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論　文　内　容　要　旨（和文）

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体内のタンパク質は、合成と分解のバランスにより恒常性が維持・調節されている。タンパク質の分解経路には、選択的に標的タンパク質を分解するユビキチン・プロテアソーム系と、非選択的に分解するリソソーム系が知られている。これらの分解経路に破綻が生じるとタンパク質の異常蓄積が起こり、癌や神経変性疾患などの難知性疾患の発症原因となることが明らかとなってきている。

我々は、乳癌において腫瘍の悪性度に関与するタンパク質 HER3 に着目した。HER3 は HER ファミリーに属す受容体チロシンキナーゼであり、これまで 4 分子（HER1、HER2、HER3 および HER4）が同定されている。これら HER ファミリーを含む膜タンパク質の多くは、ユビキチン・プロテアソーム系により分解制御されると報告がある。特にユビキチンリガーゼ(E3)が選択的にターゲットタンパク質をユビキチン化することでタンパク質の分解量が調節されている。HER3 は、そのリガンドである HRG-1 刺激下でユビキチンリガーゼ Nrdp1、Nedd4-1、Itch によりプロテアソーム系で選択的に分解されることが知られている。

本研究では、乳癌における HER3 の分解調節機構とこれに関連する因子の解明を目的に実験を計画した。まず各サブタイプの代表的乳癌細胞株である MCF-7、BT474、SKBR3、MDA-MB231 を用いて HER3 の内因性発現を確認したところ、ER 陽性細胞である MCF-7 において HER3 の高発現が認められた。ER 陽性乳癌において最も重要な増殖因子である、エストロゲン刺激（0.1nM、1nM、10nM）下でサイクロヘキシミド（CHX）による分解実験を行ったところ、1nM のエストロゲン濃度で HER3 の分解効率が最も亢進し、半減期はコントロールの 4.8 時間から 2.5 時間へ短縮した。また、酵素阻害実験からエストロゲン刺激時の HER3 の分解経路はプロテアソーム系であると同定した。この分解には、既知のユビキチンリガーゼ Nrdp1、Nedd4-1、Itch のいずれかが関わっていると考え、MCF-7 での内因性発現を検討したところ Nedd4-1 のみ発現を認めた。そこで sh-RNA により Nedd4-1 をノックダウンしたところ、エストロゲン刺激時の HER3 分解効率亢進が消失することから、Nedd4-1 を制御因子と同定した。またエストロゲン刺激による ER の分解も Nedd4-1 によって制御されることを確認した。次に ER を si-RNA によりノックダウンしたところ、コントロール、エストロゲン刺激下の双方で HER3 の分解が亢進した。最後に Nedd4-1 ノックダウン MCF-7 における増殖活性をみたところ、コントロール細胞に比較し有意に増殖活性が亢進していた。

これらの結果から ER 陽性乳癌細胞株 MCF-7 では、ER が HER3 の分解を抑制しており（本研究では実証できていないがおそらく直接会合によって）、エストロゲン刺激下では ER が急

速に分解されることにより、HER3 の分解効率も亢進すると考えられた。また、この両者の分解効率をユビキチンリガーゼである Nedd4-1 が制御しており、これら分解機構への影響を通じて癌細胞の悪性度に関与していることが示唆された。本研究は乳癌細胞における ER と HER3 の相互作用を分解機構という点で検証した初めての報告である。さらに、この分解機構に関わる Nedd4-1 の臨床的意義や治療標的としての可能性を今後検証していきたい。

（公表誌名、公表年月日、巻番号、ページ　　）

Contents

Abbreviation

ER	estrogen receptor
HRG-1	heregulin-1
Nedd4-1	neural precursor cell expressed developmentally down-regulated 4
Itch	itchy
Nrdp1	neuregulin receptor degradation protein-1
CHIP	chaperon-interacting protein

1. Introduction

HER3 is a member of the receptor tyrosine kinase family (RTK), and lacks intrinsic tyrosine kinase activity in the C-terminal tail. It is activated by Heregulin-1 (HRG-1) stimulation and plays a regulatory role in cell proliferation and migration [1-3]. Overexpression of the HER3 has been reported in breast, ovarian, pancreatic and gastric cancers, and is significantly associated with cancer malignancy [4-13]. However, the mechanisms of HER3 overexpression are still not well understood. On the other hand, previous studies have reported that HER3 is regulated by ubiquitination and degradation with HRG-1 stimulation [14-17].

Ubiquitination is controlled by regulatory proteins in the ubiquitin-conjugation system, and occurs through the three sequential classes of enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) [18-20]. Ubiquitin ligases interacts physically with the substrate to determine the protein's fate by leading to degradation. Previous studies reported that the HER family receptors are degraded with a help of their specific ubiquitin ligases [21-22]. For example, HER1 is degraded by ubiquitin ligase c-Cbl [23], HER2 is mediated by c-Cbl [24-26] or chaperon-interacting protein (CHIP) [27,28]. HER4 is ubiquitinated by WWP1[32] or Itch [33]. In the case of HER3, HRG-1 stimulation leads to proteasome-mediated degradation of HER3, and at least three ubiquitin ligases including neuregulin receptor degradation protein-1 (Nrdp1) [14-17,29], neural precursor cell expressed developmentally down-regulated 4 (Nedd4-1) [30] and Itchy (Itch) [31] have been identified the degradation process. Nevertheless, the functional relationship between the HER3- ubiquitination and hormones has been left unknown.

In our attempt to investigate breast cancer, we have been exploring the biological role of estradiol in estrogen receptor (ER) positive breast cancer. In this line, we found that estradiol promotes rapid degradation of HER3 the proteasome pathway, and an ubiquitin ligase Nedd4-1 controls this process. Furthermore, Nedd4-1 affects proliferation of MCF-7 cells through its dual action on HER3 and ER.

2. Materials and methods

2.1. Cell culture

Human embryonic kidney cells 293 T and human breast cancer cell lines MCF-7 and MDA-MB231 were purchased from ATCC, and human breast cancer cell lines SKBR3 and BT474 were gifted from by Dr. S. Hayashi (Tohoku University, Miyagi, Japan). The cells were cultured in DMEM (Wako) or RPMI 1640 (Wako) supplemented with 10% heat-inactivated FBS (Biowest), 100 units/ml penicillin G and 100 μg/ml streptomycin. For experiments evaluating the effect of 17β-estradiol (estradiol, Sigma-Aldrich), the MCF-7 cells were cultured for two days in phenol red-free DMEM (PRF-DMEM, Wako) containing 10% heat-inactivated FBS stripped of steroids by absorption to dextran-coated charcoal (DCC-FBS, Biological Industries). The cells were then cultured in a humidified 5% CO_2 incubator at 37℃

2.2. Reagents and antibodies

Reagents used were as follows: epoxomicin (Peptide); ethanol, cycloheximide and chloroquine diphosphate (Wako); dimethyl sulfoxide (DMSO, Sigma-Aldrich). Antibodies used were as follows: anti-HER3, anti-NEDD4-1 and corresponding secondary antibodies (Cell Signaling Technology); anti-Itch and anti-Nrdp1 (Santa Cruz Biothechonology); anti-ER (Thermo Scientific): anti- β actin (Sigma-Aldrich).

2.3. siRNA and shRNA mediated knockdown

Knockdown of human ER was performed using si-ER (Ambion, catalog# 4392420), along with a non-silencing control si-RNA (catalog# 4390843). MCF-7 cells were transiently transfected with 10 μM of the si-RNAs using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies) according to the manufacturer's protocol, and further cultured for 48 h before assays.

Human sh-Nedd4-1 expressing lentivirus vectors were constructed in

pRSI12-U6-sh-HTS4-UbiC-TagRFP-2A-Puro plasmid (Cellecta). To make virus particles, plasmids were transfected into 293T cells using FuGENE HD (Promega) according to the manufacturer's protocol. At 48 h post-transfection, the supernatants were collected and filtrated through a 0.45 μm syringe filter.

The lentiviral particles encoding the shRNA targeting Nedd4-1 or a scramble control were incubated with the target cells for 48 h. Transduced cells were selected for additional for 72 h in the presence of 1 μg/ml puromycin (Wako).

2.4. Cycloheximide chase assay

MCF-7 cells were plated in 6-well culture plates at a density of 4×10^5 cells/well with PRF-DMEM containing 10% DCC-FBS. After overnight incubation, the medium was replaced with serum-starved PRF-DMEM for 1.5 h. The cells were then treated with 50 μg/mL cycloheximide (CHX) for 30 min and chased with different time periods in the presence or absence of estradiol with CHX. The cells were collected at each time point then processed for immunoblotting by anti-HER3, anti-Nedd4-1, anti-ER and anti-β actin antibodies.

2.5. Western blotting

Cells were grown in PRF-DMEM containing 10% DCC-FBS in 6-well culture plates. The cultured cells were then washed twice with ice-cold PBS before they were lysed in RIPA buffer (40 mM Tris-HCl, pH7.5, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 2 mM Na_3VO_4, 50 mM NaF) containing protease inhibitor cocktail (Roche). Lysates were scraped, transferred into microtubes and centrifuged at 13,000g for 20 min at 4^0C. The supernatants were used as cell extracts. Total protein concentrations were determined using a Quick Start Bradford protein assay (Bio-Rad) using bovine serum albumin as a standard. Immunoblotting was subjected to 4-20% sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad), followed by transference to 0.45μm pore size polyvinylidene difluoride membranes (Millipore), and blotting with primary and secondary antibodies. Quantification was performed using ImageJ software.

2.6. Cell proliferation assay

Cell proliferation was detected with a Cell Counting Kit-8 (CCK-8, Dojindo) according to the manufacturer's protocol. Cells were seeded in 96-well culture plates (7×10^3 cells/well) in PRF-DMEM containing 10% DCC-FBS. After overnight incubation, the cells were replaced in the medium containing ethanol (EtOH) or 1nM estradiol reagent. At 0, 24, 48, and 72 h incubation, 10 μl of CCK-8 solution was added to the cells. After incubating the cells for 2 h at 37^0C, absorbance at 450 nm was measured using a plate reader (Thermo Scientific).

2.7. Statistical analysis

All data are expressed as mean±SD, as indicated in the figure legends. Statistical analysis was performed using the Student t-test. Significance is denoted as *, $P < 0.05$; **, $P < 0.01$. All experiments were replicated at least three times.

3. Results

3.1. HER3, ER and Nedd4-1 express in MCF-7 cells.

To confirm expression of endogenous HER3 and ER in human breast cancer cell lines (MCF-7, BT474, SKBR3 and MDA-MD-231), we examined the Western blotting results. HER3 was expressed in the MCF-7, BT474 and SKBR3 cells (Fig. 1A, upper panel), and expression of endogenous ER was confirmed in the MCF-7 and BT474 cells (Fig. 1A, second panel). We next examined whether Nedd4-1, Itch and Nrdp1 ubiquitin ligases were expressed in the four breast

cancer cell lines. Nedd4-1 was only detected in the MCF-7 cells (Fig. 1B, upper panel), and Itch was only expressed in the BT474 cells (Fig. 1B, second panel). Expression of endogenous Nrdp1 was not found in any of these breast cancer cell lines (Fig. 1B, third panel). From these results, we chose the MCF-7 cells for further study, since it expressed appreciable amounts of ER and HER3.

3.2. HER3 is rapidly degraded in the presence of estradiol via proteasome pathway.

To evaluate the degradation speed of HER3 in the presence or absence of estradiol, we performed cycloheximide (CHX) chase assay, which monitors protein amounts decrease under the *de novo* protein biosynthesis inhibition with CHX. Ethanol (EtOH) was solvent of estradiol and was used as control stimulation.

Among the several concentrations of estradiol tested, 1 nM estradiol was seems to be the most preferable concentration to see the effect of estradiol on HER3 degradation (Fig. 2A and B). As shown in Fig. 2C, the half-life of HER3 shortens from 4.8 h to 2.5 h after 1nM estradiol treatment. To identify the degradation pathway of HER3, we performed experiments using the proteasome inhibitor epoxomicin (Epx), or lysosome inhibitor chloroquine (CQ). In the presence of estradiol, Epx treatments, but not CQ, led to the remarkable accumulation of HER3 compared to control treatment (DMSO), indicating that enhanced degradation of HER3 by estradiol depends on the proteasome pathway (Fig. 2D and F). In the absence of estradiol, Epx also prevented the later degradation to some degree, indicating the contribution of the proteasome pathway in a stable condition (Fig. 2D and E). More enhanced degradation with CQ treatments might be off-target effect probably by inducing some other degradation process, although this remains to be confirmed (Fig. 2D and E). These results suggest that enhanced degradation of HER3 by estradiol is mediated through the proteasome pathway in MCF-7 cells.

3.3. Nedd4-1 regulates HER3 and ER degradation in the presence of estradiol.

To determine whether Nedd4-1 contributes to the enhanced degradation of HER3 by estradiol, we established a Nedd4-1 knockdown MCF-7 cells. sh-control MCF-7 cells or sh-Nedd4-1 MCF-7 cells were treated with CHX at indicated time points with or without 1nM estradiol. In the estradiol-stimulated condition, degradation of HER3 was suppressed in the sh-Nedd4-1 MCF-7 cells as compared to the sh-control MCF-7 cells (Fig. 3A and C), indicating that Nedd4-1 played some role in this enhanced degradation process. In the absence of estradiol, difference between the sh-Nedd4-1 MCF-7 and sh-control MCF-7 cells could not be detected (Fig. 3A and B).

For seeking the possible involvement of the same degradation process for ER, we did CHX chase assay of ER proteins in these two cells. As expected, in the estradiol-stimulated condition, ER degradation in the Nedd4-1 knockdown MCF-7 cells was suppressed (Fig. 3A and E). In the absence of estradiol, ER was similarly decreased in both of the sh-control MCF-7 and sh-Nedd4-1 MCF-7 cells (Fig. 3A and D). These results suggest that Nedd4-1 regulates HER3 and ER degradation in the estradiol-stimulated condition.

3.4. Knockdown of ER promotes the rapid degradation of HER3.

Since both HER3 and ER share the same ubiquitin ligase Nedd4-1 for their degradation under estradiol-stimulated condition, we then examined whether the amount of ER affected HER3 degradation. We transiently suppressed ER expression using si-RNA. ER knockdown promoted rapid degradation of HER3 compared to si-control after 2 h treatment in the presence of estradiol (Fig. 3F and H). The same finding was also observed in the absence of estradiol, indicating that the absence of ER, but not estradiol itself, is the key issue of rapid HER3 degradation (Fig. 3F and G). From these results, we speculated that estradiol induced rapid ER degradation, which then liberates HER3 from its inhibition by ER, eventually leading to the rapid degradation of HER3. Interestingly, these

processes appear to be regulated by Nedd4-1.

3.5. Knockdown of Nedd4-1 enhances the proliferation of MCF-7 cells.

To gain some insight into the biological role of Nedd4-1 in MCF-7 cells, we performed preliminary proliferation experiment using sh-Nedd4-1 MCF-7 cells. The Nedd4-1 knockdown in MCF-7 cells resulted in enhanced proliferation compared to the sh-control MCF-7 cells either with or without estradiol (Fig. 4A and B). These results suggest that Nedd4-1 may have a larger role in tumor biology, not only as a regulation molecule for ER and HER3 degradation upon estradiol stimulation, but also as an anti-proliferative factor in the basic cancer biology.

4. Discussion

We showed here that estradiol promotes rapid degradation of HER3 in ER-positive breast cancer MCF-7 cells.

HER3 is a member of the HER family receptors, which play roles in oncogenesis [34]. Overexpression of HER3 in several types of primary tumors or cultures cells such as breast, ovarian pancreatic and gastric cancers have been reported [4-13].

In breast cancer, HER3 contributes to tumor cell survival and proliferation, and previous reports have shown that HER3 in breast cancer cases is associated with poor prognostic factors in terms of grade, lymph node metastasis and tumor size [5,6]. Therefore, an underlying mechanism for the HER3 overexpression might be a target of drug development for breast cancer. To this end, we started experiments using have ER-positive breast cancer MCF-7 cells, which has remarkable positive HER3 expression compared to the cell lines that we evaluated (Fig. 1A).

In our HER3 CHX chase assay, we found that HER3 was degraded more rapidly in the presence of estradiol than in its absence (Fig. 2A and B). As is well-known, estradiol is a ligand of ER, and

estradiol stimulation causes rapid ER degradation as a result of ligand-receptor interaction [35-37]. In the current study, the half-life of both ER and HER3 were affected by estradiol stimulation (Fig. 2C), leading to the suspicion that the same degradation mechanism was involved in both receptors. It is known that HER3 is quickly degraded by the proteasome pathway upon Heregulin-1 (HRG-1) stimulation and, interestingly, this is also true for estradiol stimulation, as shown in our proteasome pathway inhibitor experiments (Fig. 2D-F). Moreover, ubiquitination of HER3 was observed in the estradiol-stimulated condition (data not shown).

Degradation of HER3 under HRG-1 stimulation has been associated with three ubiquitin ligases, Nedd4-1, Itch and Nrdp1 [14-17,30,31]. Nedd4-1 is the only ubiquitin ligase which was endogenously expressed in the MCF-7 cells, and it specifically contributed to the estradiol induced rapid degradation process of HER3 and ER. Interestingly, depletion of ER enhanced HER3 degradation irrespective of estradiol stimulation (Fig. 3F-H), indicating that ER might possess a function to prevent HER3 degradation through a direct interaction. Collins et al. has reported that HER3 forms a complex with ER in the presence and absence of HRG-1 [38]. We failed to prove the direct interaction between ER and HER3 in our assays, however, we speculate that formation of ER/HER3 complex could prevent HER3 from its degradation. Together, our current hypothetical schema is shown in Fig. 4C. ER prevents HER3 degradation through its interaction under an estradiol-negative condition. Upon estradiol stimulation, ER is quickly degraded, and HER3, which is now free from ER leads to prompt degradation. This ER-HER3 crosstalk would shed light on a previously unknown aspect of breast cancer research.

Finally, Nedd4-1 knockdown showed remarkable proliferative property, especially in the presence of estradiol (Fig. 4A and B). Our observation that the enhanced effect of Nedd4-1 knockdown in HRG-1 stimulated proliferation is in good accordance with a previous report [30]. Nedd4-1 contributes to estradiol-induced proliferation of MCF-7 cells through the interaction with HER3,

although this needs further experiments.

A limitation of our study is that we could not show the difference in the ubiquitination of HER3 between in the presence or absence of estradiol. The ubiquitination of HER3 seems to have occurred as very short time events and we need to establish a more precise assay system. Although we performed several experiments for evaluating PI3K/Akt and MAPK signaling pathway of HER3 to explain the biological impact of ER/HER3 interaction through a degradation process, we could not obtain significant results. It might be due to experiment settings or incorrect target signaling.

In summary, our findings showed the preliminary mechanism of estradiol-induced HER3 degradation in ER-positive breast cancer MCF-7 cells. HER3 was degraded rapidly by the proteasome pathway under estradiol stimulation, and ubiquitin ligase Nedd4-1 contributed to both HER3 degradation and tumor cell growth. Impact of Nedd4-1 on breast cancer biology should be a pertinent research topic in future.

5. Acknowledgments

We are grateful to Ms. Naoko Ogama for her excellent technical assistance, and to Dr. Tetsuo Koshizuka for his valuable advice. This work was supported by JSPS KAKENHI Grant Number 15K10059, 16K20288 and Novartis Pharma Research Grants.

6. Figure legends

Fig. 1: Expression of HER3, ER and ubiquitin ligases in breast cancer cell lines.

(A, B) Endogenous HER3, ER and ubiquitin ligases (Nedd4-1, Itch and Nrdp1) expression in the four subtypes of human breast cancer cells were analyzed by immunoblotting with HER3, ER, Nedd4-1, Itch, Nrdp1 and β actin antibody.

Fig. 2: Estradiol induces rapid degradation of HER3 via proteasome pathway.

(A) MCF-7 cells were pre-incubated for 1.5 h with 50 μg/ml cycloheximide (CHX), followed by treatment with indicated concentration of estradiol. Cells were lysed at indicated time points and subjected to immunoblotting for HER3, ER and β actin antibody. (B) The quantification of the HER3 protein levels was done using ImageJ software. Protein levels were normalized to β actin levels. (C) Half-life of HER3 was calculated based on data in A. (D) MCF-7 cells were treated with 5 mM epoxomicin, 1 mM chloroquine, or DMSO with CHX for 30 min, followed by treatment with 1 nM estradiol or EtOH. Cells were lysed at indicated time points and subjected to immunoblotting for HER3 and β actin antibody. (E, F) Quantification of the HER3 protein levels was done using ImageJ software. Protein levels were normalized to β actin levels. All values are shown as means ± SD of three independent experiments. *$P < 0.05$.

Fig. 3: Nedd4-1 regulates HER3 and ER degradation in the presence of estradiol, and knockdown of ER promotes the rapid degradation of HER3.

(A) sh-control MCF-7 cells and sh-Nedd4-1 knockdown MCF-7 cells were pre-incubated for 1.5 h with 50 μg/ml CHX, followed by treatment with EtOH or 1 nM estradiol in the presence of CHX. All protein levels were assessed by immunoblotting at indicated time points. Quantification of the HER3 (B, C) and ER (D, E) protein levels were done using ImageJ software. Protein levels were normalized to β actin levels. All values are shown as means ± SD of three independent experiments. (F) si-control MCF-7 cells and si-ER knockdown MCF-7 cells were pre-incubated for 1.5 h with 50 μg/ml CHX, followed by treatment with EtOH or 1 nM estradiol with CHX. All protein levels were assessed by immunoblotting at indicated time points. (G, H) Quantification of the HER3 protein levels was done using ImageJ software. All data from three experiments were normalized to β actin. Mean values ± SD were plotted.

Fig. 4: Knockdown of Nedd4-1 enhances proliferation of MCF-7 cells.

(A, B) sh-control and sh-Nedd4-1 knockdown MCF-7 cells were incubated for the indicated time at 37^0C with EtOH or 1 nM estradiol. Cell proliferation was measured using CCK-8 assays. These data are representative of three independent experiments (n=3). **P < 0.01. (C) Summary of findings on the effect of HER3 degradation on ER-positive breast cancer cell line MCF-7.

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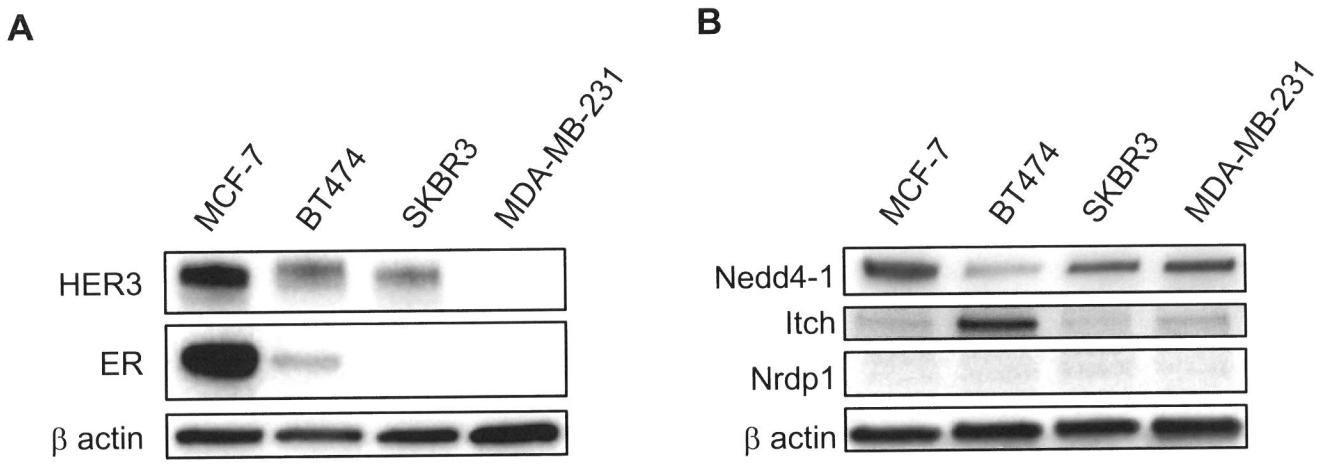
Figure.1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HER3
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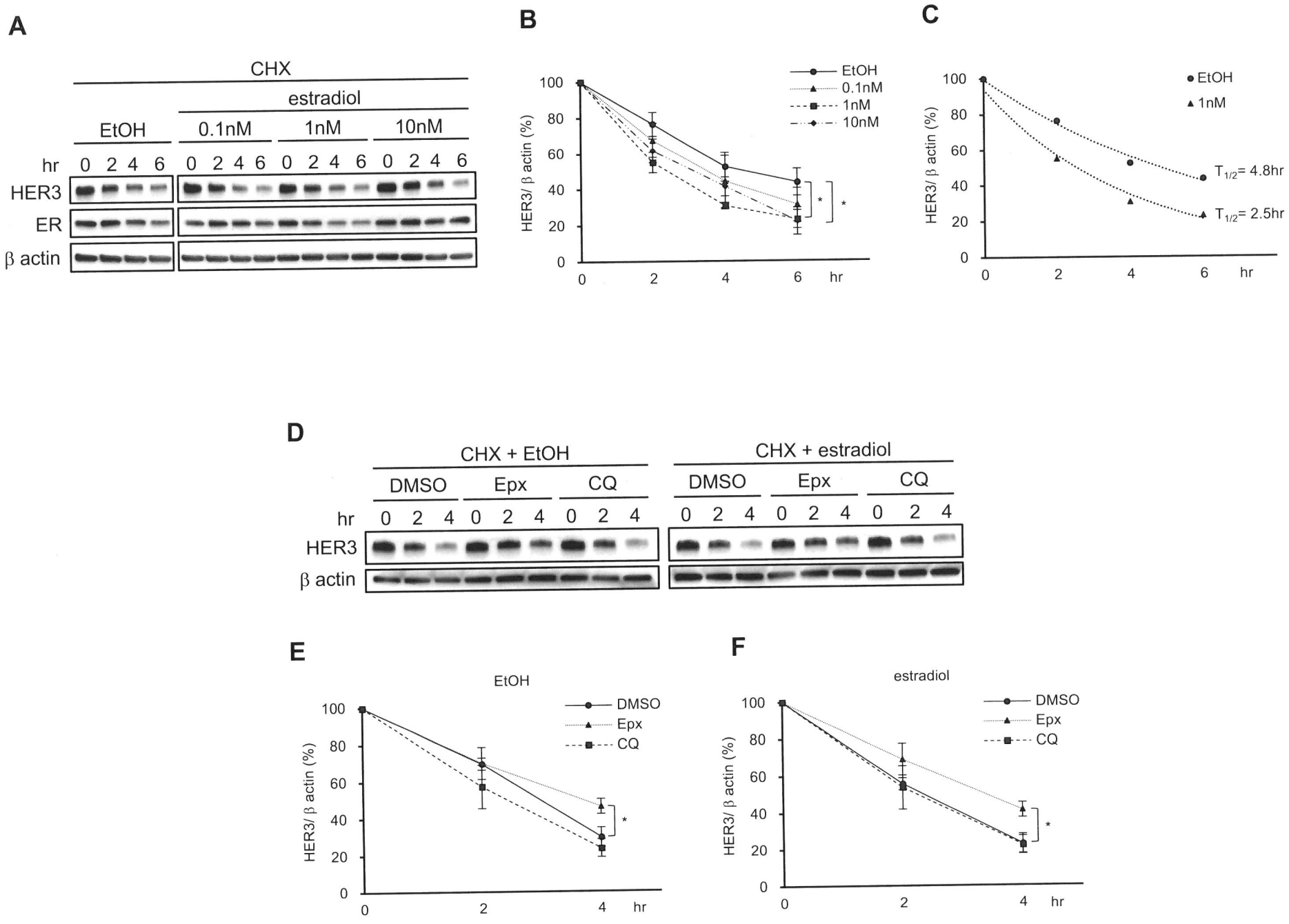
Figure.2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0.1nM
1nM
10nM
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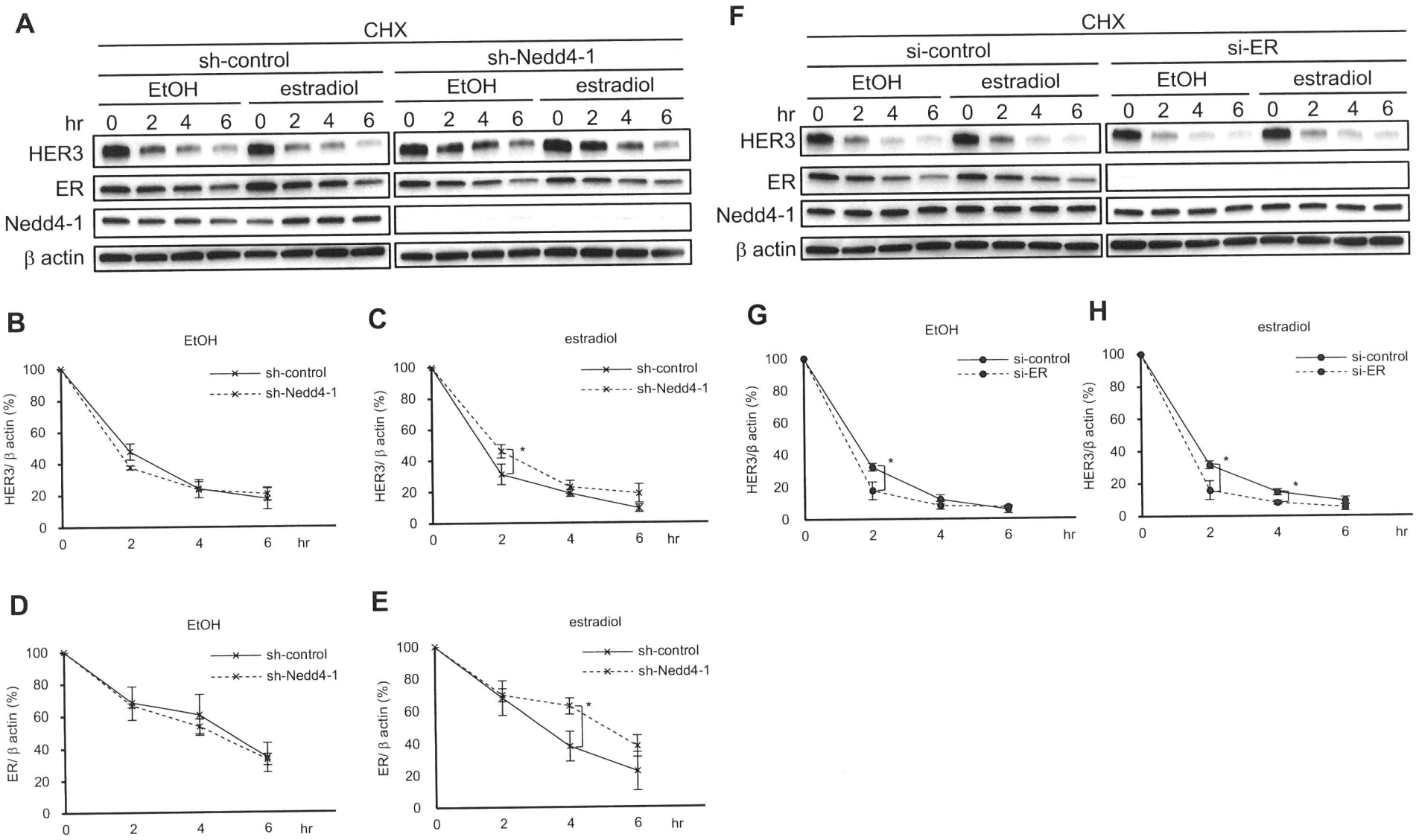
HER3
ER
β actin
B
HER3/ β actin (%)
EtOH
0.1nM
1nM
10nM
hr
C
HER3/ β actin (%)
EtOH
1nM
T1/2= 4.8hr
T1/2= 2.5hr
hr
D
CHX + EtOH
CHX + estradiol
DMSO
Epx
CQ
hr 0 2 4 0 2 4 0 2 4 0 2 4 0 2 4 0 2 4
HER3
β actin
E
EtOH
HER3/ β actin (%)
DMSO
Epx
CQ
hr
F
estradiol
HER3/ β actin (%)
DMSO
Epx
CQ
hr

Figure.3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EtOH
estradiol
EtOH
estradiol
hr 0 2 4 6 0 2 4 6 0 2 4 6 0 2 4 6
HER3
ER
Nedd4-1
β actin
F
CHX
si-control
si-ER
EtOH
estradiol
EtOH
estradiol
hr 0 2 4 6 0 2 4 6 0 2 4 6 0 2 4 6
HER3
ER
Nedd4-1
β actin
B
EtOH
sh-control
sh-Nedd4-1
HER3/ β actin (%)
hr
C
estradiol
sh-control
sh-Nedd4-1
HER3/ β actin (%)
hr
G
EtOH
si-control
si-ER
HER3/β actin (%)
hr
H
estradiol
si-control
si-ER
HER3/β actin (%)
hr
D
EtOH
sh-control
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ER/ β actin (%)
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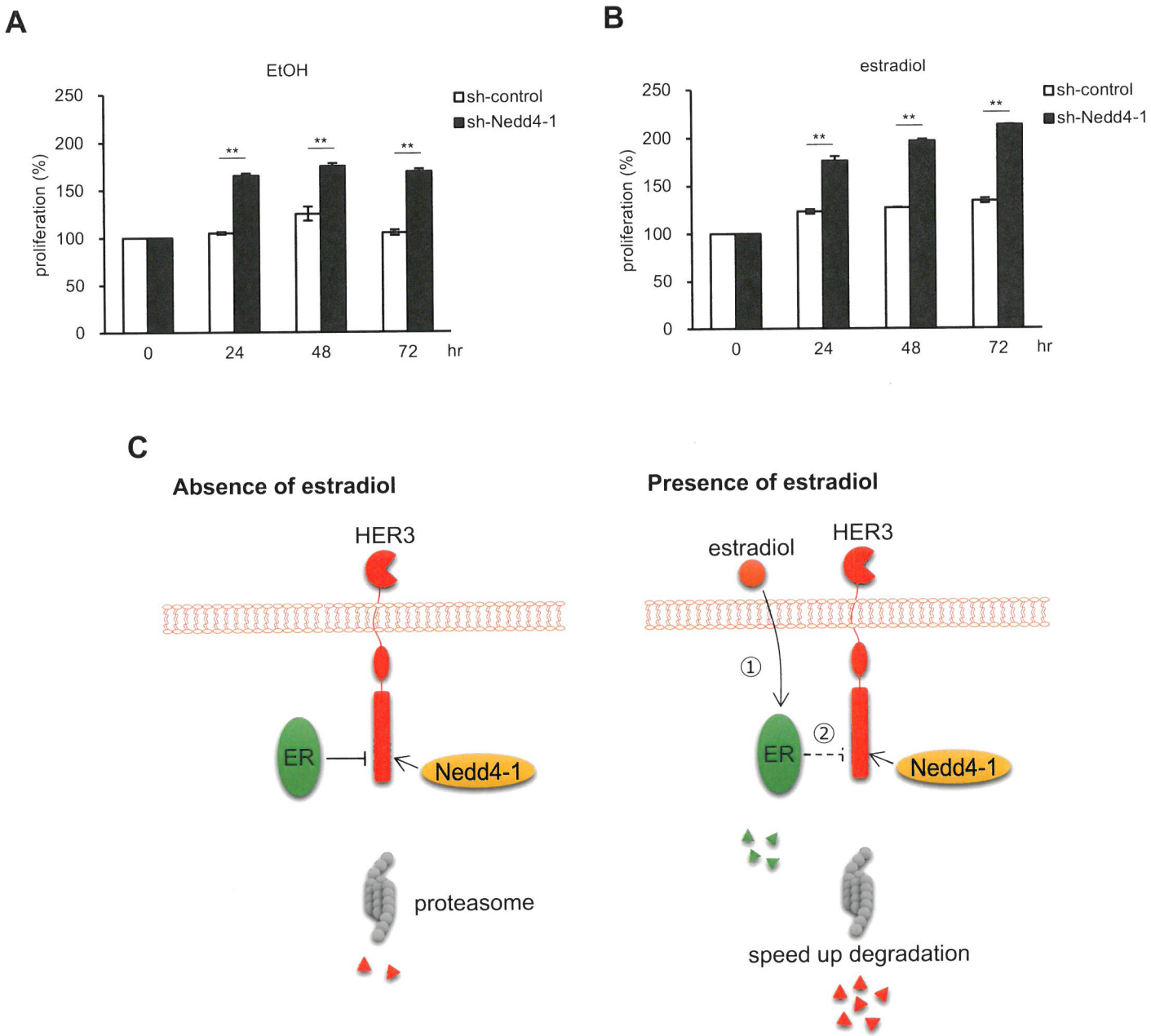
Figure.4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200
150
100
50
0
**
0
24
48
72
hr
B
estradiol
sh-control
sh-Nedd4-1
proliferation (%)
250
200
150
100
50
0
**
0
24
48
72
hr
C
Absence of estradiol
HER3
ER
Nedd4-1
proteasome
Presence of estradiol
estradiol
HER3
①
②
ER
Nedd4-1
speed up degradation