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

Running title: Dax1 as a negative regulator of Esrrb

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ABSTRACT

Self-renewal capacity and pluripotency, which are controlled by 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 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 an Esrrb-binding site 1 (ERRE1) 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 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 pluripotency and self-renewal capacity of ES cells.

INTRODUCTION

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 accelerate 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 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 NFκB family proteins including RelA, RelB and cRel. Of note, NFκB increases during differentiation of ES cells; in contrast,

Nanog inhibits NFκB 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 a N-terminal DNA-binding domain and C-terminal ligand-binding domain. 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 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 and as a result, transcriptional activity of Oct3/4 is repressed by Dax1. Since hyper-activation 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

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. Our current study together with previous investigations suggests that Oct3/4, Dax1 and Esrrb form a regulatory loop and cooperatively regulate 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 - 472), DNA-binding domain (DBD; amino acids 1 - 255), ligand-binding domain (LBD; amino acids 256 - 472) and Q23 region (amino acids 101 - 379) were constructed by inserting each cDNAs into the pGBKT7 vector (Clontech, Mountain View, CA). Since full-length Dax1, LBD and Q23 had auto-reporter activity in yeast, we used the DBD region for the screening. Plasmids of pGBKT7-Dax1-DBD and pGADT7-mouse ES cDNA library (20) were transformed into AH109 yeast strain according to the manufacture'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-galactopyramosid (X-α-Gal) (Wako Pure Chemical Industries, Osaka, Japan). Plasmid DNAs were isolated from the α-galactosidase positive yeasts and the insert 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 (HEK) 293 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, 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. pCAGIP-Esrrb, pCAGIP-Myc-Esrrb, pCAGIP-Flag-Esrrb, pCMV5-Flag-MBP-Esrrb and their derivatives were constructed by inserting corresponding coding sequences into expression vectors as described before (20). Three LXXLL motifs-mutated Dax1, which we named Dax1 LTm, was constructed by PCR using primers listed in Table S1. For mutations, the 1st LYNLL, the 2nd LYSML, and the 3rd LYSLL amino acids 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, pGL4.10-Dax1 promoter 2.1 kb (pDax1-luc (-2042/+26) was described previously (21). The promoter region of the *Dax1* gene (-546 bp region) was amplified by PCR and cloned into either pGL2 basic or pGL4.10 (Promega, Madison, WI), and these plasmids were termed pGL2-Dax1P 546 bp and pGL4.10-Dax1P 546 bp, respectively. To construct 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 either 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 1st Oct3/4-binding site (+8924 to +8931) or the 2nd Oct3/4-binding site (+34733 to +34740) were amplified by PCR and cloned into pGL4-promoter (26), and we termed these plasmids as pGL4P-Oct3/4#A and

pGL4P-Oct3/4#B. To construct Oct3/4-binding site mutated pGL4P-Oct3/4#A plasmid, the mutated Oct3/4-binding element was constructed by PCR using specific primers. PCR product was cloned into pGL4-promoter and termed pGL4P-Oct3/4#A mutant. To construct reporter plasmids of Esrrb-responsive elements of the *Esrrb* gene, approximately 500 bp sequences including either the 1st Esrrb-binding site (Esrrb-ERRE1, +9711 to +9719), the 2nd site (Esrrb-ERRE2, +31943 to +31951), the 3rd site (Esrrb-ERRE3, +33435 to +33443), or the 4th site (Esrrb-ERRE4, +34403 to +34411) were amplified by PCR and cloned into pGL4-promoter; and we termed these plasmids as 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. PCR products were cloned into pGL4-promoter and termed pGL4P-Esrrb-ERRE2 mutant and -ERRE4 mutant, respectively.

Esrrb 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.

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 48h after transfection. To establish stable transfectants, ES cells were reseeded 48h after transfection and treated with puromycin for another 3-5 days.

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

Reverse transcription-PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR). Total RNAs were isolated from ES cells with sepasol reagent (Nakalai Tesque) and converted to cDNAs by 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, qRT-PCR using Sybr Green (MxPro Mx3005P, Stratagene, La Jolla, CA) was performed, and expression levels of target genes were normalized with GAPDH. Primer sequences are listed in Table S1. No PCR products were amplified in non-RT samples (Fig. S1).

Western blot analysis and maltose binding protein (MBP) pull-down assay. ES cells lysates 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 LAS-1000 image analyzer (Fuji Film, Tokyo, Japan).

MBP pull-down 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 $MgCl_2$ and

150 mM NaCl), and the bound proteins were eluted by boiling in 2 x 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 pull-down 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 DTT, 10 μg/mL leupeptin, 10 μg/mL pepstatin and 10 μg/mL aprotinin). After incubation on ice for 15 min, samples were spun down at 1,000 rpm for 10 min to pellet the nuclei. The pellet was resuspended at 2.5×10^7 nuclei/mL in the same buffer, and then 62.5 μL of 5M 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 biotin-labeled DNA pull-down 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 non-labeled either wild-type or mutant oligonucleotide was added. The beads were washed three times with the washing buffer, and signals were detected by Western blot analysis 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

215 UCG UAU AGG-3’ for *Esrrb#1*, 5’-UGA CUA AGA UCG UCU CGA AUC-3’ for
216 *Esrrb#2*, 5’-ACC UGC ACU UCG AGA UGA UGG AGA UCC-3’ for *Dax1* and
217 5’-GCC ACA ACG UCU AUA UCA UGG-3’ for *EGFP*. ES cells (1.5×10^5 cells) in
218 a 6-cm dish were transfected with either siRNA or the siRNA expression vector using
219 lipofectamine 2000.
220
221 **Chromatin immunoprecipitation assay.** Chromatin immunoprecipitation (ChIP)
222 assay was performed according to manufacturer's protocol (Diagenode OneDay ChIP
223 Kit, Nippon Gene, Tokyo, Japan). Briefly, ES cells were fixed with formaldehyde and
224 genomic DNA was sheared. Normal murine IgG (sc-2025, Santa Cruz) and anti-Esrrb
225 antibody were added to the sheared chromatin. For the detection of precipitated
226 genomic DNA, PCR analysis was performed using specific primers, and PCR products
227 were subjected to agarose gel electrophoresis.
228
229

RESULTS

Screening of Dax1-interacting proteins. To search for Dax1-interacting proteins, we performed a yeast two-hybrid screening using 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 Dax1, was detected in undifferentiated ES cells, and their expression was reduced upon differentiation induced by LIF depletion (Fig. 1A and 1C).

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 dramatically decreased by Tet stimulation and was restored after Tet removal as described previously (21, 27) (Fig. 1B and 1D), 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 - +8931) and Oct3/4 #B (+34733 - +34740) (Fig. S2A). 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 treatment with Tet (Fig. S2B). When the Oct3/4 #A region was mutated, this element had no enhancer activity either in the presence or absence of Tet (Fig. S2C). 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 MBP pull-down assay in mammalian HEK293 cells. To determine the Esrrb-binding region of Dax1, we prepared several truncated mutants of Dax1 including full-length of Dax1 (amino acids 1 - 472), DNA-binding domain (DBD, amino acids 1 - 255), Q1a (amino acids 1 - 100), Q1b (amino acids 68 - 139), Q23e (amino acids 136 - 202), and ligand-binding domain (LBD, amino acids 256 - 472) (Fig. 2A). Esrrb was precipitated strongly with full-length Dax1, DBD, Q1a (the 1st and the 2nd LXXLL regions) and Q23e (the 3rd LXXLL region), and weakly with Q1b (the 2nd LXXLL region) and LBD (Fig. 2B), suggesting that the 3rd 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 - 433), E1 (amino acids 1 - 211), E2 (activation domain [AD], amino acids 1 - 168), E3 (amino acids 169 - 433), E4 (ligand-binding domain [LBD], amino acids 212 - 433), E5 (amino acids 93 - 433) and E7 (DNA-binding domain [DBD], amino acids 93 - 211) (Fig. 3A). Dax1 was precipitated strongly with full-length Esrrb, E3 and E4 regions, and weakly with E2 and E5 regions, but not with 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 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) (Fig. S3A and S3B). Next, we constructed LXXLL motifs-mutated Dax1 (Dax1 LTm) (Fig. S4A). As shown in Fig. S4B and S4C, 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 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 is not only a protein-protein interaction partner of Esrrb, but a downstream target of Esrrb. To explore this possibility, we first performed luciferase reporter assay. When we examined -2.1 kb of the 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 -0.5 kb of the 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 including 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 biotinylated DNA pull-down assay to examine 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 precipitate disappeared in the presence of 50-fold non-labeled 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. Current finding together with our previous observation (20) 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 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 upon Dax1 expression. As shown in Fig. 6B, two independent Esrrb RNAi decreased expression levels of endogenous Dax1 mRNA. Also, expression level of Dax1 protein was reduced by Esrrb knockdown (Fig. 6C). Interestingly, endogenous Oct3/4 was maintained in the Esrrb knockdown condition, 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 auto-regulation pathway of Esrrb gene expression. The *Esrrb* gene contains four putative Esrrb-responsive elements (Esrrb-ERRE1 to 4) in its intron regions (Fig. S5A); and Esrrb-ERRE2 and Esrrb-ERRE4 had enhanced reporter activities in A3-1 ES cells (Fig. S5B). Of note, enhancer activities of the Esrrb-ERRE2 and Esrrb-ERRE4 were reduced by either knocking-down endogenous Esrrb expression (Fig. S5C and S5D) or introducing mutations into the elements (Fig. S5E and S5F). Biotinylated DNA pull-down assay revealed that endogenous Esrrb associates with Esrrb-ERRE2 and Esrrb-ERRE4 (Fig. S5G). 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 7D). 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 between Esrrb, Dax1 and Oct3/4. We performed competitive MBP pull-down assay. As shown in Fig. 8A, Oct3/4 was co-precipitated 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 co-precipitated 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 Esrrb, we demonstrated competitive MBP pull-down assay by using the mutant Dax1. As shown in Fig. 8C, Oct3/4 was co-precipitated 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,

374 although Esrrb was not co-precipitated with MBP-Dax1 LTm. This finding is probably
375 explained as follows: Oct3/4 and Esrrb are known to form complex in ES cells (18, 29).
376 Although Oct3/4 associates with Dax1 LTm in the absence of Esrrb, Oct3/4 changes a
377 binding-partner to Esrrb in a dose-dependent manner. Taken together, these results
378 suggest that three complexes including Oct3/4-Dax1, Oct3/4-Esrrb, and Dax1-Esrrb
379 exist in cells; and Oct3/4, Dax1 and Esrrb have a competitive inhibition capacity for
380 each complex.
381
382 **Inhibition of Dax1-Esrrb interaction causes up-regulation of endodermal marker**
383 **genes.** To investigate functions of Esrrb-Dax1 complex in ES cells, Dax1 RNAi was
384 introduced into Esrrb-overexpressing ES A3-1 cells. Since the transfected cells have
385 both overexpressed Esrrb and repressed Dax1, expression level of the two genes is
386 unbalanced; and therefore Esrrb-Dax1 complex would be disrupted in the cells. When
387 we examined gene expressions of the cells, expression level of endodermal marker
388 genes (Gata4, Gata6 and Dab2), but not mesodermal (T) and ectodermal (Fgf5) genes,
389 were significantly enhanced in Dax1 RNAi-transfected Esrrb-overexpressing ES cells
390 (Fig. 9A). Enhanced expression of the endodermal marker genes also observed in
391 Esrrb- and Dax1 LTm-co-overexpressed ES cells, where Esrrb-Dax1 complex would be
392 disrupted (Fig. 9B). Previous investigations showed that Esrrb-overexpressing ES
393 cells are prone to differentiate into endoderm (5, 30). Taken together, these results
394 suggest that Esrrb enhances the expression of endodermal genes to induce
395 differentiation into endoderm, and Dax1 represses endodermal genes expression via
396 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 the subfamily 3 (estrogen receptor type). In mice, *Esrrb*-deficient embryos are embryonic lethal at 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). Conditional knockout mice of the *Esrrb* gene also exhibit head-bobbing and run in circle, which is caused by inner-ear defects (33). Of note, several germline 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 Esrrb interacts with Dax1, and the 1st and 3rd LXXLL motifs of Dax1 are crucial for the interaction. Especially, Esrrb associates with the 3rd LXXLL motif of Dax1 rather than the 1st LXXLL motif. We assume that Esrrb, Oct3/4 and Dax1 do not form a trimer complex. Instead, Esrrb or Oct3/4 exclusively binds by Dax1. Dax1 is known

421 to function as a transcriptional repressor (23, 35, 36). Transcriptional activities of
422 Oct3/4 and Esrrb are inhibited by Dax1. As described previously, hyper-activation of
423 Oct3/4 in ES cells leads to differentiation into primitive endoderm and mesoderm (10),
424 and Dax1 inhibits the over-activation and contributes to retaining self-renewal (20). A
425 similar relationship would be anticipated between Dax1 and Esrrb. Expression levels
426 of Esrrb in ES colonies are heterogeneous (mosaic-in-colony pattern) (29, 37, 38). Of
427 note, Esrrb-overexpressing ES cells are known to be prone to differentiate into
428 endoderm (5, 30). We generated Esrrb-overexpressing ES cells, and Dax1 RNAi was
429 introduced into the cells, where Esrrb-Dax1 complex would be disrupted. Expression
430 levels of self-renewal marker genes are comparable to control cells; whereas, that of
431 endodermal marker genes were enhanced. Enhanced expression of endodermal
432 markers were also observed in Esrrb- and Dax1 LTm-co-overexpressed ES cells, where
433 Esrrb-Dax1 complex would be disrupted, suggesting that Esrrb-Dax1 complex represses
434 Gata6 to prevent differentiation of ES cells. Concerning the Dax1-Oct3/4 complex,
435 Dax1 represses the transcriptional activity of Oct3/4 by inhibiting the DNA binding
436 activity of Oct3/4, since Dax1 associates with the DNA binding domain of Oct3/4 (20).
437 In case of Dax1-Esrrb complex, the association is not mediated through the
438 DNA-binding domain of Esrrb, suggesting that Dax1 will not inhibit the DNA binding
439 capacity of Esrrb.

440 Dax1 is a downstream target gene of STAT3 and Oct3/4 in ES cells (21).
441 LRH-1 and SF-1 also regulate Dax1 gene expression in ES cells and adrenocortical
442 carcinoma cells, respectively (22, 39); and the SF-1-mediated activation is repressed by
443 Dax1 via binding to a hairpin structure in the promoter region (40). Here, we found
444 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 its amino acid homology is about 70%. Esrrg is not expressed in ES cells (data not 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 among 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 their expressions are either self-regulated and/or regulated by each other (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, hyper-activation 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 knockout in ES cells leads to differentiation (44). Also, overexpression of Dax1 leads to downregulation of Sox2, Nanog, and Dax1 which are downstream target 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 study extends these findings and clarifies 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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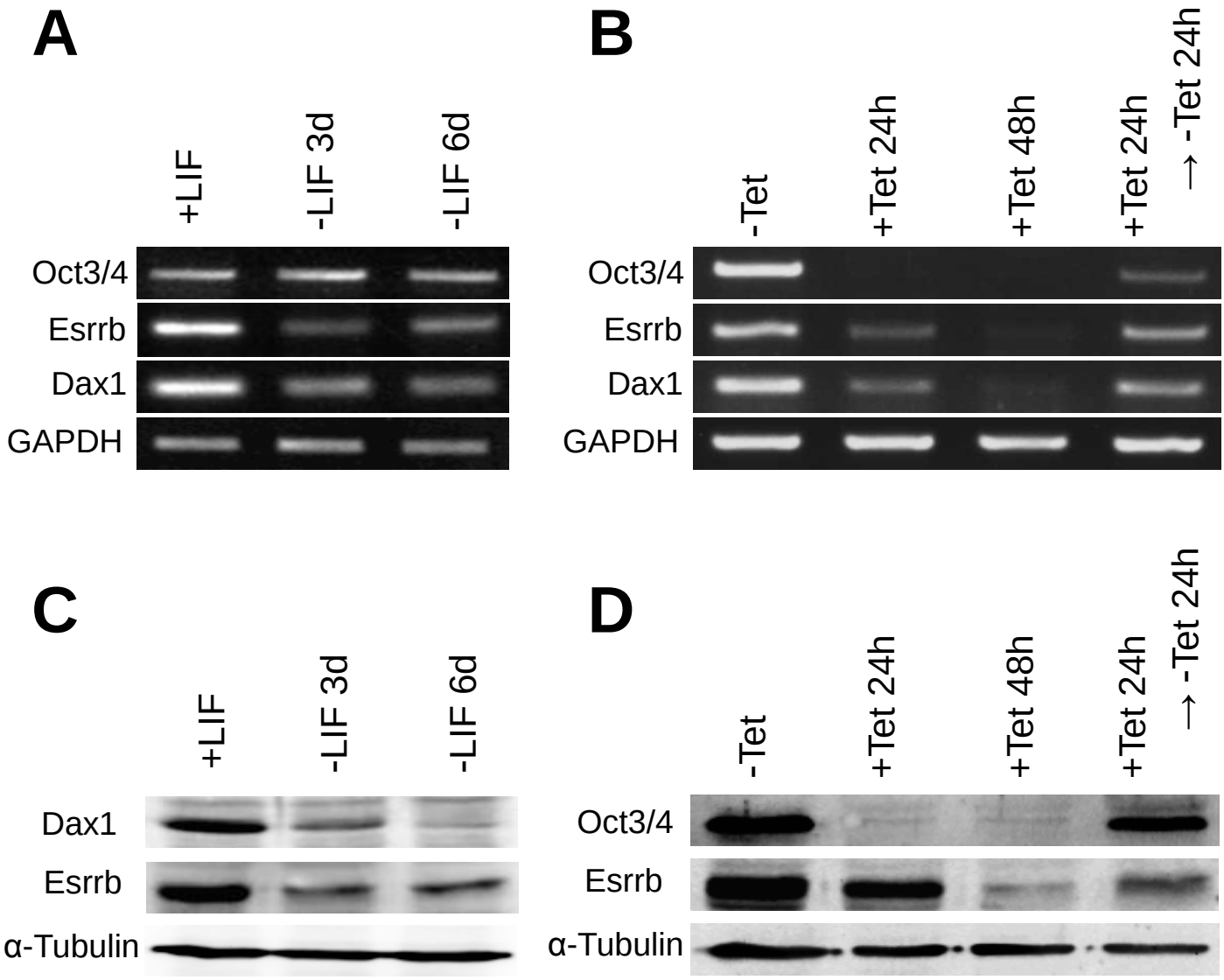


FIG. 1. Dax1 and Esrrb are expressed specifically in self-renewing ES cells. (A) Reduction of Esrrb mRNA expression by LIF withdrawal. After culture with (+) or without (-) LIF for 3 - 6 days, expression of Esrrb, as well as Oct3/4 and Dax1, in A3-1 ES cells was examined by RT-PCR analysis. (B) Reduction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

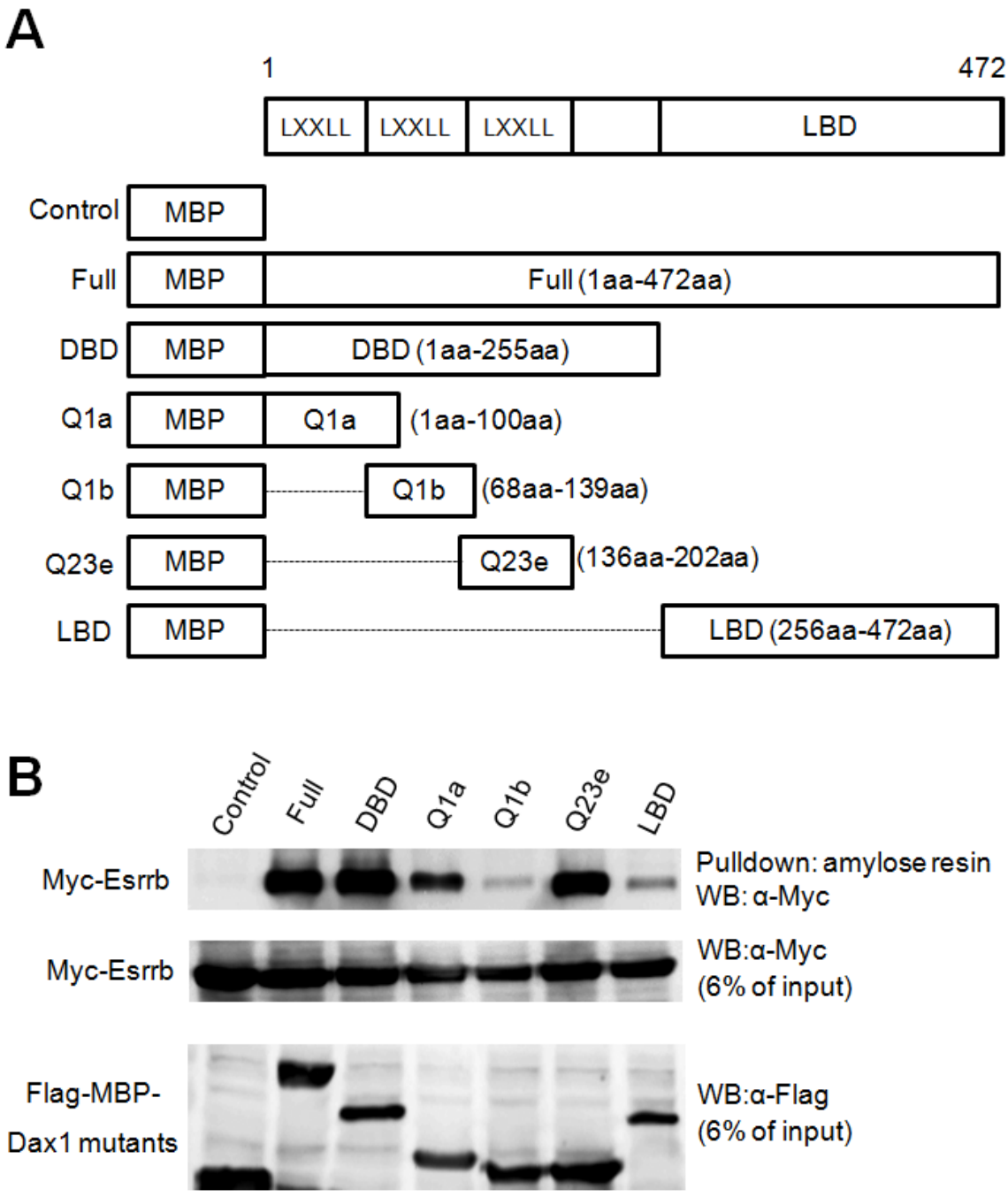


FIG. 2. Esrrb binds to LXXLL motifs of Dax1. (A) Schematic view of Flag-MBP-Dax1 and its truncated mutants. Several Flag-tagged MBP-fused constructs including full-length of Dax1 (amino acids 1 - 472), DNA-binding domain (DBD, amino acids 1 - 255), Q1a (amino acids 1 - 100), Q1b (amino acids 68 - 139), Q23e (amino acids 136 - 202) and ligand-binding domain (LBD, amino acids 256 - 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 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.

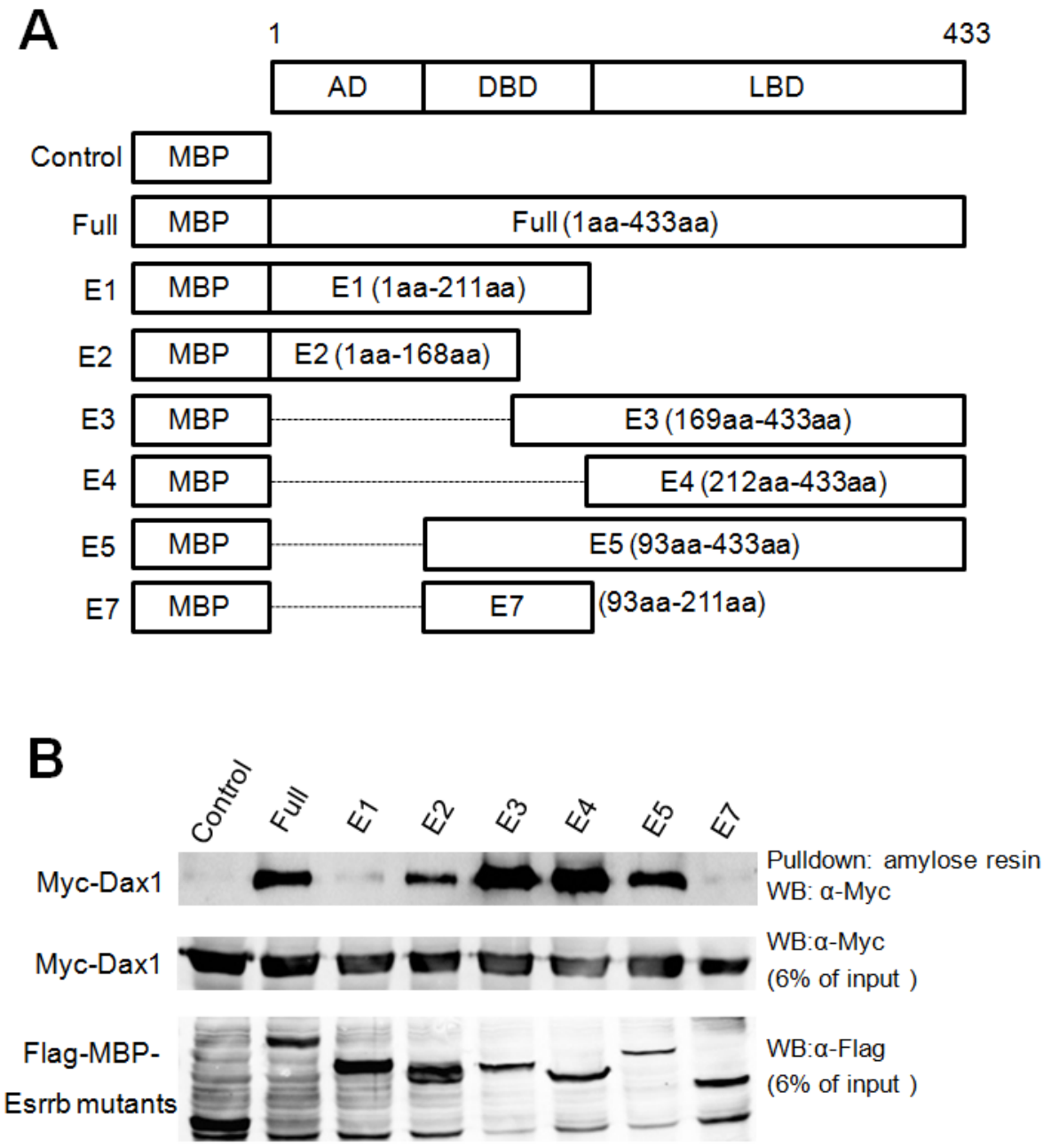


FIG. 3. Dax1 binds to the activation domain (AD), as well as the ligand-binding domain (LBD) of Esrrb. (A) Schematic view of Flag-MBP-Esrrb and its truncated mutants. Several Flag-tagged MBP-fused constructs including full-length of Esrrb (amino acids 1 - 433), E1 (amino acids 1 - 211), E2 (amino acids 1 - 168), E3 (amino acids 169 - 433), E4 (amino acids 212 - 433), E5 (amino acids 93 - 433) and E7 (amino acids 93 - 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 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.

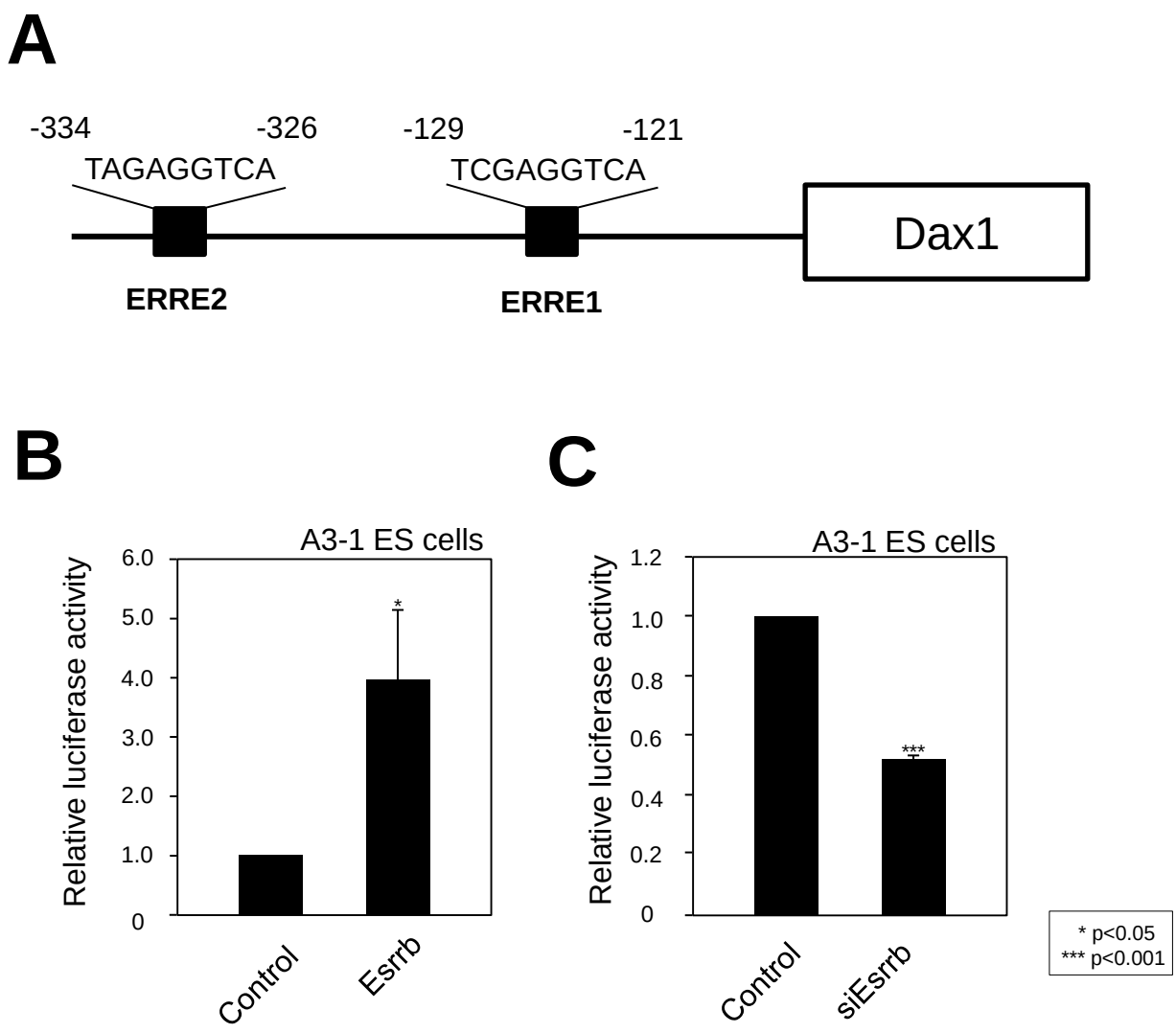


FIG. 4. Esrrb positively regulates the promoter activity of the Dax1 gene. (A) Esrrb-binding sites of the Dax1 gene. The promoter region of the Dax1 gene contains two Esrrb-binding sites called ERRE1 (-129 to -121) and ERRE2 (-334 to -326). (B) Transcriptional activity of Esrrb to the promoter region of the Dax1 gene. A3-1 ES cells were transfected with pGL4.10-Dax1P 2.1kb 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 48h after transfection. The bars represent the means and standard deviation of three independent experiments.

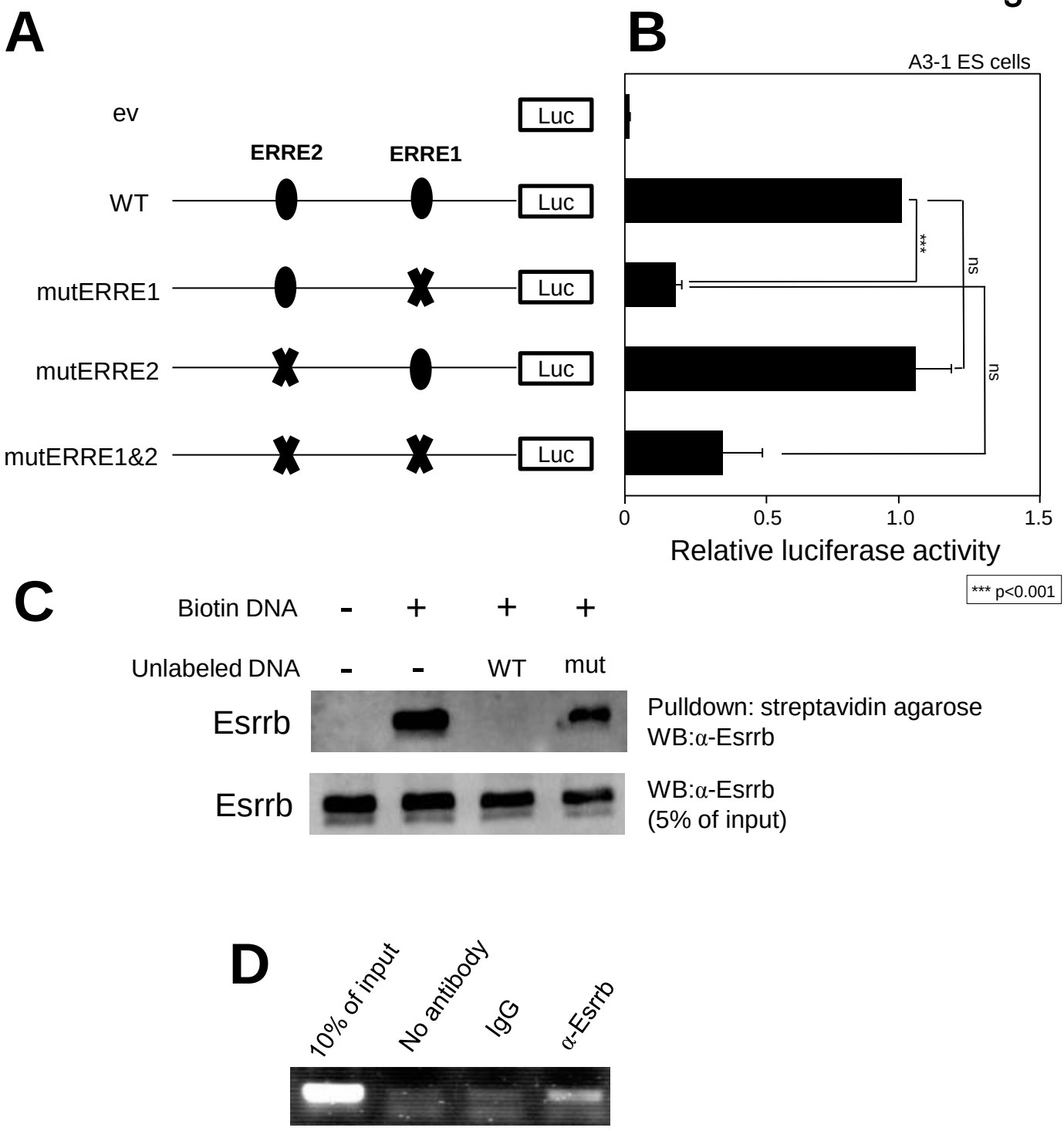


FIG. 5. Esrrb regulates promoter activity of the Dax1 gene via ERRE1. (A) Schematic view of pGL4.10-Dax1 promoter 546 bp (pGL4.10-Dax1P 546 bp) and putative Esrrb-binding sites (Dax1-ERRE1 and 2). To determine Esrrb responsive elements of the Dax1 gene, four reporter plasmids including wild-type (WT), 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 either pGL4.10 (empty vector, ev), pGL4.10-Dax1P 546 bp-WT, -mutERRE1, -mutERRE2 or -mutERRE1&2. Luciferase activity was measured 48h after transfection. The bars represent the means and standard deviations of three independent experiments. (C) Esrrb directly binds to Dax1-ERRE1 in vitro. Nuclear extracts from A3-1 ES cells were subjected to pull-down 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 non-labeled 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. 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.

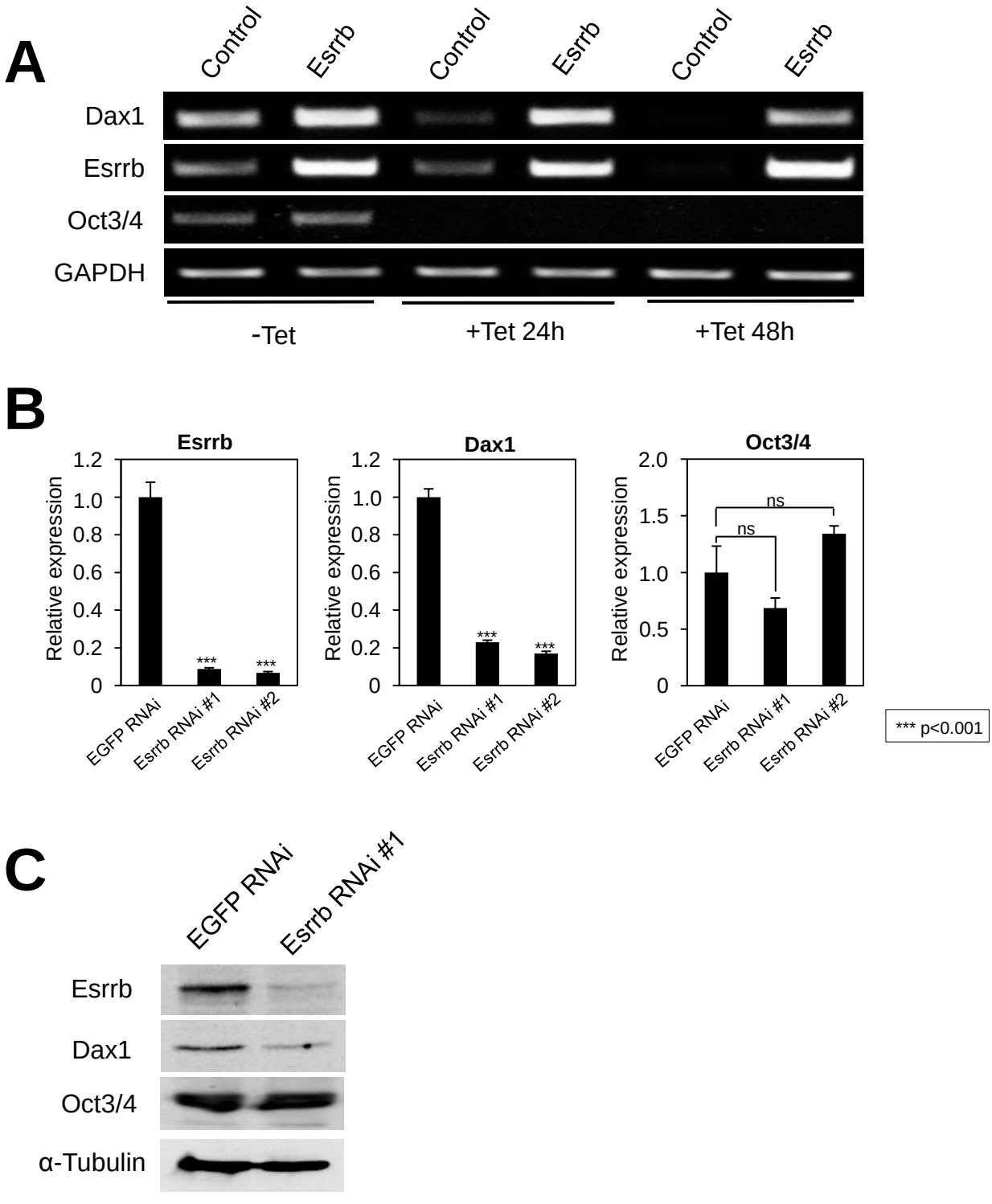


FIG. 6. Esrrb regulates expression of Dax1 in an Oct3/4-independent manner. (A) Esrrb maintains expression of Dax1 in the absence of Oct3/4. Control and Esrrb-overexpressing ZHBTc4 ES cells were cultured in the presence of LIF with (+) or without (-) 1μg/mL Tet for 24h - 48h. Expression levels of Dax1, Esrrb and Oct3/4 were examined by RT-PCR. (B) Knock-down of Esrrb leads to downregulation of Dax1 mRNA. Either EGFP dsRNA (EGFP RNAi) or Esrrb dsRNAs (Esrrb RNAi #1 and #2) was introduced into A3-1 ES cells, and expression levels of Esrrb, Dax1 and Oct3/4 were analyzed 48h after transfection. All samples were analyzed in triplicate and normalized with GAPDH expression. (C) Knock-down of Esrrb leads to downregulation of Dax1 protein

expression. Either EGFP dsRNA (EGFP RNAi) or Esrrb dsRNAs (Esrrb RNAi #1) was introduced into A3-1 ES cells, and expression levels of Esrrb, Dax1 and Oct3/4 were analyzed 48h after transfection. α-Tubulin was used as a loading control. All results are representative of three separate experiments.

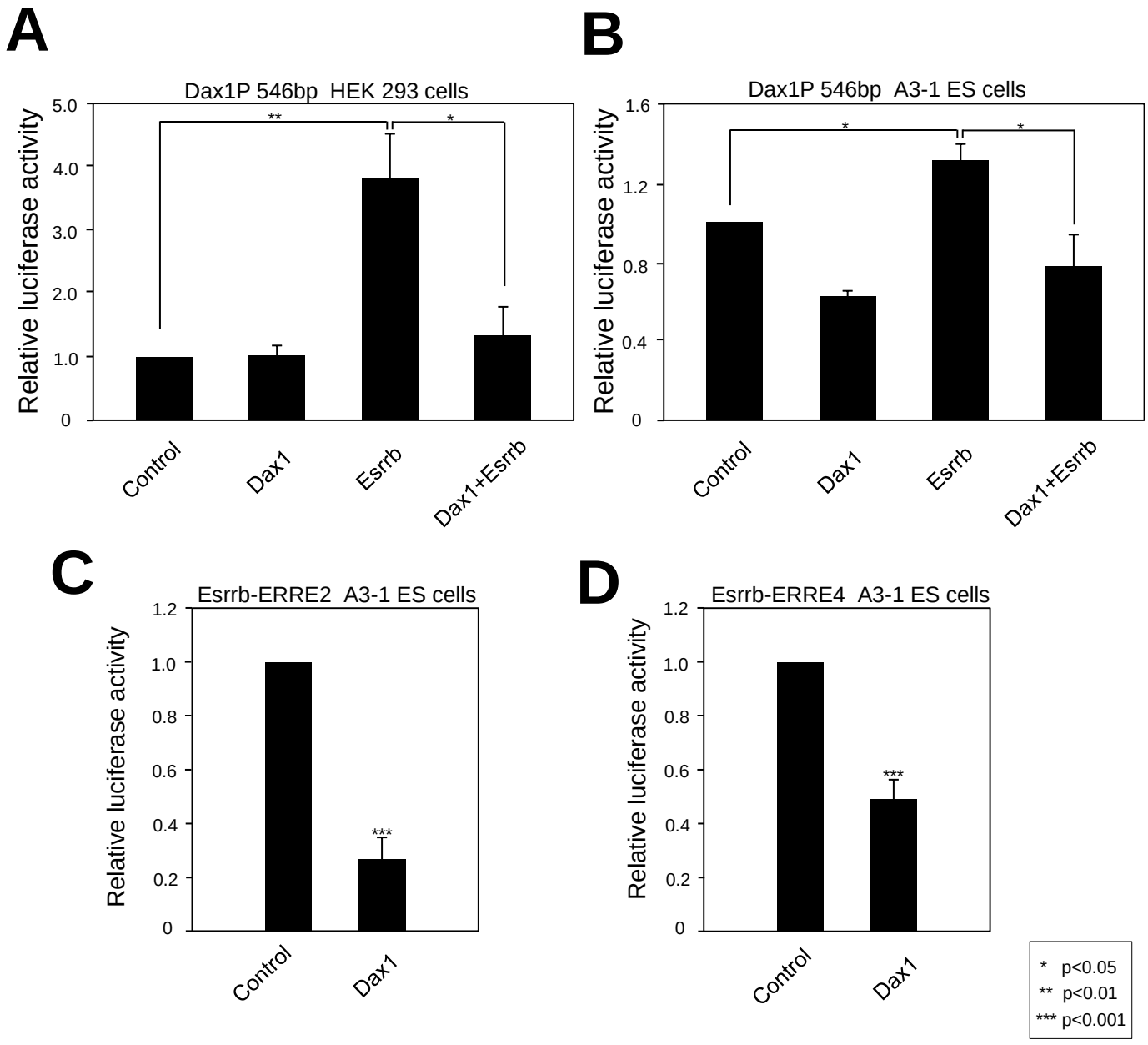
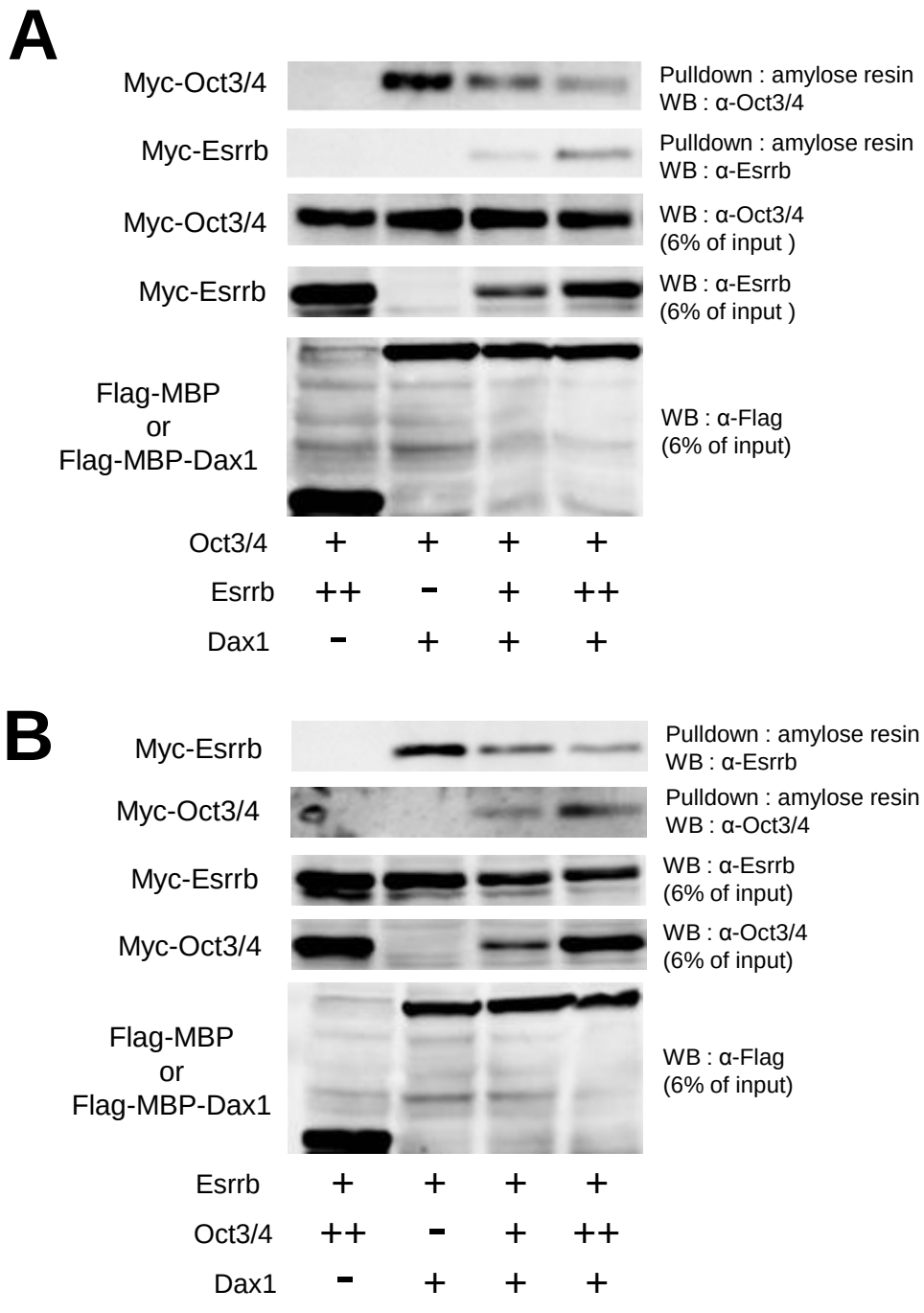


FIG. 7. Dax1 inhibits transcriptional activity of Esrrb. (A) Transcriptional activity of Esrrb upon Dax1 expression is repressed by Dax1 in HEK293 cells. Reporter plasmid carrying Esrrb-responsive element (ERRE) of the Dax1 gene (pGL2-Dax1P 546 bp) was transfected into HEK293 cells with Flag-Dax1 and/or 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 48h after transfection. The bars represent the means and standard deviations of three independent experiments.


A
Myc-Oct3/4
Pulldown : amylose resin
WB : α-Oct3/4
Myc-Esrrb
Pulldown : amylose resin
WB : α-Esrrb
Myc-Oct3/4
WB : α-Oct3/4
(6% of input)
Myc-Esrrb
WB : α-Esrrb
(6% of input)
Flag-MBP
or
Flag-MBP-Dax1
WB : α-Flag
(6% of input)
Oct3/4 + + + +
Esrrb ++ - + ++
Dax1 - + + +
B
Myc-Esrrb
Pulldown : amylose resin
WB : α-Esrrb
Myc-Oct3/4
Pulldown : amylose resin
WB : α-Oct3/4
Myc-Esrrb
WB : α-Esrrb
(6% of input)
Myc-Oct3/4
WB : α-Oct3/4
(6% of input)
Flag-MBP
or
Flag-MBP-Dax1
WB : α-Flag
(6% of input)
Esrrb + + + +
Oct3/4 ++ - + ++
Dax1 - + + +

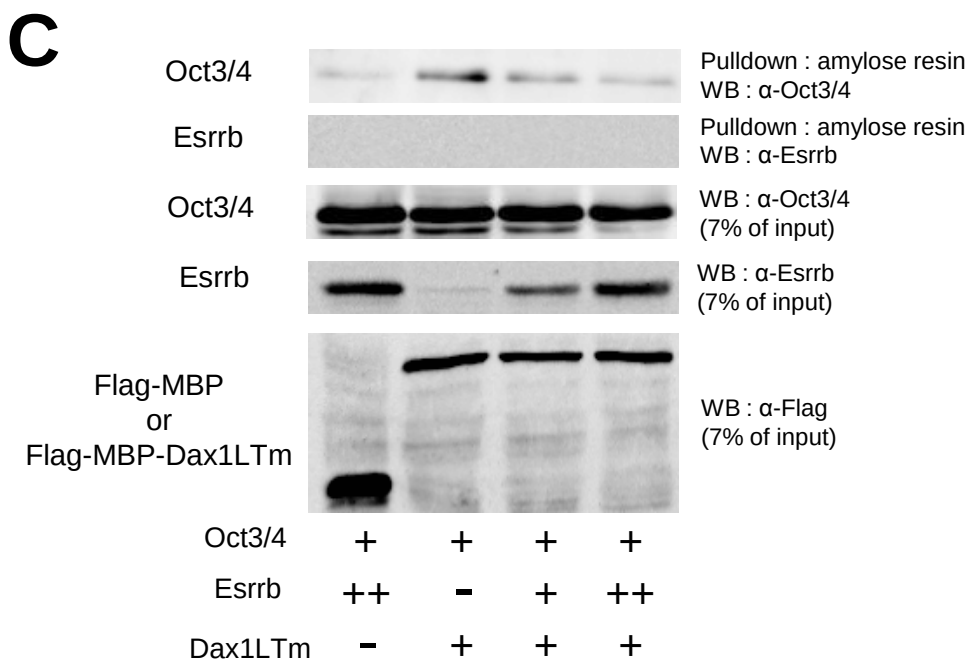


FIG. 8. Oct3/4, Dax1 and Esrrb have a competitive inhibition capacity for their interaction. (A) Oct3/4 is competed out from Dax1-Oct3/4 complex in the presence of excess amounts of Esrrb. HEK293 cells were transfected with Myc-Oct3/4 and Flag-MBP-Dax1 with or without Myc-Esrrb (0 ng, 50 ng or 100 ng). 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 competed out from Dax1-Esrrb complex 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 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.

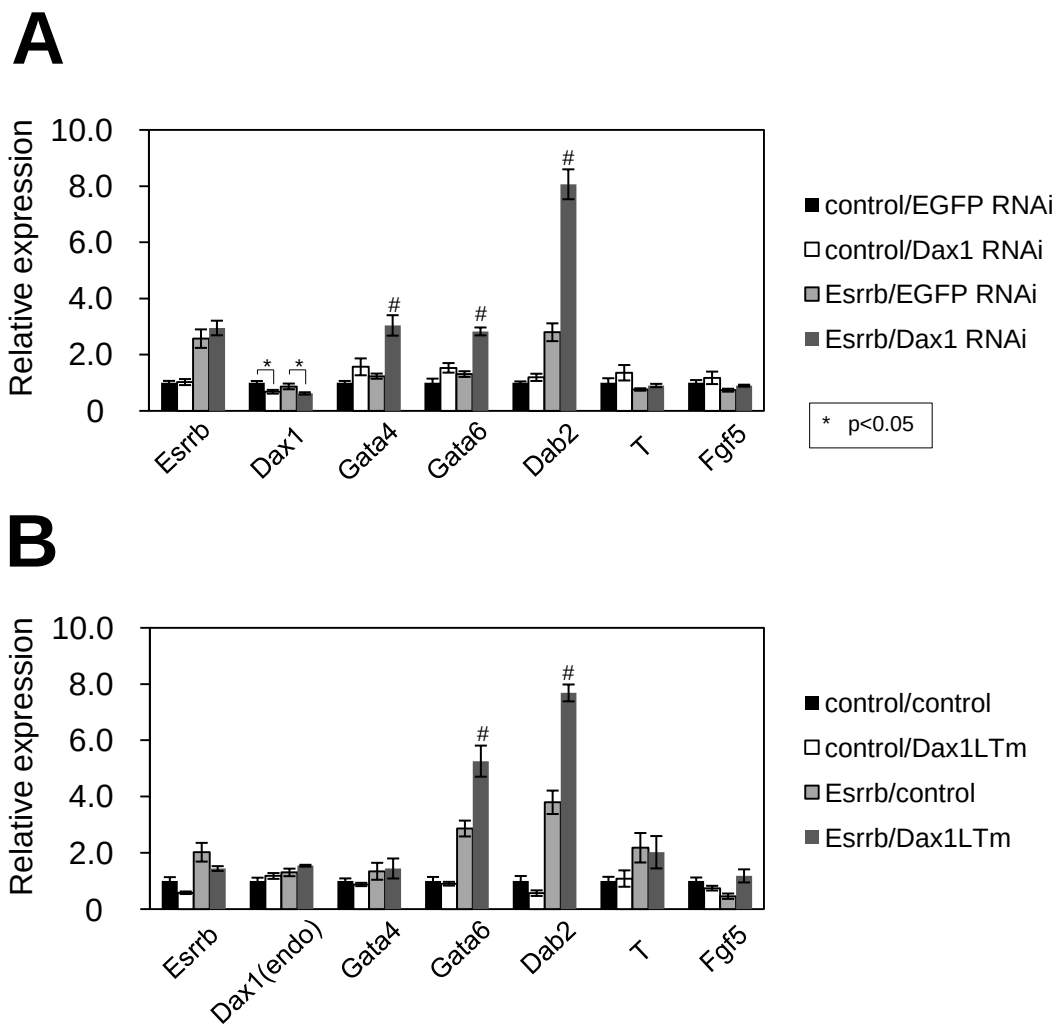


FIG. 9. Inhibition of Dax1-Esrrb interaction causes up-regulation of endodermal marker genes. (A) Control and Esrrb-overexpressing A3-1 ES cells were transfected with either EGFP dsRNA (EGFP RNAi) or Dax1 dsRNAs (Dax1 RNAi), and expression of the indicated genes was analyzed 96h after transfection. (B)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

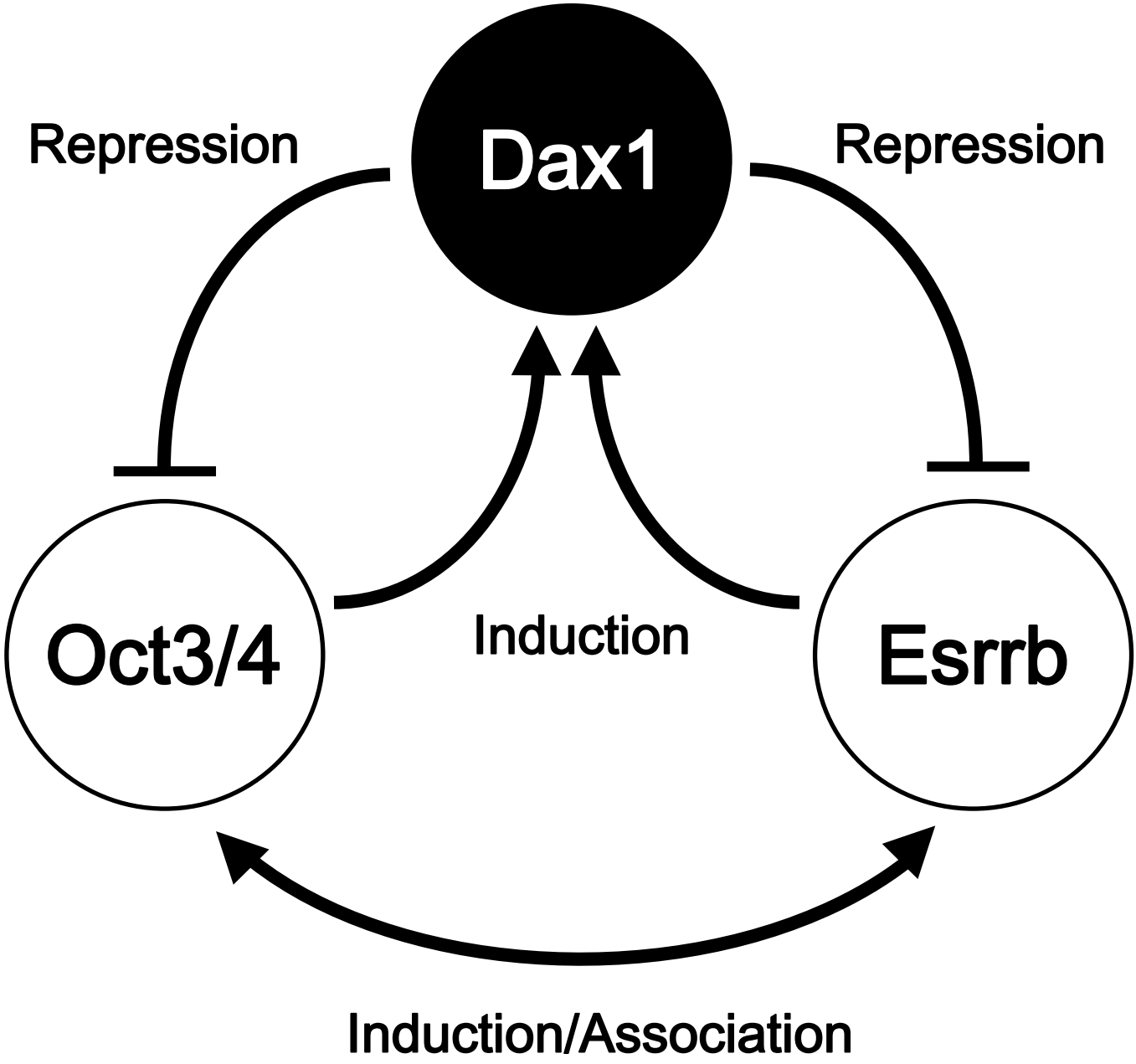


Fig. 10. Proposed model of Dax1, Esrrb and Oct3/4 interaction. Oct3/4 and Esrrb induce the expression of Dax1. Dax1 associates with Oct3/4 or Esrrb and represses transcriptional activity of them. Oct3/4 and Esrrb interact and regulate gene expression of each other. The proposed regulatory network contributes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

Supplemental Table 1. Nucleotide sequences

Oligonucleotide names		Sequences (5' to 3')
For RT-PCR analysis		
GAPDH	F	TGATGACATCAAGAAGGTGGTGAAG
	R	TCCTTGGAGGCCATGTAGGCCAT
Oct3/4	F	GCCCTGCAGAAGGAGCTAGAAC
	R	GGAATACTCAATACTTGATCT
Esrrb	F	TAGGGGTTGAGCAGGACAAG
	R	CTACCAGGCGAGAGTGTTCC
Dax1	F	CAGATCCGCTGAACTGAACA
	R	CTACGACCGCTTTCTCCATC
Gata4	F	GCCTGTATGTAATGCCTGCG
	R	CCGAGCAGGAATTTGAAGAGG
Gata6	F	GCAATGCATGCGGTCTCTAC
	R	CTCTTGGTAGCACCAGCTCA
Dab2	F	GGCAACAGGCTGAACCATTAGT
	R	TTGGTGTCGATTTCAGAGTTTAGAT
Fgf5	F	GCTGTGTCTCAGGGGATTGT
	R	CACTCTCGGCCTGTCTTTTC
T	F	CTCCAACCTATGCGGACAAT
	R	CCATTGCTCACAGACCAGAG
Dax1 promoter (for ChIP)	F	GTGCTGAGACTCTCCCTTGG
	R	AGCGCGTCCGCCTCCTCCTCTTGGA
For expression vectors		
Dax1 full length	F	GAATTCATGGCGGGTGAGGACCACCCG
	R	GCGGCCGCTCACAGCTTTGCACAGAGC
Dax1 DBD	F	GAATTCATGGCGGGTGAGGACCACCCG
	R	GCGGCCGCTACACCTGTGGATCCTTGAG
Dax1 Q1a	F	GAATTCATGGCGGGTGAGGACCACCCG
	R	GCGGCCGCTCATCGTGCCCTCGGCGCCTG
Dax1 Q1b	F	GAATTCTTTTGTGGGGAGAATCAC
	R	GCGGCCGCTATTCTCCGCAGAAACAACAGCG
Dax1 Q23e	F	GAATTCGTGTGCGGTGAAGAGCAG

	R	GCGGCCGCTAGCTGCGGTACAGGAAC
Dax1 LBD	F	GAATTCGTGTGCGAGGCAGCGTCC
	R	GCGGCCGCTCACAGCTTTGCACAGAGC
Dax1 Q23	F	GAATTCGGAGCACCTTGCTGGGGC
	R	GCGGCCGCTACTCTTTGGTGTCAATGTTCAG
Dax1 Q23-1	F	GAATTCTGCGGAGAAGAGCAC
	R	GCGGCCGCTACTCTTTGGTGTCAATGTTCAG
Dax1 Q23-2	F	GAATTCGGAGCACCTTGCTGGGGC
	R	GCGGCCGCTAGAAGTGCAGGTGATCTTG
Dax1 Q23a	F	GAATTCTGCGGAGAAGAGCAC
	R	GCGGCCGCTAGAAGTGCAGGTGATCTTG
Dax1 1st LXXLLmut	F	GAATTCATGGCGGGTGAGGACCACCCGTGGCAGGGCAGCATCCTCTACAATGCAGCGATGA
	R	TCATCGCTGCATTGTAGAGGATGCTGCCCTGCCACGGGTGGTCCTCACCCGCCATGAATTC
Dax1 2nd LXXLLmut	F	TCCTCTACTCTGCGGCCACCAACGCCAG
	R	CTGGCGTTGGTGGCCGCAGAGTAGAGGA
Dax1 3rd LXXLLmut	F	TTATACAGCGCGGCCACTAGCGCTCAGCAAACGCACGTG
	R	CACGTGCGTTTGCTGAGCGCTAGTGGCCGCGCTGTATAA
Esrrb full length	F	GAATTCATGTCGTCCGAAGACAGGCAC
	R	GCGGCCGCTCACACCTTGGCCTCCAGCATC
Esrrb E1	F	GAATTCATGTCGTCCGAAGACAGGCAC
	R	GCGGCCGCTCACTTAGTCAATGGCTT
Esrrb E2	F	GAATTCATGTCGTCCGAAGACAGGCAC
	R	GCGGCCGCTCACATCCCCACTTTGAG
Esrrb E3	F	GAATTCCTGAAGGAAGGTGTGCGC
	R	GCGGCCGCTCACACCTTGGCCTCCAGCATC
Esrrb E4	F	GAATTCATCGTCTCGAATCTACTA
	R	GCGGCCGCTCACACCTTGGCCTCCAGCATC
Esrrb E5	F	GAATTCTACATGCTTAACGCCATC
	R	GCGGCCGCTCACACCTTGGCCTCCAGCATC
Esrrb E7	F	GAATTCTACATGCTTAACGCCATC
	R	GCGGCCGCTCACTTAGTCAATGGCTT

For reporter constructs

Dax1 promoter 546bp	F	GGTACCGCTTGGCTAGCACTGAAAAA
	R	CTCGAGGCCGCTTGGGACTTATTTAT
Dax1 promoter 546bp mutERRE1 5'side	F	GGTACCGCTTGGCTAGCACTGAAAAA
	R	GTGTGGCCATTAAATCGAAAGCTC
Dax1 promoter 546bp mutERRE1 3'side	F	GAGCTTTCGATTTAATGGCCACAC
	R	CTCGAGGCCGCTTGGGACTTATTTAT
Dax1 promoter 546bp mutERRE2 5'side	F	GGTACCGCTTGGCTAGCACTGAAAAA
	R	CTTAGCTCTTAAATCTAACTTAT
Dax1 promoter 546bp mutERRE2 3'side	F	ATAAGTTAGATTTAAGAGTCTAAG
	R	CTCGAGGCCGCTTGGGACTTATTTAT
Esrrb enhancer Oct3/4#A	F	GGTACCCAGTGGCTGGTGACACTCTG
	R	CTCGAGTGATGGGATGGTGTTCTCAA
Esrrb enhancer Oct3/4#B	F	GGTACCGAAGTGGTTCTTGCCGGTTA
	R	CTCGAGGGTAGTGCGCCTCAAAAGTC
Esrrb enhancer Oct3/4#A mut 5’side	F	GGTACCCAGTGGCTGGTGACACTCTG
	R	GACCCTAAAGCGGCACTTCCAAGGAA
Esrrb enhancer Oct3/4#A mut 3’side	F	TTCCTTGGAAGTGCCGCTTTAGGGTC
	R	CTCGAGTGATGGGATGGTGTTCTCAA
Esrrb enhancer	F	GGTACCAGCCAGGGGGATACAGAGAA

ERRE1		
	R	CTCGAGTCCTTTGTGGTTTTGTTTGG
Esrrb enhancer ERRE2	F	GGTACCTGCTGGTGGTATTCAACTGC
	R	CTCGAGCAAGGATTGCACATCAAGGA
Esrrb enhancer ERRE3	F	GGTACCGAGTGTCCTGTGGCGGTTAC
	R	CTCGAGGAGCCAGTTGGTAGCCAAAG
Esrrb enhancer ERRE4	F	GGTACCCCCCTATCCAGCGTTCATTA
	R	CTCGAGGAAAAGAGGAAGGGACCAGAG
Esrrb enhancer ERRE2 mut 5’side	F	GGTACCTGCTGGTGGTATTCAACTGC
	R	TTTTCAGTTTAAATTGAAGCAGGGAT
Esrrb enhancer ERRE2 mut 3’side	F	ATCCCTGCTTCAATTTAACTGAAAA
	R	CTCGAGCAAGGATTGCACATCAAGGA
Esrrb enhancer ERRE4 mut 5’side	F	GGTACCCCCCTATCCAGCGTTCATTA
	R	TCCAAAACTTAAATTGAACTTTGGAT
Esrrb enhancer ERRE4 mut 3’side	F	ATCCAAAGTTCAATTTAAGTTTTGGA
	R	CTCGAGGAAAAGAGGAAGGGACCAGAG

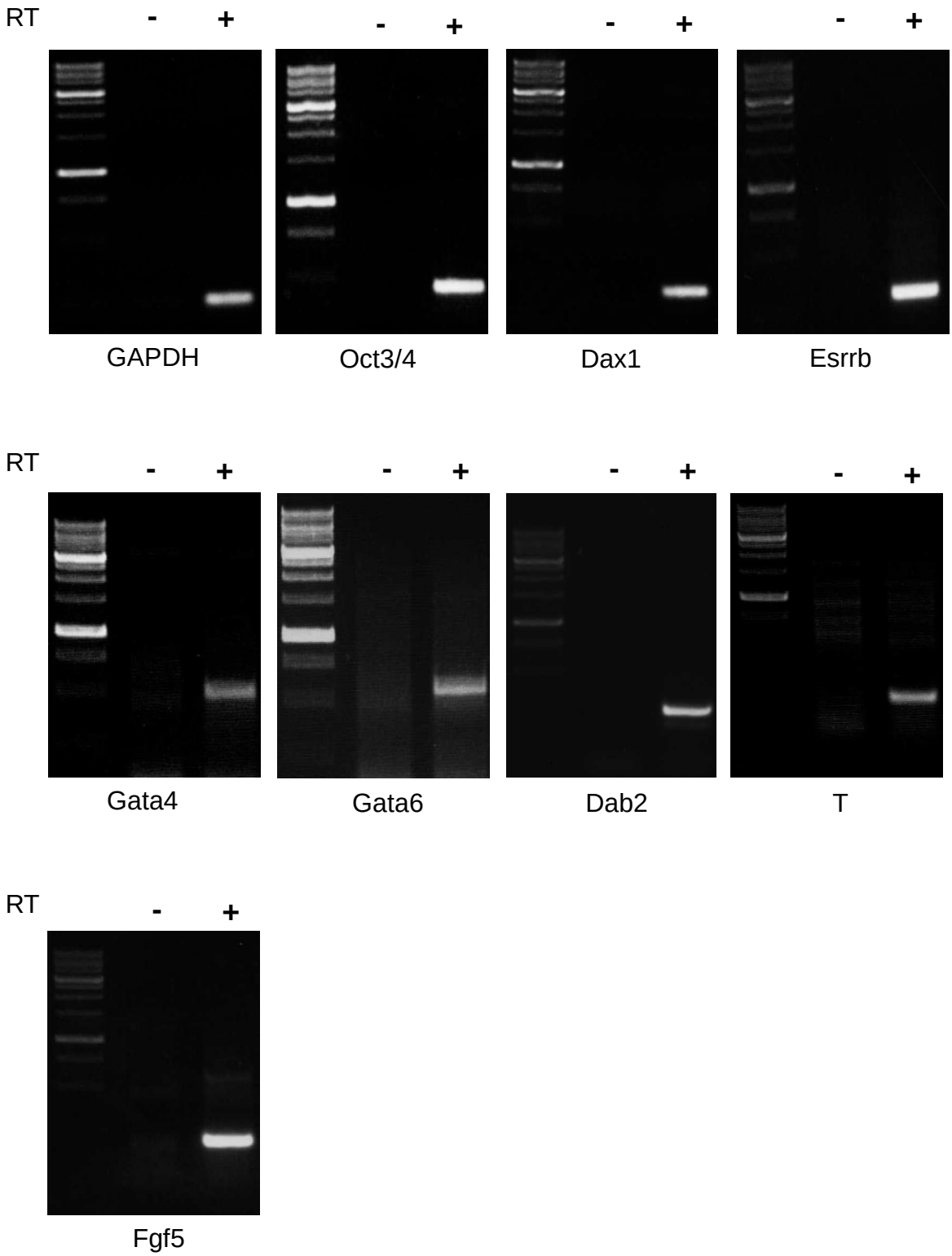
For biotin-labeled DNA pull-down assay

Dax1 ERRE1(3' biotin)	F	CTTTGAGCTTTCGAGGTCATGGCCACACA-biotin
	R	TGTGTGGCCATGACCTCGAAAGCTCAAAG
Dax1 ERRE1 competitor wild-type DNA	F	CTTTGAGCTTTCGAGGTCATGGCCACACA
	R	TGTGTGGCCATGACCTCGAAAGCTCAAAG
Dax1 ERRE1 competitor mutant DNA	F	CTTTGAGCTTTCGATTTAATGGCCACACA

	R	TGTGTGGCCATTAAATCGAAAGCTCAAAG
Esrrb ERRE2 (3’ biotin)	F	ATCCCTGCTTCAAGGTCAACTGAAAA-biotin
	R	TTTTCAGTTGACCTTGAAGCAGGGAT
Esrrb ERRE2 competitor wild-type DNA	F	ATCCCTGCTTCAAGGTCAACTGAAAA
	R	TTTTCAGTTGACCTTGAAGCAGGGAT
Esrrb ERRE2 competitor mutant DNA	F	ATCCCTGCTTCAATTTAAACTGAAAA
	R	TTTTCAGTTTAAATTGAAGCAGGGAT
Esrrb ERRE4 (3’ biotin)	F	ATCCAAAGTTCAAGGTCAGTTTTGGA-biotin
	R	TCCAAAACTGACCTTGAACTTTGGAT
Esrrb ERRE4 competitor wild-type DNA	F	ATCCAAAGTTCAAGGTCAGTTTTGGA
	R	TCCAAAACTGACCTTGAACTTTGGAT
Esrrb ERRE4 competitor mutant DNA	F	ATCCAAAGTTCAATTTAAGTTTTGGA
	R	TCCAAAACTTAAATTGAACTTTGGAT

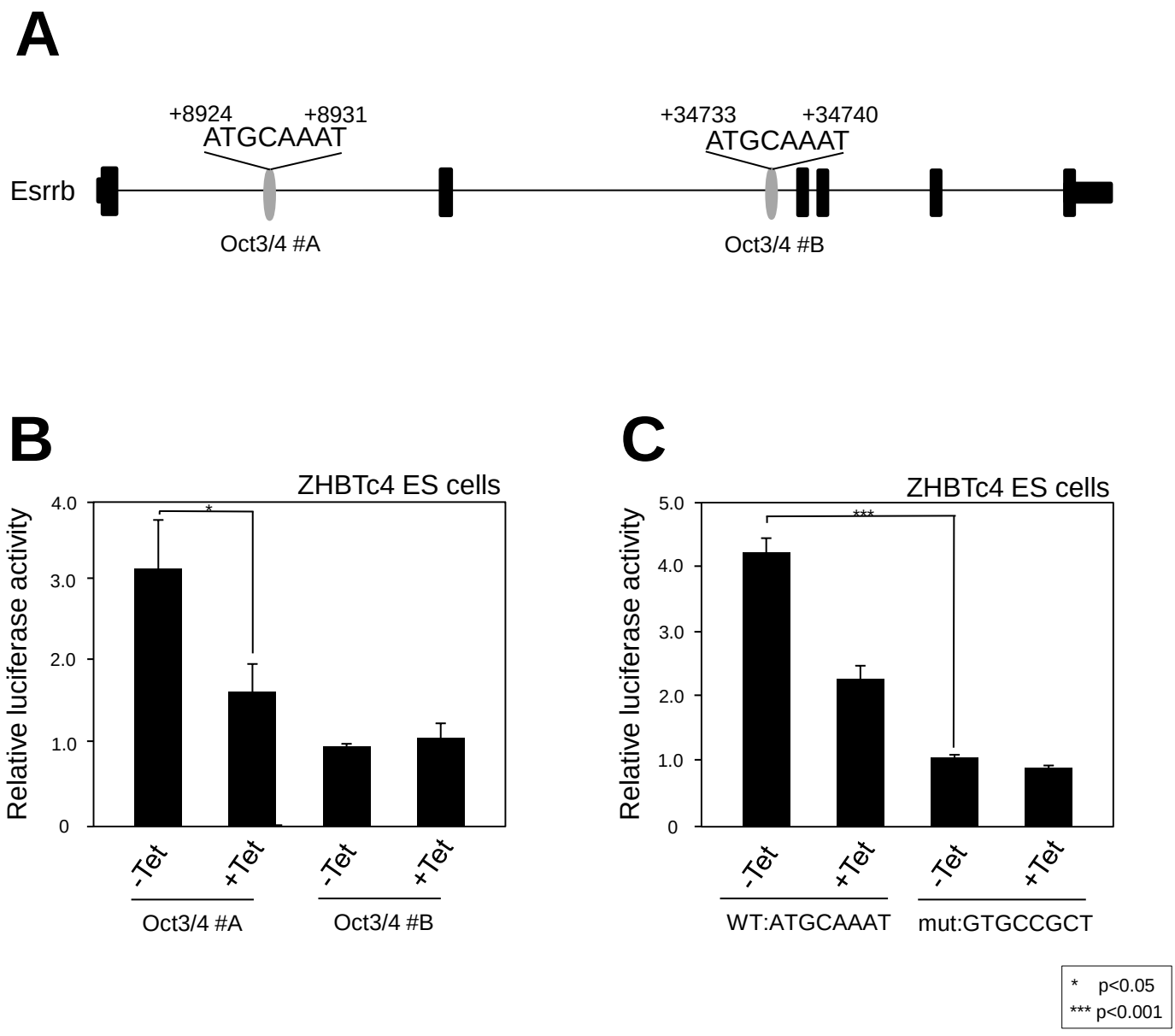
For knockdown analysis

Esrrb siRNA	F	AAAGGCAGTCTGGTTGTCGTATA
	R	AAAATATACGACAACCAGACTGC
Esrrb dsRNA #1	F	GCAGUCUGGUUGUCGUAUAGG
	R	UAUACGACAACCAGACUGCAG
Esrrb dsRNA #2	F	UGACUAAGAUCGUCUCGAAUC
	R	UUCGAGACGAUCUUAGUCAAU
Dax1 dsRNA	F	ACCUGCACUUCGAGAUGAUGGAGAUCC
	R	AUCUCCAUCAUCUCGAAGUGCAGGUGA
EGFP dsRNA	F	GCCACAACGUCUAUAUCAUGG
	R	AUGAUAUAGACGUUGUGGCUG



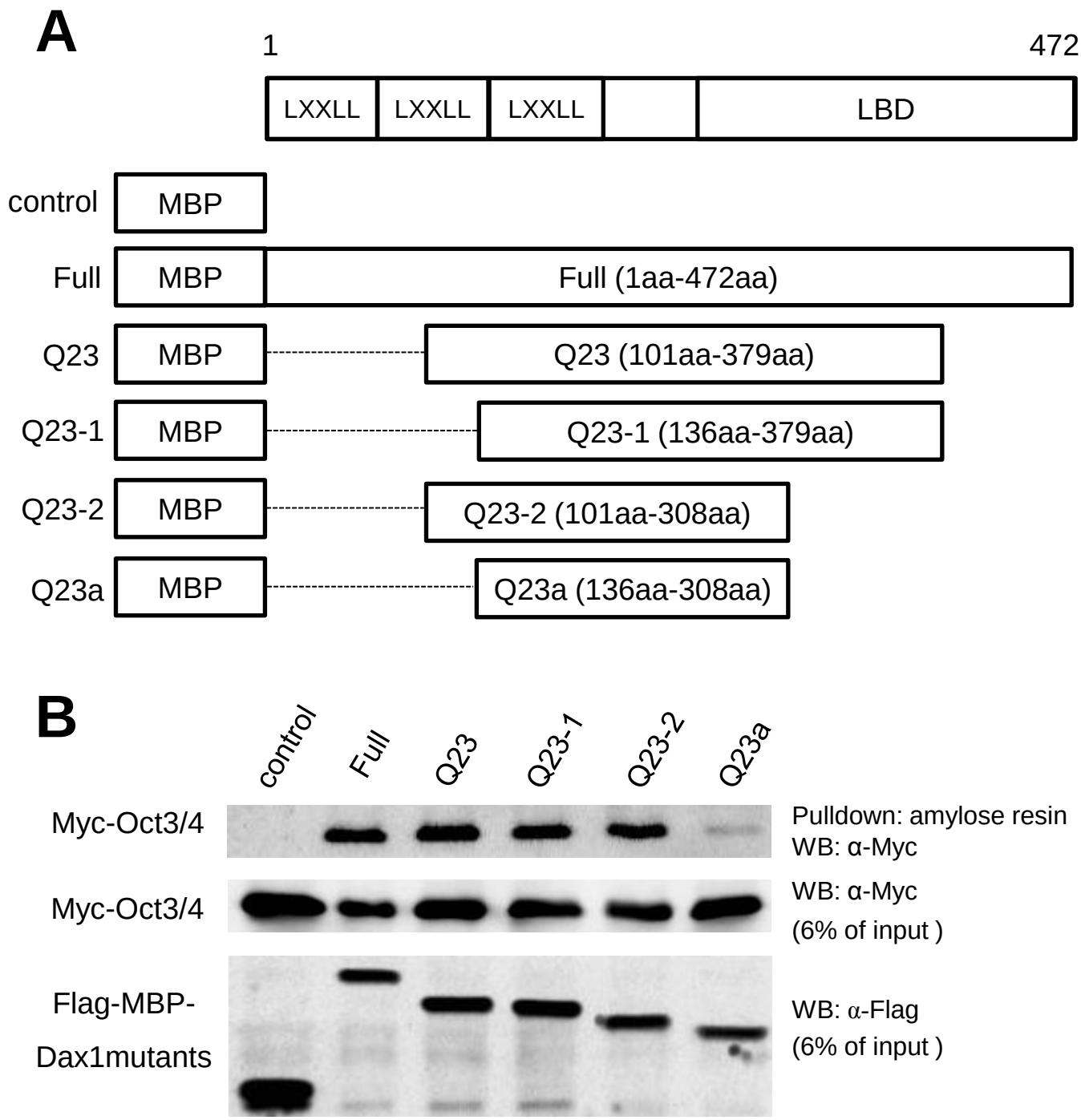
S-Fig. 1. Verification of PCR products. cDNA samples of A3-1 ES cells were synthesized with (+) or without (-) reverse transcriptase (RT), and PCR analysis was performed using specific primers. PCR products were subjected to agarose gel electrophoresis. No PCR products were obtained in non RT samples.

Supplemental Figure 1



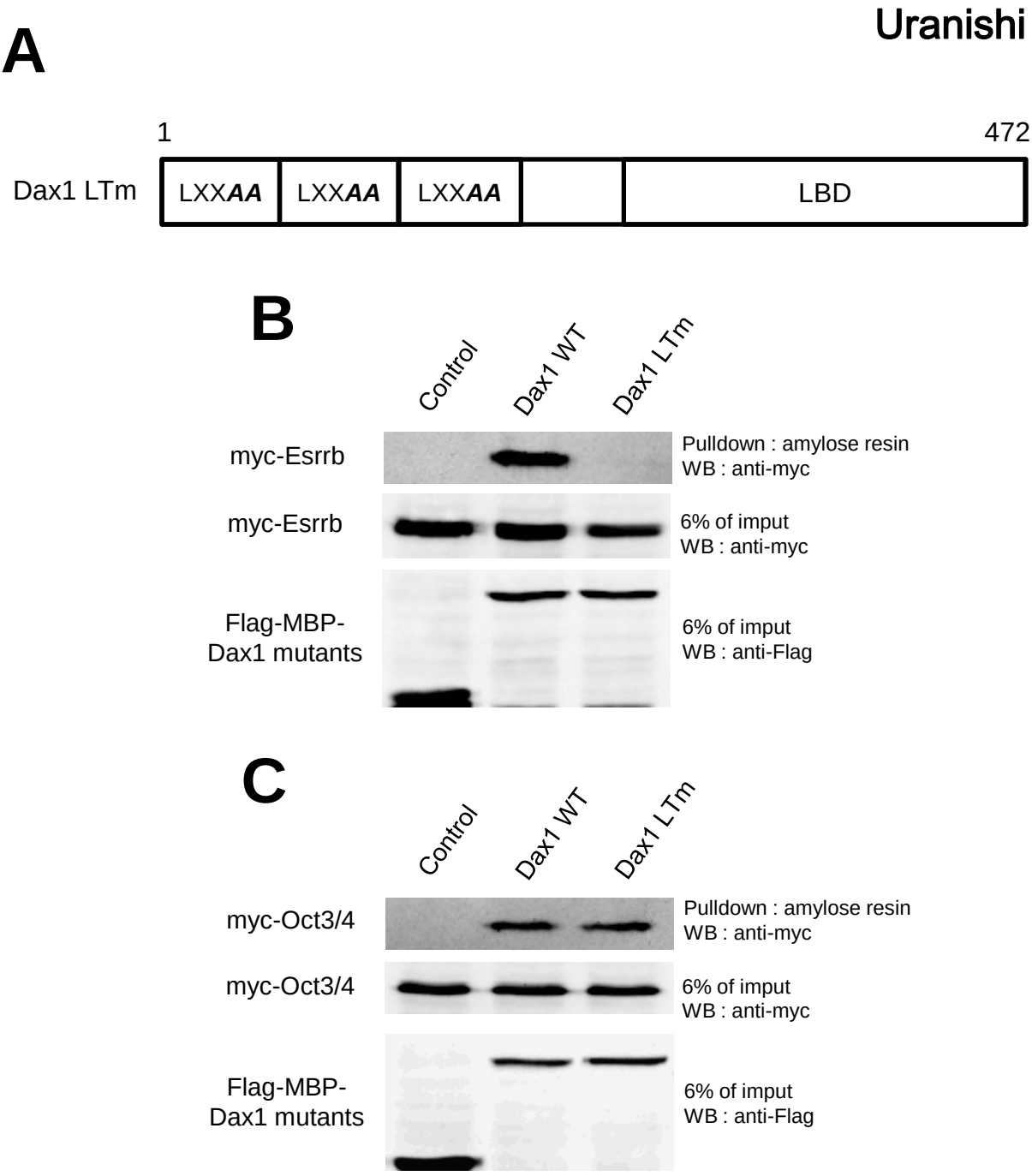
S-FIG. 2. Identification of an Oct3/4-responsive element of the Esrrb gene. (A) Putative Oct3/4-binding sites of the Esrrb gene. The intron regions contain two putative Oct3/4-binding sites, which we named Oct3/4 #A (+8924 - +8931) and Oct3/4 #B (+34733 - +34740). Schematic view is not drawn to scale. (B) Oct3/4 #A is the Oct3/4-responsive element of the Esrrb gene. ZHBTc4 ES cells were transfected with either pGL4P-Oct3/4 #A or -Oct3/4 #B. The Oct3/4 #A site, but not #B site, was activated by Oct3/4. (C) Mutation of the Oct3/4 #A site loses enhancer activity. ZHBTc4 ES cells were transfected with either wild-type of pGL4P-Oct3/4 #A (WT) or mutated one (mut). ZHBTc4 ES cells were divided into two dishes 24h after transfection and cultured with (+) or without (-) 1μg/mL Tet for another 24 hours, and luciferase activity was measured. Sequence of the Oct3/4-responsive element was changed from ATGCAAAT to GTGCCGCT. The bars represent the means and standard deviations of three independent experiments.

Supplemental Figure 2



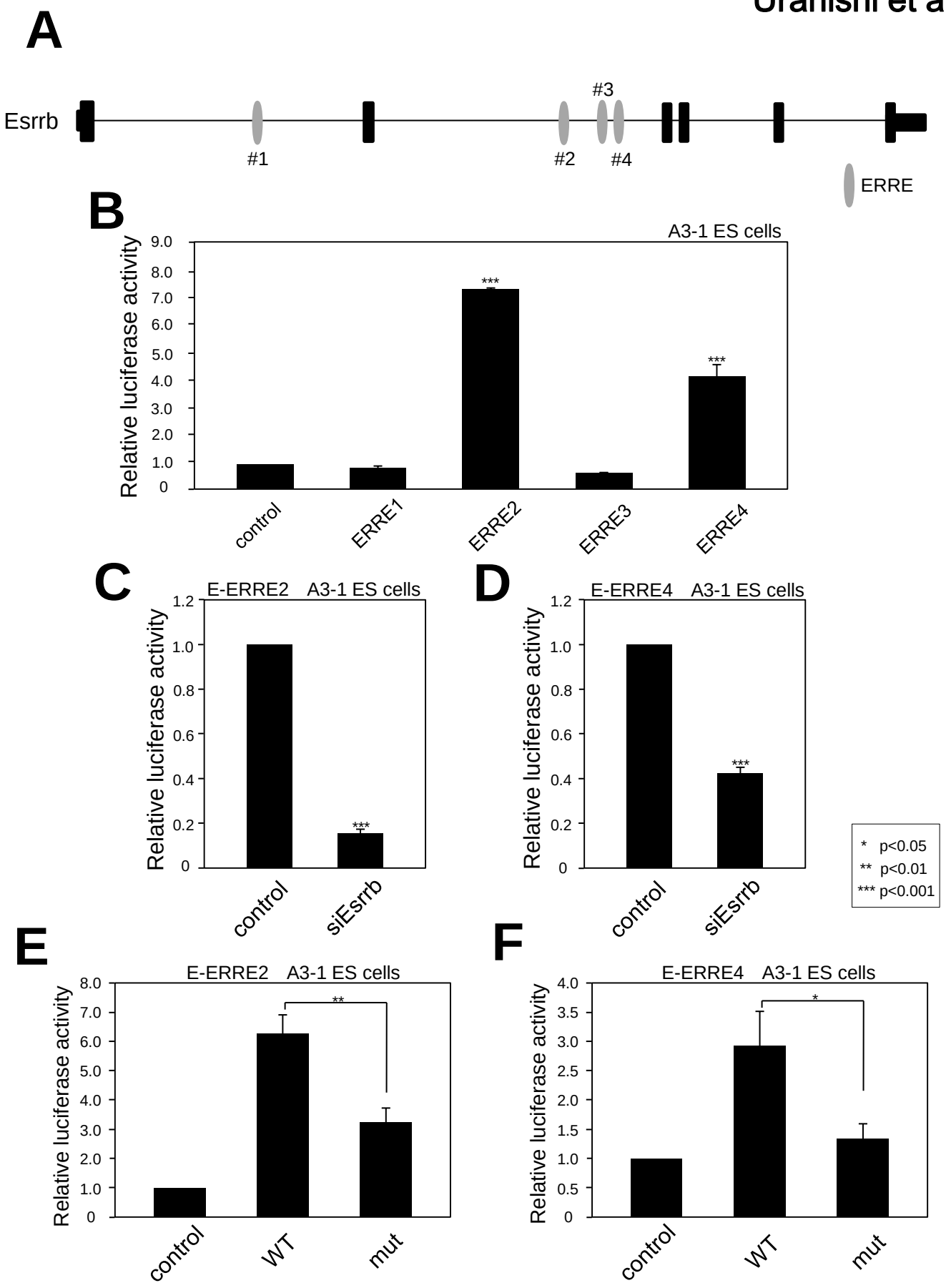
S-FIG. 3. Oct3/4 binds to both 101 aa-135 aa and 309 aa-379 aa regions of Dax1. (A) Schematic view of Flag-MBP-Dax1 and its truncated mutants. Several Flag-tagged MBP-fused constructs including full-length of Dax1 (amino acids 1 - 472), Q23 (amino acids 101 - 379), Q23-1 (amino acids 136 - 379), Q23-2 (amino acids 101 - 308) and Q23a (amino acids 136 - 308) were designed. (B) Determination of Oct3/4-interaction domains of Dax1. HEK293 cells were transfected with Myc-Oct3/4 together with Flag-MBP-Dax1 or its mutants. MBP-fused proteins were pulled down by 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.

Supplemental Figure 3

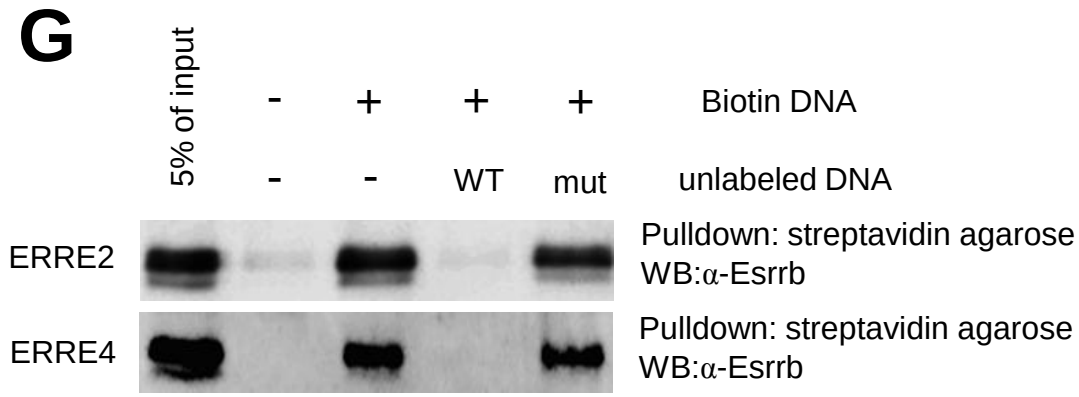


S-FIG. 4. LXXLL motifs of Dax1 are crucial for the interaction with Esrrb but not with Oct3/4. (A) Schematic view of Dax1 LTm. Amino acid sequences of the three LXXLL motifs of Dax1 were changed from LXXLL to LXXAA, and the mutated form was named Dax1 LTm. (B) Esrrb associates with wild-type Dax1 (Dax1 WT) but not Dax1 LTm. HEK293 cells were transfected with Myc-Esrrb together with Flag-MBP-Dax1 WT or -Dax1 LTm. MBP-fused proteins were pulled down by amylose resin, and precipitates were analyzed by Western blotting with anti-Myc antibody. Expression of each protein was confirmed by Western blot analysis with anti-Myc and anti-Flag antibodies. (C) Oct3/4 associates with Dax1 WT and Dax1 LTm. HEK293 cells were transfected with Myc-Oct3/4 together with Flag-MBP-Dax1 WT or -Dax1 LTm. MBP-fused proteins were pulled down by 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.

Supplemental Figure 4



Supplemental Figure 5



S-FIG. 5. Identification of Esrrb-responsive elements of the Esrrb gene. (A) Putative Esrrb-binding sites of the Esrrb gene. Intron regions of the Esrrb gene contain four putative Esrrb-binding sites, which we named Esrrb-ERRE1 (+9711 - +9719), Esrrb-ERRE2 (+31943 - +31951), Esrrb-ERRE3 (+33435 - +33443) and Esrrb-ERRE4 (+34403 - +34411). Schematic view is not drawn to scale. (B) Esrrb-ERRE2 and Esrrb-ERRE4 have an enhancer activity in ES cells. A3-1 ES cells were transfected with either pGL4-promoter (pGL4P, control), pGL4P-Esrrb-ERRE1, -ERRE2, -ERRE3 or -ERRE4. (C) Enhancer activity of ERRE2 of the Esrrb gene is repressed by knocking down of endogenous Esrrb expression in ES cells. A3-1 ES cells were transfected with pGL4P-Esrrb-ERRE2 together with or without Esrrb dsRNA (Esrrb RNAi #1). (D) Enhancer activity of ERRE4 of the Esrrb gene is repressed by knocking down of endogenous Esrrb expression in ES cells. A3-1 ES cells were transfected with pGL4P-Esrrb-ERRE4 together with or without Esrrb dsRNA (Esrrb RNAi #1). (E) Enhancer activity of Esrrb-ERRE2 is diminished by introducing mutation into the elements. A3-1 ES cells were transfected with either pGL4P (control), pGL4P-Esrrb-ERRE2 WT or -ERRE2 mutant. (F) Enhancer activity of Esrrb-ERRE4 is diminished by introducing mutation into the elements. A3-1 ES cells were transfected with either pGL4P (control), pGL4P-Esrrb-ERRE4 WT or -ERRE4 mutant. Luciferase activity was measured 48h after transfection. (G) Esrrb directly binds to both Esrrb-ERRE2 and Esrrb-ERRE4. Nuclear extracts from A3-1 ES cells were subjected to pull-down assay with biotinylated DNA. Biotin-labeled oligonucleotides (Esrrb-ERRE2 and Esrrb-ERRE4) were incubated with nuclear extracts from A3-1 ES cells either with or without 50-fold non-labeled wild-type or mutated oligonucleotide. The precipitates, as well as nuclear extracts, were analyzed by Western blotting with anti-Esrrb antibody. All results are representative of three separate experiments.