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Research Article

Repression of NFAT3 transcriptional activity by estrogen receptors

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Abstract. Nuclear factor of activated T cells 3 (NFAT3) activities have been implicated in many biological processes, such as breast cancer, cardiac hypertrophy, learning and memory, and adipocyte differentiation. However, how protein factors regulate NFAT3 transcriptional activity is poorly understood. Here, we report that regardless of estrogen, overexpression of estrogen receptor α and β (ERα and ERβ) suppresses NFAT3 transcriptional activity, whereas knockdown of endogenous ERα and ERβ enhances the activity. Estrogen further enhances ER inhibition of NFAT3-dependent transcription. ERα and ERβ interact with NFAT3 independently of the NFAT agonists phorbol myristate acetate (PMA) and ionomycin, and ERα is recruited to an NFAT3 target gene promoter. Phosphorylation of ERα at different sites differentially affects ERα modulation of NFAT3 transcriptional activity. These results suggest that ER may play a critical role in regulation of NFAT3 transcriptional activity.

Keywords. Estrogen receptor, nuclear factor of activated T cells 3, interaction, phosphorylation, transcriptional activity.

Introduction

Nuclear factor of activated T-cell (NFAT) is a family of transcription factors involved in diverse cellular functions, such as immune response, neuronal development, cardiac hypertrophy, adipocyte differentiation, angiogenesis, and cancer development [1–6]. Five members of the NFAT family have been identified in mammals, including four closely related members, NFAT1/NFATc2/NFATp, NFAT2/NFATc1/NFATc, NFAT3/NFATc4, and NFAT4/NFATc3/NFATx, and an NFAT-like factor, named NFAT5/NFATz/TONEBP. The NFAT transcription factors contain a highly conserved N-terminal regulatory NFAT homology region and a C-terminal Rel homology region for DNA binding. The regions of the NFAT transcription factors located outside the DNA-binding and regulatory domains have relatively little sequence conservation. NFAT1, NFAT2, and NFAT4 are predominantly expressed in immune cells and participate in the activation of T and B cells, whereas NFAT3 is primarily expressed in nonimmune tissues, including heart, brain, and breast [7, 8]. Consistent with the NFAT3 expression pattern, NFAT3 is implicated in cardiac hypertrophy, learning and memory, adipocyte differentiation, and breast cancer development [9–14].

NFAT1–4 are calcium-responsive transcription factors regulated by the calcium/calcineurin signaling pathway. Thus, stimulation with agonists that elicit an increase in intracellular calcium can induce NFAT transcriptional activity [15]. Recently, a few proteins

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have been shown to regulate NFAT transcriptional activity. For example, GSK3 has been demonstrated to inhibit the transcriptional activity of NFAT3 [11]. Ras can stimulate NFAT3 transcriptional activity in cardiac myocytes [16]. However, the proteins involved in regulation of NFAT3 transcriptional activity remain largely unknown.

We have previously demonstrated that NFAT3 can modulate the transcriptional activity of estrogen receptors (ERs), including ERα and ERβ [14, 17], the estrogen-activated transcription factors [18–20]. This effect is mediated by physical interaction of ERs with NFAT3. It has been reported that some transcription factors can cross-talk in a feedback way. For instance, aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor, can regulate ER signaling through interaction of AHR with ER [21], and ER can repress AHR target gene transcription also through interaction of AHR with ER [22]. We therefore decided to examine whether ER could modulate NFAT3 signaling. Here, we report for the first time that ERs can inhibit NFAT3 transcriptional activity. Estrogen further enhances ER inhibition of NFAT3-dependent transcription. Phosphorylation of ER at different sites differentially influences ER modulation of NFAT3 transcriptional activity.

Materials and methods

Plasmids. ERE-LUC (estrogen-responsive element-containing luciferase reporter) [23], NFAT-LUC (NFAT-responsive reporter) [16], and expression vectors for ERα, ERβ, FLAG-tagged NFAT3, lac-tagged ERα, and lac-tagged ERβ [14] have been described previously. The 10 ERα point mutants, ERα(S104A), ERα(S106A), ERα(S118A), ERα(S167A), ERα(S236A), ERα(S305A), ERα(K362A), ERα(T311A), ERα(S518A), and ERα(Y537A), were obtained by recombinant PCR and were verified by restriction enzyme analysis and DNA sequencing.

Cell transfection and luciferase assay. Human embryonic kidney 293T, human breast cancer MCF-7, and MDA-MB-453 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2 in air. For transfection, cells were seeded in 24-well plates in duplicate containing DMEM supplemented with 10% fetal bovine serum or phenol red-free RPMI 1640 medium supplemented with 10% charcoal-stripped fetal bovine serum. The cells were transfected with the indicated plasmids using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). A β-galactosidase reporter plasmid was used for normalization of transfection efficiency, and the respective empty vector was used to adjust the total amount of DNA. Six hours later, the transfected cells were treated with 100 nM phorbol myristate acetate (PMA) and 2 μM ionomycin, or 10 nM 17β-estradiol (E2) for 24 h. The cells were harvested, and luciferase and β-galactosidase activities were determined as described previously [14]. All experiments were repeated at least three times with similar results. Results are shown as means ± SE from a representative experiment.

SiRNA experiments. NFAT3 siRNA construct has been described previously [14]. For generation of ERα siRNA construct, a DNA fragment containing an inverted repeat of the target sequence GGCATGGAGCATCTCTACA [24], was synthesized and cloned under control of the U6 promoter into pSilencer2.1-U6neo (Ambion). For ERβ silencing, two target sequences, TTCTGGAAATCTTTGACAT (ERβ siRNA1) and GGACATAATGATTATATTT (ERβ siRNA2), were inserted into pSilencer2.1-U6neo. Plasmid pSilencer2.1-U6neo-negative control (Ambion) was used as a control vector. These constructs were trasfected into cells using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen).

Coimmunoprecipitation. 293T cells were transfected with the indicated plasmids using Lipofectamine 2000, washed with phosphate-buffered saline (PBS), lysed in 500 μl of lysis buffer (50 mM Tris at pH 8.0, 500 mM NaCl, 0.5% Nonidet P-40, 1 mM dithiothreitol, and protease inhibitor tablets from Roche Applied Science), and immunoprecipitated with anti-FLAG-agarose beads (Sigma) overnight at 4 °C. The beads were centrifuged, washed four times with the lysis buffer, and eluted in 30 μl of SDS-PAGE sample buffer. The eluted proteins were separated by SDS-PAGE, followed by Western blotting with anti-lac (Stratagene) or anti-FLAG (Sigma).

Western blotting. Transfected cells were harvested by lysis in RIPA buffer supplemented with protease inhibitors. Following removal of insoluble debris by centrifugation, supernatant was used to measure protein concentration by the Bradford procedure (Pierce). Protein samples were separated by SDS-PAGE and blotted to a nitrocellulose membrane. Blotted membranes were blocked for 1 h at room temperature or overnight at 4 °C in TBST containing 5% nonfat milk. Blots were incubated with primary antibodies diluted in TBST containing 5% nonfat milk for 1 h at room temperature. After washing

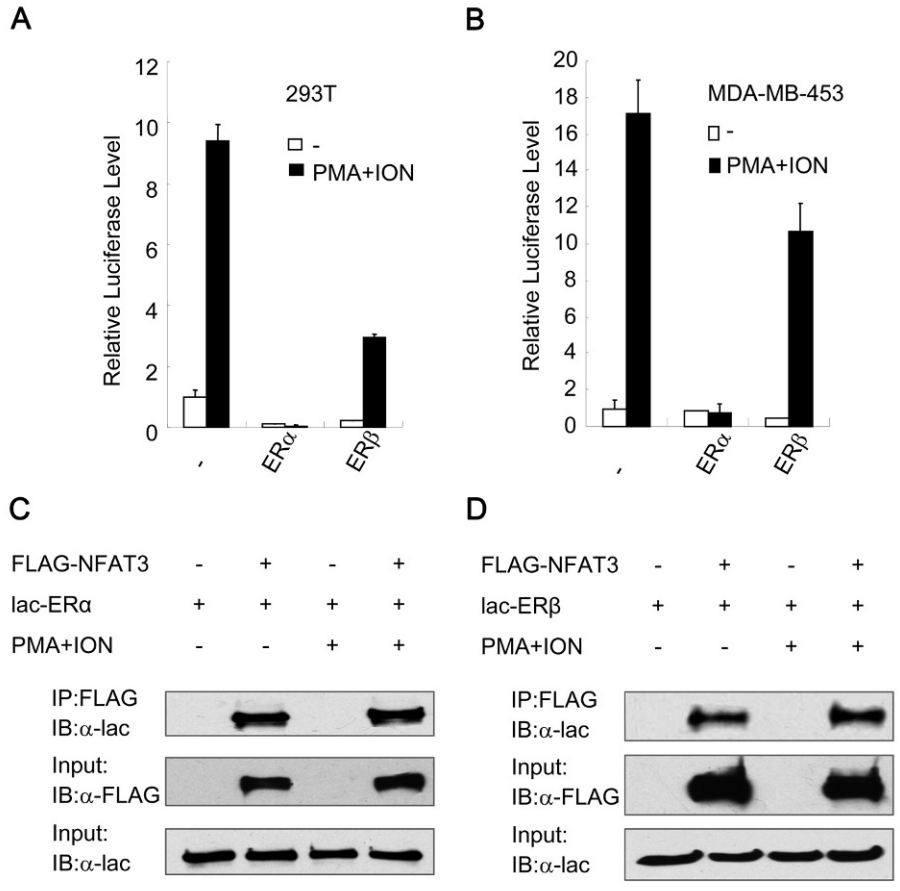

Figure 1. Overexpression of ERα and ERβ inhibits NFAT3 transcriptional activity through interaction with NFAT3. (*A* and *B*) Effects of ERα and ERβ on NFAT3-mediated transactivation. 293T (*A*) or MDA-MB-453 (*B*) cells were co-transfected with 0.1 μg of NFAT-LUC, 25 ng of the expression vector for NFAT3, and 0.5 μg of the expression vector for either ERα or ERβ, as indicated. Cells were then treated with or without 100 nM PMA plus 2 μM ionomycin (ION) for 24 h before luciferase assay. (*C* and *D*) PMA/ION-independent interaction of ERα and ERβ with NFAT3. 293T cells were co-transfected with the expression vector for either lac-tagged ERα (*C*) or lac-tagged ERβ and the expression vector for FLAG-tagged NFAT3 as indicated. Cells were then treated with or without 100 nM PMA plus 2 μM ION for 2 h. Lysates from the transfected cells were immunoprecipitated (IP) using anti-FLAG antibody (Sigma-Aldrich), and the immunoprecipitates were probed with an anti-lac antibody (Stratagene).

extensively with TBST, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody, followed by chemiluminescent detection according to the manufacturer's instructions (Pierce).

Chromatin immunoprecipitation (ChIP). ChIP assays were performed as described previously [14]. The following primers were used for ChIP PCR analysis: IL-2 promoter sense, 5′-AGGTAAAACCATTCTGAAACAGGA-3′; IL-2 promoter antisense, 5′-AGACTGACTGAATGGATGTAGGTG-3′; IL-2 upstream sense, 5′-ACCACAGAGACATAACTGTGATCA-3′; IL-2 upstream antisense, 5′-CTGACTTTCCAGGACTCTGCTTC-3′.

Subcellular fractionation. The localization of NFAT3 protein was determined by subcellular fractionation as described previously [25]. Briefly, cells were homogenized using a Dounce homogenizer, and the homogenate was centrifuged at 366 g for 10 min. The pellet was analyzed as the nuclear fraction. The supernatant was centrifuged again at 13,201 g for 10 min, and the final supernatant was analyzed as the cytoplasmic fraction.

Results

Overexpression of ERα and ERβ represses NFAT3 transcriptional activity. To determine the effects of the ERα and ERβ proteins on NFAT3 transcriptional activity, ERα- and ERβ-negative 293T cells were co-transfected with NFAT3, ERα or ERβ, and NFAT-LUC, a luciferase reporter containing three tandem repeats of a 30-bp fragment of the IL-2 promoter. As shown in Figure 1A, stimulation with the NFAT agonists PMA and ionomycin resulted in a dramatic increase in reporter activity. In the absence of PMA/ionomycin, both ERα and ERβ repressed the basal luciferase activity. ERα completely abolished activation of NFAT3 by PMA/ionomycin, whereas ERβ decreased the PMA/ionomycin-induced reporter activity (Fig. 1A). Thus, ERα had much stronger activity than ERβ. The effect of ERα and ERβ on the transactivation of NFAT3 is not restricted to a single cell type, since ERα and ERβ also inhibited the transactivation of NFAT3 in other cell lines such as the human breast cancer cell line MDA-MB-453 (Fig. 1B and see below).

NFAT agonists do not affect the interaction of NFAT3 with ERs. We have previously shown that NFAT3 interacts with ER in an estrogen-independent manner [14]. Having established that ERα and ERβ abolished or reduced activation of NFAT3 by PMA/ionomycin, we tested the effect of the NFAT agonists on the interaction of ERα and ERβ with NFAT3. 293T cells were co-transfected with FLAG-tagged NFAT3 and lac-tagged ERα or lac-tagged ERβ and grown in both the absence and presence of PMA/ionomycin. FLAG-NFAT3 was immunoprecipitated from cell lysates by an anti-FLAG antibody and analyzed for ERα or ERβ binding by Western blotting analysis with lac antibody. As shown in Figure 1C and D, both ERα and ERβ could be co-immunoprecipitated with NFAT3 independently of PMA/ionomycin.

NFAT3 is important for ER repression of the NFAT-LUC reporter activity. To determine whether NFAT3 is required for ER repression of NFAT-LUC reporter activity, 293T cells, which express endogenous NFAT3, were transfected with NFAT3 siRNA. As shown in Figure 2A, the NFAT3 siRNA effectively inhibited the expression of NFAT3 protein 48 h after transfection. Next, 293T cells were cotransfected with ERα, NFAT-LUC, and NFAT3 siRNA or control siRNA. As expected, knockdown of endogenous NFAT3 with the specific NFAT3 siRNA reduced NFAT-LUC reporter activity (Fig. 2B). Importantly, suppression of the normal expression of NFAT3 by NFAT3 siRNA almost abolished ERα inhibition of reporter activity. These data suggest that NFAT3 is a critical factor for ER repression of NFAT-LUC reporter activity.

Knockdown of endogenous ERα and ERβ enhances NFAT3 transcriptional activity. To examine the role of endogenous ERα and ERβ in suppression of NFAT3 transcriptional activity, ERα- and ERβ-positive MCF7 cells were transfected with ERα siRNA or ERβ siRNAs or universal scramble siRNA (control). As shown in Figure 3A and B, all the ERα and ERβ siRNAs effectively inhibited the expression of the corresponding genes in MCF7 cells, although ERβ siRNA2 functioned more effectively than ERβ siRNA1. Suppression of the endogenous expression of ERα in MCF7 cells by the specific ERα significantly increased NFAT-LUC reporter activity (Fig. 3C). Consistent with the knockdown levels of ERβ protein, suppression of endogenous expression of ERβ by ERβ siRNA2 increased reporter activity more than that by ERβ siRNA1. These data further suggest that ERα and ERβ can modulate NFAT3 transcriptional activity.

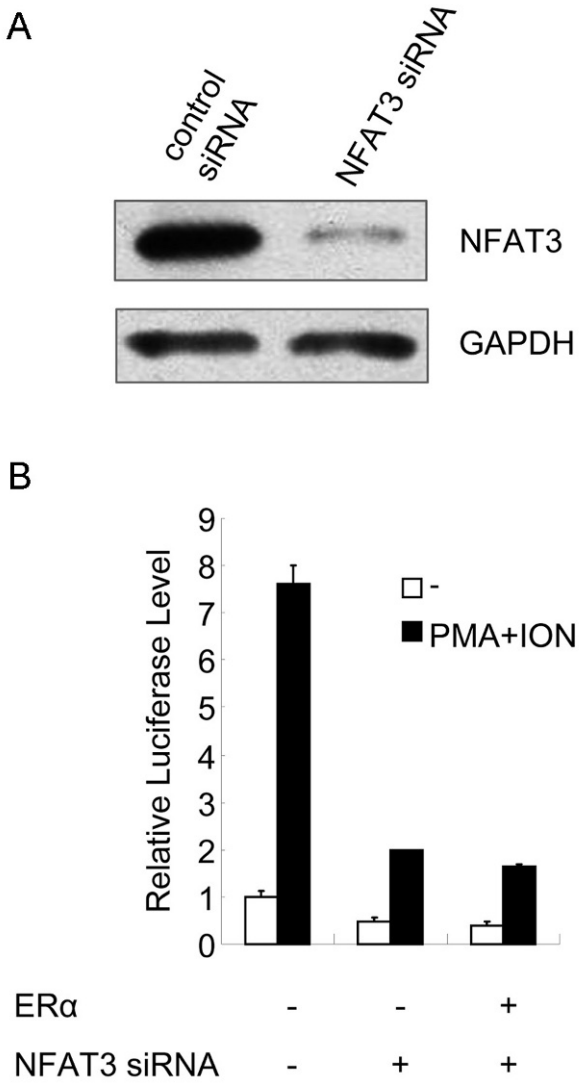

Figure 2. NFAT3 is a critical transcription factor for ERα inhibition of NFAT-Luc reporter activity. (*A*) The construct for either NFAT3 siRNA or control siRNA was trasfected into 293T cells for 48 h. Cell lysates were analyzed by immunoblotting with antibodies to NFAT3 and GAPDH. GAPDH was used as a loading control. (*B*) 293T cells were co-transfected with 0.1 μg of NFAT-LUC, 0.5 μg of the expression vector for ERα, and 0.5 μg of the construct for NFAT3 siRNA as indicated. Twenty-four hours later, cells were treated with 100 nM PMA plus 2 μM ION for 24 h before luciferase assay.

Estrogen affects ER modulation of NFAT3 transcriptional activity. ER has been shown to act as an estrogen-activated transcription factor [18–20]. To examine the effect of estrogen on ER modulation of NFAT3 transcriptional activity, 293T cells were cultured in phenol red-free medium supplemented with charcoal dextran-treated fetal bovine serum. The cells were cotransfected with NFAT3, ERα, and NFAT-LUC, and treated with 17β-estradiol (E2), PMA/ionomycin, or in combination. As shown in Figure 4A,

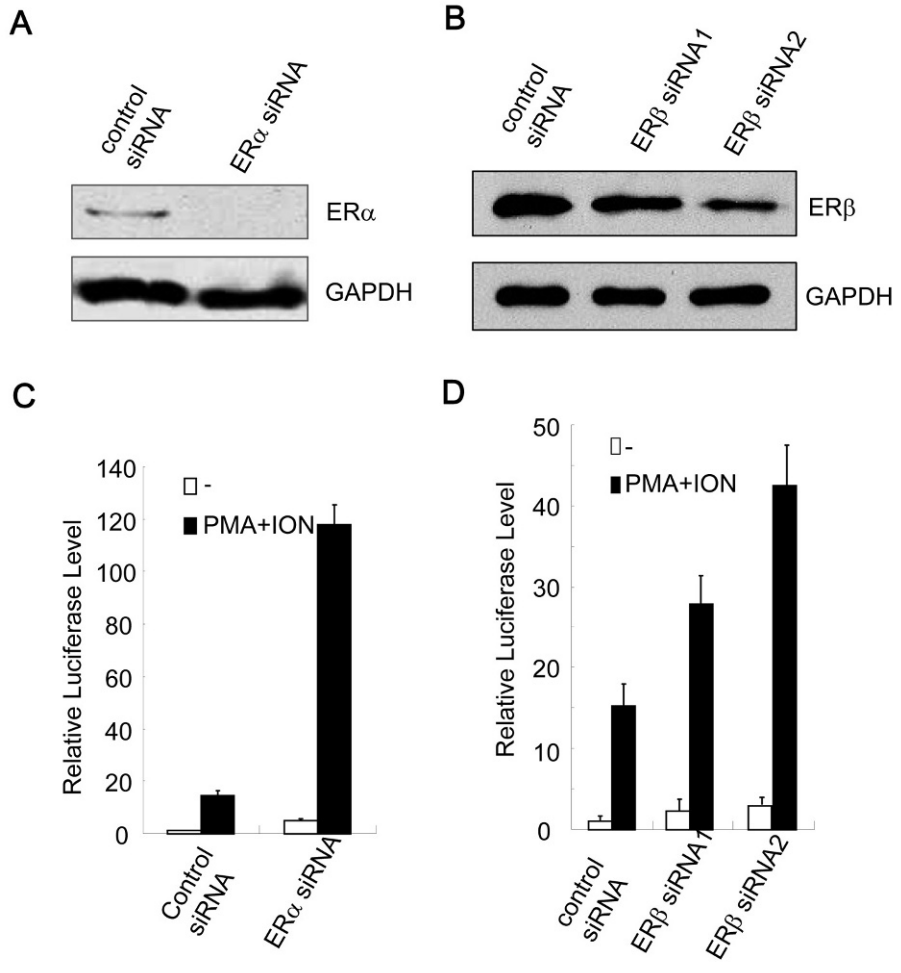

Figure 3. Knockdown of endogenous ERα and ERβ enhances NFAT3 transcriptional activity. (*A* and *B*) Immunoblotting showing the specific knockdown effect of ERα (*A*) siRNA or ERβ siRNAs (*B*) on the endogenous ERα or ERβ protein level. MCF7 cells were transfected with ERα siRNA or ERβ siRNA or scramble siRNA (control) plasmid. Forty-eight hours after transfection, whole-cell extracts were prepared and probed with anti-ERα (Santa Cruz Biotechnology), anti-ERβ (Santa Cruz Biotechnology), or GAPDH antibody (Biogenesis). (*C* and *D*) Luciferase reporter assays in the control and ERα or ERβ knockdown cells. MCF7 cells were cotransfected with with 0.1 μg of NFAT-LUC and 0.5 μg of the construct for either ERα siRNA (*C*) or ERβ siRNAs (*D*) as indicated. Twenty-four hours later, cells were treated with 100 nM PMA plus 2 μM ION for 24 h before luciferase assay.

in the absence of E2, PMA/ionomycin, or in combination, ERα still repressed basal reporter activity. Similar to stimulation with PMA/ionomycin in regular medium, stimulation with PMA/ionomycin without E2 led to a significant increase in reporter activity. In the presence of PMA/ionomycin without E2, ERα also completely abrogated activation of NFAT3 by PMA/ionomycin. Regardless of PMA/ionomycin, E2 enhanced ERα repression of the NFAT3 transcriptional activity (Fig. 4A).

To investigate whether estrogen-induced ER transcriptional activity is required for E2 repression of NFAT3 transcriptional activity, two ERα mutants, ERα(K362A) and ERα(L536A) [26–28], were first prepared and examined for their transactivation function by transient transfection with the estrogen response element-containing reporter ERE-LUC. As expected, the two mutants showed no estrogen-induced transactivation in 293T cells (Fig. 4B). Next, 293T cells were cotransfected with NFAT3, the NFAT-LUC reporter, and wild-type ERα, ERα(K362A), or ERα(L536A). As shown in Figure 4C, both in the absence and in the presence of PMA/ionomycin without E2, ERα(K362A) or ERα(L536A) still retained the majority of wild-type ERα activity in repression of NFAT3 transactivation function. However, regardless of PMA/ionomycin, E2 failed to further inhibit the the NFAT3 transcriptional activity regulated by ERα(K362A) or ERα(L536A). Notably, wild-type ERα, ERα(K362A), and ERα(L536A) were expressed at comparable levels (Fig. 4C). These data suggest that estrogen-induced ERα transcriptional activity is necessary for E2 repression of NFAT3 transcriptional activity by ER.

To determine whether protein synthesis is required for ERα repression of NFAT3 transcriptional activity, cycloheximide, a protein synthesis inhibitor, was added to 293T cells. Both in the absence and in the presence of PMA/ionomycin with or without E2, cycloheximide decreased the NFAT-LUC reporter activity (Fig. 4D). Importantly, in the presence of cycloheximide, ERα repression of NFAT3 transcriptional activity was abolished (no E2 or E2 alone) or almost abolished (PMA/ionomycin with or without

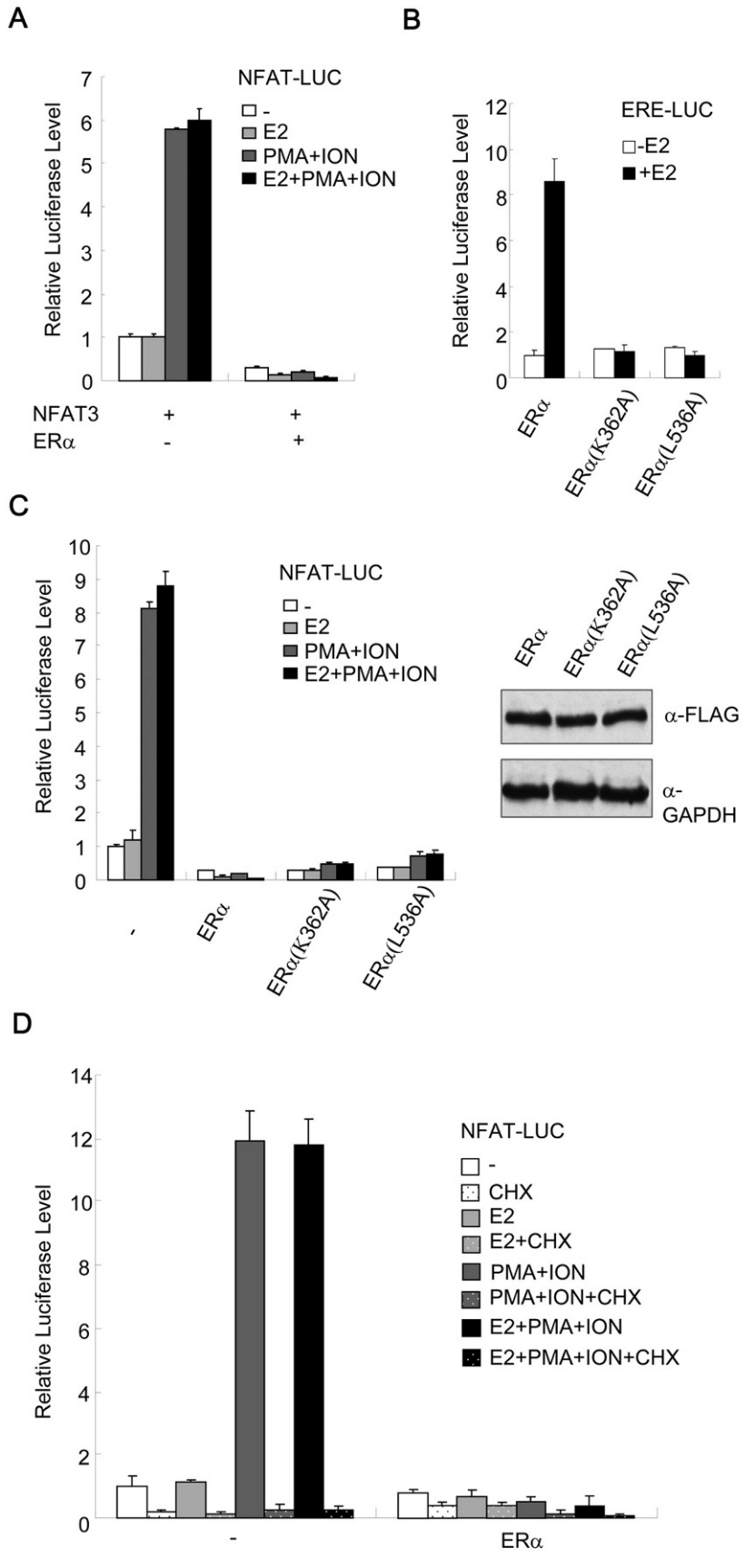

Figure 4. Effect of estrogen-induced transcription on ER modulation of NFAT3 transcriptional activity. (*A*) Effect of estrogen on ER modulation of NFAT3 transcriptional activity. 293T cells were co-transfected with 0.1 μg of NFAT-LUC, 25 ng of the expression vector for NFAT3, and 0.5 μg of the expression vector for ERα as indicated. Cells were then treated with 10 nm 17β-estradiol (E2), 100 nM PMA plus 2 μM ION, or in combination for 24 h before luciferase assay. (*B*) Effect of the two ERα mutants on E2-induced ERα transcriptional activity. 293T cells were co-transfected with 0.1 μg of ERE-LUC and 25 ng of the expression vector for FLAG-tagged ERα, ERα(K362A), or ERα(L536A) as indicated. Cells were treated with 10 nm E2 for 24 h and analyzed for luciferase activity. (*C*) Requirement of estrogen-induced ER transcriptional activity for E2 repression of NFAT3 transcriptional activity by ER. 293T cells were co-transfected with 0.1 μg of NFAT-LUC, 25 ng of the expression vector for NFAT3, and 0.5 μg of the expression vector for FLAG-tagged ERα, ERα(K362A), or ERα(L536A) as indicated. Cells were treated and analyzed as in (*A*). Expression levels of FLAG-tagged ERα, ERα(K362A), and ERα(L536A) were shown by immunoblotting with anti-FLAG on the right panel. (*D*) Effect of protein synthesis on ER repression of NFAT3 activity. 293T cells were co-transfected with 0.1 μg of NFAT-LUC and 25 ng of the expression vector for NFAT3, together with or without 0.5 μg of the expression vector for ERα. At 24 h post-transfection, the cells were treated and analyzed as in (*A*) except that 50 μg/ml cycloheximide (CHX) was used to inhibit protein synthesis as indicated.

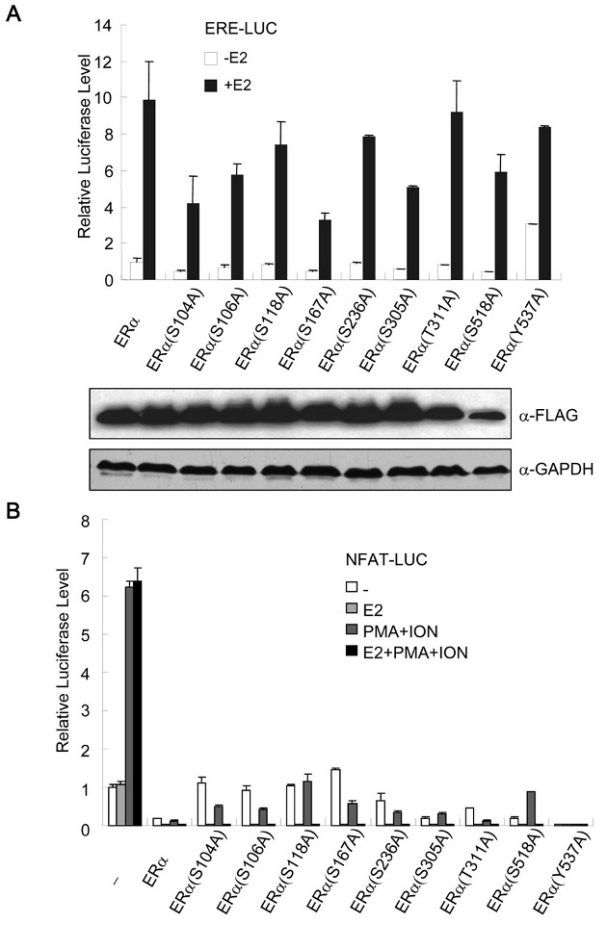

Figure 5. Effects of ERα phosphorylation on ERα modulation of NFAT3 transcriptional activity. (*A*) Effects of ERα phosphorylation on ERα transcriptional activity. 293T cells were co-transfected with 0.1 μg of ERE-LUC and 25 ng of the expression vector for FLAG-tagged ERα or its mutants as indicated. Cells were treated with or without 10 nm E2 for 24 h and analyzed for luciferase activity. Expression levels of FLAG-tagged ERα or its mutants were shown by immunobltting with anti-FLAG at the bottom. (*B*) 293T cells were co-transfected with 0.1 μg of NFAT-LUC, 25 ng of the expression vector for NFAT3, and 0.5 μg of the expression vector for FLAG-tagged ERα or its mutants as indicated. Cells were treated and analyzed as described in the legend to Figure 4A.

E2). These data suggest that protein synthesis is critical for ERα repression of NFAT3 transcriptional activity.

Phosphorylation of ERα at different sites differentially influences ERα modulation of NFAT3 transcriptional activity. Although the status of ERβ phosphorylation is largely unknown, ERα phosphorylation has been well studied [29]. ERα phosphorylation on multiple amino acid residues has been shown to differentially affect ERα transcriptional activity [29]. In view of the fact that estrogen-induced ERα transcriptional activity is necessary for E2 repression of NFAT3 transcriptional activity by ER (Fig. 4C), it is possible that ERα phosphorylation might affect ERα modulation of NFAT3 transcriptional activity. We generated nine ERα mutants in which the phosphorylation sites were mutated. As an initial step, the transcriptional activity of ERα was compared with those of the nine ERα mutants, ERα(S104A) [30], ERα(S106A) [31], ERα(S118A) [32], ERα(S167A) [33], ERα(S236A) [34], ERα(S305A) [35], ERα(T311A) [36], ERα(S518A) [37], and ERα(Y537A) [38], by transient transfection with the ERE-LUC reporter. Similar to the results reported previously [30–38], all the ERα mutants decreased E2-induced ERα transcriptional activity to varying degrees (Fig. 5A). It should be noted that ERα and the eight mutants were expressed at comparable levels except that the one mutant, ERα(Y537A), was expressed at a lower level than ERα and the eight mutants.

Next, 293T cells were cotransfected with NFAT3, NFAT-LUC, and ERα or one of the ERα mutants. As shown in Figure 5B, both in the absence and in the presence of PMA/ionomycin without E2, the ERα mutants ERα(S104A), ERα(S106A), ERα(S118A), ERα(S167A), and ERα(S236A) reduced or abolished ERα inhibition of basal or PMA/ionomycin-induced NFAT3 transcriptional activity. The ERα mutants ERα(S305A) and ERα(S518A) had little effect on ERα inhibition of basal activity but reduced ERα inhibition of PMA/ionomycin-induced NFAT3 transcriptional activity. In contrast, the ERα mutant ERα(S311A) reduced ERα inhibition of basal activity but had little effect on ERα inhibition of PMA/ionomycin-induced NFAT3 transcriptional activity. Interestingly, the ERα mutant ERα(Y537A) enhanced ERα inhibition of both basal and PMA/ionomycin-induced NFAT3 transcriptional activity. However, in the presence of E2, all the ERα mutants functioned like ERα (Fig. 5B). These results suggest that phosphorylation of ERα at different sites differentially affects ERα modulation of NFAT3 transcriptional activity.

Kinases responsible for phosphorylation of ERα affect ERα modulation of NFAT3 transcriptional activity. It has been reported that mitogen-activated protein kinase (MAPK) and protein kinase B/AKT can phosphorylate ERα at serine 118 and 167, respectively [32,33,39–41]. Because the two ERα mutants in which the phosphorylation sites at serine 118 and 167 were mutated affect ERα modulation of NFAT3 transcriptional activity (Fig. 5), we used PD98059 and LY294002, which are MAPK and PI3K/AKT inhibitors, respectively, to investigate the effects of MAPK and AKT on NFAT3 transcriptional activity by ERα. As shown in Figure 6, in the absence of E2, PMA/ionomycin, or in combination, the

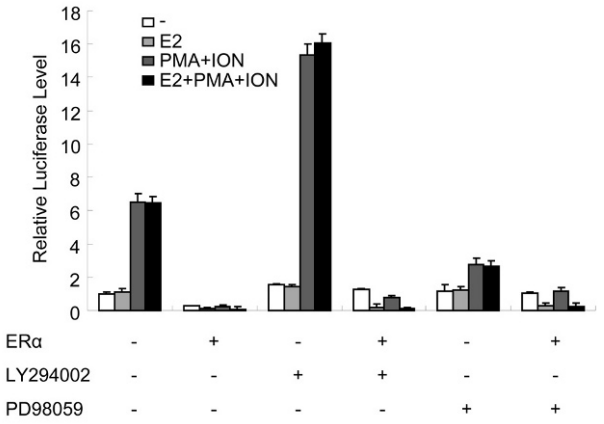

Figure 6. Effects of the inhibitors of the kinases responsible for ERα phosphorylation on ERα modulation of NFAT3 transcriptional activity. 293T cells were co-transfected with 0.1 μg of NFAT-LUC, 25 ng of the expression vector for NFAT3, and 0.5 μg of the expression vector for ERα as indicated. Cells were then treated with 10 nm E2, 100 nM PMA plus 2 μM ION, 50 μM LY294002, 20 μM PD98059, or in combination, and were analyzed for luciferase activities.

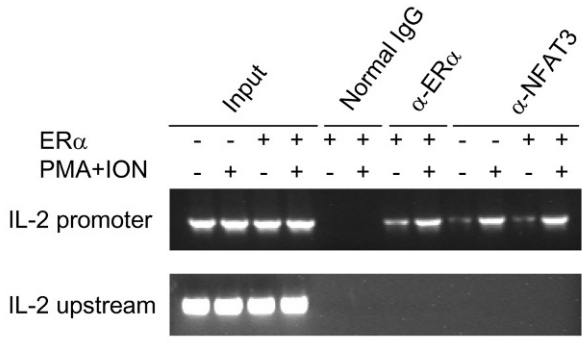

Figure 7. ERα is recruited to the IL-2 promoter. 293T cells were transfected with empty vector or the expression vector for ERα as indicated. Twenty-four hours after transfection, the cells were treated with or without 100 nM PMA plus 2 μM ION for 1 h. Soluble chromatin was prepared from the transfected cells and subjected to immunoprecipitation by using normal IgG or antibodies for NFAT3 or ERα. Immunoprecipitated DNA was PCR-amplified with primers that annealed to the proximal region of the IL-2 promoter or the region approximately 2 kb upstream of the IL-2 promoter.

MAPK and AKT inhibitors had little or no effect on basal NFAT3 reporter activity. In the presence of PMA/ionomycin with or without E2, the MAPK inhibitor PD98059 decreased PMA/ionomycin-induced NFAT3 transcriptional activity, whereas the AKT inhibitor LY294002 increased this activity. Importantly, both in the absence and in the presence of PMA/ionomycin without E2, the MAPK and AKT inhibitors reduced or abolished ERα inhibition of basal or PMA/ionomycin-induced NFAT3 transcriptional activity. However, in the presence of E2, both inhibitors almost functioned as ERα. These data suggest that the MAPK and AKT inhibitors mimic the effects of the ERα(S118A) and ERα(S167A) mutants on NFAT3 transcriptional activity (compare Fig. 6 with Fig. 5B), further confirming the effects of phosphorylation of ERα at serine 118 and 167 on NFAT3 transcriptional activity.

ERα is recruited to the NFAT3 target gene promoter. To further investigate the role of ERα as a co-repressor for NFAT3, ChIP experiments were performed using the promoter of IL-2, an NFAT3 target gene. As expected, ChIP assays using anti-NFAT3 antibody showed that NFAT3 displayed a clear recruitment to the IL-2 promoter in response to PMA/ionomycin (Fig. 7). Importantly, ERα also revealed a distinct recruitment to the IL-2 promoter. In addition, overexpression of ERα did not disrupt the promoter occupancy of NFAT3. The specificity of the ChIP analysis was indicated by the inability to detect occupancy by ERα or NFAT3 of a region approximately 2 kb upstream of the IL-2 promoter (Fig. 7). These results show the association of NFAT3 with ERα at the IL-2 promoter *in vivo*.

Effect of ERα on NFAT3 localization. To investigate whether ERα repression of NFAT3 activity could be due to sequestration of NFAT3 in the cytoplasm by ERα, the localization of NFAT3 protein was examined by subcellular fractionation, followed by immunoblotting with the anti-NFAT3 antibody. 293T cells were fractionated into cytoplasmic and nuclear fractions. As shown in Figure 8, in the absence of PMA/ionomycin, NFAT3 was both cytoplasmic and nuclear. PMA/ionomycin increased the proportion of NFAT3 in the nucleus and also slightly increased the total amount of NFAT3. Unexpectedly, overexpression of ERα promoted the nuclear accumulation of NFAT3 both in the presence and in the absence of PMA/ionomycin. These data suggest that ERα repression of NFAT3 activity is not due to sequestration of NFAT3 in the cytoplasm by ERα and that unknown nuclear repressive factor(s) associated with nuclear ERα and NFAT3 may play an important role in ERα repression of NFAT3 transcriptional activity.

Discussion

We previously reported that NFAT3 is a co-activator for ER in breast cancer cells but a co-repressor in kidney cells [14,17]. In this study, we demonstrated for the first time that ERα and ERβ can inhibit NFAT3 transcriptional activity in both breast and kidney cells, with ERα displaying relatively high inhibitory activity. Since ER can physically interact with NFAT3 and ER

is recruited to NFAT3 target gene promoter, these data suggest that ER is a novel corepressor for NFAT3.

Although ER can physically interact with NFAT3 and functionally regulate NFAT3 transcriptional activity induced by the NFAT agonists PMA and ionomycin, the agonists do not change the physical interaction of ER with NFAT3. This observation also holds for other protein-protein interactions. For example, ERα interacts with the breast cancer susceptibility protein BRCA1 independently of estrogen [42]. However, overexpression of BRCA1 can inhibit estrogen signaling by liganded ERα in various human breast and prostate cancer cell lines. Breast cancer amplified sequence 2 (BCAS2) has been identified as an ERα-interacting protein by a yeast two-hybrid system. BCAS2 associates with ERα and ERβ in a ligand-independent manner [43]. Overexpression of BCAS2 increases ER transcriptional activity.

The NFAT family includes four closely related members: NFAT1, NFAT2, NFAT3, and NFAT4 [1–6]. All NFAT members have a highly conserved N-terminal regulatory NFAT homology region and a C-terminal Rel homology region for DNA binding. The regions of the NFAT members located outside the DNA-binding and regulatory domains have relatively little sequence conservation. It is likely that ER may physically interact with other NFAT family members. Thus, we cannot exclude the possibility that ER may regulate the activities of other NFAT family members besides NFAT3. However, our observation that knockdown of endogenous NFAT3 by NFAT3 siRNA almost abolishes ER inhibition of PMA/ionomycin-induced NFAT activity suggests that, among the NFAT family members, NFAT3 is the most important protein for ER repression of NFAT activity.

Since ERα is a ligand-dependent transcriptional regulator, ERα in the presence of E2 is much more active in stimulating transcription of estrogen-responsive element (ERE)-containing genes than that in the absence of E2 [18–20]. We showed that ERα can efficiently inhibit PMA/ionomycin-induced NFAT3 transcriptional activity even in the absence of E2. Although mammalian cells tested were grown in phenol red-free medium supplemented with charcoal dextran-treated fetal bovine serum, we cannot absolutely exclude the possibility that there might be residual E2 in the medium. However, the concentration of E2 should be very low even if there was residual E2. Therefore, ERα itself may be important for inhibition of NFAT3 transcriptional activity. The fact that E2 further enhances the ERα-mediated inhibitory effect on NFAT3 transcriptional activity suggests that E2 also plays a role in NFAT3 signaling. The E2-induced effect requires E2-induced ERα transcriptional activity, because E2 fails to further inhibit NFAT3 transcriptional activity regulated by ERα(K362A) and ERα(L536A), the two mutants that abolished E2-induced ERα transactivation function. This notion is also supported by the fact that E2 can still suppress NFAT3 transcriptional activity by the ERα phosphorylation site mutants that retained E2-induced ERα transcriptional activity. E2-liganded ER, which stimulates gene transcription, has been shown to interact with co-activator proteins, such as the p160 proteins SRC-1 and GRIP-1 [18]. SRC-1 and GRIP-1 interact with ER only in the presence of E2 and not when ER is unliganded. However, in the absence of E2, ER can also associate with some coactivators, such as Cyclin D1 and XBP-1, and enhance E2-independent ER-mediated transcription [18, 23]. In addition, ER can interact with co-repressor proteins both in a ligand-independent and in a ligand-dependent manner. For example, the co-repressor SMRT interacts with ER in a ligand-independent manner, whereas the co-repressor REA interacts with ER in a ligand-dependent manner [18]. Our observations that both unliganded and E2-liganded ER repress NFAT3 transcriptional activity and that protein synthesis is critical for ER repression of NFAT3 transcriptional activity suggest that some co-activators and co-repressors of ER may play roles in ER inhibition of NFAT3 transactivation function.

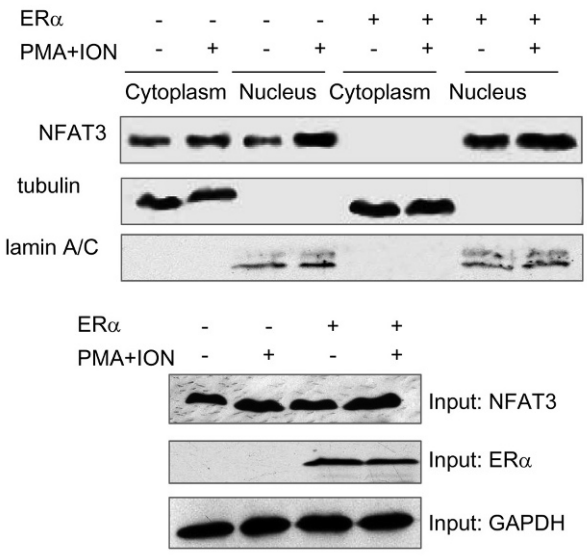

Figure 8. Effect of ERα on NFAT3 localization. 293T cells were transfected with empty vector or the expression vector for ERα as indicated. The transfected cells were treated with or without 100 nM PMA plus 2 μM ION for 12 h and then fractionated into cytoplasmic and nuclear fractions. The fractions were probed with anti-NFAT3. Lamin A/C and tubulin were used as the nuclear and cytoplasmic marker, respectively. The lysates before subcellular fractionation were probed with NFAT3, ERα, and GAPDH antibodies (Input).

Although the status of ERβ phosphorylation is poorly understood, ERα phosphorylation has been well investigated [26]. The transcriptional activity of ERα can be regulated by ERα phosphorylation at multiple sites (Ser-104, Ser-106, Ser-118, Ser-167, Ser-236, Ser-305, Thr-311, and Tyr-537) by multiple kinases. Cyclin-A-cyclin-dependent kinase 2 (Cdk2) complex phosphorylates Ser-104 and Ser-106 but not Ser-118. Ser-118 is a major ERα phosphorylation site that can be induced by estrogens such as E2, and growth factors such as EGF and IGF. Mitogen-activated protein kinase (MAPK)/ERK1/2 can phosphorylate Ser-118 in a ligand-independent manner, whereas cyclin-dependent kinase (CDK)7 phosphorylates Ser-118 in a ligand-dependent manner. Several kinases, such as AKT and p90 ribosomal S6 kinase (RSK), are responsible for ser-167 phosphorylation. Ser-236 is phosphorylated by protein kinase A (PKA). P21-activated kinase-1 (Pak1) phosphorylates ERα at the residue Ser-305. E2 activates the p38 MAPK pathway, which in turn induces ERα phosphorylation on Thr-311. The status of ERα phosphorylation on Ser-518 remains unknown. Tyr-537 is a major tyrosine phosphorylation site in ERα, and Src family tyrosine kinases may be responsible for phosphorylation of ERα on tyr-537. Our study indicated that, in the absence or presence of PMA/ionomycin without E2, most of the ERα phosphorylation mutants reduced or abolished ERα inhibition of basal or PMA/ionomycin-induced NFAT3 transcriptional activity, suggesting that ERα phosphorylation is important for ERα modulation of NFAT3 transcriptional activity under these conditions. However, the fact that the ERα(Y537A) mutant increases ERα inhibition of NFAT3 transcriptional activity in the absence or presence of PMA/ionomycin without E2 indicates that ERα phosphorylation at different sites may have opposite effects on NFAT3 signaling. Our observations also suggest that protein kinases involved in ERα phosphorylation play roles in ERα repression of NFAT3 transcriptional activity. How different kinases responsible for ERα phosphorylation regulate NFAT3 transcriptional activity remains to be investigated.

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