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Short communication

ZNF300, A RECENTLY IDENTIFIED HUMAN TRANSCRIPTION FACTOR, ACTIVATES THE HUMAN IL-2Rβ PROMOTER THROUGH THE OVERLAPPING ZNF300/EGR1 BINDING SITE

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Abstract: ZNF300 was recently identified as a member of the human KRAB/C_2H_2 zinc finger protein family. Little is known about the role of ZNF300 in human gene regulation networks. In this study, the DNA-binding property of ZNF300 was further analyzed. We found that the recombinant ZNF300 could bind to the binding site 5'-GCGGGGGCG-3' of Egr1, another member of the KRAB/C_2H_2 zinc finger protein family. Similarly, recombinant Egr1 also showed a similar binding affinity to the ZNF300 binding site 5'-CTGGGGGCG-3'. Bioinformatics analysis revealed that there is an overlapping ZNF300/Egr1 binding site in the human IL-2Rβ promoter region, which was previously known to be recognized by endogenous Egr1. Electrophoretic mobility shift assays showed that endogenous ZNF300 could also bind to this site. A transient transfection assay revealed that both ZNF300 and Egr1 could transactivate the IL-2Rβ promoter, and that the activation was abrogated by a mutation of residues in the overlapping ZNF300/Egr1 binding site. Co-expression of ZNF300 and Egr1 led to enhanced IL-2Rβ promoter activity. Thus, ZNF300 is likely to be another regulator of the human IL-2Rβ promoter.

Key words: ZNF300, Egr-1, IL-2Rβ promoter, Activation

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Abbreviations used: EMSA – electrophoretic mobility shift assay; IL-2Rβ – IL-2 receptor beta chain; KRAB – Krüppel-asscociated box; TPA – 12-o-tetradecanoylphorbol-13-acetate; ZNF300 – zinc finger 300

INTRODUCTION

Gene expression is controlled by transcriptional regulatory proteins and recruited co-factors, which bind a specific region of the target genes [1]. The KRAB/C_2H_2 zinc finger protein family is one of the largest families of mammalian trans-acting factors. It is characterized by C_2H_2 zinc finger motifs and has a highly conserved H/C linker (TGE(K/R)P(Y/F)X) [2, 3]. The study of zinc finger proteins has revealed their crucial roles in normal and pathological biological processes [4, 5].

The ZNF300 gene encodes a typical KRAB-type zinc finger protein with twelve C_2H_2 zinc finger motifs in the C-terminus [6]. Recent studies have shown that ZNF300 plays crucial roles in developmental and pathological pathways [2, 7, 8]. For example, ZNF300 is abnormally up-regulated in familial adenomatous polyposis-associated desmoids tumours compared to its levels in normal fibroblast cells. This up-regulation may contribute to the formation of desmoids tumors [7]. ZNF300 is also significantly up-regulated in undifferentiated human embryonic stem cells [2], and is associated with inflammation of the digestive tract [8]. These results indicate that ZNF300 might function as a candidate transcription factor in diverse developmental and pathological processes. However, genomic analyses indicated that ZNF300 is a *homo* inparalogue without the predicted orthologues in mouse [9]. Thus, it is difficult to study the function of ZNF300 by genetic means using an animal model.

Our recent studies have showed that ZNF300 recognizes the GC-rich DNA element (C(t/a)GGGGG(g/c)G), which is similar to the consensus binding site of Egr1 (GCG(G/T)GGGCG) [10]. Egr1, encoded by the immediate early growth response gene, is rapidly induced by growth factors to transduce the proliferative signal. It plays a crucial role in the differentiation of nerve, bone and myeloid cells, and in B cell maturation [11-14]. In this study, we further analyzed the DNA-binding property and transcriptional regulatory function of ZNF300, and found that ZNF300 could share a common DNA element with Egr1, and transactivate the IL-2Rβ promoter through an overlapping ZNF300/Egr1 binding site.

MATERIALS AND METHODS

Plasmid construction

The GST-ZF300 expression plasmid was generated via PCR amplification of the twelve zinc fingers of ZNF300 (amino acids 241-604) followed by cloning in frame with the glutathione S-transferase-coding sequence into the *Bam*H I / *Xho* I-digested pGEX-5x-2 vector (Phamacia Biotech). The GST-Egr1 expression plasmid was similarly obtained by cloning the three zinc fingers of Egr1 (amino acids 304-422) into the *Eco*R I / *Xho* I-digested pGEX-5x-2 vector. The PCR amplification of the ZNF300 ORF was inserted into the *Bam*H I / *Hin*d III-digested pcDNA3.0 vector to construct the plasmid pcDNA-ZNF300 [6].

The pcDNA-Egr1 plasmid was generated via PCR amplification of the fragment encoding the human Egr1-coding sequence cloned in pSES-Egr1 and inserted into the *Hin*d III / *Xho* I-digested pcDNA3.0 vector [5]. Human genomic DNA was extracted from normal human fresh blood and used as the template to amplify the IL-2Rβ promoter region (nt +97~-582). The PCR fragment of the IL-2Rβ promoter was digested with *Nhe* I / *Hin*d III and then inserted into the *Nhe* I / *Hin*d III-digested pGL3 basic vector to construct the pGL3-IL-2Rβ vector. To mutate the ZNF300/Egr1 site in the IL-2Rβ promoter region (underlined), the sense-primer 5'-GCCTCCTACGCTGGGATAGGCACAC ACACACAC-3' and antisense primer 5'-GTGTGTGTGTGTGCCTATCCC AGCGTAGGAGGC-3' were used, yielding pGL3-IL-2Rβ-mut via the overlapping extension PCR method with the pGL3-IL-2Rβ plasmid as the template. The sequences of all of the constructs were confirmed by DNA sequencing.

Expression and purification of the GST-ZF300 fusion protein, the GST-Egr1 fusion protein and GST

The GST-ZF300 fusion protein was expressed and purified as previously reported [10]. The GST-Egr1 expression plasmids were transformed into *E. coli* BL21 (DE3) cells. After reaching a density of 600 nm, the cells were induced with 1 mM IPTG at 25ºC for 5 h to produce the GST-Egr1 fusion protein. Then, the cells were collected and resuspended with ice-cold 1 x PBS buffer and 0.5 mM DTT. The resuspended cells were ultra-sonicated and spun, and the soluble fraction was applied to a glutathione Sepharose column that had been previously equilibrated with 1 x PBS. The column was washed in the same buffer, and the bound proteins were eluted in 30 mg/ml reduced glutathione (GSH), pH 8.0. The elution was further dialyzed in ice-cold PBS buffer and 0.5 mM DTT, and then concentrated with 10,000 M WCO Millipore (Millipore China Ltd.). The purified GST-ZF300 and GST-Egr1 fusion proteins were stored at -80ºC until use. The GST proteins were purified similarly.

Electrophoretic mobility shift assay (EMSA)

Thirty five fmol of the indicated specific (γ-^{32}P) labeled probes were incubated at 20ºC for 35 min with 600 ng of purified GST-ZF300, GST-Egr1 or GST proteins in a shift buffer consisting of 10 mM Hepes (K^+, pH 8.0), 0.06 M KCl, 4 mM $MgCl_2$, 0.1 mM EDTA, 0.25 mM DTT, 0.1 mg/ml BSA, 0.25 mM DTT, 0.1 mM $ZnCl_2$ and 10% glycerol. Samples were loaded onto 5% non-denaturing polyacrylamide gel (50 mM Tris base, 50 mM glycine, 29:1 acrylamide/ bisacrylamide (25.3%, v/v), 0.2 mM $ZnCl_2$, 40% glycerol, 0.1% APS, 0.01% TEMED) and electrophoresed in 50 mM Tris-glycine buffer. The gel was then analyzed by autoradiography. For the competition assay, oligonucleotides were synthesized with base substitution at the consensus-binding site in the probe.

To determine the dissociation constant (KD) of the ZNF300 binding site for GST-ZF300 and GST-Egr1, an increasing amount of labeled ZNF300 binding

site was mixed with an equal molar amount of the corresponding protein. The densitometry of the shifted bands was measured using BandScan 5.0, and the DNA dissociation constant (KD) was determined using the Scatchard analysis.
In the supershift experiments, the nuclear extracts were prepared as described previously [15]. Thirty five fmol of the probe was incubated with 20 μg of the nuclear extract in 1 x Gel Shift Binding Buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.05 mg/ml poly(dI-dC)) for 30 min at room temperature. Then anti-ZNF300 antibody (generated by Beijing Biosynthesis Biotechnology, China), anti-Egr1 antibody (R&D System, Inc.) or pre-immune rabbit serum (Zhongshan Biotechnology, China) were added into the binding reaction, and the whole was incubated for 1 h at room temperature. Samples were then electrophoresed on 5% non-denaturing polyacrylamide gel (1 x TGE, 25 mM Trise base, 190 mM Glysine, 1 mM EDTA, 29:1 acrylamide/bisacrylamide (25.3%, v/v), 5% glycerol), and the gel was analyzed by autoradiography.

Cell culture, transient transfection and the dual luciferase assay
A Jurkat cell line was cultured in RPMI1640 medium supplemented with 10% fetal calf serum, treated with or without 10 ng/ml TPA (Sigma). The mammalian cell line HeLa was cultured in DMEM (Dulbecco's modified Eagle's medium) with 10% Bovine Calf Serum (GIBCO, Invitrogen Corporation, USA).
The SuoHua-Sofast™ Transfection system (Xiamen Sunma Biotechnology Co., Ltd) was used for the transient transfection. The transient transfection was done with the indicated 0.05 μg of firefly luciferase reporter construct and the internal control *Renilla* luciferase reporter construct, pRL-TK (Promega) in a ratio of 10:1. The latter contains the *Renilla* luciferase gene driven by the *Herpes simplex* virus thymidine kinase (TK) promoter. Co-transfection experiments were performed on HeLa cells by adding the same amount of pcDNA3.0 vector and/or pcDNA-ZNF300 expression vector and/or pcDNA3-Egr1 expression vector together with the respective luciferase reporter construct and the internal control plasmid pRL-TK (Promega). Forty eight hours after transfection, the cells were harvested, lysed and assayed for luciferase activity according to the protocol from Promega. Triplicate samples were measured for each construct, and the average values of the ratio of firefly luciferase light units to Renilla luciferase light units were used for data analysis. The results show the mean values of three independent experiments with standard errors.

Western-blot assay
Protein samples (about 100 μg) were analyzed on 10% SDS-polyacrylamide gel and electrophoretically transferred onto nitrocellulose membrane. The membranes were blocked in 5% non-fat dry milk for 2 h and probed with anti-ZNF300 antibody (generated by Beijing Biosynthesis Biotechnology, China), anti-β-actin antibody (Zhongshan Biotechnology, China), or anti-Egr1 antibody (R&D System, Inc.). Following incubation with HRP-conjugated secondary antibody, the proteins were detected using the ECL reagent (Pierce).

RESULTS AND DISCUSSION

Recombinant ZNF300 and Egr1 share binding sites *in vitro*

In our previous study, we found that ZNF300 binds specifically to a GC-rich element (C(t/a)GGGGG(g/c)G) through its zinc finger domains [10]. That study also showed that ZNF300 has the same binding affinity to the DNA sequences CTGGGGGCG and ACGGGGGCG [10]. Further EMSA was carried out to detect whether changing the first nucleotide (an A → G, C or T change) of ACGGGGGCG would affect the binding affinity of ZNF300 to this consensus. As shown in Fig. 1A, incubating the GST-ZF300 protein with the labeled oligonucleotide wt (CTGGGGGCG) alone resulted in the formation of a shifted band (Fig. 1A, lane 2). A 200-fold molar amount of the unlabelled oligonucleotides wt, m1 (ACGGGGGCG) or m3 (GCGGGGGCG) was able to eliminate most of the shifted band (Fig. 1A, lanes 3, 4 and 6). The unlabelled oligonucleotide m2 (TCGGGGGCG) could partially compete away the shifted band (Fig. 1A, lane 5), and the unlabelled oligonucleotide m4 (CCGGGGGCG) showed the weakest competition for ZNF300 binding (Fig. 1A, lane 7). Thus, recombinant GST-ZF300 protein prefers to bind to the oligonucleotides m1 (ACGGGGGCG) and m3 (GCGGGGGCG). Further analysis revealed that the oligonucleotide m3 contained a consensus element (GCGGGGGCG) for Egr1 binding. Thus, the recombinant ZNF300 protein could bind to the consensus Egr1 binding site *in vitro*.

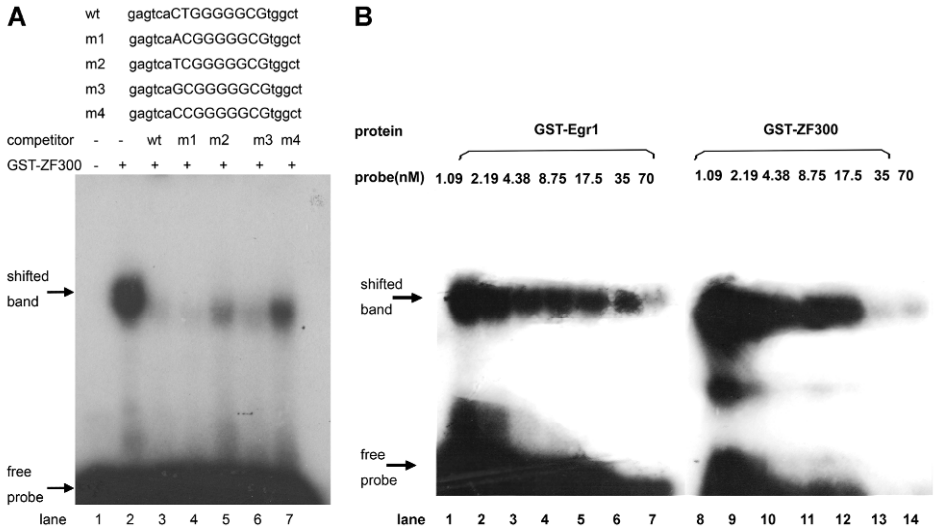

Fig. 1. ZNF300 and Egr1 share DNA sequences *in vitro*. A – EMSA was performed by incubation of the labeled oligonucleotide (wt) with GST-ZF300 in the presence or absence of the indicated unlabeled competitor: wt, m1, m2, m3 or m4. B – An equal molar amount of GST-ZF300 or GST-Egr1 protein was incubated with increasing amounts of labeled oligonucleotide (wt) for EMSA to determine the binding affinity of ZNF300 or Egr1 to the ZNF300 binding site.

To determine whether Egr1 also binds to the ZNF300 binding site in a similar manner to ZNF300, the DNA-binding affinity of the recombinant Egr1 protein GST-Egr1 to the ZNF300 binding site (CTGGGGGCG) was determined and compared with that of the recombinant ZNF300 protein. EMSAs were performed in the presence of increasing amounts of radiolabelled probe and equal molar amounts of the GST-ZF300 or GST-Egr1 protein. As shown in Fig. 1B, GST-Egr1 could bind to the ZNF300 binding site (CTGGGGGCG). The amounts of the probe in the shifted bands were further quantified by BandScan 5.0. The DNA-binding activity calculation using Scatchard analysis showed the dissociation constant (KD) of ZNF300 to be 6.33 nM versus a KD of 10.53 nM for Egr1. These results show that recombinant Egr1 and ZNF300 protein can bind to the ZNF300 binding site with similar affinities *in vitro*.

Upon TPA stimulation, endogenous ZNF300 and Egr1 bind to the IL-2Rβ promoter through the ZNF300 binding site in Jurkat cells.

It was reported that Egr1 up-regulates IL-2Rβ promoter activity through a canonical Egr1 binding site ($^{-163}$CGCCCCCAG^{-155}) and a non-canonical Egr1 binding site ($^{-155}$GCGTAGGAGGCAG^{-142}) in the IL-2Rβ promoter (Fig. 3A) [16]. Notably, the canonical Egr1 binding site ($^{-163}$CGCCCCCAG^{-155}) is also a conserved ZNF300 binding site found in the same reverse orientation. We tried to determine whether endogenous ZNF300 could also bind to this site and regulate IL-2Rβ promoter activity.

Egr1 is an immediate-early gene product induced by a variety of stimuli, including TPA. Consistent with the previous report, the expression of Egr1 was detected in TPA-treated Jurkat T cells for 12 h, but not detected in the TPA-treated Jurkat T cells for 0, 24, 48 or 72 h [16], while TPA treatment had no effect on the expression level of ZNF300 (Fig 2A). β-Actin was used for normalization of the total protein loading. We also tried to detect the binding of endogenous ZNF300 and Egr1 to the canonical ZNF300/Egr1 binding site with the labeled canonical ZNF300/Egr1 binding site from the IL-2Rβ promoter region (wt) as a probe (Fig. 2B). EMSA was performed by incubating the labeled oligonucleotide (-177 to -145) from the IL-2Rβ promoter region (wt) with nuclear extract from Jurkat cell with or without TPA treatment. Several DNA-protein complexes were detected by EMSA (Fig. 2C and D). To identify these shifted bands, anti-Egr1 antibody, anti-ZNF300 or pre-immune rabbit serum (PI) was added to the binding reaction. The Egr1:DNA complex (Fig. 2C, lanes 7, 9 and 11) supershifted by anti-Egr1 antibody (indicated by an asterisk) was only detected in Jurkat nuclear extracts treated with TPA for 12 h (Fig. 2C, lane 10 versus lane 5), consistent with the previous report [16]. Perhaps due to the high isoelectric point of the complex, the DNA:Egr1:antibody complex was retarded near the well. The DNA-protein complex supershifited by anti-ZNF300 antibody (indicated by an asterisk) was deteceted in Jurkat in the nuclear extract regardless of PMA stimulation (Fig. 2D, lane 6 versus lane 2, lane 11 versus lane 7).

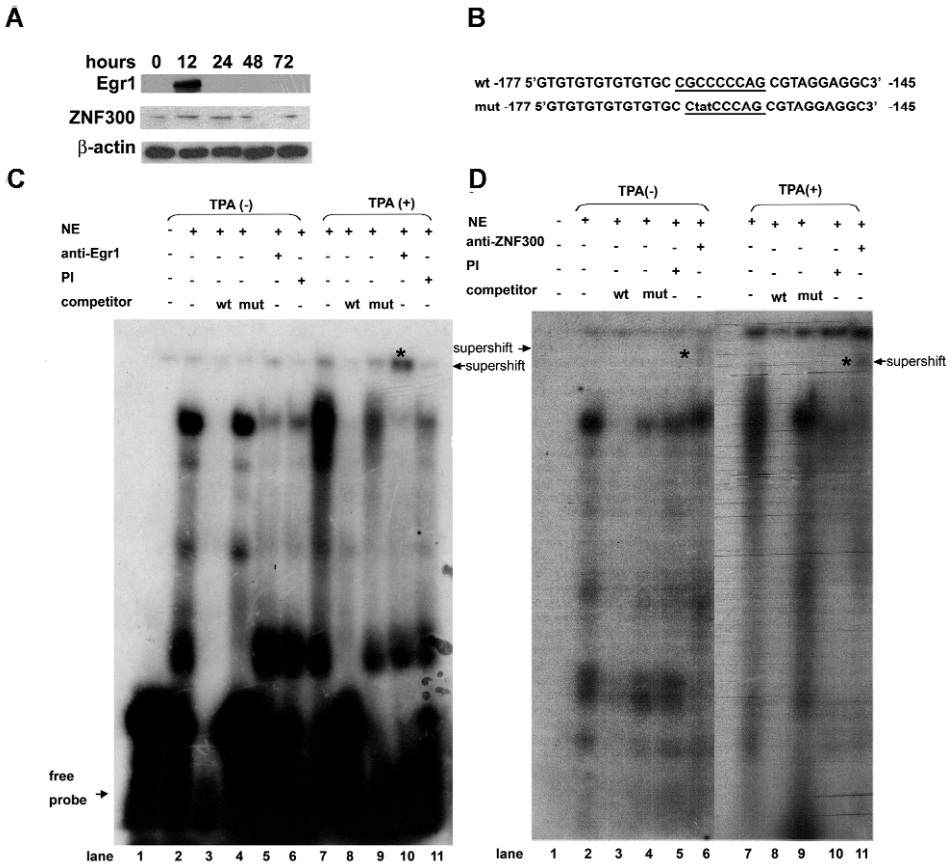

Fig. 2. Both endogenous ZNF300 and Egr1 bind to the IL-2Rβ promoter through the ZNF300/Egr1 binding site in Jurkat cells upon TPA stimulation. A – Jurkat cells were cultured with TPA stimulation for the indicated time. Western-blot analysis of the total protein extracted from Jurkat cells was performed with anti-Egr1 antibody, anti-ZNF300 antibody or anti-β-actin antibody. B – The oligonucleotide sequence containing the wild type (wt) or mutant (mut) ZNF300/Egr1 binding site (underlined) was derived from the IL-2Rβ promoter region. C and D – Both ZNF300 and Egr1 bind to the IL-2Rβ promoter through the ZNF300/Egr1 binding site in TPA-treated Jurkat cells. Nuclear extracts from untreated Jurkat cells or Jurkat cells treated with TPA for 12 h were incubated with the labeled oligonucleotide wt in the absence or presence of a 100-fold molar amount of unlabelled competitor, including wild type (wt) or mutant (mut) oligonucleotides. The identity of the Egr1 or ZNF300 complex was confirmed by supershift analysis using a specific antibody against endogenous Egr1 or ZNF300 protein. The pre-immune serum (PI) was added to the binding reaction as a negative control so that there was no supershifted band with PI. C – There were some residual signals stuck in the well in each lane, proportional to the binding complex, except in lane 10, which had little complex left due to the antibody. Here, the supershift bands are indicated with asterisks.

No supershift band was detected by the control, pre-immune serum (Fig. 2C, lane 6 and lane 11; Fig. 2D, lane 5 and lane 10), although it did reduce the complex formation (Fig. 2C, lane 2 versus lane 6, lane 7 versus lane 11; Fig. 2D, lane 2 versus lane 5, lane 7 versus lane 10). Formation of the complexes was reduced by the unlabelled probe but not by the mutated competitor, as shown in Fig. 2B (Fig. 2C, lane 3 versus lane 4, lane 8 versus lane 9, Fig. 2D, lane 3 versus lane 4, lane 8 versus lane 9), demonstrating the DNA-protein binding specificity. Taken together, this means that endogenous ZNF300 and Egr1 can bind to the IL-2Rβ promoter through the canonical ZNF300/Egr1 binding site.

ZNF300 and Egr1 co-regulate the IL-2Rβ promoter activity

Luciferase assays were carried out to detect the effects of ZNF300 and Egr1 on IL-2Rβ promoter activity. The schematic structures of the wild-type and mutant IL-2Rβ promoter cloned upstream of the firefly luciferase gene are shown in Fig. 3A. As shown in Fig. 3B, transient overexpression of ZNF300 or Egr1 could both activate the IL-2Rβ promoter significantly ($p < 0.05$; Fig. 3B). When the canonical ZNF300/Egr1 binding site in the IL-2Rβ promoter was mutated, the luciferase activity enhanced by ZNF300 was eliminated (Fig. 3B). Egr1 could still enhance the mutated IL-2Rβ promoter activity because of the non-canonical Egr1 binding site in the IL-2Rβ promoter ($p < 0.05$), while the activity enhanced by Egr1 reduced to a lower level (Fig. 3B). These results indicated that the canonical Egr1 binding site was required for the promoter activity activated by ZNF300 and Egr1.

Further studies were performed to examine the potential additive effects of ZNF300 and Egr1 on IL-2Rβ promoter activity. As shown in Fig. 3C, when equivalent plasmid concentrations of the respective expression plasmids were used, the combined expression of ZNF300 with Egr1 has a greater contribution (about 3.9-fold greater) to the IL-2Rβ promoter activity than the expression of ZNF300 or Egr1 alone *in vivo*.

The results of previous studies demonstrate that the human IL-2Rβ gene is co-regulated by several transcription factors including Ets, Sp1 and Egr1 [16, 17]. These transcription factors bind to different elements in the IL-2Rβ gene promoter region and regulate its expression. In this study, we showed that ZNF300 was involved in IL-2Rβ gene regulation by sharing a common DNA element with Egr1. Sp1 is another transcription factor that shares a DNA element with Egr1 [18].

Egr1 is an immediate-early gene product induced by different stimuli. It is not expressed in Jurkat cells without environmental stimulation, while ZNF300 is constitutively expressed in Jurkat cells. Therefore, ZNF300 may occupy the consensus binding site as a basal regulator to keep the basal expression level of the target gene. When a cell receives environmental stimulation, the transiently expressed Egr1 protein might occupy the non-canonical Egr1 binding site to activate IL-2Rβ (Fig. 3A) [16]. However, Egr1 might also co-bind to the canonical ZNF300/Egr1 binding site, and then interplay with ZNF300 to co-

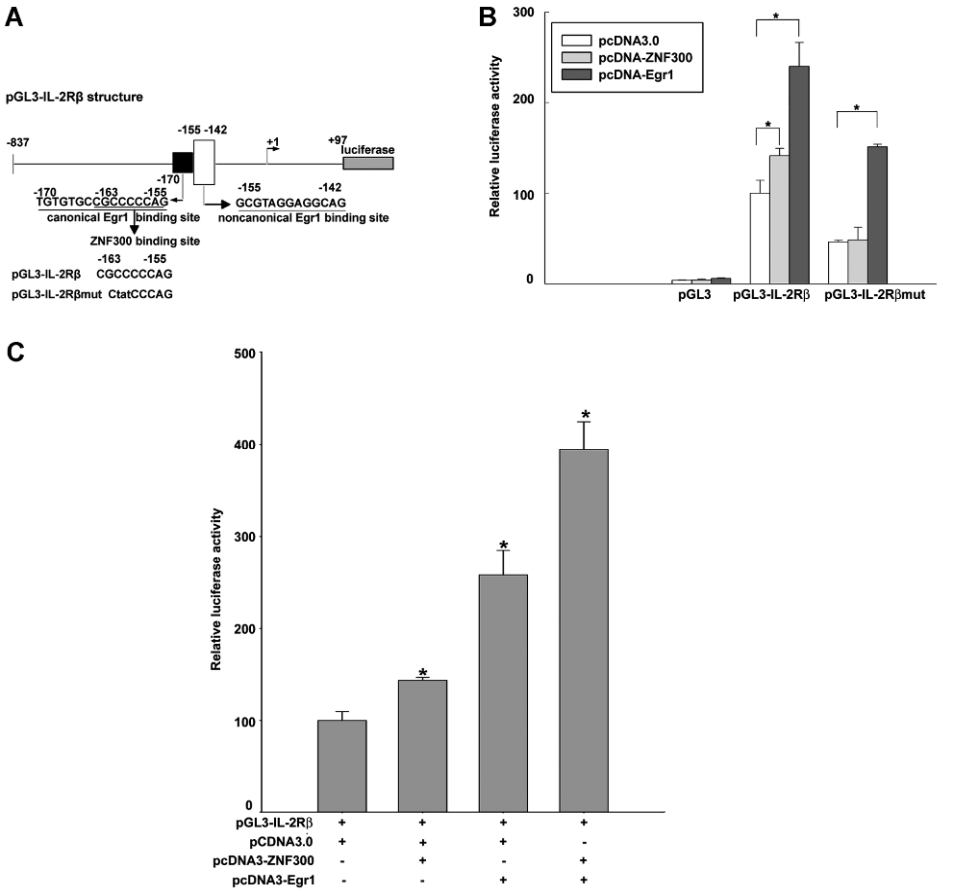

Fig. 3. Both ZNF300 and Egr1 could transacitvate the IL-2Rβ promoter through the ZNF300/Egr1 binding site. A – Schematic diagram of the reporter construct containing the wild-type or mutant IL-2Rβ promoter. The canonical Egr1 binding site/ZNF300 binding site and non-canonical Egr1 binding site are indicated. B – HeLa cells were transiently co-transfected with pGL3-IL-2Rβ construct, pGL3-IL-2Rβmut or promoter-less pGL3 reporter plasmid, and the expression plasmid pcDNA3-ZNF300 or pcDNA3-Egr1 or the empty pcDNA3.0 vector. The columns represent the means of triplicate experiments; bars, standard deviation; *, $p < 0.05$ versus control. C – Combined expression of ZNF300 with Egr1 enhances IL-2Rβ promoter activity. HeLa cells were co-transfected with the pGL3-IL-2Rβ construct and pcDNA-ZNF300, pcDNA-Egr1, or empty pcDNA3.0 vector alone or in combination. The empty pcDNA3.0 vector was used to keep the total plasmid DNA constant. As an internal control, the pRL-TK plasmid was used to normalize the transfection efficiency. The columns represent the mean of triplicate experiments; bars, standard deviation; *, $p < 0.05$ versus control.

activate the IL-2Rβ gene (Fig. 3A and C) [16]. Although binding and co-transfection studies have implied a functional role for ZNF300 and Egr1 in the regulation of the IL-2Rβ promoter, additional experiments aimed at describing the interaction of ZNF300 and Egr1 *in vivo* will contribute to an understanding of their co-activation of IL-2Rβ promoter activity.

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