Esrrb directly binds to Gata6 promoter and regulates its expression with Dax1 and Ncoa3

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Esrrb directly binds to *Gata6* promoter and regulates its expression with Dax1 and Ncoa3

Running title: Esrrb enhances Gata6 expression in ES cells

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Abstract

Estrogen-related receptor beta (Esrrb) is expressed in embryonic stem (ES) cells and is involved in self-renewal ability and pluripotency. Previously, we found that Dax1 is associated with Esrrb and represses its transcriptional activity. Further, the disruption of the Dax1–Esrrb interaction increases the expression of the extra-embryonic endoderm marker Gata6 in ES cells. Here, we investigated the influences of Esrrb and Dax1 on Gata6 expression. Esrrb overexpression in ES cells induced endogenous Gata6 mRNA and *Gata6* promoter activity. In addition, the *Gata6* promoter was found to contain the Esrrb recognition motifs ERRE1 and ERRE2, and the latter was the responsive element of Esrrb. Associations between ERRE2 and Esrrb were then confirmed by biotin DNA pulldown and chromatin immunoprecipitation assays. Subsequently, we showed that Esrrb activity at the *Gata6* promoter was repressed by Dax1, and although Dax1 did not bind to ERRE2, it was associated with Esrrb, which directly binds to ERRE2. In addition, the transcriptional activity of Esrrb was enhanced by nuclear receptor co-activator 3 (Ncoa3), which has recently been shown to be a binding partner of Esrrb. Finally, we showed that Dax1 was associated with Ncoa3 and repressed its transcriptional activity. Taken together, the present study indicates that the *Gata6*

promoter is activated by Esrrb in association with Ncoa3, and Dax1 inhibited activities of Esrrb and Ncoa3, resulting maintenance of the undifferentiated status of ES cells.

Key Words: Embryonic stem cells, Esrrb, Dax1, Gata6, Ncoa3

1. Introduction

Self-renewal capacity and pluripotency are major characteristics of mouse embryonic stem (ES) cells, and the transcription factors STAT3, Oct3/4, and Nanog are associated with ES cell properties [1-5]. In coordination with other molecules, these transcription factors regulate downstream target genes collectively forming a gene regulatory network in ES cells [6, 7]. To better understand this gene regulatory network, we previously identified the nuclear hormone receptor Dax1 as a downstream target of STAT3 and Oct3/4 [8] and demonstrated its direct interactions with Oct3/4 [9]. Further, the nuclear localization of Dax1 is enhanced by Oct3/4, and the transcriptional activity of Oct3/4 is repressed by Dax1 [9]. Dax1 has also been associated with Nanog, suggesting that it has a role in core transcriptional regulatory networks of ES cells [10-13]. The functional repression of Dax1 by siRNA leads to the induction of differentiation marker genes such as Gata4 and Gata6 in ES cells [14, 15]. Furthermore, Gata4 and Gata6 expression was reportedly induced in Cre-LoxP-mediated *Dax1* conditional knockout ES cells [16]. However, *Dax1*-deficient ES cells contributed to chimeric embryos, indicating sustained pluripotency [16]. These cells also overexpressed Zscan4c, suggesting that Dax1 is a negative regulator of Zscan4c [16].

Estrogen-related receptor beta (Esrrb) is considered to be a member of the core transcriptional regulatory network in ES cells [17, 18], and has been associated with Oct3/4 [13, 19], Nanog [10, 20], and Dax1 [21], and is reportedly required for self-renewal of ES cells [22, 23]. Moreover, Esrrb has been shown to regulate the self-renewal capacity of ES cells downstream of Nanog and Gsk3-Tcf3 pathways [24, 25].

Previously, we clarified that an interaction of Esrrb and Dax1 was mediated through the activation- and ligand-binding domains of Esrrb and LXXLL motifs of Dax1; and the transcriptional activity of Esrrb was inhibited by Dax1. We also revealed that Oct3/4, Dax1, and Esrrb are competitive binding partners, and that Dax1 negatively regulates both Esrrb and Oct3/4 [21]. In particular, we found that Esrrb overexpression and Dax1 knockdown disrupted Dax1–Esrrb interaction and increased the expression of endodermal genes, such as *Gata4*, *Gata6*, and *Dab2*. Other studies have indicated that ES cells overexpressing Esrrb are prone to differentiation into endodermal lineages, and that Esrrb enhances endodermal differentiation [22, 26]. Therefore, Esrrb has bifunctional activities as a promoter of self-renewal and an inducer of differentiation. In the present study, we showed that Gata6 is a direct target of Esrrb; and *Gata6* promoter

is activated by Esrrb together with Ncoa3, and Dax1 inhibits activities of Esrrb and Ncoa3.

2. Materials and Methods

2.1. Cell culture, plasmid construction, plasmid transfection, RT-PCR, and luciferase assays

A3-1 ES cells and human embryonic kidney (HEK) 293 cells were cultured as described previously [21, 27]. Expression vectors, including pCMV5-Flag-maltose-binding protein (MBP), pCAGIP, pCAGIP-Esrrb, pCAGIP-Myc, pCAGIP-Myc-Dax1, and pCAGIP-Myc-Dax1LTm, were generated as described previously [9, 21]. The Ncoa3 coding region and its truncated mutants were amplified using polymerase chain reaction (PCR) before being cloned into expression vectors. The *Gata6* gene promoter region (-0.9 kb) was amplified using PCR and was cloned into pGL4.10 (Promega, Madison, WI, USA) to produce the plasmid pGL4.10-Gata6P 0.9k. To construct the Esrrb-responsive element (ERRE)-mutant pGL4.10-Gata6P 0.9k plasmids, mutant ERRE1 or ERRE2 elements were generated using PCR with specific primers. PCR products were then cloned into pGL4.10 to produce pGL4.10-Gata6P 0.9k-ERRE1 mut and -ERRE2 mut vectors. All primer sequences are listed in Supplemental Table S1.

Plasmids were introduced into cultured cells using Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA). Two days after transfection, cells were treated with 1 μg/mL puromycin for 5–7 days, and RNA samples for RT-PCR were isolated and examined as described previously [21]. The cell extracts for the luciferase assays were prepared 48 h after transfection, and luciferase activity was measured using a luciferase assay kit (Promega) and an AB-2200 luminometer (ATTO, Tokyo, Japan).

2.2. Biotin-labeled DNA pull-down and chromatin immunoprecipitation assays

Biotin-labeled DNA pull-down assays were performed as described previously [21]. Briefly, biotin-labeled oligonucleotides were incubated with A3-1 ES extracts or HEK293 cell extracts that had been transfected with pCAGIP-Myc-Dax1 and/or pCAGIP-Esrrb in the presence of streptavidin–agarose (Novagen, Darmstadt, Germany). Subsequently, non-labeled oligonucleotide (either wild-type or mutant) was added for competition assays at a fifty-fold excess. Beads were then washed with a washing buffer and bound proteins were eluted by boiling in 2× sodium dodecyl sulfate sample buffer. Finally, signals were detected by western blot as described below.

Chromatin immunoprecipitation (ChIP) assays were performed according to the manufacturer's protocol (Diagenode OneDay ChIP kit; Nippon Gene, Tokyo, Japan) as described previously [21]. Immunoprecipitation was performed with normal murine IgG (sc-2025; Santa Cruz) and anti-Esrrb antibodies (PP-H6705-00; Perseus Proteomics, Tokyo, Japan).

2.3. MBP pull-down assays and western blot analyses

MBP pull-down assays were performed as described previously [9, 21]. HEK293 cell lysates and pull-down samples were subjected to western blot analysis using anti-Myc (sc-40; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Flag (F3165; Sigma-Aldrich), or anti-Esrrb antibodies followed by horseradish peroxidase-conjugated anti-mouse antibody (Millipore, Billerica, MA, USA). Blots were visualized using enhanced chemiluminescence reagents (PerkinElmer, Waltham, MA, USA).

3. Results

3.1. Esrrb directly regulates Gata6 expression in ES cells

In our previous study, Esrrb overexpression in ES cells was accompanied by an increased expression of Gata6, which is a marker of extra embryonic endodermal cells (Fig. 1A) [21]. These findings suggested Esrrb may regulate Gata6 in addition to the self-renewal genes Sox2, Nanog, and Dax1. Therefore, in the present study we examined the promoter activity of *Gata6* in the presence of LIF using reporter assays and found strong activity within the 0.9 kb *Gata6* promoter in undifferentiated ES cells (Fig. 1B). Moreover, Esrrb overexpression enhanced the promoter activity of *Gata6* in ES cells (Fig. 1C), indicating that Gata6 is a downstream target of Esrrb in ES cells.

Two putative Esrrb recognition (ERRE) sequences (TCAAGGTCA) were found in the *Gata6* promoter, and were referred to as G6-ERRE1 (−262 to −254) and G6-ERRE2 (−580 to −572; Fig. 2A). To identify ERREs that affected Gata6 expression, we constructed the ERRE-mutant *Gata6* promoters, Gata6P 0.9k ERRE1 mut and ERRE2 mut. As shown in Fig. 2B, ERRE2 mut promoter activity was less than that of the wild-type promoter and induction by exogenous Esrrb was accordingly reduced, suggesting that ERRE2 is required for activation of the *Gata6* promoter.

In further experiments, we investigated Esrrb binding to the ERRE2 element using biotinylated DNA pull-down assays, and showed that endogenous Esrrb was precipitated by the biotinylated ERRE2 oligonucleotides, and the precipitate was decreased by non-labeled ERRE2 wild-type oligonucleotides but not mutated ones (Fig. 2C). Subsequent ChIP assays demonstrated that the ERRE2 sequence of the *Gata6* promoter was precipitated by an anti-Esrrb antibody in A3-1 ES cells (Fig. 2D). Taken together, these results suggest that Gata6 is directly regulated by Esrrb in ES cells.

3.2. Promoter activation of Gata6 is repressed by Dax1

Previously, we reported that Dax1 interacts with Esrrb as a transcriptional repressor [21], and in the present experiments we examined an effect of Dax1 on enhancing activity of Esrrb against *Gata6* promoter. Specifically, whereas the enhancing activities of Esrrb were repressed by Dax1 in the present reporter assays (Fig. 3A), they were not repressed by Dax1 LTm (LXXLL motif-mutated Dax1), which does not associate with Esrrb [21] (Fig. 3B). We also investigated associations of Dax1 with this region by performing biotinylated DNA pulldown assays, and found no association between Dax1 and biotin-labeled ERRE2 (Fig. 3C). In contrast, Dax1 was successfully

pulled-down in the presence of Esrrb (Fig. 3C), suggesting that Dax1 represses *Gata6* promoter activity by interacting with Esrrb, but not DNA.

3.3. Dax1 represses transcriptional activity of Ncoa3

Ncoa3 is a known binding partner of Esrrb, and Esrrb enhances its transcriptional activity [28]. Accordingly, we found enhanced *Gata6* promoter activity in the presence of Ncoa3 or Esrrb (Fig. 4A and 4B), and additive increases in the presence of both (Fig. 4B). Additionally, we found that the enhancing activities of Ncoa3 were reduced by Dax1 LTm and by wild-type Dax1 (Fig. 4C and 4D), suggesting that *Gata6* promoter activity is induced by Ncoa3 and repressed by Dax1.

In accordance with these findings, we showed that Dax1 interacts with Ncoa3 using MBP-pulldown assays (Fig. 4E). Ncoa3 comprises several distinct motifs, including a helix loop helix motif (HLH), a PAS domain (PAS), a steroid receptor coactivator domain (SRC1), and a nuclear receptor coactivator region (Nuc) (Fig. 4F). Thus, to identify Dax1-interacting regions of Ncoa3, we prepared six truncated Ncoa3 mutants that included amino acids 1–250, 251–500, 501–751, 751–1000, 1001–1250, and 1251–1403. As shown in Fig. 4G, Dax1 associated with 251–500 and 1001–1250

regions of Ncoa3, but not the 501–751 region. As this region contains SRC1 with two LXXLL motifs, these data suggest that the LXXLL motifs are not crucial for the interaction of Ncoa3 and Dax1.

4. Discussion

At embryonic day 3.5 (E3.5) of early development, mouse blastocysts comprise an inner cell mass (ICM) and a trophectoderm, and the extraembryonic ectoderm (ExEn) differentiates from ICM. Gata6, a zinc-finger transcription factor, is important for the development of ExEn, and artificial overexpression of Gata6 in ES or neural stem cells of mice and human ES cells triggers differentiation into ExEn [29, 30]. Previous studies have shown that *Gata6*-deficient embryos exhibit early ExEn deficiencies, indicating that Gata6 is essential for ExEn development [31, 32]. In the present study we have shown that *Gata6* promoter activity is enhanced by Esrrb and that forced expression of Esrrb induces endogenous Gata6 mRNA expression. Moreover, Esrrb binds to the *Gata6* promoter region. These findings are consistent with previous findings: Esrrb induces Gata6 expression [22, 26] and binds to the *Gata6* promoter region [30]. Interestingly, *Esrrb*-deficient embryos had impaired placental formation [33], although co-expression of Esrrb and Sox2 was sufficient for multipotency of trophoblast stem cells [34], further indicating the importance of Esrrb in the development of extraembryonic tissues. We also show that the *Gata6* promoter is activated by Ncoa3 and that its activity is enhanced by Esrrb, indicating the

involvement of Ncoa3 in the development of extraembryonic tissues. In support of this, Noca3 and Noca1 were previously shown to cooperate in the regulation of placental morphogenesis [35]. Taken together, our findings indicate that Esrrb is involved in the expression of Gata6, which is an ExEn-differentiation promoting factor.

Ncoa3 is expressed in ES cells, and functional inhibition by shRNA led to morphological changes, reduced expression of the pluripotency genes Nanog, Oct3/4, and Sox2, and impaired differentiation potential [36]. Moreover, Ncoa3 reportedly binds to the *Nanog* promoter and enhances its activity via its effects on the histone modification enzymes CBP and CARM1 [36]. Ncoa3 was also associated with Esrrb and activated several genes involved in self-renewal and pluripotency [28]. Therefore, Ncoa3 is considered critical for self-renewal capability and pluripotency of ES cells. Here, we discovered a protein-protein interaction between Dax1 and Ncoa3, and Dax1 inhibits transcriptional activity of Ncoa3. Although Esrrb and Ncoa3 enhance transcription from the *Gata6* promoter, Dax1 likely represses differentiation-inducing functions of these proteins to maintain the undifferentiated status of ES cells. In fact, our data indicates that Dax1, a negative regulator of Esrrb, represses Gata6 expression, potentially influencing maintenance of undifferentiated state of ES cells. Accordingly,

Dax1-deficient ES cells tended to express Gata6 mRNA [16]. Since Esrrb is well known to enhance self-renewal capacity and maintain the undifferentiated state of ES cells [10, 20, 22, 24, 25], Esrrb has functions in the regulation of both self-renewal and differentiation. Future studies, including global investigations of Esrrb–Dax1 and/or Ncoa3–Dax1 target genes, are necessary to elucidate the full mechanisms behind the maintenance of undifferentiated ES cells.

Author disclosure statement

The authors declare no conflicts of interest.

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Figure Legends

Fig. 1. Esrrb induces Gata6 mRNA expression in ES cells

(A) Induction of Gata6 mRNA by Esrrb overexpression; A3-1 ES cells were transfected with either pCAG-IP (control) or pCAGIP-Esrrb (Esrrb), and Esrrb, Gata6, and GAPDH expression levels were examined using RT-PCR. GAPDH was used as an internal control. (B) Promoter activity of *Gata6* in undifferentiated ES cells; A3-1 ES cells were transfected with pGL4.10-Luc2 (control) or pGL4.10-Gata6P 0.9k (Gata6P 0.9k). (C) Transcriptional activation of the *Gata6* promoter by Esrrb; A3-1 ES cells were cotransfected with pGL4.10-Gata6P 0.9k and pCAG-IP (control) or pCAGIP-Esrrb (Esrrb) and luciferase activity was measured after 48 h. Data are presented as means and standard deviations for three independent experiments.

Fig. 2. Esrrb directly regulates *Gata6* promoter activity via ERRE2

(A) Esrrb binding sites on the *Gata6* promoter included ERRE1 (−262 to −254) and ERRE2 (−580 to −572). (B) Determination of Esrrb-responsive elements in the *Gata6* promoter; A3-1 ES cells were transfected with pGL4.10-Luc2 (control),

pGL4.10-Gata6P 0.9k-wild type (Wild type), ERRE1 mutant (ERRE1 mut), and ERRE2 mutant (ERRE2 mut) with pCAG-IP (control) or pCAGIP-Esrrb (Esrrb), and luciferase activities were determined after 48 h. Data are presented as means and standard deviations for three independent experiments. (C) Esrrb binds to the ERRE2 *in vitro*; biotin-labeled ERRE2 oligonucleotides were incubated with nuclear extracts from A3-1 ES cells with or without 50-fold non-labeled wild-type ERRE2 (WT) or mutant ERRE2 (mut) oligonucleotides. Precipitates were then analyzed by western blot with an anti-Esrrb antibody. (D) Esrrb binds ERRE2 *in vivo*; ChIP assays were performed using normal IgG and anti-Esrrb antibodies and precipitates were examined using RT-PCR with specific primers.

Fig. 3. Dax1 represses transcriptional activity of Esrrb

(A) Esrrb-dependent *Gata6* promoter activity was repressed by wild-type Dax1; A3-1 ES cells were transfected with pGL4.10-Gata6P 0.9k with or without pCAGIP-Esrrb or pCAGIP-Myc-Dax1. (B) Esrrb-dependent *Gata6* promoter activity was not repressed by the LXXLL motif-mutant Dax1 (Dax1 LTm); A3-1 ES cells were transfected with pGL4.10-Gata6P 0.9k with or without pCAGIP-Esrrb or pCAGIP-Myc-Dax1 LTm and

luciferase activities were determined after 48 h. Data are presented as means and standard deviations for three independent experiments. (C) Dax1 is associated with the ERRE2 via Esrrb; HEK293 cells were transfected with Myc-Dax1 and/or Esrrb, and biotinylated oligonucleotides of the ERRE2 were used for pulldown assays. Precipitates were analyzed by western blot with anti-Myc, and anti-Esrrb antibodies and protein expression levels were confirmed using anti-Myc and anti-Esrrb antibodies. All presented data are representative of three separate experiments.

Fig. 4. Dax1 represses transcriptional activation by Ncoa3

(A) Ncoa3 induced *Gata6* promoter activity; A3-1 ES cells were transfected with pGL4.10-Gata6P 0.9k and pCAGIP-Myc (control) or pCAGIP-Myc-Ncoa3. (B) Ncoa3 enhanced transcriptional activity of Esrrb; pGL4.10-Gata6P 0.9k was introduced into A3-1 ES cells with pCAGIP-Esrrb or pCAG-Myc-Ncoa3. (C and D) Ncoa3-dependent *Gata6* promoter activation was reduced by both Dax1 and Dax1LTm; A3-1 ES cells were transfected with pGL4.10-Gata6P 0.9k and pCAGIP-Myc-Dax1 (C) or pCAGIP-Myc-Dax1 LTm (D) and luciferase activities were determined after 48 h. Data are presented as means and standard deviations for three independent experiments. (E)

Dax1 interacts with full length Ncoa3; HEK293 cells are cotransfected with pCMV5-Flag-MBP-Ncoa3 and pCAGIP-Myc (control) or pCAGIP-Myc-Dax1. Subsequently, Flag-MBP-fused Ncoa3 was pulled down using amylose resin and precipitates were analyzed by western blot with an anti-Myc antibody. (F) Schematic view of full-length Ncoa3; Ncoa3 comprises a helix loop helix motif (HLH), a PAS domain (PAS), a steroid receptor coactivator domain (SRC1), and a nuclear receptor coactivator region (Nuc). (G) Determination of Dax1-interacting regions of Ncoa3; HEK293 cells were cotransfected with pCAGIP-Myc-Dax1 and expression vectors for Flag-MBP-fused Ncoa3 mutants. Flag-MBP-fused proteins were then pulled down using amylose resin and precipitates were analyzed by western blot with an anti-Myc antibody. Protein expression was analyzed by western blot with anti-Myc and anti-Flag antibodies, and the presented data represent three separate experiments.

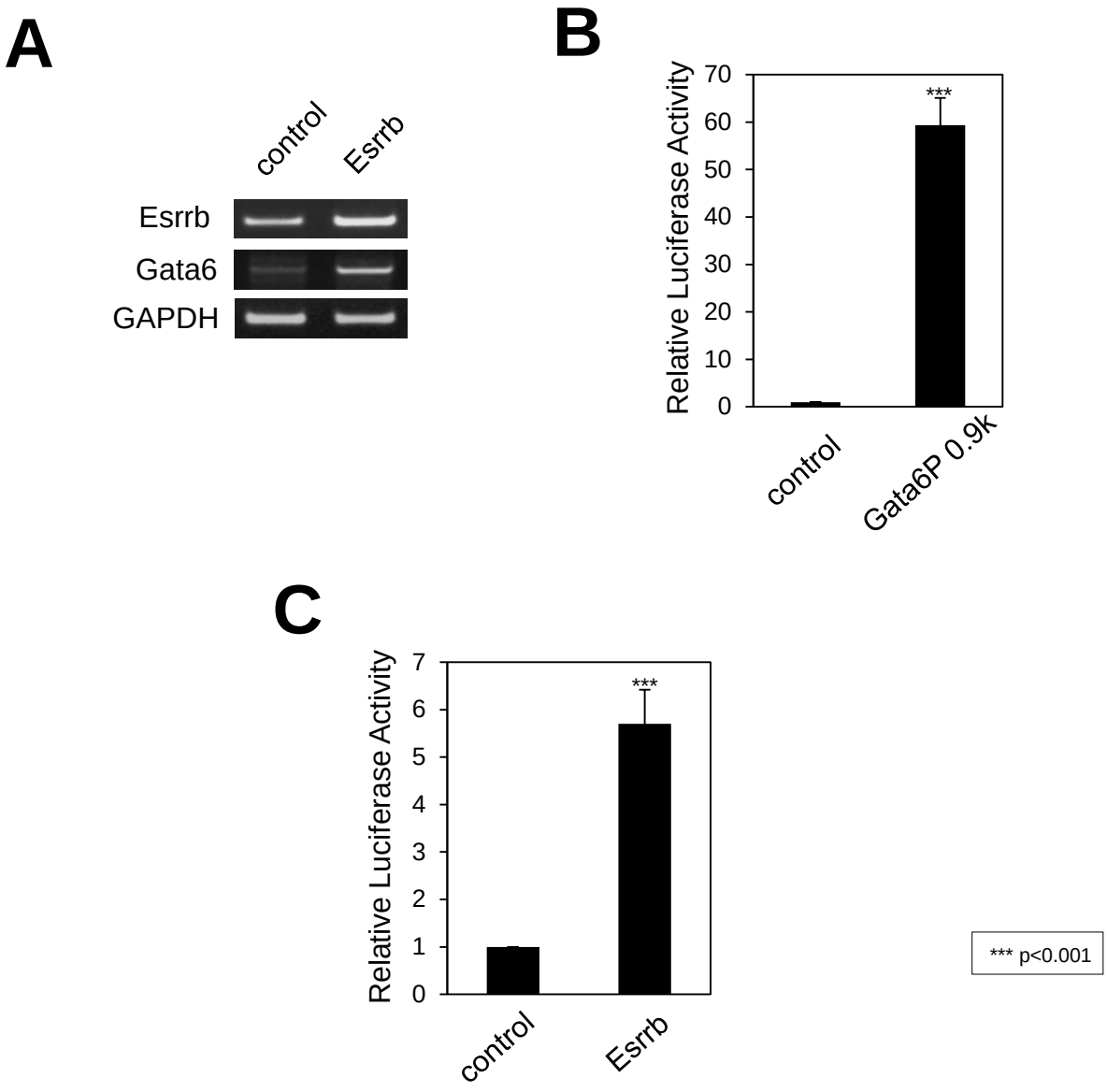
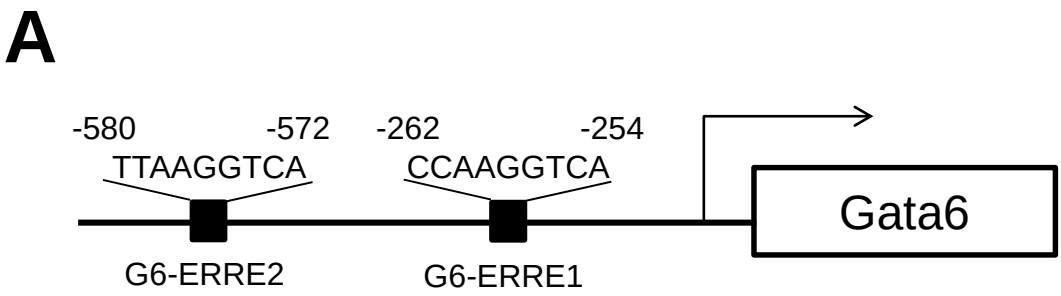
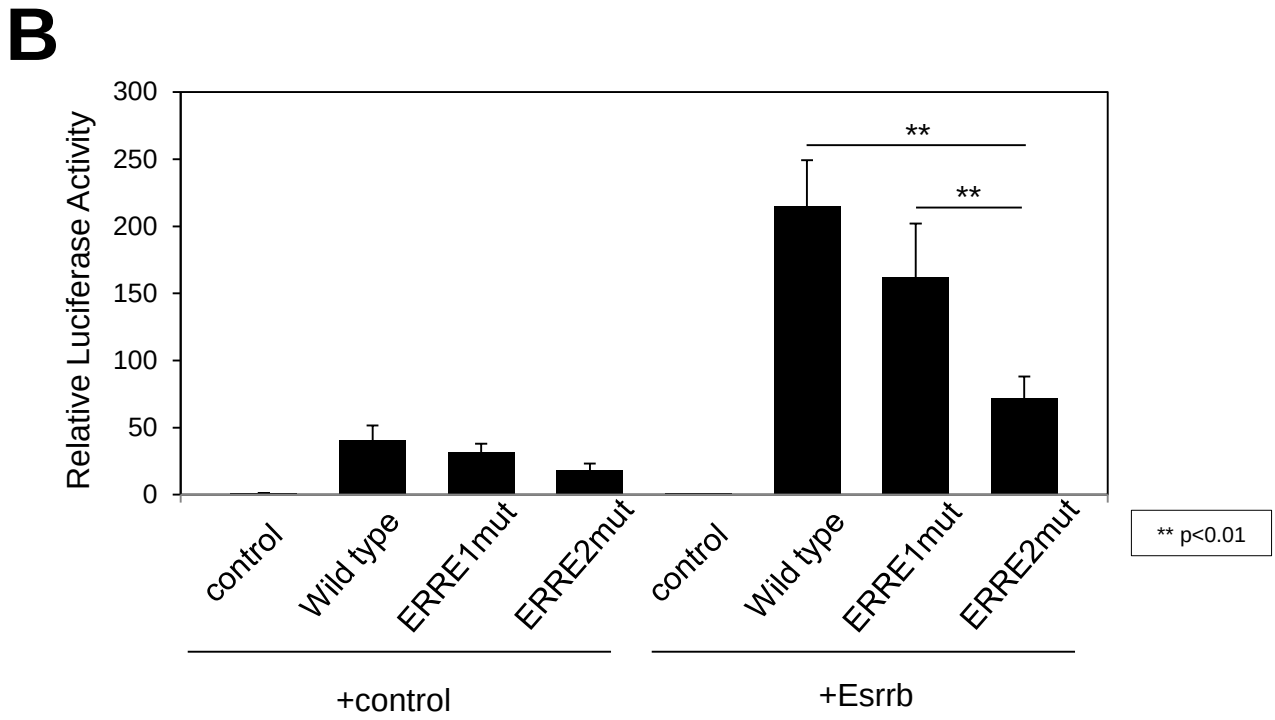
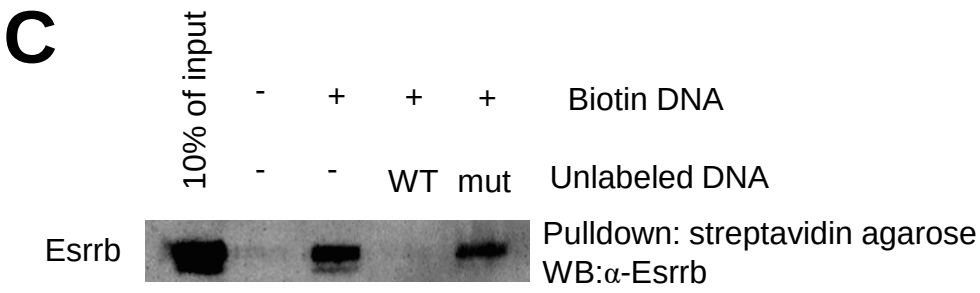

A
control
Esrrb
Esrrb
Gata6
GAPDH
B
Relative Luciferase Activity
70
60
50
40
30
20
10
0
***
control
Gata6P 0.9k
C
Relative Luciferase Activity
7
6
5
4
3
2
1
0
***
control
Esrrb
*** p<0.001

Figure 1


A
-580
-572
-262
-254
TTAAGGTCA
CCAAGGTCA
Gata6
G6-ERRE2
G6-ERRE1


B
Relative Luciferase Activity
300
250
200
150
100
50
0
**
**
control
Wild type
ERRE1mut
ERRE2mut
control
Wild type
ERRE1mut
ERRE2mut
** p<0.01
+control
+Esrrb


C
10% of input
-
+
+
+
Biotin DNA
-
-
WT
mut
Unlabeled DNA
Esrrb
Pulldown: streptavidin agarose
WB:α-Esrrb

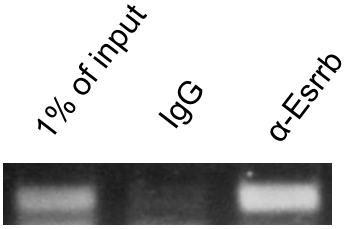

D
1% of input
IgG
α-Esrrb

Figure 2

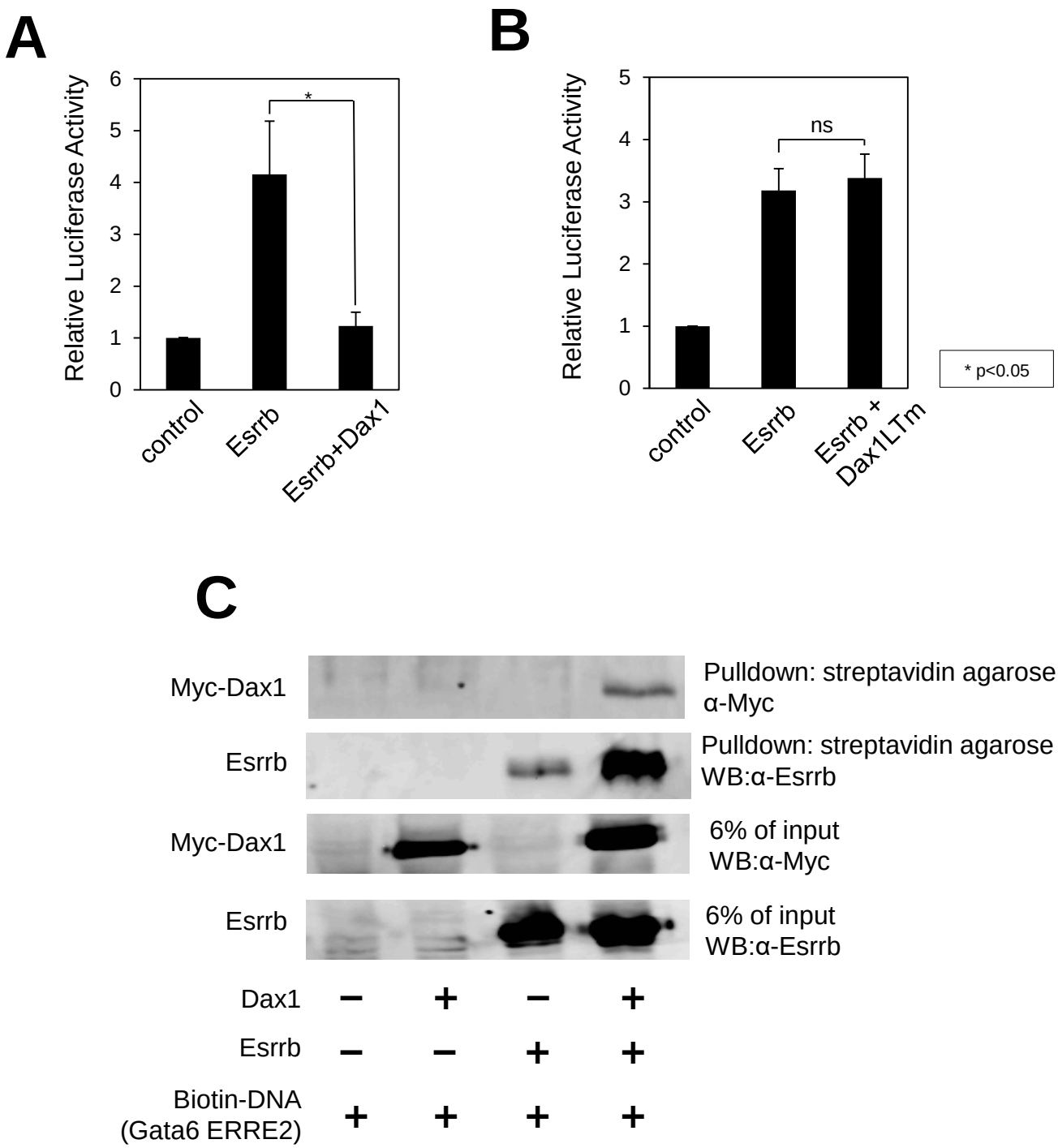

A
Relative Luciferase Activity
0
1
2
3
4
5
6
*
control
Esrrb
Esrrb+Dax1
B
Relative Luciferase Activity
0
1
2
3
4
5
ns
control
Esrrb
Esrrb + Dax1LTm
* p<0.05
C
Myc-Dax1
Pulldown: streptavidin agarose
α-Myc
Esrrb
Pulldown: streptavidin agarose
WB:α-Esrrb
Myc-Dax1
6% of input
WB:α-Myc
Esrrb
6% of input
WB:α-Esrrb
Dax1 − + − +
Esrrb − − + +
Biotin-DNA
(Gata6 ERRE2) + + + +

Figure 3

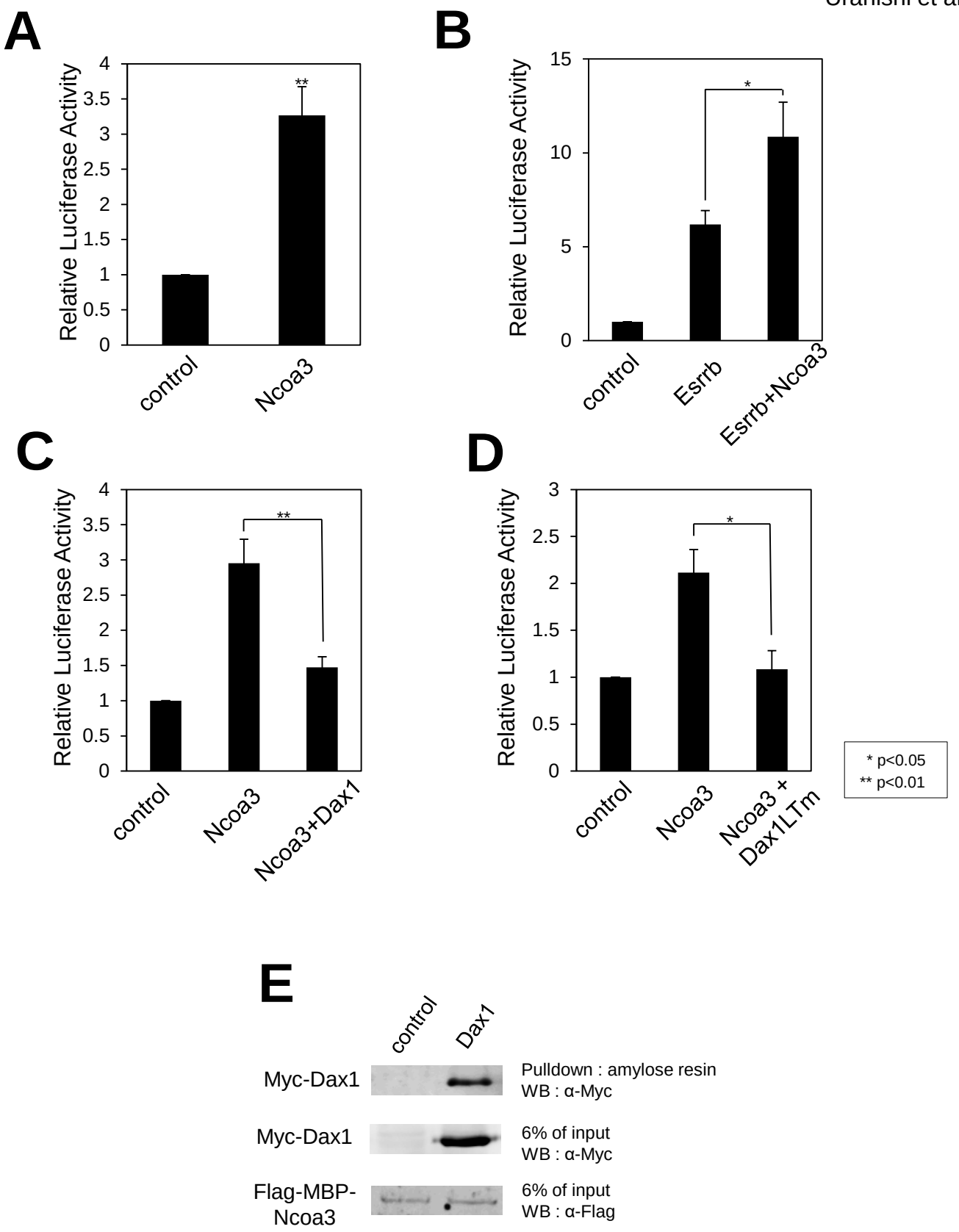
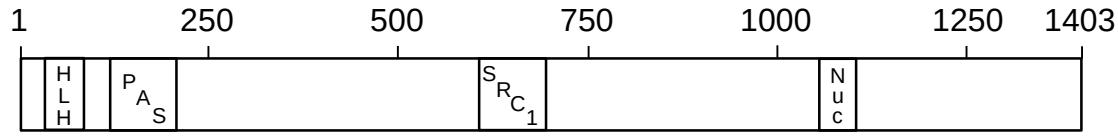

A
Relative Luciferase Activity
4
3.5
3
2.5
2
1.5
1
0.5
0
**
control
Ncoa3
B
Relative Luciferase Activity
15
10
5
0
*
control
Esrrb
Esrrb+Ncoa3
C
Relative Luciferase Activity
4
3.5
3
2.5
2
1.5
1
0.5
0
**
control
Ncoa3
Ncoa3+Dax1
D
Relative Luciferase Activity
3
2.5
2
1.5
1
0.5
0
*
control
Ncoa3
Ncoa3 + Dax1LTm
* p<0.05
** p<0.01
E
control
Dax1
Myc-Dax1
Pulldown : amylose resin
WB : α-Myc
Myc-Dax1
6% of input
WB : α-Myc
Flag-MBP-Ncoa3
6% of input
WB : α-Flag

Figure 4 A-E

F



G

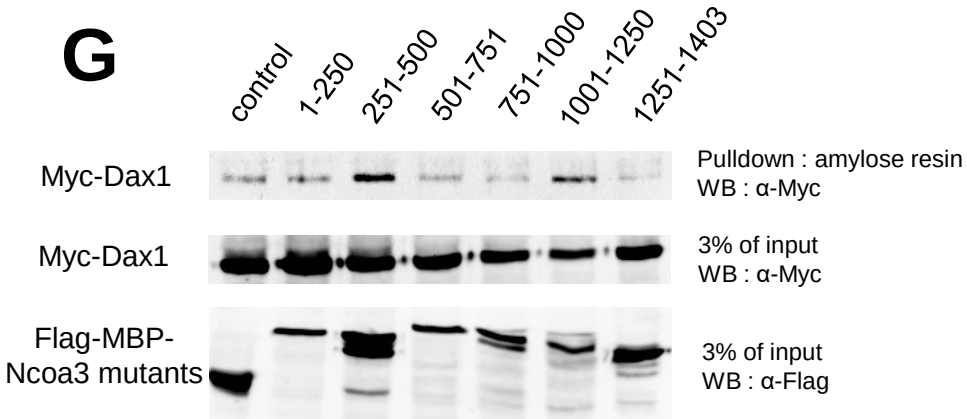


Figure 4 FG