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Dax1 Associates with Esrrb and Regulates Its Function in Embryonic Stem Cells

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Self-renewal capacity and pluripotency, which are controlled by the Oct3/4-centered transcriptional regulatory network, are major characteristics of embryonic stem (ES) cells. Nuclear hormone receptor Dax1 is one of the crucial factors in the network. Here, we identified an orphan nuclear receptor, Esrrb (estrogen-related receptor beta), as a Dax1-interacting protein. Interaction of Dax1 and Esrrb was mediated through LXXLL motifs of Dax1 and the activation- and ligand-binding domains of Esrrb. Furthermore, Esrrb enhanced the promoter activity of the *Dax1* gene via direct binding to Esrrb-binding site 1 (ERRE1, where "ERRE" represents "Esrrb-responsive element") of the promoter. Expression of Dax1 was suppressed followed by Oct3/4 repression; however, overexpression of Esrrb maintained expression of Dax1 even in the absence of Oct3/4, indicating that Dax1 is a direct downstream target of Esrrb and that Esrrb can regulate Dax1 expression in an Oct3/4-independent manner. We also found that the transcriptional activity of Esrrb was repressed by Dax1. Furthermore, we revealed that Oct3/4, Dax1, and Esrrb have a competitive inhibition capacity for each complex. These data, together with previous findings, suggest that Dax1 functions as a negative regulator of Esrrb and Oct3/4, and these molecules form a regulatory loop for controlling the pluripotency and self-renewal capacity of ES cells.

Pluripotency and self-renewal capacity are major characteristics of murine embryonic stem (ES) cells. Leukemia inhibitory factor (LIF) plays an important role for the self-renewal of ES cells, and depletion of LIF from ES cell culture medium leads to spontaneous differentiation of cells and results in a failure of self-renewal (1, 2). A large number of transcription factors function downstream of signaling by LIF, and several transcription factors, including STAT3, Oct3/4, Sox2, and Nanog, play crucial roles for pluripotency and self-renewal of ES cells (3–5). Artificial activation of STAT3, which is achieved by 4-hydroxytamoxifen stimulation of nuclear localization of the STAT3-estrogen receptor fusion protein (STAT3ER), as well as forced expression of Nanog, accelerates the self-renewal in a LIF-independent manner (6–8). *Oct3/4*-deficient embryos develop to the blastocyst stage, but their inner cell mass (ICM), from which ES cells are established, loses pluripotency, and the deletion of Oct3/4 expression in ES cells promotes differentiation into extraembryonic trophectodermal cells (9, 10). *Sox2*-deficient blastocysts form abnormal ICM and fail to achieve outgrowth (11). ES cells lacking the *Sox2* gene differentiate into trophoblast cells (12).

Actually, these transcription factors collaboratively regulate gene expression with other factors and contribute to maintenance of pluripotency and self-renewal of ES cells. For instance, Oct3/4 interacts with Sox2, and this complex enhances expression of ES cell-specific genes, including Fgf4, Lefty1, Nanog, UTF1, and Sox2 (13). β-Catenin is also a binding partner of Oct3/4, and the complex regulates expression of the *Nanog* gene (14). Nanog associates with NF-κB family proteins, including RelA, RelB, and cRel. Of note, the NF-κB level increases during differentiation of ES cells; in contrast, Nanog inhibits NF-κB activation and maintains pluripotency of ES cells (15). Nanog also physically interacts with Smad1 and represses the differentiation-inducing activity of Smad1 (16). Recently, high-throughput analyses revealed that a large number of proteins, including transcription factors, chromatin remodelers, epigenetic factors, metabolism regulators, and cell cycle regulators, associate with Oct3/4 or Nanog, and these factors form protein interaction networks for controlling pluripotency and self-renewal of ES cells (17–19).

Previously, we identified Dax1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1; Nr0b1) as an Oct3/4-interacting protein (20). Dax1 belongs to a nuclear receptor superfamily. It consists of an N-terminal DNA-binding domain (DBD) and C-terminal ligand-binding domain (LBD). The DNA-binding domain includes three LXXLL motifs, which play an important role for protein-protein interaction. The C-terminal ligand-binding domain is similar to those of other nuclear receptors; however, a specific ligand of Dax1 has not been identified, and thus Dax1 is classified as an orphan nuclear receptor. Dax1 is specifically expressed in self-renewing ES cells (21). Expression of Dax1 is regulated by several transcription factors, including STAT3, Oct3/4, and LRH-1, in ES cells (21, 22). Dax1 associates with the POU-specific domain of Oct3/4; as a result, transcriptional activity of Oct3/4 is repressed by Dax1. Since hyperactivation of Oct3/4 leads to differentiation of ES cells (10), Dax1 functions as a negative regulator of Oct3/4 to maintain self-renewal of ES cells (20).

To understand additional functions of Dax1 in ES cells, we performed a yeast two-hybrid screening and identified an orphan nuclear hormone receptor, Esrrb (estrogen-related receptor beta), as a Dax1-interacting protein, and the finding is in agreement with

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previous investigations (18, 23). Here, we discovered that Esrrb directly regulates the expression of Dax1, and Dax1 represses transcriptional activity of Esrrb. Moreover, Oct3/4, Dax1, and Esrrb have a competitive inhibition capacity for their interaction. The results of our current study, together with those of previous investigations, suggest that Oct3/4, Dax1, and Esrrb form a regulatory loop and cooperatively regulate the pluripotency and self-renewal capacity of ES cells by modulating each activity.

MATERIALS AND METHODS

Yeast two-hybrid screening. Plasmids of pGBKT7-Dax1-full length (amino acids 1 to 472), DNA-binding domain (DBD; amino acids 1 to 255), ligand-binding domain (LBD; amino acids 256 to 472), and Q23 region (amino acids 101 to 379) were constructed by inserting each cDNA into the pGBKT7 vector (Clontech, Mountain View, CA). Since full-length Dax1, LBD, and Q23 had autoreporter activity in yeast (*Saccharomyces cervisiae*), we used the DBD region for the screening. Plasmids of the pGBKT7-Dax1-DBD and pGADT7-mouse ES cDNA library (20) were transformed into the AH109 yeast strain according to the manufacturer's protocol (Clontech). The transformed yeasts were plated on minimal synthetic dropout (SD) medium lacking tryptophan, leucine, histidine, and adenine with 2 μg/ml 5-brom-4-chloro-3-indoly-α-D-galactopyranoside (X-α-Gal) (Wako Pure Chemical Industries, Osaka, Japan). Plasmid DNAs were isolated from the α-galactosidase-positive yeasts, and the insertion sequences were determined.

Cell culture. ES cell lines A3-1 and ZHBTc4 were cultured on gelatin-coated dishes with LIF-supplemented Dulbecco's modified Eagle's medium (DMEM) as described previously (6, 10, 24, 25). Esrrb-expressing ZHBTc4 ES cells were established by introduction of pCAGIP-Flag-Esrrb into ZHBTc4 ES cells and cultured in the presence of 1 μg/ml puromycin (Nacalai Tesque, Kyoto, Japan). Human embryonic kidney 293 (HEK293) cells were cultured in DMEM containing 10% fetal bovine serum.

Plasmid construction. Construction of mammalian expression vectors pCMV5-Flag-MBP, pCAG-IP, pCAGIP-Myc, and pCAGIP-Flag was described before (20). Plasmids, including pCAGIP-Oct3/4, pCAGIP-Myc-Oct3/4, pCAGIP-Myc-Dax1, pCAGIP-Flag-Dax1, and pCMV5-Flag-MBP-Dax1 and their derivatives, were described previously (20). Truncated mutants of Dax1 were amplified by PCR using primers listed in Table S1 in the supplemental material and cloned into pCMV5-Flag-MBP. The coding regions of mouse Esrrb, as well as their mutants, were amplified by PCR using primers listed in Table S1 in the supplemental material. pCAGIP-Esrrb, pCAGIP-Myc-Esrrb, pCAGIP-Flag-Esrrb, and pCMV5-Flag-MBP-Esrrb and their derivatives were constructed by inserting corresponding coding sequences into expression vectors as described before (42). Dax1 with three mutated LXXLL motifs, which we named Dax1 LTm, was constructed by PCR using primers listed in Table S1 in the supplemental material. For mutations, LYNLL (the first motif), LYSML (the second motif), and LYSLL (the third motif) amino acid sequences were changed into LYNAA, LYSAA, and LYSAA, respectively. The coding region of Dax1 LTm was cloned into either pCAGIP-Myc or pCMV5-Flag-MBP.

The reporter plasmid, the pGL4.10-Dax1 promoter (pDax1-luc [−2042/+26]) (2.1 kb), was described previously (21). The promoter region of the *Dax1* gene (bp −546 region) was amplified by PCR and cloned into pGL2 basic and pGL4.10 (Promega, Madison, WI), and these plasmids were termed pGL2-Dax1P 546 bp and pGL4.10-Dax1P 546 bp, respectively. To construct Esrrb-responsive element (ERRE)-mutated pGL4.10-Dax1P 546-bp plasmids, mutated ERRE1 and/or ERRE2 elements were constructed by PCR using specific primers. PCR products were cloned into pGL4.10 and termed pGL4.10-Dax1P 546 bp-mutERRE1, -mutERRE2, or -mutERRE1&2. To construct reporter plasmids of Oct3/4-responsive elements of the *Esrrb* gene, approximately 500-bp sequences, including either the first Oct3/4-binding site (+8924 to +8931) or the second Oct3/4-binding site (+34733 to +34740), were amplified by PCR and cloned into the pGL4 promoter (26), and we termed these plasmids pGL4P-Oct3/4#A and pGL4P-Oct3/4#B. To construct the Oct3/4-binding site mutated pGL4P-Oct3/4#A plasmid, the mutated Oct3/4-binding element was constructed by PCR using specific primers. The PCR product was cloned into the pGL4 promoter, and the product was termed the pGL4P-Oct3/4#A mutant. To construct reporter plasmids of Esrrb-responsive elements of the *Esrrb* gene, approximately 500-bp sequences, including the first Esrrb-binding site (Esrrb-ERRE1, +9711 to +9719), the second site (Esrrb-ERRE2, +31943 to +31951), the third site (Esrrb-ERRE3, +33435 to +33443), or the fourth site (Esrrb-ERRE4, +34403 to +34411), were amplified by PCR and cloned into the pGL4 promoter, and we termed these plasmids pGL4P-Esrrb-ERRE1, -ERRE2, -ERRE3, and -ERRE4, respectively. To construct Esrrb-binding site mutated pGL4P-Esrrb-ERRE2 and -ERRE4 plasmids, mutated Esrrb-binding elements were constructed by PCR using specific primers. The PCR products were cloned into the pGL4 promoter and termed the pGL4P-Esrrb-ERRE2 mutant and -ERRE4 mutant, respectively.

Esrrb small interfering (siRNA) expression vector, pFIV-H1/U6-Esrrb#3-Puro, was constructed by inserting annealed oligonucleotides against Esrrb into pFIV-H1/U6-Puro (System Biosciences, Mountain View, CA). All primer and oligonucleotide sequences used in the study are listed in Table S1 in the supplemental material.

Plasmid transfection and luciferase assay. Plasmids were introduced into cultured cells by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). One day after transfection, the medium was replaced with fresh medium. For transient-transfection experiments, samples were analyzed 48 h after transfection. To establish stable transfectants, ES cells were reseeded 48 h after transfection and treated with puromycin for another 3 to 5 days.

For a luciferase assay, cell extracts were prepared 48 h after transfection, and luciferase activities in the extracts were measured by using a luciferase assay kit (Promega) with an AB-2200 luminometer (ATTO, Tokyo, Japan).

RT-PCR and qRT-PCR. Total RNAs were isolated from ES cells with sepasol reagent (Nakalai Tesque) and converted to cDNAs by the use of ReverTraAce (Toyobo, Osaka, Japan) with oligo(dT)$_{12–18}$ primers (Nippon EGT, Toyama, Japan). Gene expression was determined by PCR using specific primers. For quantification, quantitative RT-PCR (qRT-PCR) using Sybr green (MxPro Mx3005P; Stratagene, La Jolla, CA) was performed, and expression levels of target genes were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sequences are listed in Table S1 in the supplemental material. No PCR products were amplified in non-RT samples (see Fig. S1 in the supplemental material).

Western blot analysis and maltose binding protein (MBP) pulldown assay. Lysates of ES cells were subjected to Western blot analysis using anti-Oct3/4 (sc-9081; Santa Cruz Biotechnology), anti-Dax1 (39984; Active Motif, CA), anti-Esrrb (PP-H6705-00; Perseus Proteomics, Tokyo, Japan), or anti-α-tubulin (MP Biomedicals, Solon, OH) antibodies followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Millipore, Billerica, MA). The blot was visualized by using enhanced chemiluminescence reagents (PerkinElmer, Waltham, MA) with an LAS-1000 image analyzer (Fuji Film, Tokyo, Japan).

An MBP pulldown assay was performed as described previously (20). Briefly, cell lysates were incubated overnight at 4°C with amylose resin. The beads were washed three times with a washing buffer (50 mM Tris-HCl [pH 7.5], 2 mM $MgCl_2$, 150 mM NaCl), and the bound proteins were eluted by boiling in 2× sodium dodecyl sulfate (SDS) sample buffer and subjected to Western blot analysis using anti-Myc (sc-40; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Flag (F3165; Sigma-Aldrich, St. Louis, MO), anti-Oct3/4, or anti-Esrrb antibodies.

Preparation of nuclear extracts and biotin-labeled DNA pulldown assay. Nuclear extracts were isolated from A3-1 ES cells. Cells were resuspended in a buffer consisting of 20 mM HEPES (pH 7.9), 20% glycerol, 10 mM NaCl, 0.2 mM EDTA (pH 8.0), and 1.5 mM $MgCl_2$ with protease

inhibitors (1 mM dithiothreitol [DTT], 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin). After incubation on ice for 15 min, samples were spun down at 1,000 rpm for 10 min to prepare the nuclei as a pellet. The pellet was resuspended at 2.5×10^7 nuclei/ml in the same buffer, and then 62.5 μl of 5 M NaCl was added to the 1 ml of sample. After incubation at 4°C for 30 min, the sample was centrifuged at 10,000 rpm. The supernatant was used as a nuclear extract.

For a biotin-labeled DNA pulldown assay, 20 pmol of 3′-biotinylated oligonucleotide was annealed with a complementary oligonucleotide and incubated overnight at 4°C with 60 μg of nuclear extracts in the presence of streptavidin-agarose (Novagen, Darmstadt, Germany). For competition assays, 50-fold nonlabeled oligonucleotide (either wild type [WT] or mutant) was added. The beads were washed three times with the washing buffer, and signals were detected by Western blotting as described above.

Knockdown of target genes. Double-stranded siRNAs were purchased from Operon Biotechnologies (Huntsville, AL). Their sequences were 5′-GCA GUC UGG UUG UCG UAU AGG-3′ for *Esrrb#1*, 5′-UGA CUA AGA UCG UCU CGA AUC-3′ for *Esrrb#2*, 5′-ACC UGC ACU UCG AGA UGA UGG AGA UCC-3′ for *Dax1*, and 5′-GCC ACA ACG UCU AUA UCA UGG-3′ for *EGFP*. ES cells (1.5×10^5 cells) in a 6-cm-diameter dish were transfected with either siRNA or the siRNA expression vector using Lipofectamine 2000.

ChIP assay. A chromatin immunoprecipitation (ChIP) assay was performed according to the manufacturer's protocol (Diagenode OneDay ChIP kit; Nippon Gene, Tokyo, Japan). Briefly, ES cells were fixed with formaldehyde and genomic DNA was sheared. Normal murine IgG (sc-2025; Santa Cruz) and anti-Esrrb antibody were added to the sheared chromatin. For the detection of precipitated genomic DNA, PCR analysis was performed using specific primers, and PCR products were subjected to agarose gel electrophoresis.

RESULTS

Screening of Dax1-interacting proteins. To search for Dax1-interacting proteins, we performed a yeast two-hybrid screening using a cDNA library of self-renewing A3-1 ES cells and identified several transcription factors, including Rxrb (retinoid X receptor beta), LRH-1 (liver receptor homolog 1), Nanog, and Esrrb (estrogen-related receptor beta). Among them, we obtained Esrrb at the highest frequency; therefore, we further analyzed the relationship between Dax1 and Esrrb.

Esrrb and Dax1 are expressed specifically in self-renewing ES cells. First, we examined whether expression of Esrrb correlates with Dax1 expression in A3-1 ES cells. RT-PCR and Western blot analyses revealed that expression of Esrrb, as well as of Dax1, was detected in undifferentiated ES cells, and their expression was reduced upon differentiation induced by LIF depletion (Fig. 1A and C).

We also examined Esrrb expression in ZHBTc4 ES cells (Oct3/4 conditional knockout ES cells) (10). Tet stimulation completely repressed the expression of Oct3/4, which was recovered by removal of Tet. Esrrb and Dax1 expression was dramatically decreased by Tet stimulation and was restored after Tet removal as described previously (21, 27) (Fig. 1B and D), indicating that expression of Esrrb and Dax1 correlated with levels of Oct3/4 in ES cells.

Since Dax1 is characterized as a direct downstream target of Oct3/4 (21), we examined the possibility that Esrrb is also a downstream target of Oct3/4. The *Esrrb* gene contains two putative Oct3/4-binding sites, which we named Oct3/4 #A (+8924 to +8931) and Oct3/4 #B (+34733 to +34740) (see Fig. S2A in the supplemental material). A luciferase assay demonstrated that the Oct3/4 #A region, but not the #B region, has enhancer activity in ZHBTc4 ES cells, and the enhanced activity was reduced after

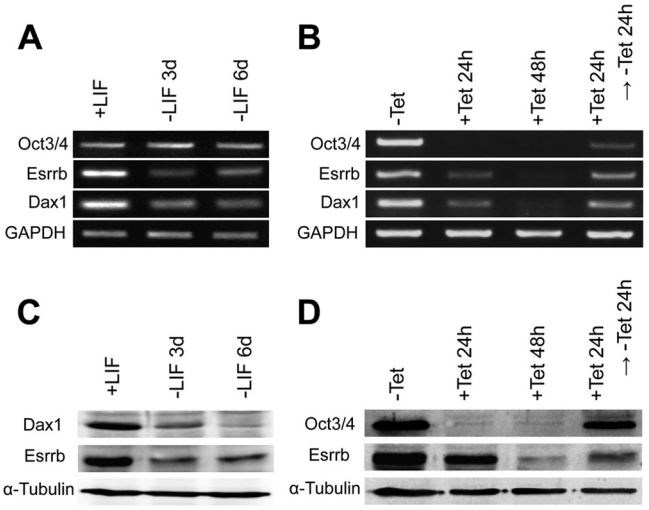

FIG 1 Dax1 and Esrrb are expressed specifically in self-renewing ES cells. (A) Reduction of Esrrb mRNA expression by LIF withdrawal. After culture with (+) or without (−) LIF for 3 days (3d) to 6 days (6d), expression of Esrrb, as well as of Oct3/4 and Dax1, in A3-1 ES cells was examined by RT-PCR analysis. (B) Reduction of Esrrb mRNA expression after repression of Oct3/4 expression. ZHBTc4 ES cells were cultured in the presence of LIF with (+) or without (−) tetracycline (Tet [1 μg/ml]) for 24 h to 48 h. Expression of the indicated genes was examined by RT-PCR. To restore the expression of Oct3/4, the culture medium of Tet-treated cells was charged to a Tet-free medium, and the cells were cultured for another 24 h. GAPDH was used as an internal control. (C) Reduction of Esrrb protein expression by LIF withdrawal. A3-1 ES cells were cultured as described for panel A, and expression of Esrrb and Dax1 was examined by Western blotting. (D) Reduction of Esrrb protein expression after repression of Oct3/4 expression. ZHBTc4 ES cells were cultured as described for panel B, and expression of Esrrb and Oct3/4 was examined by Western blotting. α-Tubulin was used as a loading control. All results are representative of three separate experiments.

treatment with Tet (see Fig. S2B in the supplemental material). When the Oct3/4 #A region was mutated, this element had no enhancer activity in either the presence or absence of Tet (see Fig. S2C in the supplemental material). Taken together, these results suggest that one of the regulators of Esrrb is Oct3/4 in ES cells.

Identification of Esrrb as a Dax1-interacting protein. Esrrb and Dax1 are known to be component molecules of the Oct3/4-centered transcription factor network in ES cells (18). Next, we confirmed the interaction between Dax1 and Esrrb and determined the interaction regions by using a MBP pulldown assay in mammalian HEK293 cells. To determine the Esrrb-binding region of Dax1, we prepared several truncated mutants of Dax1, including full-length Dax1 (amino acids 1 to 472), DNA-binding domain (DBD; amino acids 1 to 255), Q1a (amino acids 1 to 100), Q1b (amino acids 68 to 139), Q23e (amino acids 136 to 202), and ligand-binding domain (LBD; amino acids 256 to 472) (Fig. 2A). Esrrb was precipitated strongly with full-length Dax1, DBD, Q1a (the first and the second LXXLL regions), and Q23e (the third LXXLL region) and weakly with Q1b (the second LXXLL region) and LBD (Fig. 2B), suggesting that the third LXXLL region of Dax1 strongly associates with Esrrb. To determine the Dax1-binding region of Esrrb, we prepared several truncated mutants of Esrrb, including full-length Esrrb (amino acids 1 to 433), E1 (amino acids 1 to 211), E2 (activation domain [AD], amino acids 1 to 168), E3 (amino acids 169 to 433), E4 (ligand-binding domain [LBD], amino acids 212 to 433), E5 (amino acids 93 to 433), and

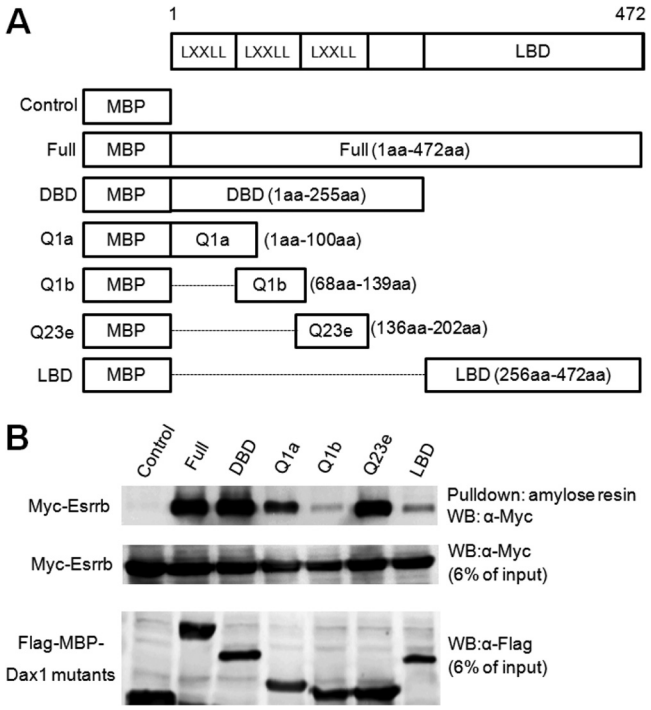

FIG 2 Esrrb binds to LXXLL motifs of Dax1. (A) Schematic view of Flag-MBP-Dax1 and its truncated mutants. Several Flag-tagged MBP-fused constructs, including full-length Dax1 (amino acids [aa] 1 to 472), DNA-binding domain (DBD; amino acids 1 to 255), Q1a (amino acids 1 to 100), Q1b (amino acids 68 to 139), Q23e (amino acids 136 to 202), and ligand-binding domain (LBD; amino acids 256 to 472), were designed. (B) Determination of Esrrb interaction domains of Dax1. HEK293 cells were transfected with Myc-Esrrb together with Flag-MBP-Dax1 or its mutants. MBP-fused proteins were pulled down by amylose resin, and precipitates were analyzed by Western blotting (WB) with anti-Myc antibody. Expression of each protein was confirmed by Western blotting with anti-Myc and anti-Flag antibodies. All results are representative of three separate experiments.

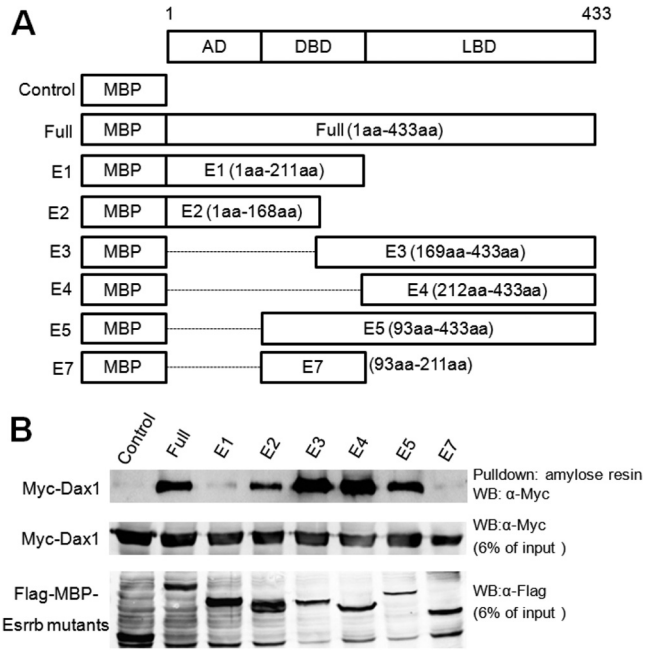

FIG 3 Dax1 binds to the activation domain (AD), as well as to the ligand-binding domain (LBD), of Esrrb. (A) Schematic view of Flag-MBP-Esrrb and its truncated mutants. Several Flag-tagged MBP-fused constructs, including full-length Esrrb (amino acids 1 to 433), E1 (amino acids 1 to 211), E2 (amino acids 1 to 168), E3 (amino acids 169 to 433), E4 (amino acids 212 to 433), E5 (amino acids 93 to 433), and E7 (amino acids 93 to 211), were designed as described in Materials and Methods. (B) Determination of Dax1-interaction domains of Esrrb. HEK293 cells were transfected with Myc-Dax1 together with Flag-MBP-Esrrb or its mutants. MBP-fused proteins were pulled down by the use of amylose resin, and precipitates were analyzed by Western blotting with anti-Myc antibody. Expression of each protein was confirmed by Western blotting with anti-Myc and anti-Flag antibodies. All results are representative of three separate experiments.

E7 (DNA-binding domain [DBD], amino acids 93 to 211) (Fig. 3A). Dax1 was precipitated strongly with the full-length Esrrb, E3, and E4 regions and weakly with the E2 and E5 regions but not with the E1 and E7 regions (Fig. 3B), suggesting that Dax1 binds to the AD and LBD regions of Esrrb, but a part of the DBD region of Esrrb may act as an inhibition domain for the association of Dax1 and Esrrb.

LXXLL motifs of Dax1 are crucial for the interaction with Esrrb but not for the interaction with Oct3/4. The results described above show that Esrrb binds to the LXXLL region of Dax1. Importantly, Oct3/4 also binds near the LXXLL region of Dax1 (20) (see Fig. S3A and B in the supplemental material). Next, we constructed LXXLL motif-mutated Dax1 (Dax1 LTm) (see Fig. S4A in the supplemental material). As shown in Fig. S4B and C in the supplemental material, Dax1 LTm was not able to interact with Esrrb, whereas the mutant associated with Oct3/4. These results revealed that LXXLL motifs of Dax1 are crucial for the interaction with Esrrb but not for the interaction with Oct3/4.

Esrrb regulates Dax1 expression in ES cells. Esrrb is a transcriptional regulator, and its recognition DNA sequence is known to be TCAAGGTCA (28). Interestingly, we discovered that the promoter region of the *Dax1* gene contains two putative Esrrb binding sites, which we named Dax1-ERRE1 (−129 to −121) and Dax1-ERRE2 (−334 to −326) (Fig. 4A). This finding indicates that Dax1 not only is a protein-protein interaction partner of Esrrb but also is a downstream target of Esrrb. To explore this possibility, we first performed a luciferase reporter assay. When we examined the kb −2.1 promoter region of the *Dax1* gene, the promoter activity increased by overexpression of Esrrb in A3-1 ES cells (Fig. 4B). We also examined the activity of the kb −0.5 promoter region of the gene in A3-1 ES cells and found that the enhanced promoter activity of the region decreased by knocking down of Esrrb (Fig. 4C). These results suggest that Esrrb positively regulates the promoter activity of the *Dax1* gene.

To determine which ERRE sites are involved in regulation of Dax1 expression, we examined four reporter plasmids that included wild-type (WT), ERRE1-mutated (mutERRE1), ERRE2-mutated (mutERRE2), and ERRE1 and -2-mutated (mut ERRE1&2) promoters (Fig. 5A). As shown in Fig. 5B, enhanced promoter activity was reduced when ERRE1, but not ERRE2, was mutated, suggesting that ERRE1 is required for the activation of the *Dax1* promoter.

Next, we performed a biotinylated DNA pulldown assay to look for an interaction of Esrrb with the Dax1-ERRE1 element. Nuclear extracts of undifferentiated ES cells were incubated with a biotin-labeled oligonucleotide carrying Dax1-ERRE1 and precipitated by streptavidin-agarose. Endogenous Esrrb was precipitated by biotinylated Dax1-ERRE1 oligonucleotides, and the pre-

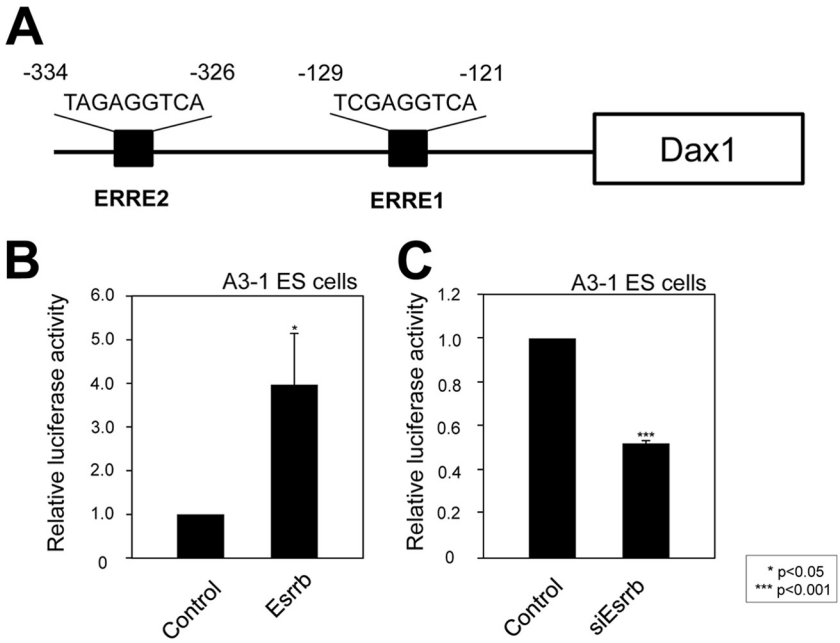

FIG 4 Esrrb positively regulates the promoter activity of the Dax1 gene. (A) Esrrb-binding sites of the Dax1 gene. The promoter region of the Dax1 gene contains two Esrrb-binding sites called ERRE1 (−129 to −121) and ERRE2 (−334 to −326). (B) Transcriptional activity of Esrrb corresponding to the promoter region of the Dax1 gene. A3-1 ES cells were transfected with pGL4.10-Dax1P 2.1-kb plasmid and either pCAG-IP (control) or pCAGIP-Esrrb. (C) Repression of the Dax1 gene promoter activity by knocking down of Esrrb. A3-1 ES cells were transfected with pGL2-Dax1P 546bp plasmid and either control vector (pFIV-H1/U6-Puro) or Esrrb knockdown vector (pFIV-H1/U6-Esrrb#3-Puro). Luciferase activity was measured 48 h after transfection. The bars represent the means and standard deviations of the results of three independent experiments.

cipitate disappeared in the presence of 50-fold nonlabeled wild-type ERRE1 oligonucleotides, suggesting that endogenous Esrrb binds to the Dax1-ERRE1 element *in vitro* (Fig. 5C).

To examine further whether endogenous Esrrb binds to the element *in vivo*, we performed chromatin immunoprecipitation assays. As shown in Fig. 5D, ERRE1 of the *Dax1* gene was precipitated by anti-Esrrb antibody in A3-1 ES cells. Taken together, these results suggest that Dax1 is a direct downstream target of Esrrb in ES cells.

Esrrb regulates expression of Dax1 in an Oct3/4-independent manner. The current findings, together with our previous observation (21), revealed that Dax1 is a downstream target of both Oct3/4 and Esrrb. Here, we examined whether Esrrb is able to regulate Dax1 expression in the absence of Oct3/4. For this, we established Esrrb-overexpressing ZHBTc4 ES cells. Expression of Dax1, as well as of Oct3/4, in the control ZHBTc4 ES cells was repressed in the presence of Tet; however, expression of Dax1 was maintained in Esrrb-overexpressing ZHBTc4 ES cells even in the absence of Oct3/4 (Fig. 6A).

Next, we verified the effect of Esrrb knockdown on Dax1 expression. As shown in Fig. 6B, two independent Esrrb RNA interference (RNAi) assays showed decreased expression levels of endogenous Dax1 mRNA. Also, the expression level of Dax1 protein was reduced by Esrrb knockdown (Fig. 6C). Interestingly, endogenous Oct3/4 was maintained under Esrrb knockdown conditions, suggesting that Esrrb is able to regulate Dax1 expression in an Oct3/4-independent manner.

Esrrb regulates expression of Esrrb in ES cells. We also found an autoregulation pathway of Esrrb gene expression. The *Esrrb* gene contains four putative Esrrb-responsive elements (Esrrb-ERRE1 to -4) in its intron regions (see Fig. S5A in the supplemental material), and Esrrb-ERRE2 and Esrrb-ERRE4 had enhanced reporter activities in A3-1 ES cells (see Fig. S5B in the supplemental material). Of note, the enhancer activities of Esrrb-ERRE2 and Esrrb-ERRE4 were reduced by either knocking down endogenous Esrrb expression (see Fig. S5C and D in the supplemental material) or introducing mutations into the elements (see Fig. S5E and F in the supplemental material). A biotinylated DNA pulldown assay revealed that endogenous Esrrb associates with Esrrb-ERRE2 and Esrrb-ERRE4 (see Fig. S5G in the supplemental material). Taken together, these findings indicate that Esrrb is able to regulate its expression via Esrrb-ERRE2 and Esrrb-ERRE4 in ES cells.

Dax1 inhibits transcriptional activity of Esrrb. To understand the significance of the interaction between Dax1 and Esrrb, we examined the effects of Dax1 on the transcriptional activity of Esrrb using reporter plasmids carrying either the promoter region of the *Dax1* gene or Esrrb-responsive elements (ERRE2 and ERRE4) of the *Esrrb* gene.

The promoter activity of the *Dax1* gene was enhanced by Esrrb and the enhanced activity was repressed by Dax1 in HEK293 cells (Fig. 7A). Similarly, promoter activity was stimulated by Esrrb and the activity was suppressed by Dax1 in A3-1 ES cells (Fig. 7B). Unlike the case of HEK293 cells, Dax1 promoter activity was suppressed by Dax1 itself in A3-1 ES cells, indicating that Dax1 represses endogenous Esrrb transcriptional activity. Also, enhancer activities of ERRE2 and ERRE4 regions of the *Esrrb* gene, where Esrrb directly binds, were repressed by overexpression of Dax1 in ES cells (Fig. 7C and D). These data suggest that Dax1 functions as a negative regulator of Esrrb.

Oct3/4, Dax1, and Esrrb have a competitive inhibition capacity for their interaction. Next, we examined the relationship be-

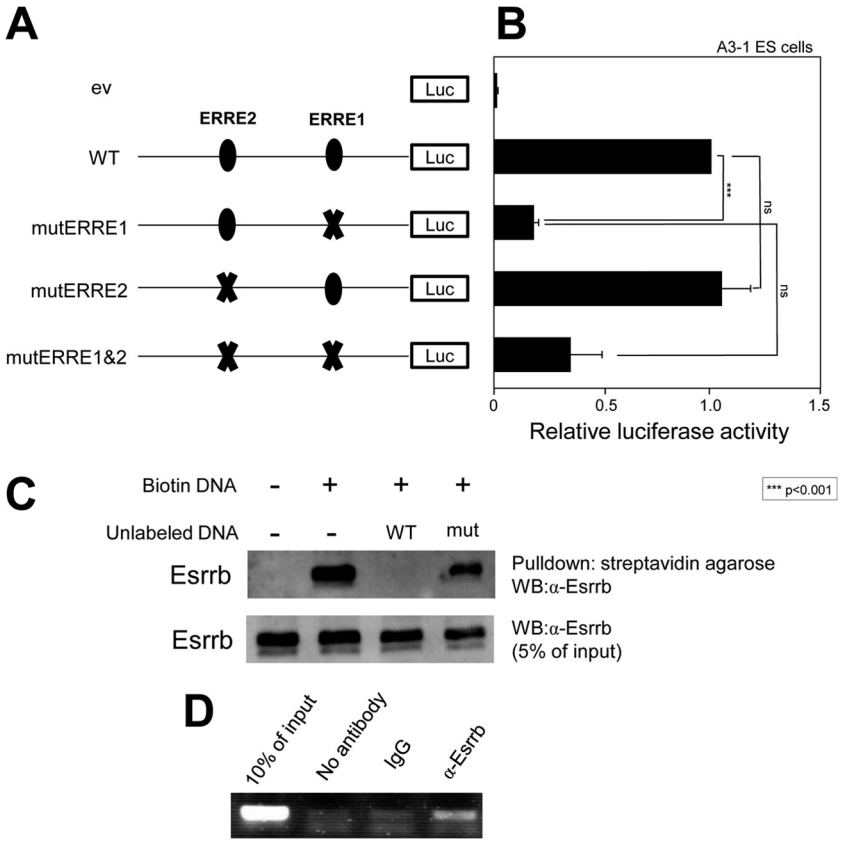

FIG 5 Esrrb regulates promoter activity of the Dax1 gene via ERRE1. (A) Schematic view of pGL4.10-Dax1 promoter (546 bp; pGL4.10-Dax1P 546 bp) and putative Esrrb-binding sites (Dax1-ERRE1 and -2). To determine the Esrrb-responsive elements of the Dax1 gene, four reporter plasmids that included wild-type (WT), ERRE1-mutated (mutERRE1), ERRE2-mutated (mutERRE2), and ERRE1- and -2-mutated (mutERRE1&2) promoters were designed. The mutated sequence of ERRE was changed from AGGTCA to ATTTAA. (B) ERRE1 is involved in promoter activity of the Dax1 gene. A3-1 ES cells were transfected with pGL4.10 (empty vector [ev]), pGL4.10-Dax1P 546 bp-WT, -mutERRE1, -mutERRE2, or -mutERRE1&2. Luciferase activity was measured 48 h after transfection. The bars represent the means and standard deviations of the results of three independent experiments. (C) Esrrb directly binds to Dax1-ERRE1 *in vitro*. Nuclear extracts from A3-1 ES cells were subjected to a pulldown assay with biotinylated DNA. The biotin-labeled Dax1-ERRE1 oligonucleotide was incubated with nuclear extracts from A3-1 ES cells either with or without 50-fold nonlabeled wild-type Dax1-ERRE1 or mutated Dax1-ERRE1 oligonucleotide. The precipitates, as well as nuclear extracts, were analyzed by Western blotting with anti-Esrrb antibody. (D) Esrrb binds to Dax1-ERRE1 *in vivo*. A chromatin immunoprecipitation (ChIP) assay was performed using normal IgG and anti-Esrrb antibody. The precipitates were examined by PCR using specific primers, and PCR products were subjected to 1.5% agarose gel electrophoresis. All results are representative of three separate experiments.

tween Esrrb, Dax1, and Oct3/4. We performed a competitive MBP pulldown assay. As shown in Fig. 8A, Oct3/4 was coprecipitated with MBP-Dax1 in the absence of Esrrb, whereas the amount of precipitated Oct3/4 was reduced in the presence of Esrrb in a dose-dependent manner. Similarly, Esrrb was coprecipitated with MBP-Dax1 in the absence of Oct3/4, and the amount of precipitated Esrrb was reduced in the presence of Oct3/4 in a dose-dependent manner (Fig. 8B), suggesting that Esrrb or Oct3/4 exclusively binds by Dax1.

Since Dax1 LTm binds to Oct3/4 but not to Esrrb, we demonstrated a competitive MBP pulldown assay by using the mutant Dax1. As shown in Fig. 8C, Oct3/4 was coprecipitated with MBP-Dax1 LTm in the absence of Esrrb, whereas the amount of precipitated Oct3/4 was reduced in the presence of Esrrb in a dose-dependent manner, although Esrrb was not coprecipitated with MBP-Dax1 LTm. This finding can probably be explained as follows: Oct3/4 and Esrrb are known to form a complex in ES cells (18, 29). Although Oct3/4 associates with Dax1 LTm in the absence of Esrrb, Oct3/4 changes a binding partner to Esrrb in a dose-dependent manner. Taken together, these results suggest that three complexes, including Oct3/4-Dax1, Oct3/4-Esrrb, and Dax1-Esrrb, exist in cells and that Oct3/4, Dax1, and Esrrb have a competitive inhibition capacity for each complex.

Inhibition of Dax1-Esrrb interaction causes upregulation of endodermal marker genes. To investigate functions of the Esrrb-Dax1 complex in ES cells, Dax1 RNAi was introduced into Esrrb-overexpressing ES A3-1 cells. Since the transfected cells have both overexpressed Esrrb and repressed Dax1, the expression levels of the two genes are unbalanced, and therefore the Esrrb-Dax1 complex would be disrupted in the cells. When we examined gene expression of the cells, the expression levels of endodermal marker genes (Gata4, Gata6, and Dab2), but not mesodermal (T) and ectodermal (Fgf5) genes, were significantly enhanced in Dax1 RNAi-transfected Esrrb-overexpressing ES cells (Fig. 9A). Enhanced expression of the endodermal marker genes was also observed in Esrrb- and Dax1 LTm-co-overexpressed ES cells, where the Esrrb-Dax1 complex would be disrupted (Fig. 9B). Previous investigations showed that Esrrb-overexpressing ES cells are prone to differentiate into endoderm (5, 30). Taken together, these results suggest that Esrrb enhances the expression of endo-

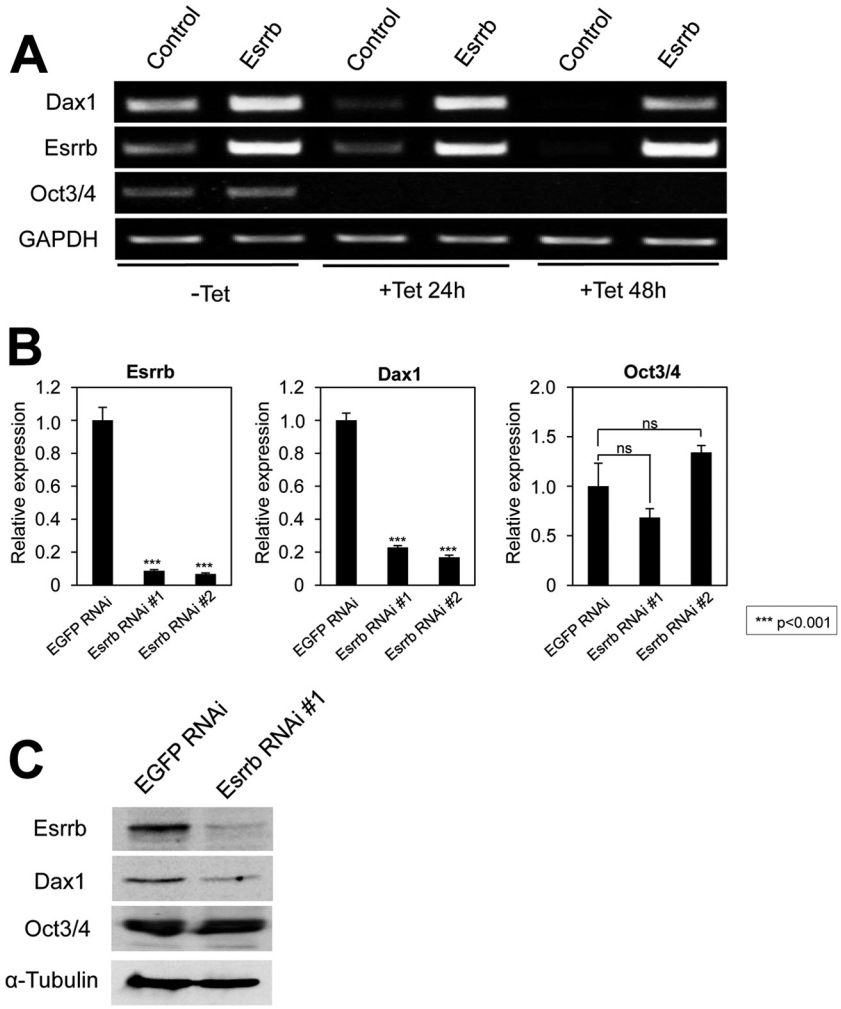

FIG 6 Esrrb regulates expression of Dax1 in an Oct3/4-independent manner. (A) Esrrb maintains expression of Dax1 in the absence of Oct3/4. Control and Esrrb-overexpressing ZHBTc4 ES cells were cultured in the presence of LIF with (+) or without (−) 1 μg/ml Tet for 24 h to 48 h. Expression levels of Dax1, Esrrb, and Oct3/4 were examined by RT-PCR. (B) Knockdown of Esrrb leads to downregulation of Dax1 mRNA. Either enhanced green fluorescent protein (EGFP) double-stranded RNA (dsRNA) (EGFP RNAi) or Esrrb dsRNAs (Esrrb RNAi #1 and #2) were introduced into A3-1 ES cells, and expression levels of Esrrb, Dax1, and Oct3/4 were analyzed 48 h after transfection. All samples were analyzed in triplicate, and data were normalized to GAPDH expression. (C) Knockdown of Esrrb leads to downregulation of Dax1 protein expression. Either EGFP dsRNA (EGFP RNAi) or Esrrb dsRNAs (Esrrb RNAi #1) were introduced into A3-1 ES cells, and expression levels of Esrrb, Dax1, and Oct3/4 were analyzed 48 h after transfection. α-Tubulin was used as a loading control. All results are representative of three separate experiments.

dermal genes to induce differentiation into endoderm and that Dax1 represses endodermal gene expression via association with Esrrb and prevents differentiation of ES cells.

DISCUSSION

Esrrb (estrogen-related receptor beta) is a member of the nuclear hormone receptor superfamily and belongs to subfamily 3 (estrogen receptor type). In mice, *Esrrb*-deficient embryos are embryonically lethal at embryonic day 10.5 (E10.5) because of placental hypoplasia, suggesting that Esrrb is involved in placental formation (31). Complementation analysis of placental defects of the *Esrrb*-deficient embryo with wild-type tetraploid embryos revealed that *Esrrb*-deficient mice mature to adults, but the number of germ cells is significantly reduced in male and female gonads, showing that Esrrb is involved in proliferation of gonadal germ cells (32). In addition, the mice exhibit circling behavior and head-tossing (32). Mice with conditional knockout of the *Esrrb* gene also exhibit head-bobbing and run in circles, which is caused by inner-ear defects (33). Of note, several germ line mutations of the *Esrrb* gene in individuals cause an autosomal-recessive, nonsyndromic hearing impairment, indicating that Esrrb is essential for inner-ear development (29, 34). Taken together, these findings indicate that Esrrb has an important role during early embryogenesis, as well as organogenesis. In the present study, we revealed that Esrrb, together with Oct3/4 and Dax1, regulates self-renewal of ES cells.

Esrrb interacts with Oct3/4, and the association of Esrrb and Oct3/4 enhances the promoter activity of the *Nanog* gene (29). Oct3/4 is able to bind to Dax1, as described in our previous study (20) as well as our current study. Here, we found that Esrrb interacts with Dax1 and that the first and third LXXLL motifs of Dax1 are crucial for the interaction. In particular, Esrrb associates with

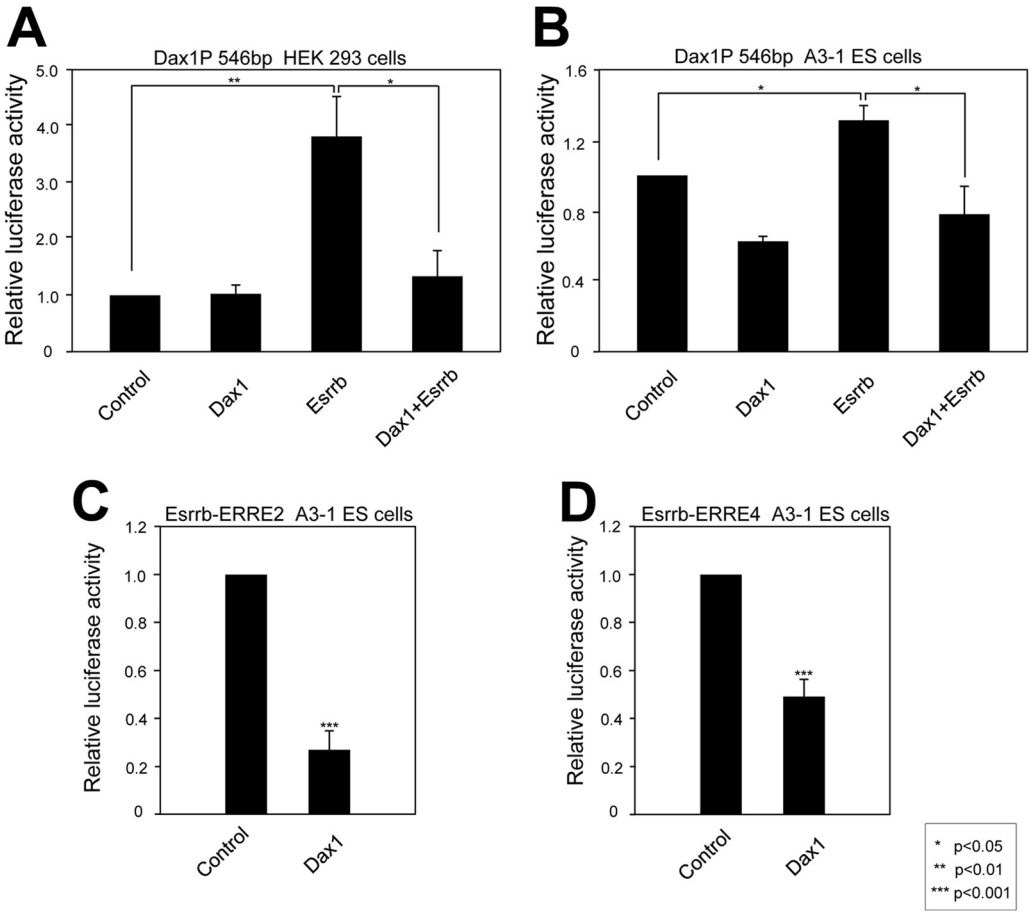

FIG 7 Dax1 inhibits transcriptional activity of Esrrb. (A) Transcriptional activity of Esrrb upon Dax1 expression was repressed by Dax1 in HEK293 cells. Reporter plasmid carrying the Esrrb-responsive element (ERRE) of the Dax1 gene (pGL2-Dax1P 546 bp) was transfected into HEK293 cells with Flag-Dax1 and/or Myc-Esrrb. (B) Transcriptional activity of Esrrb upon Dax1 expression is repressed by Dax1 in A3-1 ES cells. pGL2-Dax1P 546 bp was transfected into A3-1 ES cells with Flag-Dax1 and/or Myc-Esrrb. (C) Enhancer activity of the Esrrb-ERRE2 was repressed by Dax1. Reporter plasmid pGL4P-Esrrb-ERRE2 was transfected into A3-1 ES cells with or without Myc-Dax1. (D) Enhancer activity of the Esrrb-ERRE4 was repressed by Dax1. Reporter plasmid pGL4P-Esrrb-ERRE4 was transfected into A3-1 ES cells with or without Myc-Dax1. Luciferase activity was measured 48 h after transfection. The bars represent the means and standard deviations of the results of three independent experiments.

the third LXXLL motif of Dax1 rather than with the first LXXLL motif. We assume that Esrrb, Oct3/4, and Dax1 do not form a trimer complex. Instead, Esrrb or Oct3/4 is exclusively bound by Dax1. Dax1 is known to function as a transcriptional repressor (23, 35, 36). Transcriptional activities of Oct3/4 and Esrrb are inhibited by Dax1. As described previously, hyperactivation of Oct3/4 in ES cells leads to differentiation into primitive endoderm and mesoderm (10), and Dax1 inhibits the overactivation and contributes to retaining self-renewal (20). A similar relationship between Dax1 and Esrrb would be anticipated. Expression levels of Esrrb in ES colonies are heterogeneous (mosaic-in-colony pattern) (29, 37, 38). Of note, Esrrb-overexpressing ES cells are known to be prone to differentiate into endoderm (5, 30). We generated Esrrb-overexpressing ES cells, and Dax1 RNAi was introduced into the cells, where the Esrrb-Dax1 complex would be disrupted. Expression levels of self-renewal marker genes were comparable to those of control cells, whereas those of endodermal marker genes were enhanced. Enhanced expression of endodermal markers was also observed in Esrrb- and Dax1 LTm-co-overexpressed ES cells, where the Esrrb-Dax1 complex would be disrupted, suggesting that the Esrrb-Dax1 complex represses Gata6 to prevent differentiation of ES cells. Concerning the Dax1-Oct3/4 complex, Dax1 represses the transcriptional activity of Oct3/4 by inhibiting the DNA-binding activity of Oct3/4, since Dax1 associates with the DNA-binding domain of Oct3/4 (20). In the case of the Dax1-Esrrb complex, the association is not mediated through the DNA-binding domain of Esrrb, suggesting that Dax1 does not inhibit the DNA-binding capacity of Esrrb.

Dax1 is a downstream target gene of STAT3 and Oct3/4 in ES cells (21). LRH-1 and SF-1 also regulate Dax1 gene expression in ES cells and adrenocortical carcinoma cells, respectively (22, 39), and the SF-1-mediated activation is repressed by Dax1 via binding to a hairpin structure in the promoter region (40). Here, we found that Dax1 is a downstream target gene of Esrrb. Esrrb recognized the ERRE1 of the *Dax1* gene and enhanced the promoter activity, and the Esrrb-mediated activation of the *Dax1* gene promoter was suppressed by Dax1 itself. In MCF7 breast cancer cells, expression of Dax1 is regulated by Esrrg, but not Esrrb, via ERRE1 (41). Esrrb and Esrrg belong to the same family, and their amino acid homology is about 70%. Esrrg is not expressed in ES cells (data not

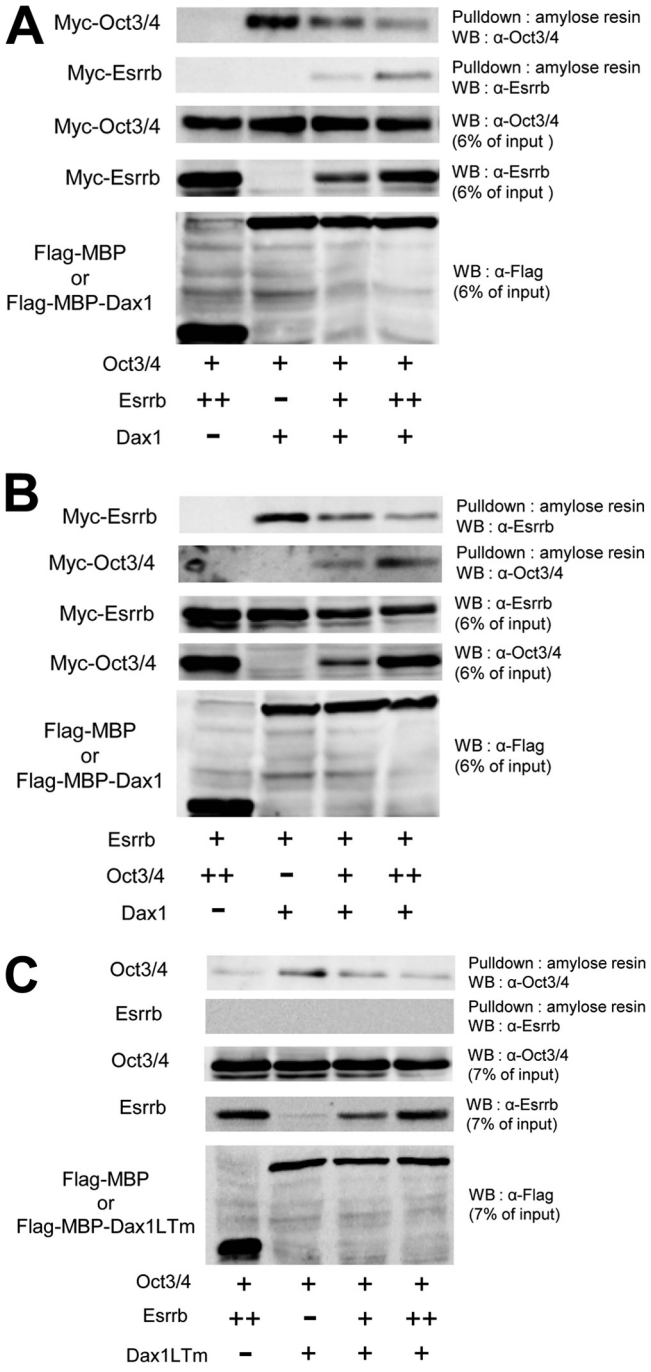

FIG 8 Oct3/4, Dax1, and Esrrb have a competitive inhibition capacity for their interaction. (A) Oct3/4 is eliminated from the Dax1-Oct3/4 complex as the result of competition in the presence of excess amounts of Esrrb. HEK293 cells were transfected with Myc-Oct3/4 and Flag-MBP-Dax1 with or without Myc-Esrrb (0 ng, 50 ng, or 100 ng). Flag-MBP-Dax1 was pulled down by amylose resin, and precipitates were analyzed by Western blotting with either anti-Oct3/4 or anti-Esrrb antibodies. (B) Esrrb is eliminated from the Dax1-Esrrb complex as the result of competition in the presence of excess amounts of Oct3/4. HEK293 cells were transfected with Myc-Esrrb and Flag-MBP-Dax1 with or without Myc-Oct3/4 (0 ng, 100 ng, or 600 ng). Flag-MBP-Dax1 was pulled down by amylose resin, and precipitates were analyzed by Western blotting with either anti-Oct3/4 or anti-Esrrb antibodies. Expression of each protein was confirmed by Western blotting with anti-Oct3/4, anti-Esrrb, and anti-Flag antibodies, respectively. (C) Oct3/4 does not associate with Dax1 LTm in the presence of an excess amount of Esrrb. HEK293 cells were transfected with Oct3/4 and Flag-MBP-Dax1 LTm with or without Esrrb (0 ng, 100 ng, or 200 ng). Flag-MBP-Dax1 LTm was pulled down by amylose resin, and precipitates were analyzed by Western blotting with either anti-Oct3/4 or anti-Esrrb antibodies. All results are representative of three separate experiments.

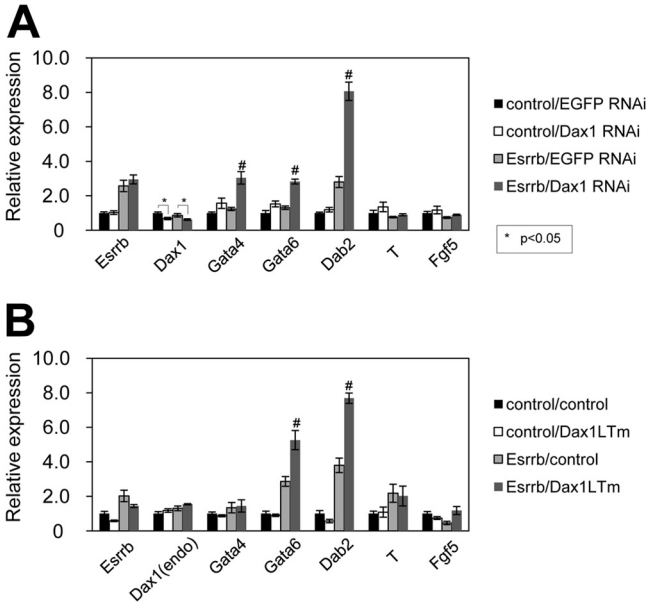

FIG 9 Inhibition of Dax1-Esrrb interaction causes upregulation of endodermal marker genes. (A) Control and Esrrb-overexpressing A3-1 ES cells were transfected with either EGFP dsRNA (EGFP RNAi) or Dax1 dsRNAs (Dax1 RNAi), and expression of the indicated genes was analyzed 96 h after transfection. (B) Control and Esrrb-overexpressing A3-1 ES cells were transfected with either pCAGIP-Myc or Myc-Dax1LTm, and expression of the indicated genes, including endogenous Dax1 [Dax1(endo)], was analyzed 96 h after transfection. All samples were analyzed in triplicate, and data were normalized to GAPDH expression. A value that is significantly different from the other values are indicated by # ($P < 0.05$).

shown). Interestingly, Esrrg-mediated activation of the *Dax1* promoter is repressed by Dax1 in MCF7 cells. These findings indicate that an autoregulatory loop that controls Dax1 gene expression by the estrogen receptor-related receptors is conserved among several cell types.

Here, we propose a novel regulatory loop that includes Dax1, Esrrb, and Oct3/4 for controlling pluripotency and self-renewal of murine ES cells (Fig. 10). Expression of Dax1 is induced by Oct3/4 and Esrrb (21). Oct3/4 and Esrrb associate with each other (18, 29), and expression of each is self-regulated or is regulated by expression of the other or both (42). Also, three complexes, including Oct3/4-Dax1, Oct3/4-Esrrb, and Dax1-Esrrb, would exist in cells, and Oct3/4, Dax1, and Esrrb have a competitive inhibition capacity for each complex. Transcriptional activities of Oct3/4 and Esrrb are repressed by the direct association with Dax1 (20). As described above, hyperactivation of Oct3/4 disrupts self-renewal of ES cells (10) and overexpression of Esrrb impairs pluripotency of ES cells (30), showing that appropriate levels of expression and/or activation of Oct3/4 and Esrrb are important for ES cells. Dax1 functions as a negative regulator of Oct3/4 and Esrrb, and it plays essential roles for maintaining pluripotency and self-renewal capacity of ES cells. In fact, knockdown of Dax1 induces expression of differentiation markers (43), and Dax1 conditional

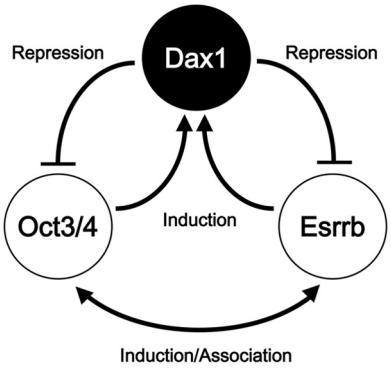

FIG 10 Proposed model of Dax1, Esrrb, and Oct3/4 interaction. Oct3/4 and Esrrb induce the expression of Dax1. Dax1 associates with Oct3/4 or Esrrb and represses their transcriptional activity. Oct3/4 and Esrrb interact, and each regulates the gene expression of the other. The proposed regulatory network contributes to the control pluripotency and self-renewal capacity of ES cells.

knockout in ES cells leads to differentiation (44). Also, overexpression of Dax1 leads to downregulation of Sox2, Nanog, and Dax1, which are downstream targets of Oct3/4 and Esrrb (20). Recent studies have shown that Oct3/4 and Dax1, as well as Esrrb, are part of the core protein network in ES cells (17, 18). During the revision of the manuscript, extensive studies of Esrrb were published. Esrrb is a direct downstream target of Nanog and able to substitute for functions of Nanog in ES cells (45). Also, Esrrb is a target gene of Gsk3/Tcf3 signal, and Esrrb is necessary and sufficient to control self-renewal of ES cells downstream of Gsk3 inhibition (46). Our current report extends these findings and clarifies the biological significance of these interactions. Further investigation of the complex protein network in ES cells will further our understanding of the molecular mechanism of pluripotency and the self-renewal capacity of ES cells.

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