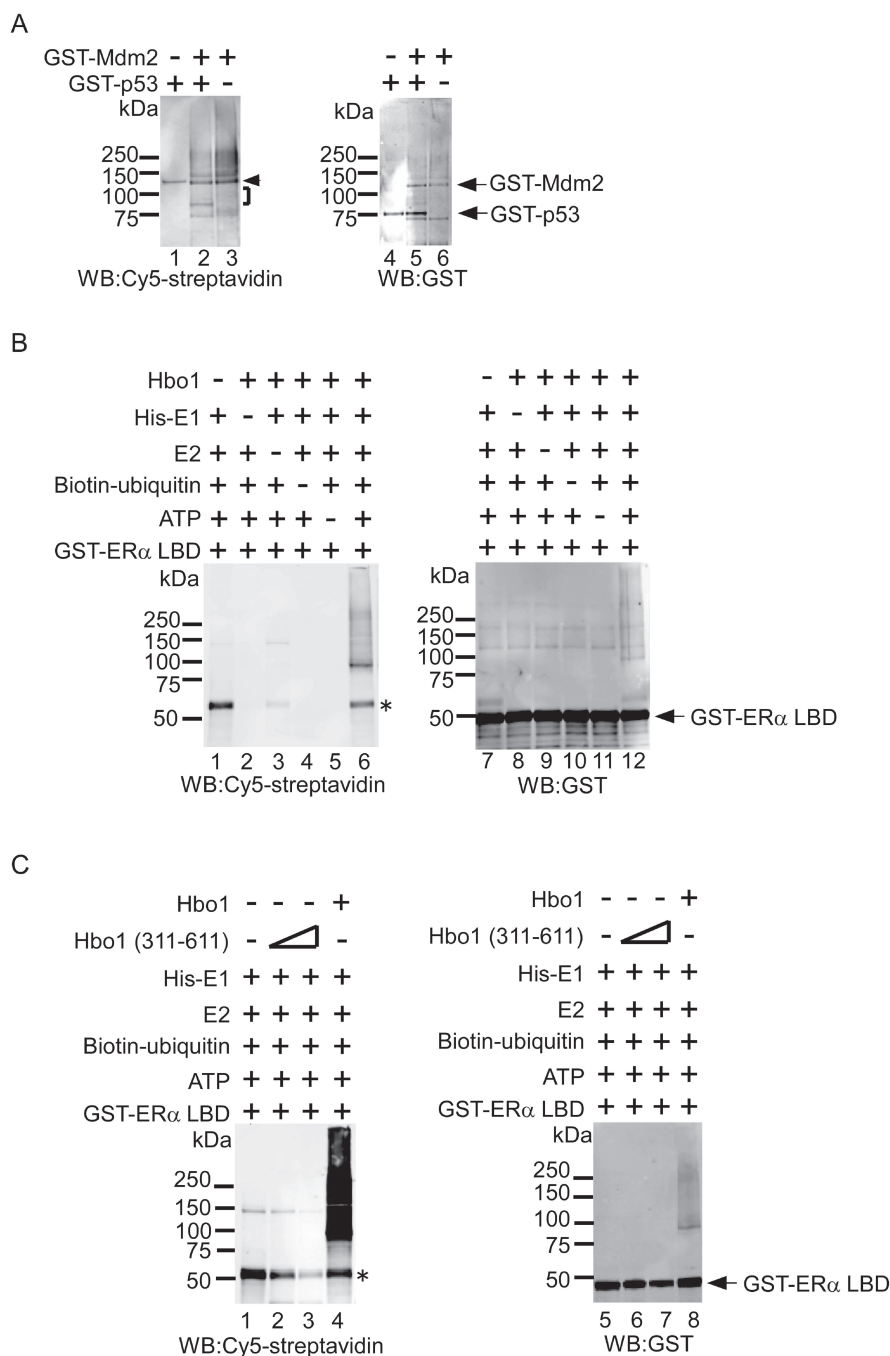


Fig. 4. Ubiquitination of ER α LBD by Hbo1. (A) GST-tagged p53 (2 μ g) and GST-tagged Mdm2 (E3 ligase) were mixed in the indicated combination in the presence of E1, E2 (UbcH5c), biotinylated ubiquitin, and ATP for 2 hours, followed by affinity purification of GST-tagged p53 and Mdm2 proteins with Glutathione Sepharose under stringent condition and by immunoblotting with Cy5-streptavidin (left panel) or anti-GST antibody (right panel). Arrowhead: ubiquitinated E1. Bracket: ubiquitinated GST-p53. (B) Commercial Hbo1 protein (1.2 μ g) was incubated in the listed combinations. GST-tagged ER α LBD protein was affinity-purified with Glutathione Sepharose under stringent condition, and assayed for ubiquitin (left panel) and GST (right panel). (C) No enzyme (lane 1), Recombinant His-Hbo1 (amino acids: 311–611) protein (lane 2: 1 μ g; lane 3: 2.5 μ g), and commercial Hbo1 (1.2 μ g, lane 4) were assayed in the ubiquitination reaction, and GST-ER α LBD protein was stringently affinity-purified with Glutathione Sepharose. The purified proteins were separated, transferred onto membrane, and ubiquitin and GST were detected with Cy5-conjugated streptavidin (left panel) or with anti-GST antibody (right panel), respectively. The identity of the band between 50 and 75 kDa (*) is unknown.

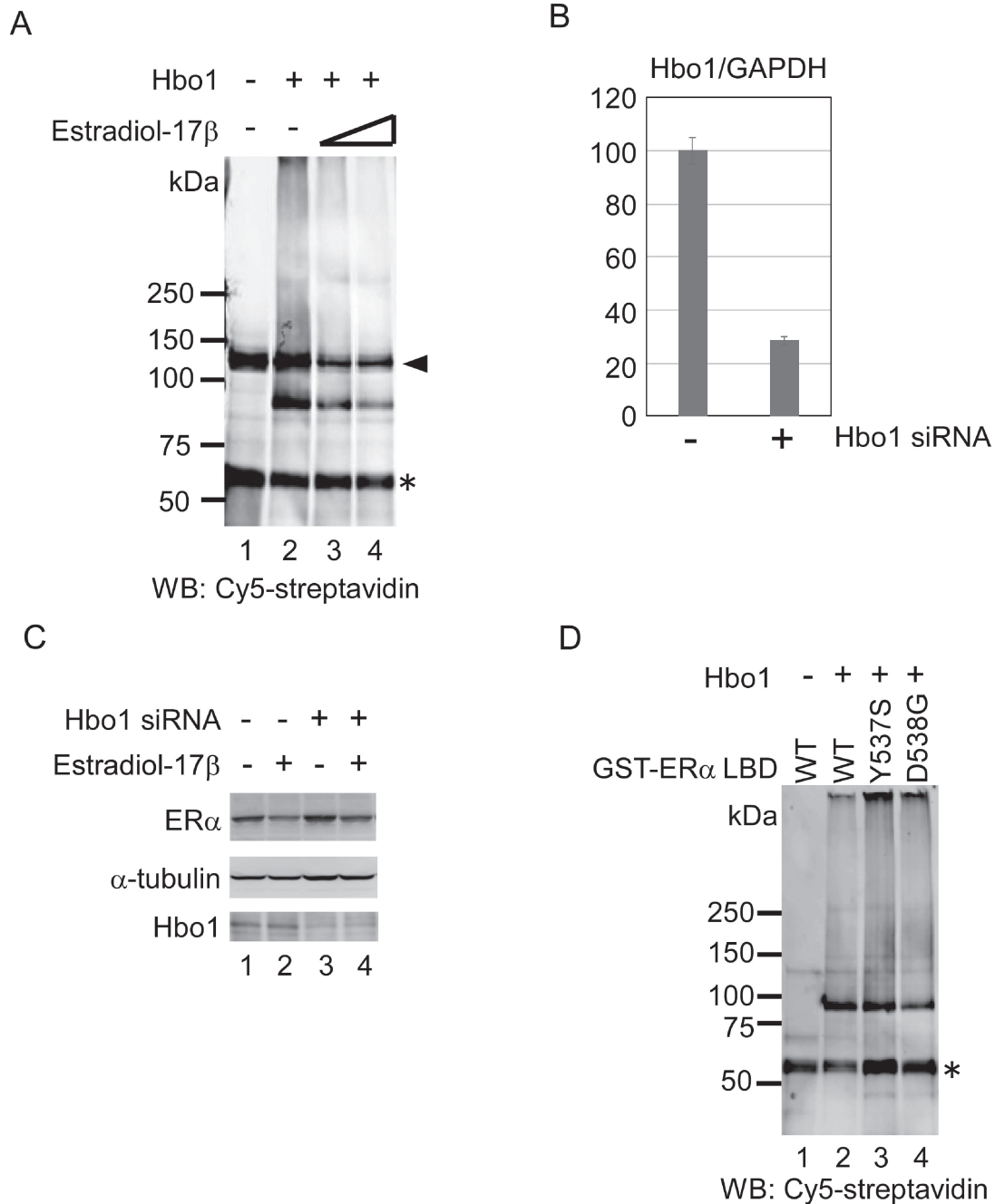


Fig. 5. Modulation of Hbo1 E3 ligase activity. (A) Commercial Hbo1 protein (1.2 μ g) was incubated with GST-ER α LBD in the ubiquitination reaction in the presence of estradiol-17 β (lane 2: vehicle; lane 3: 20 nM; lane 4: 200 nM). GST-tagged ER α LBD protein was purified and probed with Cy5-conjugated streptavidin to detect ubiquitin. The identity of the band between 50 and 75 kDa (*) is unknown. Arrowhead: ubiquitinated E1. (B) Expression of Hbo1 mRNA was analyzed by qRT-PCR following Hbo1 knock-down. A ratio of the Hbo1 mRNA to GAPDH mRNA in the control was arbitrarily set to 100. Each value was represented as a mean \pm SD. (C) MCF-7 cells were transfected with Hbo1 siRNA for 48 hours. Post-transfection, the cells were treated with or without 10^{-8} M estradiol-17 β for 24 hours. The cell lysates were probed with anti-ER α , anti- α -tubulin and anti-Hbo1 antibodies. (D) Commercial Hbo1 protein (1.2 μ g) was incubated with GST-ER α LBD (lane 2), GST-ER α LBD-Y537S (lane 3), and GST-ER α LBD-D538G (lane 4) in the ubiquitination reaction. GST-tagged ER α LBD protein were analyzed as in Fig. 5A. Asterisk (*): origin unknown.

proteins were examined for ubiquitination by Hbo1 (Fig. 5D). The ER α LBD-Y537S and ER α LBD-D538G proteins were ubiquitinated by Hbo1 (lanes 3 and 4) more than the wild-type (lane 2). Notably, the ER α LBD-Y537S showed a more robust ubiquitination than the wild-type, whereas ER α LBD-D538G showed a modest increase in ubiquitination.

Discussion

Intrinsic E3 ligase activity of Hbo1. Here, we report analysis of ER α ubiquitination by Hbo1. ER α LBD is preferentially ubiquitinated by Hbo1. Hbo1 has intrinsic ubiquitin E3 ligase activity for ER α LBD. We discovered two factors which regulates E3 ligase activity of Hbo1 for ER α : the ligand, estradiol-17 β and the hyperactive ER α mutants derived from metastatic breast cancers. The ligand inhibited E3 ligase activity of Hbo1 for ER α , whereas the hyperactive ER α mutants were better substrates for the E3 ligase activity. Hbo1 regulated basal and estrogen-induced turnover of ER α .

Ubiquitination of ER α hyperactive mutants by Hbo1. The hyperactive ER α mutants, ER α LBD-Y537S and ER α LBD-D538G, which activate ER α -dependent transcription in the absence of the ligand, were ubiquitinated more than the wild-type ER α by Hbo1 (Fig. 5D). Of interest, the marked difference in ubiquitination between the ER α LBD-Y537S and ER α LBD-D538G mutants was similar to that of transcriptional activation by ER α -Y537S and ER α -D538G in the absence of the ligand (Fig. 3⁴⁵) and Fig. 2a⁴⁶), respectively, suggesting that ER α ubiquitination by Hbo1 contributed to transcriptional activation by ER α . It is speculated that the enhanced ubiquitination of the ER α mutants by Hbo1 induces cyclic proteasome-mediated turnover of ER α , leading to potentiation of estrogen signaling.⁵⁰ Ubiquitin-mediated degradation of ER α by Hbo1, in cooperation with histone acetylation by Hbo1, may lead to transcriptional activation. ER α -dependent genes, such as E2F1, RRM2, CTSD, and NRG1, which are activated by Hbo1,³¹ may be subjected to dual enhancement by its two enzymatic activities, HAT and ubiquitin E3 ligase. In this context, Hbo1 might contribute to enhancing hormonal resistance of breast cancers with ER α mutations.

Inhibition of Hbo1-mediated ER α ubiquitination of by the ligand. It seems puzzling that Hbo1 promoted ligand-induced turnover of ER α , although the ligand, estradiol-17 β , inhibits Hbo1 E3 ligase activity *in vitro*. One explanation is that the interaction of the ligand with the ER α LBD masks

LBD, facilitates ubiquitination at regions other than LBD by Hbo1, and induces subsequent proteasome-dependent degradation. Thus, the binding of the ligand could enhance ER α ubiquitination by Hbo1. The extensive ubiquitination of ER α by Hbo1 (Fig. 1B) supports the notion. Lysines 302 and 303 in the hinge region are important for ligand-induced degradation of ER α ³⁵) and may be target sites for ubiquitination by Hbo1.

Possible E3 ligase activity of MYST HATs.

Several HATs have ubiquitination-linked enzymatic activity. p300/CBP has E3/E4 ligase activity for p53.^{51),52)} PCAF has E3 ligase activity toward Mdm2⁵³⁾ and Gli1.⁵⁴⁾ The results of the present study demonstrated that the MYST domain, that is, the HAT domain, has an intrinsic ubiquitin E3 ligase activity. It is conceivable that other members of the MYST family HATs, such as MOF,⁵⁵⁾ MOZ/MORF,⁵⁶⁾ and yeast MYST proteins,⁵⁷⁾ have E3 ligase activity as well. Some functions of MYST family HATs might be attributable to E3 ligase activity, instead of HAT activity. For instance, Esa1 is essential for viability in budding yeast, but its HAT activity is dispensable for viability.⁵⁸⁾ The essential function of Esa1 may be its E3 ligase activity.

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