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Ligand-dependent transcription of estrogen receptor \( \alpha \) is mediated by
the ubiquitin ligase EFP

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Abstract

Estrogen-mediated ubiquitylation and subsequent degradation of the estrogen receptor α (ERα) appears to be involved in the transcriptional activity of ERα. We show that the estrogen-responsive finger protein (EFP) interacts with and ubiquitylates ERα. EFP promoted the ubiquitylation of ERα in vitro and in vivo and consequently promoted the degradation of ERα. The interaction between EFP and ERα was greatly enhanced in the presence of estrogen. The action of EFP on ERα in the presence of estrogen resulted in a robust interaction between ERα and Tip60, one of the transcriptional coactivators, leading to activation of ERα transcriptional activity. However, a dominant negative mutant of EFP lacking the RING domain prolonged the half-life of ERα and inhibited the transcription by ERα. Our results indicate that EFP functions as a cofactor for ERα-mediated transcription, thus suggesting that ERα-mediated transcription is closely linked to the ubiquitylation of ERα.
Introduction

Estrogen is a hormone that plays crucial roles in cell growth and differentiation and in regulation of the female reproductive system. The multifunctional effects of estrogen are elicited through interaction with the estrogen receptor (ER). ERα and ERβ belong to the steroid hormone superfamily of nuclear receptors, which act as ligand-dependent transcriptional factors [1]. Native unliganded ERα occurs in complex with the molecular chaperone Hsp90 and cochaperones, contributing to maintenance of the receptor stability. When bound to estrogen, ERα undergoes conformational changes, dissociations from heat shock proteins, a receptor dimerization, DNA bindings at the estrogen responsive element (ERE) of the target, interactions with coactivators, and the subsequent recruitment of transcription factors to be formed by either up-regulation or down-regulation of target gene transcription [2]. A number of coactivators of nuclear receptors have been identified, including members of the p160 family (SRC-1, TIF-2 and SRC-3), p300/CBP and Tip60, which exhibit histone acetyltransferase activities and recruit components of the general transcription machinery [3, 4].

Furthermore, the involvement of ubiquitin-proteasome components in ERα-mediated transcription has been suggested by the results of studies utilizing proteasome inhibitors [5]. Upon treatment of cells with 17β-estradiol, ubiquitylation of ERα is enhanced and the degradation of ERα by a proteasome-dependent proteolytic pathway is required for efficient ERα transactivation [6]. Ubiquitin conjugation is catalyzed by enzymes designated as ubiquitin-activating enzyme (E1),
ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). The resulting covalent
ubiquitin ligations form polyubiquitylated conjugates that are rapidly detected and
degraded by 26S proteasome. E3 has so far been shown to include members of the
HECT (homologous to E6-AP carboxyl terminus), RING-finger, and U-box families of
proteins [7-9].

Here we provide evidence that the estrogen-responsive finger protein (EFP)
promotes the ubiquitylation and proteasome-mediated degradation of liganded ERα. We
show that EFP binds to and ubiquitylates ERα to activate transcription, suggesting that
EFP may function as a cofactor for ERα-induced transcription.
Materials and methods

Cell culture. HEK293T and HeLa cells (ATCC) were cultured under an atmosphere of 5% CO₂ at 37°C in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum (Invitrogen). MCF-7 cells (ATCC) were cultured under the same conditions with 0.01 mg/ml insulin (Sigma).

Cloning of cDNAs and plasmid construction. Human EFP and ERα cDNAs were amplified from MCF-7 cDNA by the polymerase chain reaction (PCR) with KOD plus (TOYOBO) using primers 5’-GCCATGGGCAGCTGTGCCCCCTG-3’ (EFP-sense), 5’-TGCCTACCTTGGGGAGCAGATGGA-3’ (EFP-antisense), 5’-ATGACCATGACCCTCCACACAAAA-3’ (ERα-sense), and 5’-TCAGACTGTGGCAGGGAAACCCTC-3’ (ERα-antisense). The amplified fragments were subcloned into pBluescript II SK⁺ (Stratagene). The FLAG-tagged EFP cDNA and FLAG- or HA-tagged ERα cDNA were then subcloned into pCGN, pcDNA3 (Invitrogen), pFastBacHT (Invitrogen), pBTM116 and pACT2 (Clontech). Site-specific mutagenesis was performed with a Quick Change kit (Stratagene). Human Tip60 cDNA was obtained from Dr. Ikura (Tohoku University). The deleted mutants of ERα cDNA were generated by PCR.

Baculovirus expression system. The plasmids pFastBacHT containing the relevant cDNAs were subjected to recombination with the baculoviral genome in DH10BAC, and the resulting recombinant viral genome was introduced into Sf9 cells by transfection in order to generate recombinant baculoviruses.
Transfection, immunoprecipitation, and immunoblot analysis. HEK293T cells were transfected by the calcium phosphate method [9]. Immunoblot analysis was performed with the following primary antibodies: anti-c-Myc (1 μg/ml; 9E10, Covance), anti-FLAG (1 μg/ml; M2 or M5, Sigma), anti-HA (1 μg/ml; HA.11/16B12, Babco), anti-hexa-histidine (1 μg/ml; H-15, Santa Cruz), anti-Hsp90 (1 μg/ml; 68, TDL), anti-EFP (1 μg/ml; 2, TDL), anti-ubiquitin (1 μg/ml; P4D1, Santa Cruz) and anti-ERα (1 μg/ml; H-184, Santa Cruz).

In vitro pull-down assay. Recombinant His$_6$-EFP (0.5 μg) and recombinant His$_6$-ERα (0.5 μg) were mixed in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1% Nonidet P-40 for 1 h at 4°C. The binding mixtures were incubated with anti-EFP antibody for 1 h at 4°C. Protein G-Sepharose, which was equilibrated with the same solution, was added to the mixture and then rotated for 1 h at 4°C. The resin was separated by centrifugation, washed five times with lysis buffer, and then boiled in SDS sample buffer. Immunoblot analysis was performed with anti-ERα antibody.

β-galactosidase assay using yeast two-hybrid system. β-galactosidase assays were performed as previously described [10]. Incubations were allowed to proceed for 5 h at 30°C and then the OD$_{420}$ was taken to calculate the β-galactosidase activity using the following equation: 1000 × OD$_{420}$/ (t x V x OD$_{600}$), where t is the elapsed time (min) and V is 0.1 ml x concentration factor (concentration factor = 5 in this experiment).

In vitro ubiquitylation assay. An in vitro ubiquitylation assay was performed as previously described. Reaction mixtures were incubated for 2 h at 30°C and then subjected to immunoblot analysis with a mouse monoclonal antibody to ubiquitin or
ERα [11].

**Pulse-chase experiments.** Transfected HEK293T cells were metabolically labeled with Trans$^{35}$S (ICN, PerkinElmer) at a concentration of 100 mCi/ml for 1 h and then 17β-estradiol ($10^{-8}$ M, SIGMA) was added, and this was then chased. Cell lysates were immunoprecipitated with anti-HA antibody followed by purification with protein G-Sepharose, separated by SDS-PAGE, and exposed and quantified by BAS-2000 (Fuji Film, Kanagawa, Japan).

**Luciferase assay.** MCF-7 cells were cultured for 48 h in phenol red-free MEM containing dextran-coated charcoal-treated 10% FCS (Hyclone) before transfection. 17β-estradiol ($10^{-8}$ M) was added 48 h after transfection, and then after 12 h cells were assayed for luciferase activity with a Dual-Luciferase Reporter Assay System (Promega).
Results

Interaction of EFP with ERα

The Efp gene has been reported to have an ERE, and its mRNA is actually induced by estrogen treatment [12]. We investigated whether EFP physically interacts with ERα. We expressed FLAG-tagged EFP, together with/without hemagglutinin epitope (HA)-tagged ERα, in HEK293T cells. Cell lysates were subjected to immunoprecipitation with an antibody to FLAG, and the resulting precipitates were subjected to immunoblot analysis with an antibody to HA or FLAG. HA-ERα was co-precipitated by the antibody to FLAG in the presence of FLAG-EFP (Fig. 1A). To examine the interaction by an in vitro pull down assay, we generated and purified recombinant hexahistidine epitope (His₆)-tagged EFP and ER. The recombinant His₆-EFP was mixed with His₆-ERα and then immunoprecipitated with an antibody to EFP, and the resulting precipitates were subjected to immunoblot analysis with an antibody to ERα or EFP. His₆-ERα was selectively co-precipitated by the antibody to EFP in the presence of His₆-EFP (Fig. 1B). These results suggest that EFP specifically interacts with ERα. To quantify the affinity between EFP and ERα in the presence or absence of estrogen (17β-estradiol), we used a yeast two-hybrid system. EFP interacted weakly with ERα in a yeast two-hybrid assay in the absence of estrogen but interacted strongly with ERα in the presence of estrogen (Fig. 1C). Next we examined the ligand dependency of this interaction by using the mammalian cell line MCF-7 that
endogenously expressed ERα. It has been reported that 17β-estradiol causes proteasome-dependent degradation of ERα [13]. Thus, we investigated whether endogenous EFP interacts with endogenous ERα in MCF-7 cells in the presence of the proteasome inhibitor MG132. A small amount of ERα was detected in the immunoprecipitation performed with an anti-EFP antibody in the presence of 17β-estradiol but not in the immunoprecipitation with an anti-HA antibody as a negative control (Fig. 1D). The combination of 17β-estradiol and MG132 more greatly enhanced the endogenous interactions in vivo (Fig. 1D). These results suggest that estrogen contributes to the interaction between EFP and ERα in MCF-7 cells.

To identify the region of ERα responsible for the interaction with EFP, a series of ERα deletion derivatives were generated (Fig. 1E). Deletion of the N-terminal 296 amino acids (ERα(297-595)) did not affect the binding to EFP, whereas the deletion of C-terminal 255 amino acids (ERα(1-340)) abolished the binding to EFP. The region containing amino acids 297-534 of ERα, not comprising the coactivator binding region in AF-2, is required for the interaction with EFP (Fig. 1F).

*Ubiquitin ligase activity of EFP*

To determine whether EFP has an E3 ligase activity, we performed in vitro ubiquitylation assays. Immunoblot analysis of the reaction mixtures with antibodies to ubiquitin and EFP revealed the ubiquitylation of His$_6$-EFP only in the presence of E1, E2 (UbcH5C), ubiquitin, ATP, and EFP itself (Fig. 2A). The lack of any of these
components prevented self-ubiquitylation of EFP. EFP was thus shown to be a bona fide E3 ligase.

To further investigate the region of EFP responsible for ubiquitylation, a deletion mutant lacking the RING domain (ΔRING) and a RING domain mutant in which the first cysteine was substituted by alanine (C8A) were generated (Fig. 2B). *In vitro* ubiquitylation assays indicated that an intact RING domain is indispensable for E3 ligase activity of EFP (Fig. 2C).

To analyze the class of polyubiquitin chains supported by E3 ligase activity of EFP, three mutant ubiquitins in which lysine was substituted by arginine at position 48 and/or 63, namely UbK48R, UbK63R and UbK48/63R, were purified. According to *in vitro* ubiquitylation assays, EFP underwent polyubiquitylation with UbK63R but not with UbK48R or UbK48/63R, implying that EFP predominantly uses the lysine at position 48 on ubiquitin for the formation of a polyubiquitylation chain (Fig. 2D and E). To further confirm polyubiquitylation of EFP through K48-based chains, recombinant proteins of mutants UbK48 and UbK63, each having one lysine residue at positions 48 and 63, respectively, and all other lysines substituted by arginine, were generated. EFP mediated the polyubiquitylation by UbK48 but not by UbK63, indicating the lysine at position 48 on ubiquitin is sufficient for the formation of a polyubiquitylation chain by EFP (Fig. 2E).

*EFP ubiquitylates ERα in vitro and in vivo*
To determine whether EFP ubiquitylates ERα, we assayed E3 ligase activity of His6-EFP against His6-ERα. Polyubiquitylation of His6-ERα was observed only in the additional presence of the His6-EFP (Fig. 3A). However, polyubiquitylation of ERα was not detected in the presence of EFP(C8A) (Fig. 3B). To further investigate the ubiquitylation of ERα in cells by in vivo ubiquitylation assay, FLAG-ERα, EFP and HA-ubiquitin were expressed in HEK293T cells. FLAG-ERα was ubiquitylated extensively when cotransfected with EFP (Fig. 3C).

To examine the possible effect of EFP on the degradation of ERα in vivo, we transfected HEK293T cells with vectors for HA-ERα and for FLAG-EFP or FLAG-EFP(ΔRING) as a dominant negative form. Pulse-chase analysis in the presence of estrogen revealed that the expression of EFP exhibits the degradation of ERα, whereas overexpression of EFP(ΔRING) delays the degradation of ERα (Fig. 3D and E). These results thus suggest that EFP contributes to the turnover of ERα in cells.

_EFP enhances activation of transcription by ERα_

To examine the effect of EFP on ERα-induced transcription in MCF-7 cells, we measured the relative luciferase activity of MCF-7 cells transfected with an _ERE_ reporter construct with basal HSV-thymidine kinase gene promoter (pTK-ERE-Luc). The relative luciferase activity of cells with the addition of 17β-estradiol or with the overexpression of EFP was increased compared with that of mock-transfected cells. The combination of the addition of 17β-estradiol with the overexpression of EFP resulted in
a further increase of luciferase activity, whereas EFP(ΔRING) inhibited the activity even in the presence of 17β-estradiol, suggesting that EFP acts as a positive regulator for transcriptional activation by ERα (Fig. 4A).

Transcriptional regulation by ERα involves protein-protein interactions among the receptor and its associated coactivators, including Tip60. It is known that interaction between ERα and coactivators enhances ERα-mediated transcriptional initiation. To explore the interdependence between EFP and coactivators in activation of the ERE promoter, we examined the effect of EFP on Tip60-induced transcription in MCF-7 cells. Overexpression of Tip60 activated the ERE promoter in the presence of 17β-estradiol, and the combination of EFP and Tip60 resulted in greater activation of the ERE promoter (Fig. 4B). Similar results were obtained for another coactivator, p300 (data not shown). These findings suggest that EFP assists the ERα-mediated transcription together with coactivators.

To clarify the relationship between the transcriptional activity and the ubiquitylation, we examined the level of physical interaction between ERα and coactivators, especially Tip60. Physical interaction between ERα and Tip60 was detectable by coimmunoprecipitation in the presence of 17β-estradiol (Fig. 4C). We thus expressed Myc-EFP(WT/C8A), HA-ERα and FLAG-Tip60 in HEK293T cells, followed by immunoprecipitation with an antibody to FLAG and detection with an anti-HA antibody. The interaction of ERα with Tip60 was more greatly enhanced in cells expressing EFP(WT), whereas the interaction was reduced in cells expressing the dominant negative EFP(C8A), indicating that EFP is likely to promote the interaction between
ER$\alpha$ and Tip60 (Fig. 4D).
Discussion

Here we have demonstrated that EFP directly interacts with ERα in an estrogen-dependent manner and ubiquitylates ERα and that the ubiquitylation by EFP regulates the transcription of ERα including the interaction with coactivators, followed by the degradation of ERα. Previous studies indicated that the inhibition of proteasomal degradation markedly increased estrogen-dependent transcription on an ERE-responsive reporter in HeLa cells transfected with ERα. Blocking ligand-induced degradation of ERα by proteasome inhibitors resulted in prolonged simulation of ER-responsive gene transcription [14]. These observations suggest that proteasomal degradation of ERα leads to attenuation of transactivation but do not rule out the possibility of ubiquitylation to initiate the transcription of ERα. We have shown that EFP functions as a cofactor by recruiting coactivators that have histone acetyltransferase activities, such as Tip60. Previous studies have shown that transcriptional regulation by ERα involves interactions among the receptor, coactivators and RNA polymerase II. Tip60 is one of the nuclear receptor coactivators and enhances transactivation though liganded ERα in collaboration with other coactivators, p300/CBP [15]. It has been reported that Skp2 binds to c-Myc and thereby mediates its ubiquitylation. Skp2 also increases the transcriptional activity of c-Myc, suggesting that Skp2 is a transcriptional cofactor [16]. Moreover, it has been shown that HectH9-mediated ubiquitylation of Myc is required for transactivation of multiple target genes by recruitment of the coactivator p300 [17]. EFP-mediated ERα ubiquitylation may be crucial for recruitment of the transcriptional...
cofactors as well.

It has been shown that Efp-/- mice of both sexes were viable and fertile and that the uterus, which expressed a high level of ERα in wild-type mice, exhibited significant underdevelopment [18]. We have demonstrated that the transcriptional activation of ERα is enhanced by EFP. Therefore, these phenotypes of Efp-/- mice can be explained by the balance of interaction between EFP and ERα.

Recently, another E3 ligase, CHIP, has been reported to induce ubiquitylation of unliganded ERα, and CHIP probably degrades unfolded and misfolded ERα to maintain quality control of ERα in the cytoplasm [19]. The ubiquitylation of ERα by CHIP is independent of estrogen and serves for the degradation of misfolded ERα. EFP ubiquitylates ERα dependent on estrogen to up-regulate its transcriptional activity. These findings demonstrate that the role of EFP is different from that of CHIP with regards to the ubiquitylation of ERα.

In breast and endometrial carcinomas which are classified as estrogen-responsive malignant tumors, estrogen is one of the significant factors with regard to anti-tumor therapies and their prognoses. Our findings suggest that the expression levels of EFP are correlated with the histological grade of an estrogen-responsive tumor, and results of large-scale clinicopathological analyses of EFP should contribute to the clinical knowledge of breast, ovarian or endometrial carcinomas.
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References


[15] T. Ikura, V.V. Ogryzko, M. Grigoriev, R. Groisman, J. Wang, M. Horikoshi, R. Scully, J. Qin, Y. Nakatani, Involvement of the TIP60 histone acetylase complex in


**Figure Legends**

Fig. 1. EFP binds to ERα in vivo and in vitro. (A) In vivo binding assay between EFP and ERα. FLAG-EFP and HA-ERα expression vectors were transfected into HEK293T cells. Cell lysates (WCL) were subjected to immunoprecipitation (IP) with antibodies to FLAG, immunoblotted (IB), and probed with antibodies to HA or FLAG. (B) In vitro pull-down assay between EFP and ERα. Recombinant His₆-EFP and His₆-ERα were mixed in the combination as indicated. The reaction mixture was immunoprecipitated with anti-EFP antibody and immunoblotted with anti-ERα. (C) L40 yeast cells were cotransfected with pBTM116-EFP and pACT2-ERα, and then three independent clones were selected, grown with 17β-estradiol (10⁻⁷ M) or with ethanol as a mock, and harvested. The β-galactosidase activities of cell lysates were calculated in ONPG assays. (D) Interaction between endogenous EFP and ERα in MCF-7 cells. Cells were incubated with 17β-estradiol (10⁻⁸ M) and/or MG132 (10⁻⁵ M) for 3 h, and the cell lysates were immunoprecipitated with anti-EFP antibody or with anti-HA antibody as a control and then the resulting precipitates were subjected to immunoblot analysis with antibodies to EFP and ERα. (E) Schematic representation of ERα deletion mutants. A/B, N-terminal region including activation function 1; C, a conserved DNA binding domain, D; a hinge region containing nuclear localization signal; E/F, C-terminal region including activation function 2. (F) The region of ERα which interacts with EFP. HEK293T cells were transfected with vectors for HA-EFP, and either full-length ERα or deleted mutants tagged with the FLAG epitope. Cell lysates were
immunoprecipitated with anti-FLAG antibody, and then immunoblotted with anti-HA antibody.

Fig. 2. Ubiquitin ligase activity of EFP. (A) *In vitro* ubiquitylation assays were performed and the reaction mixtures were subjected to immunoblot analysis with antibodies to ubiquitin (upper panel) or to EFP (lower panel). (B) Schematic representation of the domain structures of EFP(WT), EFP(ΔRING) and EFP(C8A). (C) Requirement of the RING domain in EFP for E3 ligase activity. Equimolar amounts of the indicated EFP derivative (WT, C8A or ΔRING) proteins were assayed for ubiquitylation activity in the presence of E1 and E2 (UbcH5C). (D) Specificity of lysine residue on ubiquitin for polyubiquitylation by EFP. Recombinant wild-type and mutant ubiquitins (UbK48R, UbK63R) were used for *in vitro* ubiquitylation assays. (E) Lysine 48 on ubiquitin is sufficient for polyubiquitylation by EFP. Recombinant wild-type and mutant ubiquitins (UbK48/63R, UbK48 and UbK63) were used for *in vitro* ubiquitylation assays.

Fig. 3. EFP mediates the ubiquitylation of ERα *in vitro* and *in vivo*. (A) *In vitro* ubiquitylation assay for ERα by EFP. The reaction mixture was also subjected to immunoblot analysis with antibodies to ERα (upper panel) or to EFP (lower panel). (B) Requirement of the RING domain of EFP for ERα. EFP derivatives (WT and C8A) were assayed for the polyubiquitylation of ERα by *in vitro* ubiquitylation assays. (C) *In vivo* ubiquitylation assay for ERα with EFP. Expression vectors for EFP, FLAG-ERα
and HA-Ub were transfected into HEK293T cells, and the cell lysates were immunoprecipitated with anti-FLAG antibody and then anti-HA immunoblot analysis was performed to detect the ubiquitylation of ERα. (D) Pulse chase analysis of ERα with EFP. HEK293T cells that had been transfected with an expression plasmid encoding HA-ERα and either FLAG-EFP or FLAG-EFP(ΔRING) were radiolabeled with [35S]-methionine, immunoprecipitated with anti-HA antibody, and then subjected to SDS-PAGE and autoradiographed. (E) Quantification of the autoradiogram in Fig. 3D was performed by BAS-2000, and the amount of HA-ERα at 0 h was defined as 100%.

Fig. 4. EFP enhances ERα-mediated transcriptional activation. (A) MCF-7 cells were transfected with pTK-ERE-Luc, pRL-TK, plus FLAG-EFP(WT or ΔRING) with 10⁻⁸ M 17β-estradiol or mock (ethanol). The luciferase activities represent the average of three independent transfections normalized with the internal control. (B) MCF-7 cells expressing FLAG-EFP or mock were transfected with pTK-ERE-Luc, pRL-TK, plus HA-Tip60 as indicated with 10⁻⁸ M 17β-estradiol or mock (ethanol), and then the luciferase activities were measured. (C) FLAG-Tip60 and HA-ERα expression vectors were transfected into HEK293T cells. Cells incubated with or without 10⁻⁸ M 17β-estradiol for 6 h. Cell lysates were subjected to immunoprecipitation with antibodies to FLAG, immunoblotted, and probed with antibodies to HA or FLAG. (D) Expression vectors encoding FLAG-Tip60, HA-ERα and Myc-EFP(WT or C8A) were transfected into HEK293T cells, and then the cells were incubated with 10⁻⁸ M
17β-estradiol for 6 h. Cell lysates were subjected to immunoprecipitation with an antibody to FLAG, immunoblotted, and probed with antibodies to HA or FLAG.
Nakajima, Fig. 2
A

B

C

D

Nakajima, Fig. 4