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Title: **DUSP22 / LMW-DSP2 regulates estrogen receptor α-mediated signaling through dephosphorylation of Ser-118**

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Running title: Interactions between ERα and DUSP22 / LMW-DSP2

Key words: β-estradiol; estrogen receptor; phosphatase; transcriptional regulation; breast cancer

Abstract

In the previous study, we demonstrated the involvement of dual specificity phosphatase 22 (DUSP22 / LMW-DSP2) in regulating the LIF/IL-6/STAT3-mediated signaling pathway. In this study, we show β-estradiol (E2)-induced DUSP22 mRNA expression in estrogen receptor α (ERα)-positive breast cancer cells, while E2-induced phosphorylation and activation of ERα was suppressed by overexpression of DUSP22 but not catalytically inactive mutants. Furthermore, small-interfering RNA-mediated reduction of DUSP22 expression enhanced ERα-mediated transcription and endogenous gene expression. In fact, DUSP22 associated with ERα *in vivo* and both endogenous proteins interacted in ERα-positive breast cancer T47D cells. These results strongly suggest that DUSP22 acts as a negative regulator of the ERα-mediated signaling pathway.

Introduction

Estrogen receptor α (ERα) is a ligand-activated transcriptional factor that is a member of the nuclear receptor superfamily (Mangelsdorf et al., 1995). Estrogens play important roles in the differentiation and development of various organs, maintenance of proper cellular function in a wide variety of tissues, and are also risk factors for breast and endometrial cancer (Couse and Korach. 1999). The ERα interacts with estrogen response elements (EREs) in the target gene promoters and directly regulates their transcription (Mangelsdorf et al., 1995). The ERα has two well characterized transcriptional activation functions (AFs): AF-1, which is located in the N-terminal A/B region and may be activated in a ligand-independent manner, and AF-2, which is located in region E and whose activity is ligand-dependent. AF-1 and AF-2 can activate transcription independently and synergistically act in a promoter- and cell type-specific manner (see Figure 4, Mangelsdorf et al., 1995, Tora et al., 1989).

The ERα, like other members of the steroid hormone receptor superfamily, is phosphorylated on multiple serine residues (Castano et al., 1998, Lannigan, 2003). Serine phosphorylation is essential for transcriptional activation in response to α-

estradiol (E2) binding and occurs on Ser-118 and Ser-167. Ser-118 is shown to be the major phosphorylation site required for full activation of ERα and to a lesser extent on Ser-104 and Ser-106, all located in the AF-1 domain of the receptor (Lannigan, 2003). TFIIH kinase Cdk7 (Chen et al., 2000), mitogen-activated protein kinase (MAPK) (Kato et al., 1995) and glycogen synthethase kinase-3 (GSK-3) (Medunjanin et al., 2005) have been reported to phosphorylate Ser-118, and cyclin A-Cdk2 was associated with Ser-104 and -106 phosphorylation (Rogatzki et al., 1999). The molecular mechanisms by which the phosphorylation of ERα is regulated still remain to be clarified. Although protein phosphatase 2A (PP2A) and PP5 have been recently shown to associate with ERα directly and inhibit its transcriptional activity (Lu et al., 2003, Ikeda et al., 2004).

Dual specificity phosphatases (DSPs) / MAP kinase phosphatases (MKPs) are known to regulate MAP kinases-mediated signaling pathways, including ERK, JNK and p38 MAPK (Theodosiou A and Ashworth A. 2002). In previous studies, we cloned a distinct class of DSPs with low molecular weight (LMW-DSP1, -DSP2, -DSP4, -DSP6, -DSP10 and DSP11) (Aoyama et al., 2001, Aoki et al., 2001), which contained a single catalytic domain but lacked a putative common docking site for MAPKs, the cdc25 homology domain. The first LMW-DSP to be cloned was the VH1 protein from *Vaccinia* virus (Guan et al., 1991). A related phosphatase was cloned in mammalian cells and termed VHR, for VH 1-related (Ishibashi et al., 1992). Both VH1 and VHR differ from other DSPs in that they are much smaller, only 19 and 21 kDa, respectively. VH1 has been reported to dephosphorylate both MAP kinases and STAT1 (Najarro et al.,

2001), while VHR seems to be specific for ERK and JNK (Denu and Dixon, 1995, Alonso et al., 2001). LMW-DSP-2 dephosphorylated and deactivated p38 MAPK and JNK, but not ERK (Aoyama et al., 2001). LMW-DSP2 is also designated as VHX (Alonso et al., 2002) and JSP1 (Shen et al., 2001), JKAP (Chen et al., 2002). JSP1 and JKAP has been shown to positively regulate JNK activation. These results indicate that LMW-DSP2 may play a regulatory role in the JNK or p38 MAPK pathways. This gene is now designated as DUSP22. DUSP22 has been also demonstrated to belong to a new subgroup of small DSPs anchored at the membranes by an N-terminal myristic acid moiety (Alonso et al., 2004). However, the physiological function of LMW-DSPs has remained unclear as it seems to be less efficient than many other MAPK-specific DSPs. Interestingly, DUSP22 has been shown to regulate T cell antigen receptor signaling through ERK2 (Alonso et al., 2002). Recently, we have also demonstrated that DUSP22 regulates interleukin 6 (IL-6)-/leukemia inhibitory factor (LIF)-mediated signaling by dephosphorylating STAT3 (Sekine et al., 2006).

We show here that ERα is a substrate of DUSP22, being dephosphorylated at Ser-118 through a direct interaction with DUSP22. This suggests an important function of DUSP22 in the regulation of ERα-dependent transcription in breast cancer cells by negative feedback.

Results

DUSP22 mRNA expression is induced in breast cancer cells in an ERα-dependent manner

Recently, we have demonstrated that DUSP22 / LMW-DSP-2 regulates interleukin 6 (IL-6)-/leukemia inhibitory factor (LIF)-mediated signaling by dephosphorylating STAT3 (Sekine et al., 2006). In our previous study, we have shown that E2 suppressed IL-6-induced STAT3 activation and STAT3-mediated gene expression through estrogen receptors in breast cancer cells (Yamamoto et al., 2000). These facts let us to examine whether DUSP22 expression is induced by E2 breast cancer cells and participates in the E2-mediated suppression of STAT3 activation. Furthermore activation of ERα is also tightly regulated by its phosphorylation status as well as STAT3 (Tora et al., 1989, Castano et al., 1998), indicating the possibility that DUSP22 may be involved in the regulation of ERα-mediated signaling.

To investigate the involvement of DUSP22 in ERα-mediated signaling, we first examined whether DUSP22 expression was regulated by estrogen in human breast

cancer cell lines using quantitative real-time PCR. As shown in Figure 1a and b, a significant induction of DUSP22 mRNA expression was observed in ERα-positive breast cancer MCF-7 and T47D cell lines. ERα-mediated cathepsin D mRNA expression was also observed in these cells. However, any significant induction of DUSP22 as well as cathepsin D mRNA expression was not observed in the ERα-negative HBL100 cell line (Figure 1c). We attempted to examine the changes of protein level of DUSP22 by E2 stimulation. Unfortunately, we could not detect alteration of the endogenouos protein level of DUSP22 in the total lysates of these cells, because of the low detection sensitivity of anti-DUSP22 antibody immunoblotting. These results indicate that expression of DUSP22 mRNA is induced in breast cancer cells in an ERα-dependent manner.

DUSP22 regulates ERα-mediated transcriptional activation

To examine the effect of DUSP22 on ERα-induced transcriptional activation, a transient transfection assay was used. The ERα-mediated transcriptional responses were measured by using the reporter construct Vit-LUC in which two copies of an

estrogen response element (ERE) drive expression of the LUC gene (Matsuda et al., 2001). 293T or HeLa cells were transfected with Vit-LUC together with ERα and treated with E2, and LUC activity was determined. When cells were co-transfected with DUSP22, transcriptional activation of Vit-LUC decreased in a dose-dependent manner compared with that of mock transfectants (Figure 2a and b). We further examined whether DSP activity of DUSP22 was necessary for suppression of ERα activation. Two amino acids in DUSP22, namely Asp (D)-57 and Cys (C)-88, have previously been shown to participate in the catalytic mechanism of DSP activity (Aoyama et al., 2001). Wild-type (WT) or catalytically inactive Asp/Ala (D/A) or Cys/Ser (C/S) forms of DUSP22 together with ERα and Vit-LUC were co-transfected into 293T cells. A decrease of ERα activation was not observed when cells were co-transfected with DUSP22 D/A or C/S mutant (Figure 2a and b), suggesting that DSP activity of DUSP22 is essential for deactivation of ERα. To examine the effect of DUSP22 on the ERα-mediated transcriptional activation under more physiological conditions, we transfected the DUSP22 WT, D/A or C/S mutant together with Vit-LUC into MCF-7 cells and treated the cells with E2. As shown in Figure 2c, E2-mediated

ERα activation was markedly decreased by expression of DUSP22 WT but not by the D/A or C/S mutant in MCF-7 cells, indicating that DUSP22 negatively regulates ERα-mediated transcription through its DSP activity. Similarly, we also tested the effects of expression of another DUSP, DUSP19 / LMW-DSP1 / SKRP1 (Aoki et al., 2001, Zama et al., 2002) , which has been shown to regulate JNK activation (Zama et al., 2002) on E2-induced ERα activation in 293T cells (data not shown). However, we could not observe any significant alteration of ERα activation by expression of DUSP19, suggesting that DUSP22 specifically acts ERα-mediated signaling. Importantly, DUSP22 has been shown to be modified by the attachment of a myristate to the N-terminal Gly-2 (Alonso et al., 2004). Therefore, N-terminally tagged DUSP22 constructs lacks myristoylation of Gly-2 (Alonso et al., 2002). We then tested both N-terminal or C-terminal tagged DUSP22 constructs on ERα-mediated transcription. However, expression of either N-terminal or C-terminal tagged construct similarly suppressed ERα-mediated transactivation (data not shown), suggesting that myristoylation of DUSP22 has no effect on the DUSP22-mediated suppression of ERα activation.

Reduction of endogenous DUSP22 enhances ERα-mediated transcriptional activation in breast cancer cells

To further explore whether DUSP22 represses ERα-mediated transcriptional activation, we used small interfering RNA (siRNA) to reduce endogenous expression of DUSP22 in HeLa or MCF-7 cells. A specific siRNA for DUSP22 or a control siRNA was transfected into ERα-transfected HeLa or MCF-7 cells. Total RNA isolated from the transfected cells was subjected to quantitative real-time PCR analysis, which confirmed a reduction of DUSP22 mRNA expression. As shown in Figure 3a and b, approximately 70% reduction in HeLa cells or 60% reduction in MCF-7 cells of the DUSP22 mRNA expression was observed following DUSP22 siRNA expression. We then determined the effects of DUSP22 siRNAs on ERα-induced Vit-LUC activation in these cells. As shown in Figure 3c and d, siRNA-mediated reduced expression of DUSP22 also resulted in a significant enhancement of ERα-induced Vit-LUC activation in these cells. We next examined the effects of DUSP22 siRNA on other steroid receptor signalings such as androgen receptor (AR) and glucocorticoid receptor (GR).

The AR-mediated transcriptional responses were measured by using the androgen dependent reporter construct –285PB-LUC (Matsuda et al., 2001). HeLa cells were transfected with control or DUSP22 siRNA together with –285PB-LUC and AR, and treated with dihydrotestosterone (DHT), and LUC activity was determined. Interestingly, siRNA-mediated reduced expression of DUSP22 resulted in a significant reduction of DHT-induced –285PB-LUC activation in HeLa cells (Figure 3e), suggesting that DUSP22 may act AR-mediated signaling positively. We also tested the effect of DUSP22 siRNA on GR-mediated signaling in HeLa cells. The GR-mediated transcriptional responses were measured by using the reporter construct GRE-LUC (Yamamoto et al., 2000). HeLa cells were transfected with control or DUSP22 siRNA together with GRE-LUC and treated with dexamethasone (DEX), and LUC activity was determined. As shown in Figure 3f, siRNA-mediated reduced expression of DUSP22 resulted in no alteration of DEX-induced GRE-LUC activation in HeLa cells, indicating that DUSP22 has no effect on GR-mediated signaling. We further examined the effects of DUSP22 siRNA on ERα-mediated cathepsin D mRNA expression in MCF-7 cells. As shown in Figure 4a, siRNA-mediated reduced expression of DUSP22 resulted

in a significant enhancement of E2-induced cathepsin D mRNA expression in MCF-7 cells. These results strongly suggest that DUSP22 regulates ERα-mediated transcriptional activation in HeLa and MCF-7 cells. Because DUSP22 affects ERα-mediated transcriptional activation, it seemed plausible that DUSP22 might alter ERα-mediated transcription by influencing the ability of ERα to bind to DNA. We then examined the effects of DUSP22 siRNA on the ERα DNA binding activity to ERE. To this end, we performed DNA pulldown assays using immobilized ERα Consensus Oligonucleotide. As shown in Figure 4b, after E2 stimulation, the ERα-DNA complex formation was observed in MCF-7 cells. Furthermore, this complex formation was enhanced by siRNA-mediated reduced expression of DUSP22, suggesting that DUSP22 negatively regulates ERα-DNA complex formation.

DUSP22 physically interacts with ERα

One of the mechanisms that would be consistent with the data described above is a direct interaction with ERα by DUSP22, which would trigger its deactivation. First this possibility was tested in co-immunoprecipitation experiments. Expression vectors

encoding FLAG-tagged ERα and Myc-tagged DUSP22 or its inactive mutant D/A or C/S, were transiently transfected into 293T cells. The transfected 293T cells were lysed and subjected to immunoprecipitation with anti-FLAG antibody. Immunoprecipitates were then used in Western blot analysis with anti-Myc antibody. As shown in Figure 5a, ERα interacted with DUSP22 WT and C/S. However, DUSP22 D/A showed a very weak binding potential to ERα, suggesting that the DUSP22 binding site is in close proximity to Asp-57. These results also indicate that the interaction between ERα and DUSP22 is not required for its DSP activity.

To further confirm that endogenous DUSP22 interacts with ERα *in vivo*, co-immunoprecipitation experiments were performed using cell extracts obtained from T47D cells, in which both proteins could be detected using specific antibodies. As shown in Figure 5b, anti-DUSP22 immunoprecipitates from cell extracts of T47D cells contained ERα protein. This result suggests that endogenous DUSP22 interacts and forms a complex with ERα in T47D cells.

To further delineate the domains in ERα that mediate protein-protein interactions between ERα and DUSP22, co-immunoprecipitation experiments were performed with

a series of mutant ERα proteins (Figure 5c). As shown in Figure 5d, the AF-1 domain of ERα (12-175) did not interact with DUSP22. Both the DNA binding (176-395) and the AF-2 domain of ERα (396-595) interacted with DUSP22, although the DNA binding domain of ERα (176-395) showed a weak interaction with DUSP22. These data suggest that the C-terminal region containing the DNA binding and AF-2 domain is required for ERα to interact with DUSP22.

DUSP22 dephosphorylates ERα Ser-118

It is well known that phosphorylation of ERα modulates the activity of both AF-1 and AF-2 (Tora et al., 1989, Castano et al., 1998). Among the sites of potential phosphorylation, Ser-118 in the ERα AF-1 domain has been particularly intensively studied with regard to the state of its phosphorylation and consecutive potentiation of ERα activity. Thus, the effect of DUSP22 on ERα Ser-118 phosphorylation was examined. First, we examined whether E2 stimulation induces ERα Ser-118 phosphorylation and complex formation between ERα and DUSP22 in 293T cells. Expression vectors for ERα together with or without DUSP22 WT were transfected into

293T cells and treated with E2. The transfected 293T cells were lysed and subjected to immunoprecipitation with anti-FLAG antibody. Immunoprecipitates were then used in Western blot analysis with anti-Myc antibody or anti-phospho-ERα Ser-118. As shown in Figure 6a, ERα strongly interacted with DUSP22 WT in the presence of E2, whereas E2-induced phosphorylation of ERα Ser-118 decreased by expression of DUSP22 WT. We further confirmed the effect of DUSP22 on E2-induced ERα Ser-118 phosphorylation. As shown in Figure 6b, E2-induced ERα Ser-118 phosphorylation decreased by expression of DUSP22 in a dose-dependent manner. DUSP22 WT, D/A or C/S mutant was also co-transfected with FLAG-tagged ERα into 293T cells and treated with E2. Upon co-expression of LMW-DSP WT, E2-induced ERα Ser-118 phosphorylation remarkably decreased, compared with mock, DUSP22 D/A and C/S transfectants (Figure 6c). Comparable expression of DUSP22 WT, D/A and C/S mutant was confirmed by immunoblotting with anti-Myc antibody. These results indicate that DUSP22 dephosphorylates ERα Ser-118 and negatively regulates E2-induced ERα transcriptional activation. We next examined the effect of DUSP22 siRNA on E2-induced phosphorylation of ERα Ser-118 in MCF-7 cells. As shown in

Figure 7a, DUSP22 siRNA treatment significantly enhanced E2-induced phosphorylation of ERα Ser-118. These results indicate that endogenous DUSP22 dephosphorylates ERα and negatively regulates E2-mediated ERα transcriptional activation in MCF-7 cells. To further confirm the dephosphorylation action of DUSP22 on ERα, recombinant GST fusion proteins containing full-length DUSP22 WT were expressed in *Escherichia coli* and purified. GST-DUSP22 WT exhibited catalytic activity against an artificial substrate, *p*-nitrophenylphosphate (Aoyama et al., 2001, data not shown). 293T cells that had been co-transfected with ERα were treated with E2, and the Ser-118 phosphorylated ERα was immunoprecipitated. The indicated amounts of the recombinant GST or GST-DUSP22 WT fusion proteins were added to the immune complexes and incubated. As shown in Figure 7b, the Ser-118 phosphorylation level of ERα was clearly reduced by GST-DUSP22 WT. Incubation of the immune complexes with empty GST resulted in no reduction in Ser-118 phosphorylation levels of ERα.

Discussion

Estrogen receptors are transcription factors that regulate gene expression by both estrogen-dependent and estrogen-independent pathways (Mangelsdorf et al.,1995). Kinase-mediated ER phosphorylation contributes to transcriptional activation of the ER by both these pathways (Couse and Korach. 1999, Tora et al., 1989). However, the roles of phosphatases in the regulation of ER-mediated signaling pathway are less well defined. PP2A associates with ERα inhibiting phosphorylation of Ser-118, a site important for ligand-independent activation of ERα (Lu et al., 2003). The Ser/Thr phosphatase PP5 binds to and suppresses ER-mediated transcriptional activation (Ikeda et al., 2004). Although the phosphatase Cdc25B stimulates ERα activity, the stimulation is independent of the phosphatase activity of Cdc25B (Ma et al., 2001). Protein phosphatase magnesium-dependent 1d also stimulates ERα activity through an indirect interaction (Proia et al., 2006). We here show the first example of an estrogen-induced DSP whose activity is required for optimal regulation of ERα-mediated transcription. Our study shows not only that overexpression of DUSP22 reduces ERα activity. Reduction of endogenous DUSP22 expression in MCF-7 breast cancer cells enhances

endogenous ERα transcriptional activity by influencing DNA binding activity of ERα. Furthermore, DUSP22 directly interacts with ERα *in vivo* and dephosphorylates ERα Ser-118. These results strongly indicate that DUSP22 plays an important in the ERα-mediated transcription through a negative feedback loop in breast cancer cells.

DUSP22 is abundantly expressed in testis (Aoyama et al., 2001, Aoki et al., 2001). Recently, we have shown that DUSP22 regulates IL-6-/LIF-mediated transcription by dephosphorylating STAT3 (Sekine et al., 2006). In mammals, STAT3 is expressed in developing acrosomes of round spermatids in the adult testis (Murphy et al., 2005). In testes, the addition of LIF to cocultures enhances the *in vitro* survival of both primary Sertoli cells and proliferating gonocytes without affecting cell proliferation (Jenab and Morris, 1998). IL-6 and IFN-γ activate STAT3 and induce c-*fos* gene expression in Sertoli cells (Jenab and Morris, 1997). These findings indicate that DUSP22 modulates STAT3-mediated signaling in testis. Estrogen is also thought to have a regulatory role in the testis because estrogen biosynthesis occurs in testicular cells and the absence of ERα caused adverse effects on spermatogenesis and steroidogenesis (Couse et al.,2001, Carreau et al., 2002). Moreover, several chemicals that are present in the environment,

designated xenoestrogens, because they have the ability to bind and activate ERβ, are known to affect testicular gene expression (Safe, 2004). We have previously demonstrated that estrogen suppresses IL-6-/LIF-mediated transcription through a direct interaction between ERβ and STAT3 (Yamamoto et al., 2000). The ERβ-dependent protein inhibitor of activated STAT3 (PIAS3) induction is also involved in ERβ-dependent repression of STAT3 transcriptional activation (Wang et al., 2001). Our present study suggests that ERβ-dependent DUSP22 induction may participate in ERβ-mediated repression of STAT3 transcriptional activation. Therefore, estrogens regulate spermatogenesis and steroidogenesis in testis by modulating STAT3 activity via a direct interaction with ERβ and DUSP22 induction. Furthermore, their activities are also regulated by DUSP22 through a direct interaction with ERβ. In this study, we also proposed the possibility that DUSP22 may positively regulate AR-mediated signaling. This finding may support an important role in spermatogenesis and steroidogenesis in testis by modulating STAT3 activity. Because androgens has been also shown to modulate STAT3 activation (Matsuda et al., 2001).

DUSP22 has been originally shown to regulate JNK activation (Aoyama et al., 2001,

Shen et al., 2001, Chen et al., 2002). E2 is also reported to induce apoptosis through JNK activation in ER-positive MCF-7 cells (Altiok et al., 2006). Although we could not detect any activation of JNK using anti-phosho-JNK antibody, our siRNA experiments revealed that siRNA-mediated reduced expression of DUSP22 enhanced E2-induced cell growth in MCF-7 cells (data not shown). This result suggests that DUSP22 may also regulate JNK signaling pathway in breast cancer cells.

In summary, we demonstrated that DUSP22 is an important regulator of ERα function and thereby may have critical roles in spermatogenesis and the progression of diseases, such as breast cancer or prostate disease. More detailed understanding of the interaction between ERα and DUSP22 is therefore important as this new information may provide new therapeutic approaches for these and other pathological conditions.

Materials and methods

Reagents and antibodies-17β-estradiol (E2), dihydrotestosterone (DHT) and dexamethasone (DEX) was purchased from Wako Chemicals (Osaka, Japan). Expression vectors, ERβ (HEGO), AR, Vit-LUC, -285PB-LUC, GRE-LUC were kindly provided by Dr. P. Chambon (Institut de Chimie Biologique, Strasbourg, France) Dr. F. Saatcioglu (Univ. Oslo, Oslo, Norway). Epitope-tagged ERβ and its mutants were described previously (Matsuda et al., 2001, Yamamoto et al., 2002). Epitope-tagged wild-type DUSP22 (DUSP22 WT) and its D/A or C/S mutant were described previously (Aoyama et al., 2001, Aoki et al., 2001). Anti-Myc, anti-GST, anti-ERβ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG M2 monoclonal antibody and rabbit polyclonal anti-FLAG antibody were purchased from Sigma (St Louis, MO). Anti-phospho-ERβ (pERβ) Ser118 was purchased from Cell Signaling Technology (Beverly, MA). Anti-DUSP22 antibody was described previously (Aoyama et al., 2001).

Cell culture, transfection, and luciferase assays - Human breast cancer cell line, MCF-7, T47D and HBL100 were kindly provided from Cell Resource Center for Biomedical Research (Tohoku Univ., Sendai, Japan) maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Before stimulation, the cells were cultured for 24 h in phenolred free DMEM containing 10% charcoal-stripped FCS followed by treatment with E2 (Matsuda et al., 2001). Human cervix carcinoma

cell line, HeLa, and MCF-7 was maintained in DMEM containing 10 % FCS (Matsuda et al., 2001, Sekine et al., 2006) and transfected with Vit-LUV together with or without ERβ using jetPEI (PolyPlus-transfection, Strasbourg, France) according to the manufacturer's instructions. Before stimulation, the cells were cultured for 24 h in phenol red free DMEM containing 10% charcoal-stripped FCS followed by treatment with E2. The cells were harvested and assayed for the luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Human embryonic kidney carcinoma cell line, 293T, was maintained in DMEM containing 10% FCS and transfected by the standard calcium precipitation protocol (Sekine et al., 2004). The cells were harvested 36 hrs after transfection and lysed in 50 μl of Reporter Lysis Buffer (Promega) and assayed for luciferase and β-galactosidase activities according to the manufacturer's instructions. Luciferase activities were normalized to the β-galactosidase activities. Three or more independent experiments were carried out for each assay.

Transfection of Small Interfering RNA (siRNA) - siRNAs targeting DUSP22 and control siRNA used in this study was described previously (Sekine et al., 2006). HeLa or MCF-7 cells were plated on a 24-well plate at 2 x 10^4 cells/well, and then incubated with an siRNA-Lipofectamine 2000 (Invitrogen, Carlsbad, CA) mixture at 37 °C for 4 hrs, followed by addition of fresh medium containing 10% charcoal-stripped FCS. The cells were then transfected with or without Vit-LUC, -285PB-LUC or GRE-LUC together with or without ERβ or AR using jetPEI as described the above. Twenty-four

hrs after transfection, the cells were treated with the indicated concentration of steroid hormone for the indicated periods.

Immunoprecipitation and immunoblotting - The immunoprecipitation and Western blotting assays were performed as described previously (Sekine et al., 2005). The cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml each of aprotinin, pepstatin and leupeptin). The immunoprecipitates from cell lysates were resolved on SDS-PAGE and transferred to PVDF transfer membrane (PerkinElmer; Boston, MA). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore; Bedford, MA).

DNA binding assay - Cell extracts from cells treated with or without E2 (10^{-7} M) for the indicated period were prepared as described (Sekine et al., 2006). To measure ERα DNA binding, cell extracts were treated with the immobilized ER Consensus Oligonucleotide (Santa Cruz Biotechnology) -Sepharose conjugate in binding buffer (20 mM HEPES, 0.5 mM EDTA, 1 mM dithiothreitol, 2 μg / ml poly (dI-dC)) and rotated for 2 h at 4°C. Samples were centrifuged, and the pellets were washed three times with binding buffer. Proteins were eluted from the beads by boiling in sample buffer, and SDS-PAGE was performed as described above.

RNA isolation and quantitative real-time PCR – MCF-7, T47D, HBL100 or siRNA-transfected HeLa cells were treated with or without the indicated concentration of E2 for the indicated time (Sekine et al., 2006). Total RNAs were prepared by using Iso-Gen (Nippon Gene, Tokyo, Japan) and used in RT-PCR. RT-PCR was performed using RT-PCR high -Plus- Kit (TOYOBO, Tokyo, Japan). Quantitative real-time PCR analyses of DUSP22, cathepsin D, as well as the control G3PDH mRNA transcripts were carried out using the assay-on-demandTM gene-specific fluorescently labeled TaqMan MGB probe in an ABI Prism 7000 sequence detection system (Applied Biosystems, Tokyo, Japan).

In Vitro Dephosphorylation Assay – The glutathione-*S*-transferase (GST) fusion protein containing DUSP22 WT (GST-DUSP22) were purified on glutathione-Sepharose beads and eluted with neutralized glutathione as described previously (Aoyama et al.,2001). Enzymatic activity of the GST-DUSP22 fusion protein was also determined using *p*-nitrophenylphosphate as described (Aoki et al., 1996).

Statistical Analysis – Statistical analysis was performed using Student's *t* test, and $p<0.05$ is considered significant.

Abbreviations

ER, estrogen receptor; LMW-DSP, low molecular weight- dual specificity phosphatase; DUSP, dual specificity phosphatase; E2, β-estradiol; STAT, signal transducer and activator of transcription; LUC, luciferase; siRNA, small interfering RNA; RT, reverse transcription; PCR, polymerase chain reaction; GST, glutathione *S*- transferase.

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Legends to figures

Figure 1. Estrogen induced mRNA expression of DUSP22 in breast cancer cells. Human breast cancer cell line, MCF-7 (a), T47D (b) and HBL100 (c) were maintained in phenol red free DMEM containing 10% charcoal-stripped FCS. Cells were treated or untreated with E2 (10^{-7}M) for the indicated periods. Both DUSP22 and cathepsin D expression levels were quantified by reverse transcription and quantitative real-time PCR analysis. Data represent the levels of DUSP22 and cathepsin D mRNA normalized to that of a G3PDH internal control and are expressed relative to the value at time zero. Data represent the mean of duplicate PCR determinations, which in general varied by <10%. Shown is a representative experiment, which was repeated at least twice with similar results.

Figure 2. DUSP22 deactivated E2-induced ER-mediated transcriptional activation. (a) Human embryonic kidney carcinoma cell line, 293T, was maintained in phenol red free DMEM containing 10% FCS and transfected in a 24-well plate were

transfected with ERα (50ng) and Vit-LUC (100 ng) and/or indicated amounts of empty vector, WT, D/A or C/S of DUSP22 by the standard calcium precipitation protocol. At 36 h after transfection, the cells were treated with E2 (10^{-9}M) for additional 12 h. The stimulated cells were harvested, and luciferase activities were measured. Total cellular protein collected from parallel cultures was analysed for DUSP22 by Western blot analysis (*lower panel*). (b) Human cervix carcinoma cell line, HeLa cells in a 24-well plate were transfected with ERα (50ng) and Vit-LUC (50 ng) and/or indicated amounts of empty vector, WT, D/A or C/S of DUSP22 using jetPEI. At 36 h after transfection, the cells were treated with E2 (10^{-9}M) for additional 12 h. The cells were harvested and assayed for the luciferase activity using the Dual-Luciferase Reporter Assay System. Total cellular protein collected from parallel cultures was analysed for DUSP22 by Western blot analysis (lower panel). (c) Human breast cancer cell line, MCF-7 cells in a 24-well plate were transfected with Vit-LUC (50 ng) and/or indicated amounts of empty vector, WT, D/A or C/S of DUSP22 using jetPEI. At 36 h after transfection, the cells were treated with E2 (10^{-7}M) for additional 12 h. The cells were harvested and assayed for the luciferase activity using the Dual-Luciferase Reporter Assay System.

The results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the S.D. At least three independent experiments were carried out for each assay. *, $p < 0.05$ Total cellular protein collected from parallel cultures was analysed for DUSP22 by Western blot analysis (lower panel).

Figure 3. Reduction of endogenous DUSP22 enhances ERα-mediated transcriptional activation in breast cancer cells. (a and b), HeLa (a) or MCF-7 (b) cells in a 24-well plate were transfected with control siRNA or siRNA targeting DUSP22 (25 pmol) using Lipofectamine 2000 (Invitrogen). DUSP22 expression levels were quantified by reverse transcription and quantitative real-time PCR. Data represent the levels of DUSP22 mRNA normalized to that of a G3PDH internal control and are expressed relative to the value at time zero. Data represent the mean of duplicate PCR determinations, which in general varied by <10%. Shown is a representative experiment, which was repeated at least twice with similar results. (c) siRNA-tranfected HeLa cells were then transfected with ERα (50ng) and Vit-LUC (50ng) using jetPEI (PolyPlus-transfection). At 36 h after transfection, the cells were treated with E2 (10^{-9}M) for additional 12 h. The cells were then harvested, and luciferase activities were measured. The results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the S.D. *, $p < 0.05$ (d) siRNA-transfected MCF-7 cells in a 24-well plate were then transfected

with Vit-LUC (50 ng) using jetPEI (PolyPlus-transfection). At 36 h after transfection, the cells were treated with E2 (10^{-7}M) for additional 12 h. The cells were then harvested, and luciferase activities were measured. The results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the S.D. *, $p< 0.05$ (e) siRNA-tranfected HeLa cells were then transfected with AR (10 ng) and –285PB-LUC (0.2 μg) using jetPEI (PolyPlus-transfection). At 36 h after transfection, the cells were treated with DHT (10^{-8}M) for additional 12 h. The cells were then harvested, and luciferase activities were measured. The results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the S.D. *, $p< 0.05$ (f) siRNA-tranfected HeLa cells were then transfected GRE-LUC (0.2 μg) using jetPEI (PolyPlus-transfection). At 36 h after transfection, the cells were treated with DEX (10^{-7}M) for additional 12 h. The cells were then harvested, and luciferase activities were measured. The results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the S.D. **, $p> 0.1$

Figure 4. Reduction of endogenous DUSP22 enhances E2-induced gene expression and E2-induced DNA binding activity of ERα in breast cancer cells. (a) siRNA-transfected MCF-7 cells in a 24-well plate were treated with E2 (10^{-7}M) for the indicated periods. Cathepin D expression levels were quantified by reverse transcription and quantitative real-time PCR analysis. Data represent the levels of cathepsin D mRNA normalized to that of a G3PDH internal control and are expressed

relative to the value at time zero. Data represent the mean of duplicate PCR determinations, which in general varied by <10%. Shown is a representative experiment, which was repeated at least twice with similar results. (b) siRNA-transfected MCF-7 cells in a 6-well plate were treated with E2 (10^{-7}M) for the indicated periods. Pulldown assays were carried out using the immobilized ERα Consensus oligonucleotide-Sepharose. Specifically bound proteins were eluted and subjected to Western blot analysis using anti-ERa antibody (upper panel). Densitometric quantification of the above results was also shown (middle panel). Relative intensity of DNA-bound ERα was normalized to total ERα of the same sample. An aliquot (1%) of the input samples was analysed for ERα by Western blot analysis (lower panel).

Figure 5. DUSP22 and ERα physically interact in vivo. (a) 293T cells ($1x10^7$ cells) were transfected with FLAG-tagged ERα (7.5 μg) together with or without WT, D/A or C/S of Myc-tagged DUSP22 (10 μg). At 48 h after transfection, the cells were lysed, and immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-Myc (upper panel) or anti-FLAG antibody (middle panel). Total cell lysates (1%) were blotted with anti-Myc (bottom panel). (b) Human breast cancer T47D cells ($2x10^7$) were lysed, and immunoprecipitated with control IgG or anti-DUSP22 antibody and immunoblotted with anti-ERα antibody (upper panels) or anti-DUSP22 antibody (lower

panels). (c) Domain structure of ERα and mutant fragments are schematically shown. D, 293T cells ($1x10^7$) were transfected with a series of Myc-tagged ERα mutants (10 μg) and DUSP22 (10 μg). At 48 h after transfection, cells were lysed and immunoprecipitated with anti-FLAG antibody, and immunoblotted with anti-Myc antibody (upper panel) or anti-FLAG antibody (middle panel). Total cell lysates (1%) were blotted with anti-Myc antibody (lower panel).

Figure 6. DUSP22 dephosphorylated ERα on Ser-118. (a) 293T cells ($1x10^7$ cells) were transfected with FLAG-tagged ERα (7.5 μg) together with or without DUSP22 WT (10 μg). At 36 h after transfection, the cells were treated with or without E2 (10^{-7}M) for 30 min. The cells were then lysed and immunoprecipitated with anti-FLAG antibody, and immunoblotted with anti-FLAG antibody (upper panel), anti-ERα antibody (middle panel) or anti-pERα Ser-118 antibody (lower panel). Total cell lysates (1%) were blotted with anti-Myc antibody (bottom panel). (b) 293T cells ($1x10^7$) were transfected with FLAG-tagged ERα (7.5 μg) and/or DUSP22 WT (5-15 μg). At 36 h after transfection, the cells were treated or untreated with E2 (10^{-7}M) for

30 min. The cells were then lysed and immunoprecipitated with anti-FLAG antibody, and immunoblotted with anti-pERα Ser-118 antibody (upper panel) or anti-FLAG antibody (middle panel). Total cell lysates (1%) were blotted with anti-Myc antibody (lower panel). (c) 293T cells ($1x10^7$) were transfected with FLAG-tagged ERα (10 μg) and/or Myc-tagged DUSP22 WT, D/A or C/S (10 μg). At 36 h after transfection, the cells were treated or untreated with E2 (10^{-7}M) for 30 min. The cells were then lysed and immunoprecipitated with anti-FLAG antibody, and immunoblotted with anti-pERα Ser-118 antibody (upper panel) or anti-FLAG antibody (middle panel). Total cell lysates (1%) were blotted with anti-Myc antibody (lower panel).

Figure 7. Reduction of endogenous DUSP22 enhances E2-induced phosphorylation of ERα Ser-118 and *in vitro* dephosphorylation of ERα Ser-118 by DUSP22.

(a) siRNA-transfected MCF-7 cells in a 6-well plate were treated with E2 (10^{-7}M) for the indicated periods. The cells were then lysed and immunoprecipitated with anti-ERα antibody, and immunoblotted with anti-pERα Ser-118 (upper panel) or anti-ERα antibody (middle panel). Densitometric quantification of the above results was also shown (lower panel). Relative intensity of pERα was normalized to total ERα of the same sample. (b) 293T cells ($1x10^7$) were transfected with Myc-tagged ERα (7.5 μg).

At 48 h after transfection, cells were then lysed, immunoprecipitated with anti-Myc antibody and subjected to an *in vitro* dephosphorylation assay by incubation with the indicated GST or GST-LMW-DSP WT (15-30 μg). After termination of the incubation, proteins were separated by SDS-PAGE and analyzed with anti-pERα Ser-118 antibody (upper panel). The same blot was reprobed with anti-Myc antibody after stripping.

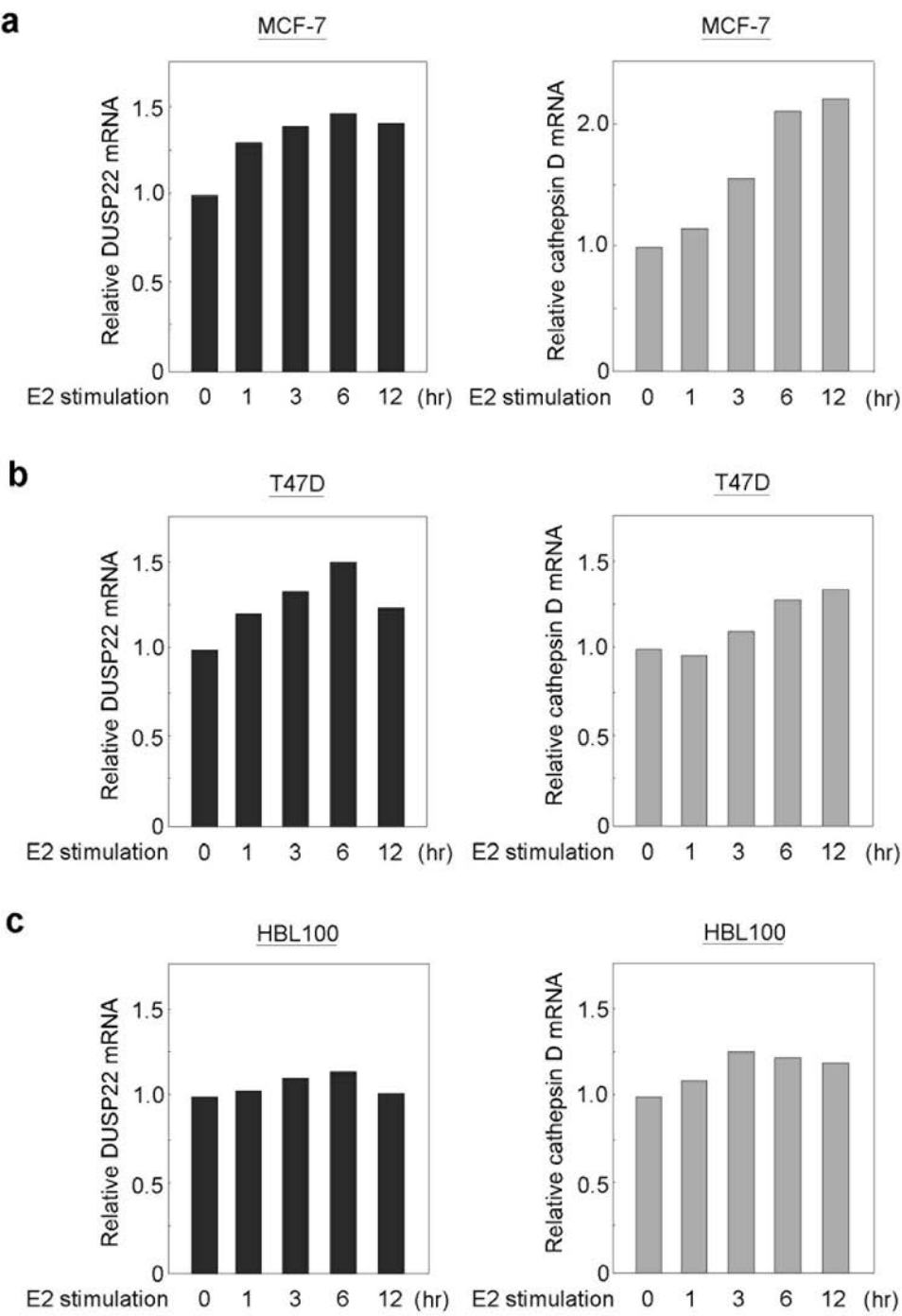
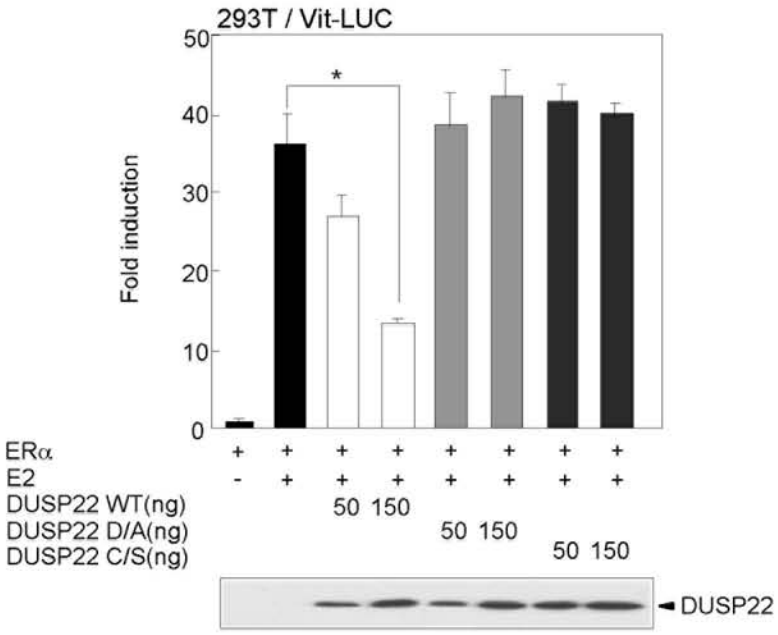
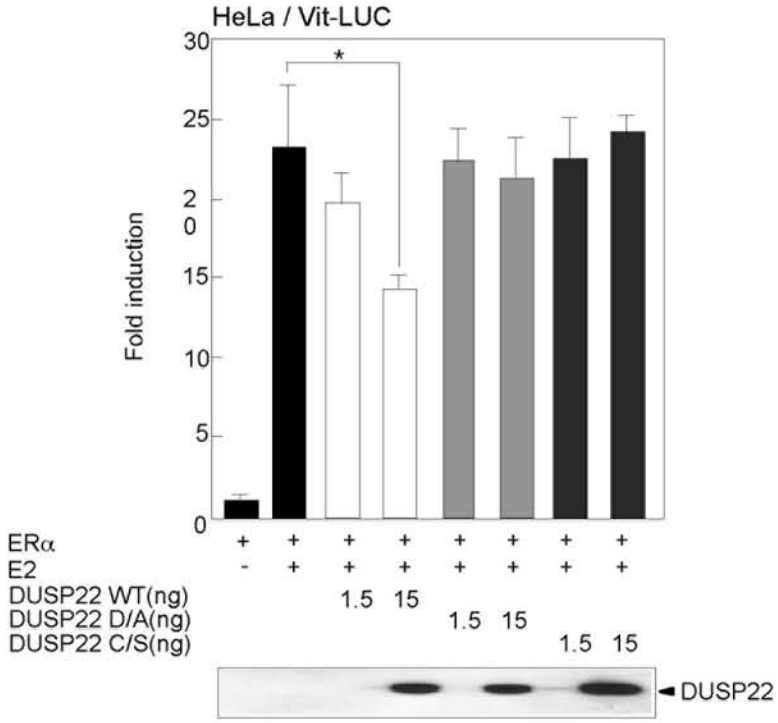

a
MCF-7
Relative DUSP22 mRNA
1.5
1.0
0.5
0
E2 stimulation 0 1 3 6 12 (hr)
MCF-7
Relative cathepsin D mRNA
2.0
1.0
0
E2 stimulation 0 1 3 6 12 (hr)
b
T47D
Relative DUSP22 mRNA
1.5
1.0
0.5
0
E2 stimulation 0 1 3 6 12 (hr)
T47D
Relative cathepsin D mRNA
1.5
1.0
0.5
0
E2 stimulation 0 1 3 6 12 (hr)
c
HBL100
Relative DUSP22 mRNA
1.5
1.0
0.5
0
E2 stimulation 0 1 3 6 12 (hr)
HBL100
Relative cathepsin D mRNA
1.5
1.0
0.5
0
E2 stimulation 0 1 3 6 12 (hr)

Fig. 1

a


293T / Vit-LUC
Fold induction
50
40
30
20
10
0
*
ERα
E2
DUSP22 WT(ng)
DUSP22 D/A(ng)
DUSP22 C/S(ng)
50 150
50 150
50 150
DUSP22

b


HeLa / Vit-LUC
Fold induction
30
25
20
15
10
5
0
*
ERα
E2
DUSP22 WT(ng)
DUSP22 D/A(ng)
DUSP22 C/S(ng)
1.5 15
1.5 15
1.5 15
DUSP22

c

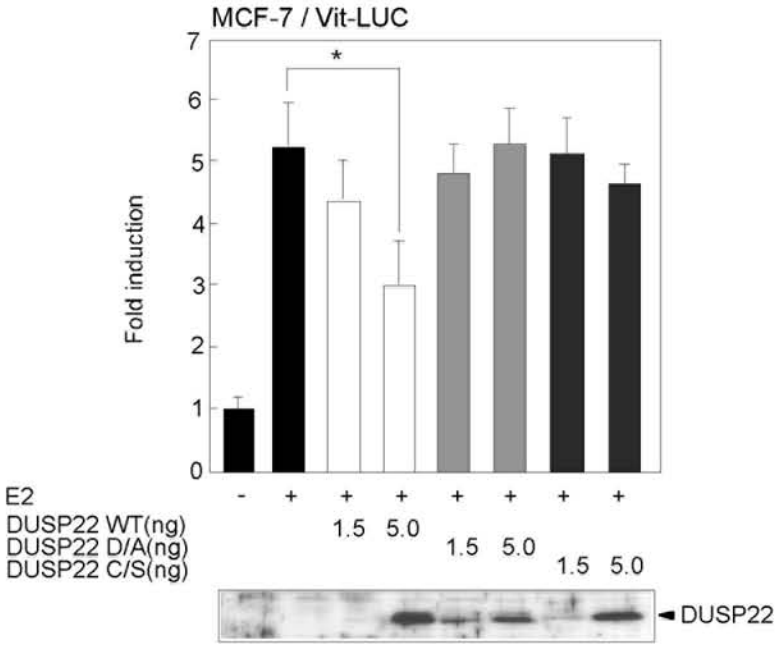

MCF-7 / Vit-LUC
Fold induction
7
6
5
4
3
2
1
0
*
E2
DUSP22 WT(ng)
DUSP22 D/A(ng)
DUSP22 C/S(ng)
1.5 5.0
1.5 5.0
1.5 5.0
DUSP22

Fig. 2

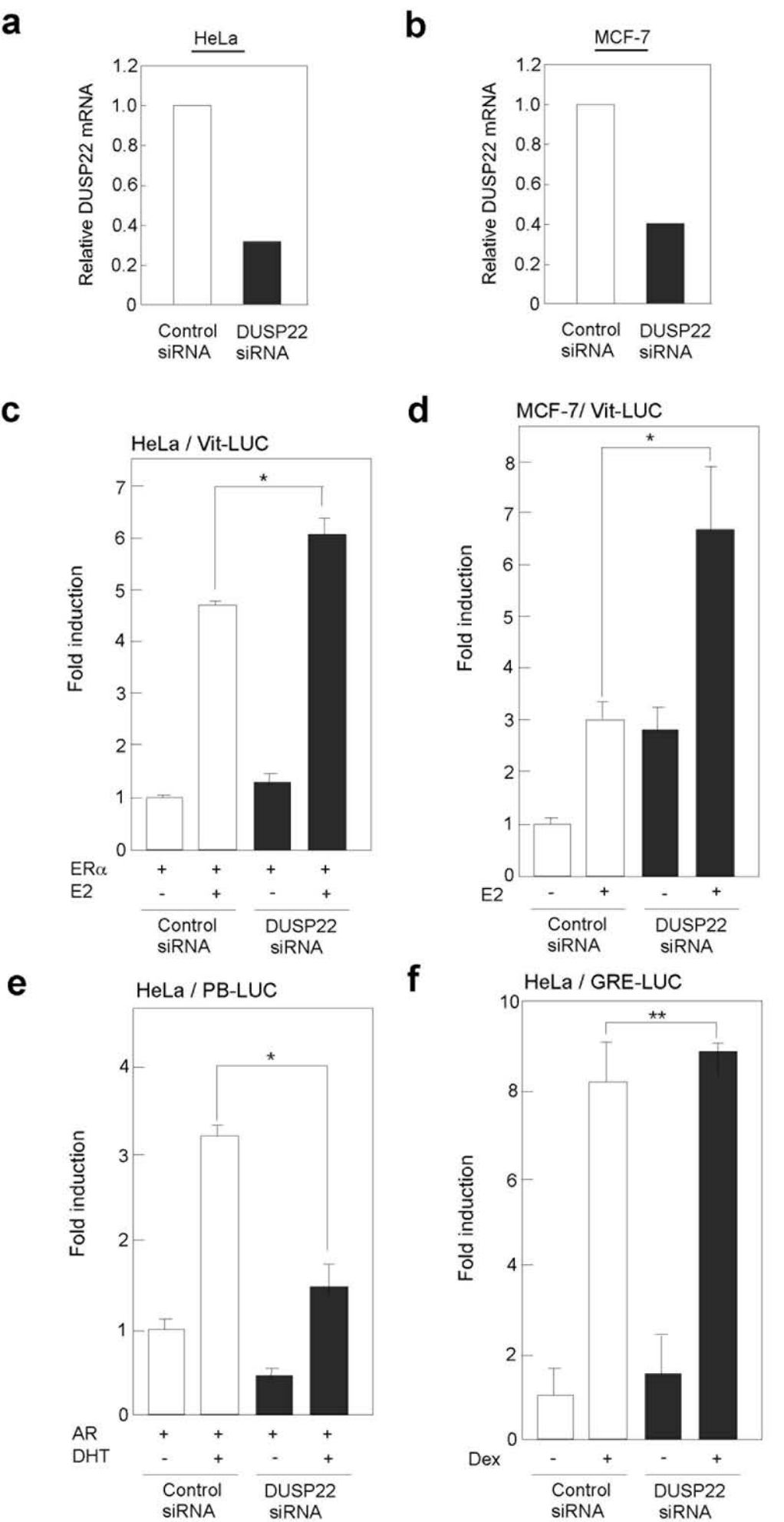
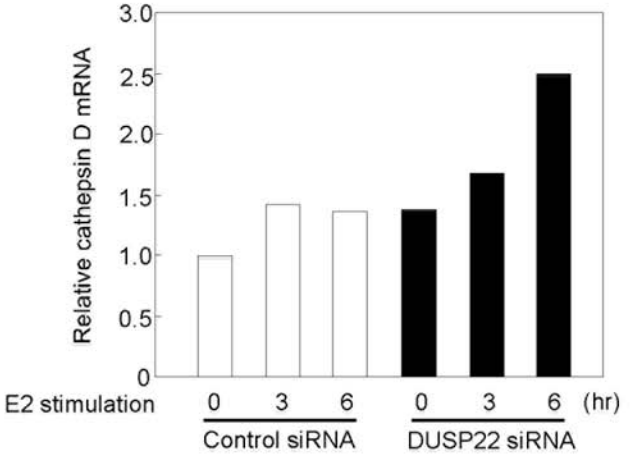

a
HeLa
Relative DUSP22 mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0
Control siRNA
DUSP22 siRNA
b
MCF-7
Relative DUSP22 mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0
Control siRNA
DUSP22 siRNA
c
HeLa / Vit-LUC
Fold induction
*
7
6
5
4
3
2
1
0
ERα + + + +
E2 - + - +
Control siRNA
DUSP22 siRNA
d
MCF-7/ Vit-LUC
Fold induction
*
8
7
6
5
4
3
2
1
0
E2 - + - +
Control siRNA
DUSP22 siRNA
e
HeLa / PB-LUC
Fold induction
*
4
3
2
1
0
AR + + + +
DHT - + - +
Control siRNA
DUSP22 siRNA
f
HeLa / GRE-LUC
Fold induction
**
10
8
6
4
2
0
Dex - + - +
Control siRNA
DUSP22 siRNA

Fig. 3

a


Relative cathepsin D mRNA
3.0
2.5
2.0
1.5
1.0
0.5
0
E2 stimulation
0 3 6 0 3 6 (hr)
Control siRNA
DUSP22 siRNA

b

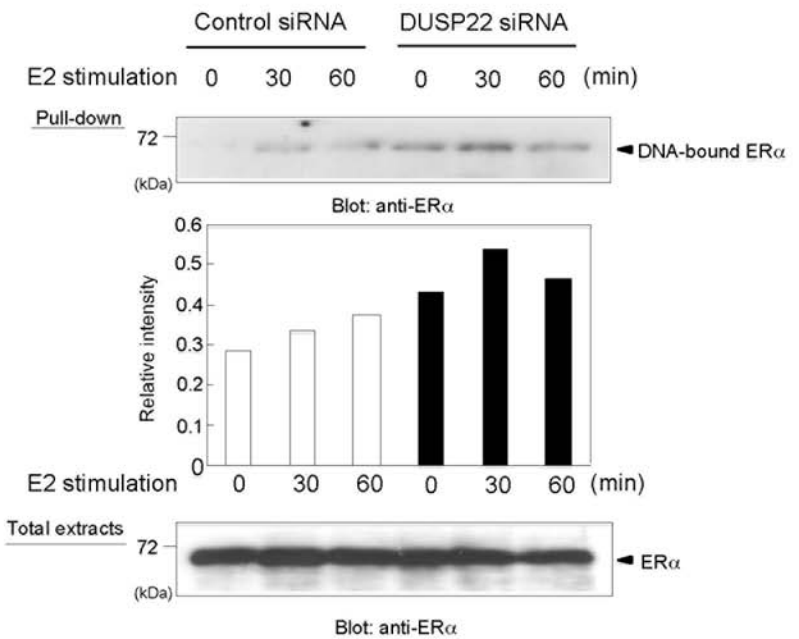

Control siRNA
DUSP22 siRNA
E2 stimulation 0 30 60 0 30 60 (min)
Pull-down
72
(kDa)
DNA-bound ERα
Blot: anti-ERα
Relative intensity
0.6
0.5
0.4
0.3
0.2
0.1
0
E2 stimulation 0 30 60 0 30 60 (min)
Total extracts
72
(kDa)
ERα
Blot: anti-ERα

Fig. 4

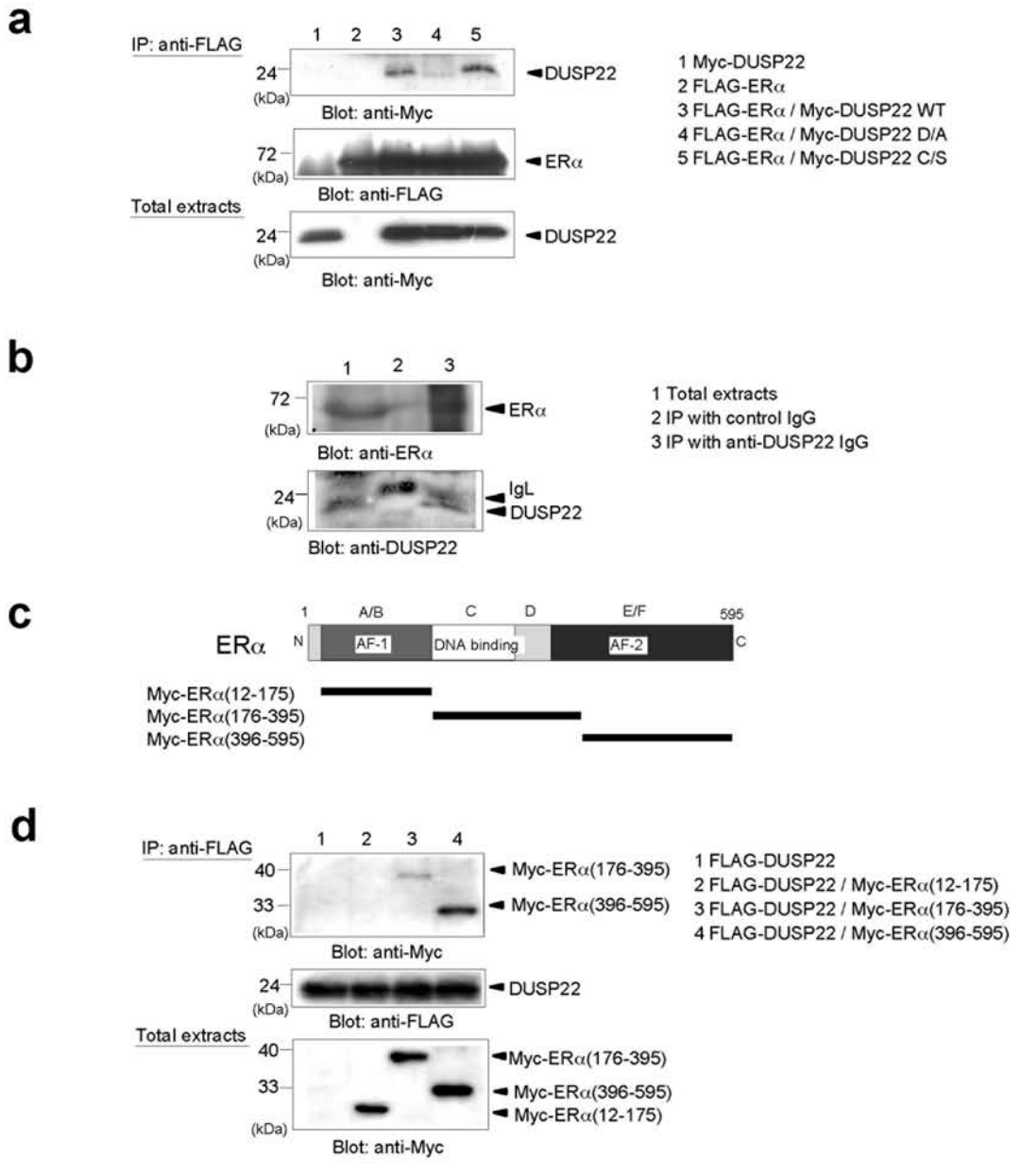

a
IP: anti-FLAG
1 2 3 4 5
24
(kDa)
DUSP22
Blot: anti-Myc
72
(kDa)
ERα
Blot: anti-FLAG
Total extracts
24
(kDa)
DUSP22
Blot: anti-Myc
1 Myc-DUSP22
2 FLAG-ERα
3 FLAG-ERα / Myc-DUSP22 WT
4 FLAG-ERα / Myc-DUSP22 D/A
5 FLAG-ERα / Myc-DUSP22 C/S
b
1 2 3
72
(kDa)
ERα
Blot: anti-ERα
24
(kDa)
IgL
DUSP22
Blot: anti-DUSP22
1 Total extracts
2 IP with control IgG
3 IP with anti-DUSP22 IgG
c
1
A/B
C
D
E/F
595
ERα
N
AF-1
DNA binding
AF-2
C
Myc-ERα(12-175)
Myc-ERα(176-395)
Myc-ERα(396-595)
d
IP: anti-FLAG
1 2 3 4
40
33
(kDa)
Myc-ERα(176-395)
Myc-ERα(396-595)
Blot: anti-Myc
24
(kDa)
DUSP22
Blot: anti-FLAG
Total extracts
40
33
(kDa)
Myc-ERα(176-395)
Myc-ERα(396-595)
Myc-ERα(12-175)
Blot: anti-Myc
1 FLAG-DUSP22
2 FLAG-DUSP22 / Myc-ERα(12-175)
3 FLAG-DUSP22 / Myc-ERα(176-395)
4 FLAG-DUSP22 / Myc-ERα(396-595)

Fig. 5

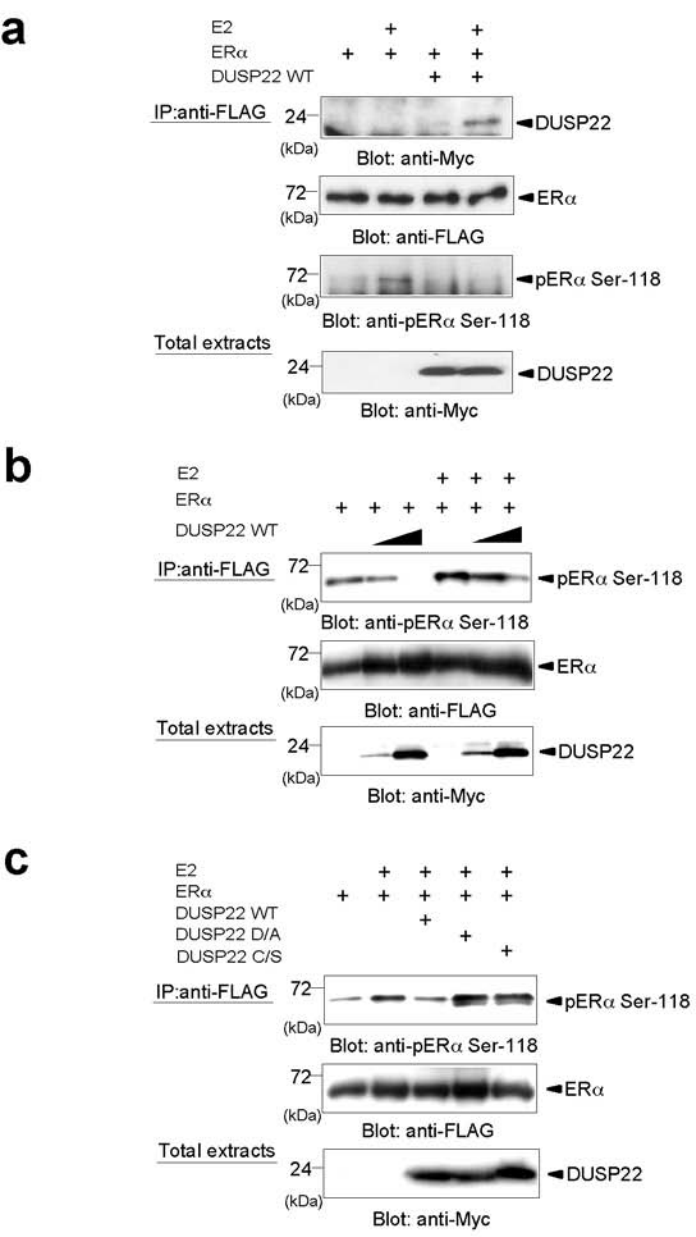

a
E2
ERα
DUSP22 WT
IP:anti-FLAG
24
(kDa)
DUSP22
Blot: anti-Myc
72
(kDa)
ERα
Blot: anti-FLAG
72
(kDa)
pERα Ser-118
Blot: anti-pERα Ser-118
Total extracts
24
(kDa)
DUSP22
Blot: anti-Myc
b
E2
ERα
DUSP22 WT
IP:anti-FLAG
72
(kDa)
pERα Ser-118
Blot: anti-pERα Ser-118
72
(kDa)
ERα
Blot: anti-FLAG
Total extracts
24
(kDa)
DUSP22
Blot: anti-Myc
c
E2
ERα
DUSP22 WT
DUSP22 D/A
DUSP22 C/S
IP:anti-FLAG
72
(kDa)
pERα Ser-118
Blot: anti-pERα Ser-118
72
(kDa)
ERα
Blot: anti-FLAG
Total extracts
24
(kDa)
DUSP22
Blot: anti-Myc

Fig. 6

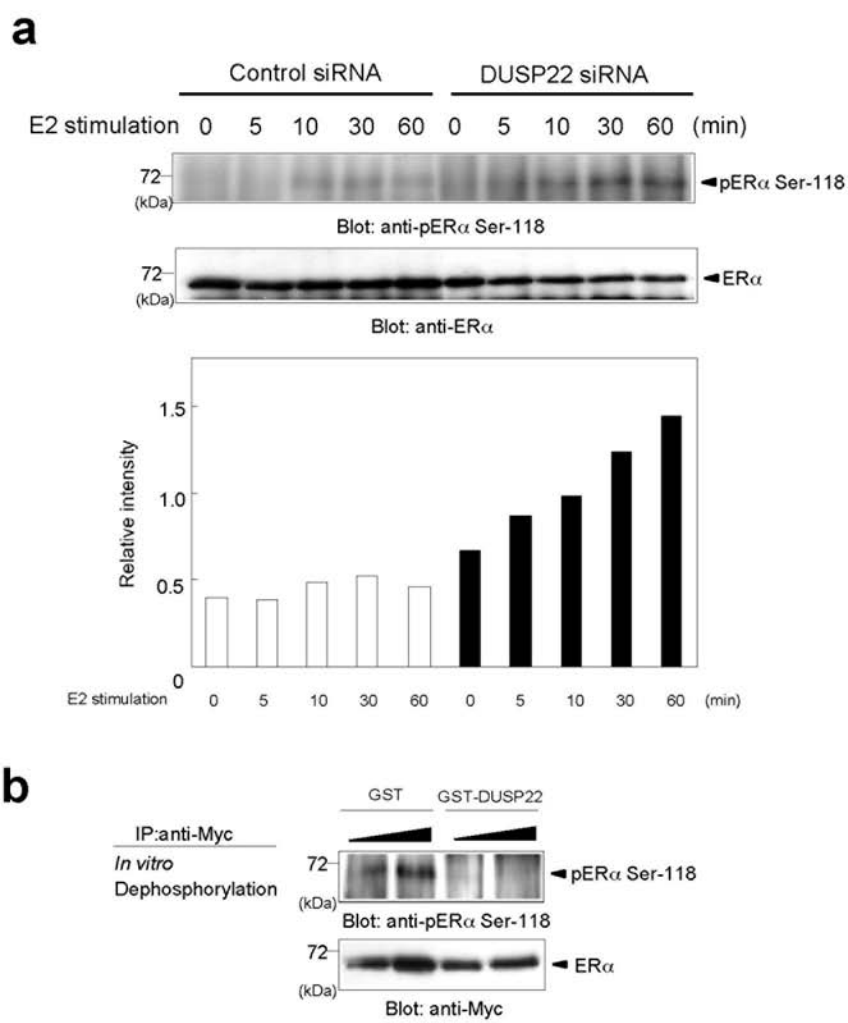

a
Control siRNA
DUSP22 siRNA
E2 stimulation 0 5 10 30 60 0 5 10 30 60 (min)
72
(kDa)
pERα Ser-118
Blot: anti-pERα Ser-118
72
(kDa)
ERα
Blot: anti-ERα
Relative intensity
1.5
1.0
0.5
0
E2 stimulation 0 5 10 30 60 0 5 10 30 60 (min)
b
GST
GST-DUSP22
IP:anti-Myc
In vitro
Dephosphorylation
72
(kDa)
pERα Ser-118
Blot: anti-pERα Ser-118
72
(kDa)
ERα
Blot: anti-Myc

Fig. 7