IDENTIFICATION OF NBRE (NERVE GROWTH FACTOR RESPONSIVE ELEMENT) OF THE TCL1 PROMOTER AS A NOVEL NEGATIVE REGULATORY ELEMENT

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Running Title: TCL1-NBRE as a Novel Negative Regulatory Element

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The serine/threonine kinase, Akt (Protein Kinase B) plays a central role in the regulation of intra-cellular cell survival. Recently, we demonstrated that the protooncogene TCL1, overexpressed in human T-cell prolymphocytic leukemia, is an Akt kinase co-activator.

Tightly restricted TCL1 gene expression in early developmental cells suggested that the TCL1 gene is regulated at a transcriptional level. To characterize the TCL1 gene regulation, we cloned the 5'-promoter of the TCL1 gene located at human chromosome 14q32. The 5'-TCL1 promoter region contains a TATA box with cis-regulatory elements for Nur77/NGFI-B, (NBRE, Nerve Growth Factor Responsive element, CCAAGGTCA), NFκB, and FKHRL (Fork Head Transcription Factor). Nur77/ NGFI-B, an orphan receptor super family transcription factor implicated in T cell apoptosis, is a substrate for Akt. We hypothesized that TCL1 transactivity is regulated through Akt-induced phosphorylation of Nur77/NGFI-B *in vivo*.

In EMSA with CHIP assays, wild type Nur77, but not S350A mutant Nur77, could specifically bind to TCL1-NBRE. A luciferase assay demonstrated that TCL1-NBRE is required for inhibition of TCL1 transactivity upon NGF/PDGF stimulation, which activates Akt and phosphorylates Nur77. Using a CHIP assay with RT-PCR, NGF stimulation inhibited binding of endogenous Nur77 to TCL1-NBRE, in turn, suppressing TCL1 gene expression.

The results together establish that TCL1-NBRE is a novel negative regulatory element of Nur77/NGFI-B. To the best of our knowledge, TCL1-NBRE is the first direct target of Nur77 involving the regulation of intracellular cell death–survival. This Akt-induced inhibitory mechanism of TCL1 should play an important role in immunological and/or neuronal development *in vivo*.

Serine threonine kinase Akt, viral homologue of v-Akt, is an important intracellular regulatory molecule that controls apoptosis (1-3). Recently, we demonstrated that the protooncogene TCL1, whose function was previously unknown, is an Akt kinase co-activator that physically binds, and hence activates Akt (4, 5). These studies together provided not only a molecular basis, but also therapeutic implications for human T-cell prolymphocytic leukemia (T-PLL) in which the TCL1 oncogene is over expressed due to a chromosomal translocation (6-10).

In T cell prolymphocytic leukemia, both TCL1 and TCL1b genes on human chromosome 14q23.1 are activated by translocations and inversions at 14q32.1, juxtaposing them to regulatory elements of T cell receptor genes. In addition to T-PLL, in human diseases, TCL1 is over expressed in EBV infected B cell lymphoma, ataxia-telangiectasia, teratoma, and/or AIDS related lymphoma (10-13). In physiological conditions, however, TCL1 expression is tightly

restricted to early developmental T cells (CD3 negative) as well as early B cells with negative IgM expression (14-16).

Three major isoforms of the human TCL1 oncogene are present in both the human and mouse genome, namely TCL1, TCL1b, and MTCP1 (9,13). Under physiological conditions, TCL1 and TCL1b are highly expressed at early developmental stages in several fetal tissues including thymus, kidney, lung (TCL1), and spleen (TCL1b). After birth, expression of all TCL1 family members is mainly restricted to lymphoid tissues, although TCL1b mRNA is also found in the placenta. It is notable that in mice both TCL1b and TCL1 mRNAs are abundant in oocytes and two-cell embryos, but rare in various adult tissues and lymphoid cell lines (14,17,18). Tightly restricted physiological expression of TCL1 family proteins suggested that TCL1 gene expression is regulated at a transcriptional level (14,16,19).

To clarify molecular regulation of TCL1 gene expression, we cloned 1123 base pairs of the 5' promoter sequence of TCL1 (including 931 from base pairs of the 5'-TCL1 promoter region) from human chromosome 14q32. Nucleotide sequence analysis revealed that a Nur77 (NGFI-B) binding site {NBRE, Nerve Growth Factor response element, CCAAGGTCA (30)} is located within the 5'- proximal promoter.

Nur77 (NGFI-B, Nerve growth factor induced-B, TR3) was originally identified as a NGF induced ligand-dependent transcriptional activator from PC12 cells (rat pheochromocytoma cells) (20). Unlike other orphan receptor super family transcription factors, it is believed that ligands were not required for transactivation through Nur77/NGFI-B (21). Additional subfamilies of NGFI-B transcription factor have been identified from neuronal cells as Nurr1 (Nur-related factor 1), and NOR-1 (neuron-derived orphan receptor 1) (22-24). Subsequent studies demonstrated that Nur77 plays a pivotal role in T-cell apoptosis *in vivo* (25,26). Recent studies further demonstrated that Akt interacts with Nur77 and hence induces phosphorylation of Nur77 (27,28).

Despite intensive studies, a direct gene target of Nur77, which regulates the cell death survival machinery, is yet to be determined (21). Therefore, it is noteworthy that the cis-regulatory element of Nur77 (NBRE) is well conserved within the TCL1 5'-proximal promoter region of human, mouse, and rattus (21). These observations lead us to hypothesize that the TCL1 gene can be regulated through NBRE by Akt-induced phosphorylation of Nur77/NGFI-B *in vivo*.

By EMSA and luciferase reporter assays, we demonstrated that Nur77/NGFI-B bound to the NBRE site of the TCL1 promoter (TCL1-NBRE), and negatively regulated TCL1 gene expression by Akt stimulation. In PC12 cells, NGF stimulation induced endogenous Nur77 phosphorylation, diminished binding of Nur77 with the TCL1-NBRE, in turn, and suppressed TCL1 transactivation. The results together demonstrated that TCL1-NBRE is a novel functional target of Nur77/NGFI, which could play an important role for the restricted expression of the TCL1 gene *in vivo*.

EXPERIMENTAL PROCEDURES

Cloning and Construction of 5' TCL1 Promoter Reporter Constructs

Using the following primers for PCR （Forward primer: 5'-ATCATCGAGCTC CAGGCTGGAGCTGGTTTCCATG-3', Reverse primer: 5'-ATCATCAGATCTCGTCCAAA TACACGAACTTCTCCC-3'), we amplified 1123 base pairs (-931 to +192 of TCL1 proximal promoter region) from human 293 kidney fibroblasts (ATCC). The resulted TCL1 promoter fragment was cloned into Sac I and Bgl II sites of luciferase reporter construct PGL3 (Promega), and designated as pGL3-TCL1.

NBRE mutant was generated by digesting with BstE II (within the binding sequence of NBRE of TCL1 promoter), treated with Mung Bean Nuclease (New England Biolabs), and ligated back into PGL3 (PGL3-mt-NBRE or pGL3-mt-NBRE-470). The resulted nucleotide

sequences were mutated from AAGGTCA to AAGCCC at the TCL1-NBRE site. Mutation of cis-regulatory element of FKHRL (pGL3-mt-FKHRL) was introduced by Quick change (Stratagene) using pairs of primers (Forward primer: 5'-GTTACTGCAAAGCGAAGTGAAATTG-3' and Reverse primer:5'-CAATTTCACTTCGCTTTGCAGTAAC-3'). Truncation mutant of TCL1 promoter (PGL3-TCL1-470) was generated by amplifying by the pairs of primers (Forward primer: 5'-CGGGGTACCTGATCCCATAAGAT GAGAG-3', Reverse primer: 5'-ATCATCAGATCT CGTCCAAATACACGAACTTCTCCC -3'). The resulted truncated promoter fragment (from -470 to +192) was digested with Kpn I and Bgl II and subcloned into PGL3 Luciferase reporter construct (pGL3-TCL1-470). The nucleotide sequences of all the luciferase reporter constructs were confirmed by Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) using ABI PRISM 310 DNA sequence analyzer (Applied Biosystems).

Luciferase Reporter Assays

PC12 cells (rat pheochromocytoma cells, ATCC) or 293T cells (human kidney fibroblasts, ATCC) were cultured in the charcoal treated 10% FBS in 24-well plates (Corning). One microgram of indicated luciferase reporter constructs were transfected (wild type or mutant forms of TCL1 promoter as indicated) by Fugene 6 transfection reagent （ Roche). Thirty-six hours after transfection, the cells were serum starved and for additional 12 h, stimulated with NGF {50 ng/mL, murine NGF (7S), 13290-010, Invitrogen} or PDGF (100ng/mL, PDGF-AB, p3326, Sigma) as indicated. Luciferase reporter assays were performed using Dual Luciferase kit (Promega). Twenty four hours after transfection, cells were treated with 20μM wortmannin (Calbiochem, #KY-12420) or 20μM PD98059 (Biomol, #EI-360) or DMSA for 30 minutes at 37^{o}C as indicated. Cells were then stimulated with 50 ng/ml PDGF for 15 minutes and luciferase reporter activities were measured. Statistical analysis was performed using student t-test and $P < .05$ was considered to be significant.

Generation of Recombinant Nur77

GST-Nur77-DBD (DNA Binding Domain) fusion construct in pGEX-4T (pGEX-4T-Nur77-DBD) was generated by PCR amplification using pairs of primers (Forward primer:
primer:5'-CGGGATCCGCACCCGTAACCTCC AC-3' and Reverse primer:5'-CGGAAT TCTCAGGGTTTTGAAGGTAGC-3') with wild type Nur77 cDNA as a template and subcloned into pGEX-4T-2 vector (Amersham Pharmacia). The resulted pGEX-4T-Nur77-DBD expression vector was transformed into BL21 cells (DE3, Invitrogen) and the GST-Nur77-DBD recombinant protein was harvested, dialyzed in a dialysis buffer (20mM Tris-HCl, pH 8.0, 150mM NaCl, and 10% glycerol). The protein concentrations were determined by Bradford Assay (Bio-rad).

Electric Mobility Band Shift Assays

EMSA （ Fig. 2A ） was performed as described in (29) with the following modifications. 5' end of the following oligos (5'-GAAAGGGCCAAGGTCACCCCGGTGC-3' and 3'-CTTTCCCGGTTCCAGTGGGGCC ACG-5') were labeled with IRDye (IRD700, LI-COR Bioscience, Nebraska) and annealed to generate double stranded probes for EMSA assay. 293T cells were cultured in charcoal treated FBS, transfected with indicated Nur77 expression vectors {pCS-myc-Nur77-WT or pCS-myc-Nur77-S350A in which Serine 350 was mutated to Ala (28, 43) in Fig. 2A using calcium phosphate method (4), cultured on 100 mm dish (CellBIND Surface, Corning). Twenty four hours after transfection the cells were serum starved for additional 16 hours, stimulated (or non-stimulated) with PDGF (50ng/mL, PDGF-AB, p3326, Sigma) for 20 minutes. Forty-eight hours after transfection, the cells were harvested and lysed with Hepes cell lysis buffer [20mM Hepes-NaOH pH7.4, 1mM EDTA, 150mM NaCl, 1mM DTT, 10% glycerol, 0.5mM PMSF, 5μg/mL leupeptin] by freeze thaw method. Twenty micrograms of cellular extracts were incubated with {0.1%TritonX100, 4% Glycerol, 1mM EDTA, 10mM βME, 10mM Tris HCl (pH

7.4), 1μg poly (dI-dC), and 5 μg BSA〕in the presence of 20 pmols of IRD700 labeled probes for 30 min at 4 °C. The resulted samples were resolved onto 5% acrylamide-0.5X TBE gels and DNA protein complexes were visualized using Infrared Imaging system（Odyssey, LI-COR Bioscience, Nebraska, USA）.

EMSA (Fig. 2B, D, E, and 4B) was performed essentially described in (30) using fluorescence labeled double stranded TCL1-NBRE probes (5'-CGGCCAAGGTCA CCCCGGCCAAGGT CACCCCA-3') using 5' EndTag Labeling Kit (Vector Laboratories Inc. CA). 293T cells were cultured in charcoal treated FBS, transfected with indicated Nur77 expression vectors {pCMV-Flag-Nur77-WT or pCMV-Flag-Nur77-S350A in which Serine 350 was mutated to Ala (28, 43) using calcium phosphate method (4), cultured on 100 mm dish (CellBIND Surface, Corning). Twenty four hours after transfection, the cells were serum starved for additional 16 hours, and stimulated (or non-stimulated) with PDGF (50ng/mL, PDGF-AB, p3326, Sigma) for 20 minutes as indicated. The cells were then harvested and lysed with Hepes cell lysis buffer [20mM Hepes-NaOH pH7.4, 1mM EDTA, 150mM NaCl, 1mM DTT, 10% glycerol, 0.5mM PMSF, 5μg/mL leupeptin] by freeze thaw method. Thirty microgram of cell lysates were incubated with {20 mM HEPES (pH 7.5), 90 mM NaCl, 1.5 mM $MgCl_2$, 10 μM $ZnCl_2$, 10 mM DTT, 0.02% TritonX100, 10% Glycerol, 1mM PMSF, 5μg/mL leupeptin, 10 mM NaF, 1 mM Na_3VO_4, 2.5 μg poly dI-dC, and 5 μg BSA} in the presence of 30 picomoles of Fluorescein labeled TCL1-NBRE probes for 30 min at 4˚C. The resulted samples were resolved onto 4.5% acrylamide-0.5X TBE gels and DNA protein complexes were visualized by Biorad Imaging system (Bio-Rad VersaDoc 5000). GST antibody (0-5 μg, Clonetech) was used for the supershift experiment (Fig. 2E). Eighty picomoles of recombinant GST-Nur77-DBD protein were used for the cold competition experiments（Fig. 2D）combined with 500 to 1000 fold molar excess of unlabeled TCL1-NBRE probes as indicated. Thirty microgram of PC 12 cell derived cell extract with 3 μg of anti-Nur77 antibody (M-210, sc5569, Santa Cruz), anti-NOR1 (C-19, sc-22519, Santa Cruz), anti- Nurr1 (E-20, sc-990, Santa Cruz), or rabbit normal serum as a control were used for super shift experiment (Fig. 4B).

In Vitro CHIP (Chromatin Immunoprecipitation) Assay

293 cells (human kidney fibroblast cell line, ATCC) were transiently transfected with 5 μg of luciferase reporter constructs (pGL3-TCL1 or pGL3-mt-NBRE) with Nur77 {pCS-myc-Nur77-WT or pCS-myc-Nur77-S350A (28, 31)} by calcium phosphate method. Forty-eight hours after transfection, cells were fixed with 1% formaldehyde and treated with 0.125 M Glycin to terminate the fixation reaction. Cells were lysed with cell lysis buffer (5mM PIPES pH 8.0, 85mM KCl, 0.5% NP-40, 1 mM PMSF, and 5 μM Luepeptin) at 4°C for 20 min, centrifuged at 14000 rpm for 30 min at 4°C. The resulted celular extracts were lysed using Nuclear Lysis Buffer (20 mM Hepes-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, and 0.1% SDS), immunoprecipitated with protein agarose conjugated anti-myc antibody (9E10, sc-40, Santa Cruz, Biotechnology) for 16 h. at 4˚C. The resulted immune complexes were extracted and used for subsequent PCR reaction with pairs of primers (Forward primer: 5'-TCCTGGTGTC GACTGTGAGT-3', Reverse primer: 5'-AGCTC CTGGGAACGCAGAC-3') to detect fragment of TCL1-NBRE fragment by resolving onto 1.0 % TAE agarose gel. Equal amount of NBRE expression was verified by PCR using the same sets of primers using 5 μl of cellular extracts as a template from the transfected cells and visualized on 1.0% agarose gel.

In vivo Chromatin Immunoprecipitation Assay

In vivo CHIP assays were performed essentially using QuikChip kit (IMGENEX) with minor modification using 3 μg of anti-Nur77 antibody (M-210, sc5569, Santa Cruz), anti-NOR1 (C-19, sc-22519, Santa Cruz), anti-Nurr1 (E-20, sc-990, Santa Cruz), or rabbit normal serum as a control. PC12 cell line (rat

pheochromocytoma cells, ATCC) was cultured on 100 mm dish (CellBIND Surface, Corning) with charcoal treated 10% FBS and 5% horse serum. The cells were serum starved for 24 h before NGF stimulation {50ng/mL, murine NGF (7S), 13290-010, Invitrogen} for additional 6 h, and harvested for the subsequent PCR reaction. The 250 base pair genomic TCL1 promoter DNA fragments were detected by two-step PCR using the following primer sets {first primer sets (5'-TGACCTCATGGTAAACATAGC-3') and (5'-ACATATAGCGAGCGCATCAG-3')};
second primer sets {(5'-ACCTCATGGTAAAC ATAGCG-3') and (5'-GTCTATACACACTGCA TATGC-3')}. The resulted PCR products were loaded onto 1.5% TBE agarose gel and the DNA fragments were visualized.

RT-PCR

PC 12 cells (ATCC) were cultured on 100 mm dish (CellBIND Surface, Corning) with charcoal treated 10% FBS and 5% horse serum. The cells were serum starved for 16 h, then stimulated with NGF {50 ng/ml, murine Nerve Growth Factor (7S), 13290-010, Invitrogen} for indicated time periods. Total RNA was isolated with TRIzol reagent (Invitrogen). mRNA was isolated using Oligotex-dT30 (Takara) for generating the first strand cDNA using First-strand cDNA synthesis kit (Takara). RT-PCR was performed using pairs of primers (TCL1 ; 5'-TGGGTTCACTGTGAGGGTGT C-3' and 5'-GTAGAGCTGCCACATGAGAG G-3', G3PDH; 5'-AAGTATGATGACATCAAG AAGG-3' and 5'-AGTTACAGGAGACAACC TGG-3') with EX taq polymerase (Takara) using thermal cycler (iCycler, Biorad).

Immunobloting

293 cells (Fig. 2A and B) were cultured in 100 mm dish (CELLBIND, Corning) in charcoal treated FBS and transfected with 7.5 μg expression vectors (or sham transfected) using calcium phosphate method. Twenty four hours after transfection, the cells were serum starved for additional 16 h, then stimulated with (or without) PDGF (50 ng/ml, PDGF-AB, p3326, Sigma) as indicated for 8 min., and lysed with Brij lysis buffer in the presence of 1mM Na_3VO_4 and 10mM NaF (4). Twenty microgram of cell lysate were loaded onto 7.5% SDS-PAGE (Daiichikagaku), and immunoblotted by indicated antibodies {anti-Akt, #9272; anti-phospho-Ser473 Akt, #9271, Cell Signaling, anti-cMyc, 9E10, Santa Cruz Biotech.(Fig 2A), or anti-Flag M2, Sigma} using ECL (Amersham). To detect phosphorylated Nur77 (Fig.2B), cell lysates were precleaned, immunoprecipitated with anti-Flag antibody (M2, Sigma) using ProA/proG mixture (4), and immunoblotted with anti-phospho antibody (#9601, Cell Signaling).

To detect non-phosphorylated (or phosphorylated) Akt and Nur77 (Fig. 4 and 5), PC12 cells were harvested at indicated time points after NGF stimulation {50 ng/mL, murine NGF (7S), 13290-010, Invitrogen}, lysed with Brij97 lysis buffer (4), resolved onto SDS-PAGE, and immunoblotted by TCL1 polyclonal antibody (#533, generated by recombinant human TCL1), anti-Akt Abs, or anti-phospho-Ser473 Akt Abs using ECL (Amersham). To detect phosphorylated Nur77, PC 12 cells were harvested, lysed, then immunoprecipitated with anti-Nur77 antibody (M-210, sc5569, Santa Cruz), and immunoblotted by anti-phospho antibody (#9601, Cell Signaling).

RESULTS

Structure of the TCL1 Promoter Region

To clarify the molecular regulation of TCL1, we cloned 1123 base pairs of the TCL1 5'-proximal promoter (from -931 to +192) from human 293 cells (ATCC). Sequence analysis of the 5'-TCL1 promoter region revealed a TATA box with the following transcriptional regulatory elements of FKHRL, Nur77, MyoD, IRF, NFκB, or Sp1 (**Fig. 1A**). Recently, it was reported that Akt activation directly phosphorylates and regulates Nur77 (NGFI-B, Nerve growth factor induced - B, or TR3) and FKHRL (Fork Head Transcription Factor) (27,28,31). In this regard it was striking that a putative FKHRL consensus binding site (CAAAATAA, located at from -857

to 850) as well as a Nur77/NGFI-B binding site (NBRE, Nerve Growth Factor Responsive element, CCAAGGTCA, located from -340 to -332) were present within 1000 of the 5'-TCL1 proximal promoter region (**Fig. 1B**).

Nur77 is an orphan receptor super family transcription factor that is implicated in neuronal development and apoptosis (20). It is noteworthy that none of the molecular targets of Nur77/NGFI-B in cell death machinery have been identified in literature (21). Recognition sequences of NGFI-B orphan receptor super family transcription factors present within the 5' proximal TCL1 promoter were well conserved (underlined) in humans (CCAAGGTCA, -396 to -388 from ATG), the mouse (ACCTGGTCA, -336 to -328), and rat (ACGAGGTCA, from -402 to -394 from ATG)(32,33)(**Fig. 1C**). The observation led us to hypothesize that TCL1 gene expression is regulated via the Akt-Nur77 (NGFI-B)/NBRE-TCL1 functional regulatory loop.

Wild type Nur77, but not S350A mutant Nur77, could specifically interact with TCL1-NBRE.

A recent study demonstrated that Nur77, an orphan receptor super family transcription factor, is a substrate of Akt phosphorylation both *in vitro* and *in vivo*. Activated Akt could induce phosphorylation of Nur77, which decreased DNA binding activity of Nur77, and as a consequence, dissociated from DNA (27,28).

To evaluate the functional role of TCL1-NBRE in TCL1 gene regulation, we transfected wild type Nur77 (pCMV-Nur77) or mutant Nur77 {pCMV-Nur77-S350A, in which serine residue at 350 of Nur77 was replaced by Alanine (28, 33)} into 293 cells. In an EMSA assay using double stranded wild type TCL1-NBRE (5'-GAAAGGGCCAAGGTCA CCCCGGTGC-3') as probes, Nur77-WT transfected cellular extracts, but not Nur77-S350A transfected cellular extracts, could bind to wild type TCL1-NBRE probes (**Fig 2A**, upper panel, lanes 3 and 4, wild type-Nur77 and S350A-Nur77, respectively). Both wild type and S350A Nur77 expressed equally in the transfected cells by immunodetection (**Fig. 2A**, lower panel)

PDGF stimulation, which induced phosphorylation of Akt (**Fig. 2B**, lower panel, top row, compare lane 2 vs. lane 4, before and after PDGF stimulation) and Nur77 (lower panel, third row, lanes 2 and 4), could reduce the DNA-protein complex formation of TCL1-NBRE (**Fig. 2B**, upper panel, compare lane 2 vs. lane 4, before and after PDGF stimulation, indicated by the right arrow). Please note that phosphorylation of wild type Nur77 was potently induced compared to S350A Nur77 (lower panel, bottom row, lane 2 vs. lane 4, wild type vs. S350A Nur77 transfected cells, respectively). The results were consistent in two independent experiments using a TCL1-NBRE probe (data not shown).

Consistently, in CHIP assays, wild type Nur77 (pCS-Nur77) could interact with a DNA fragment of TCL1-NBRE (CCAAGGTCA of PGL3-TCL1), but not with the mutant NBRE (CCAAGCCC of PGL3-mt-NBRE) (**Fig. 2C**, upper row, compare lane 2 vs. lane 3, wild type vs. mt-NBRE, respectively). Nur77-S350A (pCS-Nur77-S350A) failed to bind to wild type TCL1-NBRE (upper row, lane 4) or mutant TCL1-NBRE (upper row, lane 5). An equal amount of NBRE construct was transfected to these cells shown by PCR detection (middle row, lanes 1-5).

To examine the specific binding of Nur77 with TCL1 NBRE, we generated a recombinant fusion protein of DNA Binding Domain of Nur77 (GST-Nur77-DBD). Cold competition experiments in EMSA demonstrated that Nur77 could specifically bind to TCL1-NBRE (**Fig. 2D**, lanes 2-4). Moreover, anti-GST antibody supershited the DNA-protein complexes in a dose escalation manner, suggesting that Nur77 could specifically bind to TCL1-NBRE probes (**Fig. 2E**, lanes 2-6). The observations (Fig. 2A-E) together demonstrated that Nur77 could specifically recognize and bind to the TCL1-NBRE (AAGGTCA from -340 to -332).

TCL1-NBRE is a novel regulatory element in TCL1 transactivation.

Nur77, an orphan receptor super family transcription factor, was a substrate for Akt (27,28). None of the target genes for Nur77 linked to cell death and survival machinery have been identified; therefore, it was striking that NBRE {cis-regulatory elements of Nur77, CCAAGGTCA, (19, 20)} was well conserved within the TCL1 5'-proximal promoter of human, mouse, and rattus (see Fig. 1C). The observation together lead us to hypothesize that Akt-induced phosphorylation of Nur77, in turn, can regulate TCL1 transactivity.

To examine the functional role of TCL1-NBRE for TCL1 transactivation, we subcloned 1123 base pairs of the TCL1 proximal promoter fragment into the PGL3 luciferase reporter construct (including 931 base pairs of 5' promoter sequences of TCL1) from human chromosome 14q32. PC12 cells were transfected with PGL3-TCL1 (or indicated mutant) and subsequently stimulated by 50 ng/mL NGF. In luciferase reporter assays, mutation of FKHRL showed a modest increase in TCL1 transactivation, but retained the inhibitory effect upon NGF stimulation {**Fig. 3A**, compare 2nd vs. 4th sets of the panels; wild type (PGL3-TCL1) vs. FKHRL mutation (PGL3- mt-FKHRL), respectively}. In contrast to the wild type TCL1 promoter (**Fig. 3A**, PGL3-TCL1), introduction of mutation into TCL1-NBRE (**Fig. 3A**, pGL3-mt-NBRE) or double mutation of NBRE and FKHRL (**Fig. 3A**, PGL3-mt-FKHRL -mtNBRE) showed no inhibition of TCL1 transactivity after NGF stimulation.

Consistently, PDGF stimulation inhibited TCL1 transactivation of PGL3-TCL1-470 (consisted with -470 to +192 of TCL1 promoter, which lacks additional upstream regulatory sequences) in 293 human kidney fibroblast cell lines {(**Fig. 3B,** PGL3-TCL1(-470)}. However, mutation of TCL1-NBRE within this truncated contract resulted in no inhibition after PDGF stimulation {**Fig. 3B**, PGL3-mt-NBRE (-470)}. The results together demonstrated that TCL1-NBRE is required for the inhibition of TCL1 transactivity upon NGF/PDGF stimulation.

The PDGF induced inhibitory effects on TCL1 transactivities were Akt activation dependent, since PI3K inhibitor treatment (20μM wortmannin), which then inhibited Akt activation, compromised PDGF-induced TCL1 inhibition (**Fig. 3D,** before and after PDGF stimulation). In contrast, the MEK inhibitor (20 μM PD98059, **Fig. 3E**, before and after PDGF stimulation) or DMSO (control, **Fig. 3C**) treatment could inhibit TCL1 reporter activity in a PDGF-TCL1 reporter experiment. The results together suggested the notion that TCL1 transactivity could be regulated primarily by Akt-induced phosphorylation of Nur77.

Activation of Akt induces Nur77 phosphorylation, in turn, decreases endogenous Nur77 binding with TCL1-NBRE.

We next elucidated endogenous Nur77 could bind to DNA complex with TCL1-NBRE and be functional in the regulation of TCL1 transactivity. PC12 cells (rat pheochrmocytoma cells) were chosen, since Nur77/NGFI was originally identified from PC12 cells (20), and NBRE within the TCL1 promoter sequences was well conserved in human and rattus (Fig. 1C). Using PC12 cells, in which Nur77 was endogenously expressed, EMSA was performed to examine the formation of endogenous Nur77 protein with TCL1-NBRE.

First we confirmed that NGF stimulation induced Akt phosphorylation at Serine 473 (**Fig. 4A**, third row, before and after NGF stimulation), threonine 308 (data not shown), and Nur77 in PC12 cells (**Fig. 4A**, top row, before and after NGF stimulation).

EMSA experiment demonstrated that endogenous Nur77 formed DNA-protein complexes with TCL1-NBRE in a serum starved condition. However, after NGF stimulation, which activated Akt-induced Nur77 phosphorylation (Fig. 4A), resulted in diminishing the formation of DNA-protein complexes of endogenous Nur77 with TCL1-NBRE (**Fig. 4B**, compare lane 7 vs. lane 8,

without vs. with NGF stimulation, respectively). Relative intensities of the formation of DNA-protein complexes were shown on the right side of the panel (3.8 vs. 1.0, before and after NGF stimulation, respectively). The results were consistent when authentic NBRE probes (AAAAGGTCA) that were used in an EMSA experiment (data not shown).

The NGFI-B subfamily constitutes with Nur77 (also called NGF-I-B or TR3), Nurr1 (Nur-related factor 1), and NOR-1 (Neuron-derived Orphan Receptor 1) (22-24). The NGFI-B family shares over 90 % homology in their DNA binding domain; hence, it is logical other isoforms of NGFI-B/Nur77 could also target TCL1-NBRE. Accordingly, in the presence of anti-Nur77, anti-NOR1, or anti-Nurr1 antibodies, DNA-protein complexes were dramatically diminished in their intensities, suggesting that endogenous NGFI-B subfamily transcription factors (Nur77, NOR1, and Nurr1) could specifically recognize and bind to TCL1-NBRE probes (**Fig. 4B**, lane 2-5, control, anti-Nur77, anti-NOR1, or anti-Nurr1 antibodies, respectively). The results demonstrated that endogenous Nur77 and its subfamily proteins could bind to a DNA complex with TCL1-NBRE and be functional.

NGF stimulation decreases binding of Nur77 with TCL1-NBRE, and consequently, suppresses TCL1 gene expression.

Next we performed CHIP assays using PC12 cells to examine the physical interaction of endogenous Nur77 with TCL1-NBRE in a cellular environment. In PC12 cells, endogenous Nur77/NGFI-B physically interacted with TCL1-NBRE (**Fig. 5A**, lane 3), but this interaction could be abrogated after NGF stimulation **(Fig. 5A**, lane 1). In accord with EMSA (Fig. 4B), CHIP assays demonstrated that all three isoforms of the endogenous NGFI-B subfamily (Nur77, NOR1, or Nurr1) transcription factors could interact with TCL1-NBRE in serum starved PC 12 cells (**Fig. 5B**, lanes 1-3, Nur77, NOR1, or Nurr1, respectively).

Moreover, we attempted to clarify the functional relationship of the physical interaction of endogenous Nur77 with TCL1-NBRE in TCL1 transactivation. mRNA was isolated from PC12 cells (0, 1, and 4 hours after NGF-stimulation) for RT-PCR to quantitate TCL1 gene expression in the absence or presence of NGF stimulation, which is known to stimulate Akt. Consistent with luciferase reporter assays, in PC12 cells NGF stimulation decreased TCL1 expression detected by RT-PCR (**Fig. 5C**, lane 1 vs. 2, before vs. 4 hours after NGF stimulation, respectively) with G3PDH as an internal control (**Fig. 5C**, lane 4 vs. 5, before vs. 4 hours after NGF stimulation, respectively). Moreover, consistent with the inhibitory effect observed in RT-PCR, the protein levels of TCL1 expression also decreased after NGF stimulation in a time dependent manner (**Fig. 5D**, bottom row, 0, 10 min, 4 hrs, and 18 hrs after NGF stimulation, respectively), which correlated well with activated Akt (third row) and phosphorylated Nur77 (top row). The results demonstrated that NGF-induced activation of endogenous Nur77 by Akt, diminished Nur77 binding to TCL1-NBRE, in turn, suppressed endogenous TCL1 gene activation in PC12 cells. The results supported the notion that TCL1 transactivation is regulated via an Akt-Nur77-TCL1-NBRE negative feedback loop.

DISCUSSION

We have demonstrated that the protooncogene TCL1 physically interacts with Akt and functions as a co-activator of Akt, which enhances Akt kinase activity (4,5,10). Protooncogene TCL1 was originally identified from the chromosomal breakpoint in human T-cell prolymphocytic leukemia (T-PLL), a rare form of an adulthood chronic leukemia. In human T-PLL, the TCL1 gene is activated due to chromosomal translocations involving a T-cell receptor gene and either the 14q32.1 or the Xq28 regions (13,34).

In pathological conditions, TCL1 is over expressed in several human diseases including

Burkitt's lymphoma, ataxia-telangiectasia (A-T), and AIDs related lymphomas(11,14,35). In contrast, the physiological expression of TCL1 is tightly restricted to early developmental cells (14,17). The observation suggested that the TCL1 gene is regulated at the transcriptional level (36,37). To clarify as to how the TCL1 gene is regulated, we cloned 931 base pairs of the 5'-promoter of human TCL1 located at chromosome 14q32. We found that the 5'-TCL1 proximal promoter region contained a TATA box with cis-regulatory elements of NBRE (Nerve Growth Factor Responsive element, CCAAGGTCA, from -340 to -332), NFκB (GCCCCGCCCC, from -121 to -110), and FKHRL (Fork Head Transcription Factor, CAAAATAAA, from -857 to -850). Differential expression between TCL1 and TCL1b are consistent in that the TCL1b gene, located adjacent to the TCL1 oncogene on human chromosome 14q32, contains no TATA box and GC rich sequences with YY1 with c-myc, typical for a housekeeping gene. Recent studies clarified that Sp1 sites around the TCL1 transcriptional start site play a regulatory role for gene activation of the TCL1 oncogene (19). In contrast to TCL1 and TCL1b, physiological expression of MTCP1, the third member of the TCL1 family oncogene, is still unclear. However, a recent study suggested that SEB treatment induced MTCP gene expression in murine T cells *in vivo* (38).

It was striking that the proximal 5'-promoter of TCL1, an Akt kinase co-activator, bears NBRE, the cis-regulatory element of Nur77/NGFI-B (Nerve Growth Factor I-B) that is phosphorylated by Akt. The NGFI-B gene was originally identified by differential hybridization that is rapidly induced in PC12 cells by NGF, which is required for the development and survival of sympathetic and neural crest-derived sensory neurons (20,21). Importantly, subsequent *in vivo* studies demonstrated that Nur77, originally identified in neuronal cells, plays a pivotal role in the TCR activation that induced cell death in developmental thymocytes (21,25,26). It is also noteworthy that in some circumstances, Nur77 is shown to translocalize to mitochondria to regulate cytochrome *c* release for cell death (39). The presence of cis-regulatory elements of Akt substrate Nur77/NGFI-B within the proximal TCL1 promoter prompted us to hypothesize that TCL1 gene expression can be regulated through the Akt-Nur77-TCL1 regulatory loop.

Nur77/NGF1-B bears the DNA-binding domain and the region comprising the ligand-binding domain near the COOH-terminus end. The DNA-binding domain of Nur77 recognizes the NBRE element (NGFI-B response element, TTTTAAAAGGTCATGC)(33). Despite the importance of *in vivo* T cell apoptosis, direct molecular targets of Nur77/NGFI-B, which bind, and hence regulate cell death machinery, have not been identified. In literature, the only molecular target of Nur77/NGFI-B, though not relevant to cell death machinery, was found in steroid 21-hydroxylase promoter sequences. Steroid 21-hydroxylase is one of a group of related cytochrome p-450 enzymes that is required for steroid hormone biosynthesis (40,41). In addition, Fas-L, (42), or NDG 1, 2 were also suggested to be upregulated by overexpressing Nur77 (43); however, it is of note that these effecter molecules were not directly targeted and/or regulated by Nur77/NGFI-B.

In EMSA and transfected cells, we demonstrated that wild type Nur77, but not S350A Nur77 (28, 31), could bind to TCL1-NBRE. Consistently, in CHIP assays using 293 cells transfected with the TCL1-NBRE reporter construct of wild type Nur77, exogenous wild type Nur77, but not S350A mutant, could bind to TCL1-NBRE. The affinity of wild type Nur77 with TCL1 NBRE was fairly weak, as human TCL1-NBRE has first two nucleotide bases that were substituted from the core NBRE sequences (AAAAGGTCA to CCAAGGTCA) (33). Our preliminary observation suggested a dissociation constant of Nur77 with TCL1 NBRE as 5 μ M range (MN and MH unpublished observation).

Nur77/NGFI-B belongs to orphan nuclear receptor superfamily transcription factors with

three subfamily members (Nur77, NOR1, and Nurr1) (20,22,23). Over 90 % homology in their DNA binding domains were conserved among the Nur77/NGFI-B family (21,24). Based on the highly homologous DNA binding domain of Nur77/NGFI-B family members, it is logical that other Nur77/NGFI-B family members (NOR1 and Nurr1) could also bind to TCL1-NBRE. In CHIP assays and EMSA using PC12 cells, we showed that all three Nur77/NGFI-B subfamily transcription factors could interact with TCL1-NBRE. The result suggested that three isoforms of Nur77/NGFI-B subfamily transcription factors may differentially regulate TCL1 gene expression in various cellular environments *in vivo*.

Moreover, given the conserved binding motif among the orphan receptor super family proteins, it is possible that other orphan receptor superfamily transcription factors (other than the Nur77/NGFI-B subfamily) may also physically and/or functionally regulate TCL1 gene expression *in vivo*.

Functionally, TCL1-NBRE is required to inhibit TCL1 transactivation upon NGF/PDGF stimulation in luciferase reporter assays. Introduction of mutation of NBRE showed no inhibitory effect upon NGF/PDGF stimulation. The observation is consistent in that the non-phosphorylated form of Nur77 could bind to TCL1-NBRE, hence activating TCL1 transactivity. Upon PDGF/NGF stimulation, Nur77 was phosphorylated and it decreased binding to TCL1-NBRE, hence translocated to cytosole (27,28). Consistently, phosphorylation of Nur77 could inhibit TCL1 transactivity both in luciferase reporter assays and in CHIP assays. The results together supported the notion that TCL1-NBRE plays a key inhibitory role in TCL1 gene regulation *in vivo*.

In addition to NBRE, we localized a cis-regulatory element of FKHRL at the 5'-TCL1 promoter region (from -857 to -850). Recent investigation revealed that Akt induced phosphorylation of FKHRL (31). The presence of FKHRL upstream of human TCL1-NBRE strongly suggested that both FKHRL and Nur77, both of which were the substrate of Akt, could play a role in NGF-induced TCL1 gene expression *in vivo*. Consistent with previous reports, in luciferase reporter assays using TCL1-PGL3, increasing the amount of FKHRL inhibited TCL1 transactivation as a dose responsive manner. Moreover, we showed that the introduction of a FKHRL mutation modestly, but reproducibly enhanced TCL1 promoter activity in luciferase reporter assays. However, introduction of double mutation of both NBRE and FKHRL compromised the enhancement of TCL1 transactivity in luciferase reporter assays. It is of note that in contrast to the NBRE mutation, introduction of FKHRL mutation within -931 TCL1 promoters retained inhibition after NGF stimulation in PC12 cells. The results suggested that both Nur77 and FKHRL, both of which are substrates for Akt, coordinately, but differentially, regulated TCL1 gene expression *in vivo*. Since Akt interacts with and phosphorylates over ten intracellular signaling molecules (3), it is likely that additional regulatory mechanisms may also play a role in TCL1 gene regulation, which negatively regulates TCL1 gene expression upon NGF (or PDGF) stimulation *in vivo*.

EMSA using either endogenous Nur77 (derived from PC12 cells) or exogenous Nur77 (overexpression experiment in 293 cells) showed binding ability to TCL1-NBRE. We demonstrated that endogenous Nur77 could bind to NBRE only in a serum starved condition, but dissociated after NGF stimulation, which is known to activate Akt kinase. It is reported that phosphorylation of Nur77 by MAP family kinase induces translocation to the cytosole from nucleus, which was correlated with diminishing Nur77 binding activity in PC12 cells (44,45). Therefore, other kinases might also phosphorylate other serine threonine residues of Nur77, which, in turn, suppresses TCL1 transactivation in a negative feedback manner.

The present studies together establish that the TCL1 gene, an Akt kinase co-activator which interacts with Akt and enhances its kinase activity, is a novel functional target of

Nur77/NGFI-B that regulates cell death-survival machinery. TCL1-NBRE regulates TCL1 gene expression through the Akt-Nur77/NGFIB-TCL1 regulatory loop. In this scenario, TCL1 induces Akt phosphorylation, inhibits interaction of endogenous Nur77 with NBRE, and in turn, suppresses TCL1 gene expression *in vivo*. Given the role of Nur77 in thymic developmental stages and the restricted expression of TCL1 in early development, this novel regulatory mechanism may play a role in early embryogenesis and/or early immunological development *in vivo.*

Acknowledgement

We thank A. Toker (BIDMC) for PC12 cells, K. Imada (Kyoto Univ.) for 293T cells, Y. Gotoh (Tokyo Univ.) for Nur77 expression vectors and antibody, Milbrant (Washington Univ.) for Nur77 cDNA , H. Shida (Hokkaido Univ.) for rat genomic DNA, J. Tanaka and M. Imamura (Hokkaido Univ.) for K562 cells, M-H Stern (Curie) for SupT 11 cells, Y. Nakatani (Harvard Medical School), P. Farnham (Univ. Wisconsin), K. Ozato (NIH) for their technical suggestions, and S. Shibata for her secretary assistance.

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FIGURE LEGENDS

Figure 1. Structure and Nucleotide Sequences of the Human TCL1 Promoter Region

A. Schematic presentation of the 5' TCL1 promoter region. The predicted transcription start site was indicated as +1 (19). Putative transcriptional regulatory elements present in the TCL1 promoter region are shown {FKHRL (Fork Head Transcription Factor), NBRE (Nerve Growth Factor Responsive Element), MyoD, IRF (Insulin Responsive Element), NFκB, Sp1, or TATA box}. The positions of primer pairs to generate short version of luciferase constructs are underlined {PGL3-TCL1(-470) forward primer: 5'-ATCATCGAGCTCCAGGCTGGAGC TGGTTTCCATG-3', reverse primer: 5'-ATCATCAGATCTCGTCCAAATACACGAAC TTCTC CC-3'}. Please note that NBRE and FKHRL which bind and are phosphorylated by Akt (28, 29, 32) are present within 931 base pairs of the 5'-proximal promoter of TCL1, an Akt kinase co-activator, which physically interacts and activates Akt.

B. A nucleotide sequence alignment of the TCL1 5'-proximal promoter is presented with cis-regulatory elements of FKHRL (from -857 to -850), Nur77/NGFI-B (from -340 to -332), MyoD (from -256 to -248), IRF1/IRF2 (from -176 to -164) NFκB (from -122 to -113), Sp1 (from -86 to -83, -67 to -64, +40 to +43, and +53 to +56), or TATA box (from -30 to -26) (boxed). Promoter analysis was performed by TFSEARCH (http://cbrc.jp/htbn/nph-tsearch). Numbers were nucleotide distances from the predicted transcription start site (19).

C. Comparison of the nucleotide sequences of an authentic NBRE consensus sequence

{AAAAGGTCA, (19, 20)} vs. a human TCL1-NBRE sequence (CCAAGGTCA, from -396 to -388), mouse (ACCTGGTCA, from -340 to -332), and rat (ACGAGGTCA, from -402 to -394) present within the proximal TCL1 promoter sequences were shown. Numbers were nucleotide distances from ATG (translation start site). Numbers in parenthesis were from the predicted transcription start site (19).

Figure 2. In EMSA assays, wild type Nur77 could bind to the NBRE site of the TCL1 proximal promoter region.

A. Wild type Nur77, but not S350A Nur77 {serine residue at amino acid 350 of Nur77, was replaced by Alanine (28)}, could bind to TCL1-NBRE (CCAAGGTCA) in EMSA assays. An equal amount of wild type or S350A Nur77 were expressed in this experiment as determined by immunoblotting (lower panels).

B. 293 cells (ATCC) were transfected with indicated expression vectors, serum starved, then stimulated with (or without) PDGF. In EMSA using wild type TCL1-NBRE probes, wild type Nur77, but not S350A Nur77 could form DNA-protein complex with TCL1-NBRE in a serum starved condition (indicated by the right arrow on the top panel). The formation of a DNA-protein complex reduced its intensities after PDGF stimulation (upper panel, compare lane 2 vs. lane 4, before and after PDGF stimulation, respectively). Upon PDGF stimulation, phosphorylation of Akt (lower panel, top row, lanes 2 and 4) and Nur77 (lower panel, third row, lanes 2 and 4) were induced. An equal amount of expression of wild type Nur77 or S350A were shown by immunodetection (lower panel, bottom row, lanes 1-4).

C. A CHIP assay using 293 cells transfected with wild type Nur77 (pCS-Nur77-WT) and wild type TCL1-NBRE (pGL3-TCL1-Wild type) resulted in specific band which indicated that wild type Nur77 could bind to a wild type TCL1-NBRE element (top panel, lane 2). However, wild type Nur77 did not interact with mutant NBRE (pGL3-TCL1-mt-NBRE) (top panel, lane 3). Similarly, no interactions were observed from double transfected cells of S350A Nur77 with wild type Nur77 (top panel, lane 4), or S350A Nur77 with NBRE mutant (top panel, lane 5). The right arrow indicated the position of 210 base pair PCR products of TCL1-NBRE from the TCL1 promoter region (5'-GAAAGGGCCAAGGTCACCCCGGTGC-3', position from -340 to -332 of the TCL1 5'-promoter). Equal amounts of NBRE expression were detected by PCR from the cellular extracts and visualized on 1.5% agarose gel (middle panel, lanes 1-5). Wild type and mutant Nur77 were equally expressed by Western blot analysis (bottom panel, lanes 2 and 3 vs. 4 and 5, wild type Nur77 vs. S350A Nur77, respectively).

D. In EMSA using recombinant Nur77, increasing the amount of cold probes dramatically reduced the formation of DNA-protein complex, suggesting specific interactions of Nur77 with TCL1-NBRE (lane 1; probe only, lane 2; positive DNA protein complex, lanes 3 and 4; in the presence of 500 and 1000 fold molar excess of cold competitor, respectively). The right arrow indicated a specific DNA-Protein complex. An equal amount of recombinant protein was used in each lane shown by commassie blue staining of the samples (lower panel).

E. The DNA-protein complexes were super-shifted in the presence of anti-GST antibody in a dose escalation manner (0, 1, 2.5, 5 μg of GST antibody, lanes 3-6), suggesting that Nur77 could specifically recognize and bind to TCL1-NBRE (AAGGTCA). GST protein alone did not form DNA-protein complex with TCL1-NBRE (lane7), hence no supershift bands were observed (lane 8).

Figure 3. TCL1-NBRE is a negative regulatory element of TCL1 transactivation in luciferase reporter assays.

A. TCL1 transactivity was measured in PC12 cells in the presence or absence of NGF stimulation. Wild type TCL1 promoter (PGL3-TCL1) showed over 100 fold of luciferase reporter activity compared to control vector (PGL3) (1st and 2nd sets of the panels, respectively). NGF stimulation could dramatically inhibit TCL1- reporter activity (PGL3-TCL, 2nd set of the panel, open vs. filled bar, unstimulated vs. NGF stimulated, respectively). In contrast, an introduction of mutation of TCL1-NBRE (pGL3-mt-NBRE) showed no inhibition after NGF stimulation (3rd set of the panel, open vs. filled bar, unstimulated vs. NGF stimulated, respectively). Mutation of FKHRL showed a modest increase in TCL1 transactivation, but retained the inhibition of TCL1 transactivity after NGF stimulation (pGL3-mt-FKHRL, fourth set of the panel, open vs. filled bar, unstimulated vs. stimulated with NGF, respectively). Double mutation of both NBRE and FKHRL showed no inhibition after NGF stimulation (pGL3-mt-FKHRL-mt-NBRE, 4th sets of the panel). FKHRL, NBRE (Nur77), NFκB, or the TATA binding region present in the wild type TCL1 promoter were boxed. X indicated that mutations of indicated cis-elements were introduced in each luciferase reporter construct (see Experimental Procedure). Relative luciferase reporter activities of indicated constructs were shown on the right side measured by a dual luciferase reporter system with triplicate measurement (Promega).

B. Luciferase activities (before and after PDGF stimulation) using 293 cells were measured by a dual luciferase reporter system (Promega) using pGL3 luciferase constructs shown on the left side. Wild type TCL1 promoter activity (PGL3-TCL1-470) was dramatically reduced after PDGF stimulation (top panel, open vs. filled bar, unstimulated vs. PDGF stimulated, respectively). However, introduction of mutation of TCL1-NBRE (pGL3-mt-NBRE-470) compromised the inhibitory effect on TCL1 transactivities after PDGF stimulation (lower panel). Results were consistent in other sets of independent experiments.

C. Statistical analysis was performed by student t-test and the p value < 0.5 was considered as statistically significant.

D-E. Wortmannin (PI3K inhibitor, panel **D,** before and after PDGF stimulation), but not PD98059 (MEK inhibitor, panel **E**) or DMSO (control, panel **C**) treatment, compromised PDGF-induced inhibition of TCL1 transactivity in luciferase reporter assays using PGL3-TCL1-470. The results suggested that inhibition of PGL3-TCL1 reporter activity upon PDGF stimulation was primarily mediated through the PI3K-Akt activation pathway.

Figure 4. NGF stimulation induced phosphorylation of Akt and Nur77, and consequently, inhibited Nur77 binding to TCL1-NBRE.

A. NGF stimulation on PC 12 cells increased Akt kinase activity (pSer473, third row, compare lane 1 and 2 before and after NGF stimulation, respectively) and phosphorylation of endogenous Nur77 (top row, compare lane 1 and 2, before and after NGF stimulation, respectively) as previously reported. Shown on the right are relative intensities of Nur77 phosphorylation by imageJ (ver. 1.34) (1.0 vs. 3.8, before and after NGF stimulation, respectively) (top right panel). Relative intensities of phospho-Ser 473Akt were 1.0 vs. 4.8 before and after NGF stimulation, respectively (lower right panel).

B. In EMSA, cell lysate from serum starved PC12 cells could form DNA-protein complexes with TCL1-NBRE probes. In the presence of anti-Nur77, anti-NOR1, or anti-Nurr1 antibodies, DNA-protein complexes formation diminished their intensities (compare lane 2-5, control antibody, anti-Nur77, anti-NOR1, or anti-Nurr1 antibodies, respectively). NGF stimulation, which activates Akt and its substrate Nur77 (see Fig. 4A), could inhibit Nur77 binding to TCL1-NBRE (compare lanes 7 vs. 8, before vs. after NGF stimulation, respectively). Relative intensities of the binding of TCL1 NBRE (area pixels) were shown on the right panel (compare lanes 7 vs. 8, 3.8 vs. 1.0, before and after NGF stimulation, respectively). Quantitation of the signals (area pixel) were performed using ImageJ (ver. 1.34S) software and the numbers (fold increase compared to the control) are shown on the right side.

Figure 5. NGF stimulation inhibited Nur77 binding to TCL1-NBRE, in turn, suppressed TCL1 gene expression.

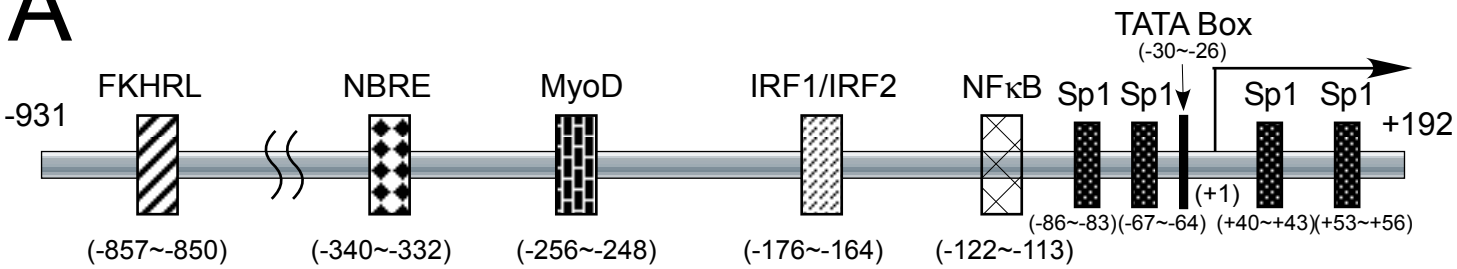
A. CHIP assays in PC12 cells (rat pheochromocytoma cells that express endogenous Nur77) demonstrated that endogenous Nur77 interacted with NBRE of TCL1 promoter sequences in serum starved cells (lane 3). Moreover, NGF stimulation of PC12 cells, which presumably activates Akt and Nur77 (see panel D) abrogated the interaction of Nur77 with NBRE-TCL1 (lane 1).

B. Three subfamilies of NGFI-B nuclear receptor are known (Nur77, NOR1, and Nurr1). In CHIP assays, using specific antibodies against Nur77, NOR1, Nurr1, or control, all three subfamilies of NGFI-B nuclear receptor (Nur77, NOR1, Nurr1, or control), could interact with TCL1-NBRE in serum starved PC12 cells (lanes 1-3, Nur77, NOR1, or Nurr1, respectively).

C. mRNA was isolated for RT-PCR to quantitate the endogenous expression of TCL1 with G3PDH as an internal control. NGF stimulation inhibited TCL1 mRNA expression in PC12 cells (lanes 1 and 2, before and after NGF stimulation, respectively).

D. To examine the effect on protein levels, PC 12 cells were used for immunodetection by anti Akt (fourth panel), anti-phospho-Ser473 Akt (third row), or anti-phospho antibody (top row) after immunoprecipitated with anti-Nur77 antibody (Santa Cruz) at indicated time points after NGF stimulation. NGF stimulation induced activation of Nur77 (top row), and phosphor-Ser 473Akt (third row), which inversely correlated with TCL1 protein expression detected by the TCL1 antibody as a time dependent manner (bottom row, 0, 10 min., 4hr., and 18 hr. after NGF stimulation, respectively)

A



B

-931 CAGGCTGGAG CTGGTTTCAC ATGTTTTTCA TTGTGCATGA CTTTCTTAAT TGCCTTAGTG

-871 GGAATGCGTT ACTGCAAAAT AAAGTGAAAT TGTTTTCCTT ATTGCTTGTA ATTTACAGAG

FKHRL

-811 GGGTGGTTTT GATCATATAA ACCAGGCACT TTTCAAATCC ATTGATCTTA GGGTGGAAGG

-751 AGGGATTTCT TTTTAAGTAT TCATTTCGAA CTATGCTTTC ACTAGTAAAC TCAAAGCCCA

-691 AGGGCAAAAA CCCATGATTC TCCATTTTGT AATAGCTATT AGATGTCCAC AGCACATCAG

-631 ACCTGTGGAA AACGCATGCT TTGTGTATGG AAGAGGGAGA GGGAGCGTCA GGAGTTGTCC

-571 TTTGCCAAAA AACGTACTCT GACACTCAAA GGCACAGGCT GGTGGAGATC CAGGGAACCC

-511 GGGAAAGCCC AGGCCTAACT CAGGCCCGCG CGGGTCTCTT CTGATCCCAT AAGATGAGGA

Forward primer

-451 GTGGGTCCTG GCTCTGGGCA TCCTGGTGTC GACTGTGAGT TCCCAGCAGA GGCCCAGAGT

-391 CCCGGTCCGG CAGCCGAGGG AAGCGGGGGG GTCTTCCAGA AGAAGAAAGG GCCAAGGTCA

NBRE

-331 CCCCGGTGCC TCTCCAGCAG CAGCAGAGGG CGGCGGTCGG TGTCGCTGCT GGCCGGGGCC

-271 TCGAGGAAGG CGCGGCCAG CTGGGGCCGG GTCTGCGTTC CCAGGAGCTG CCACCGTTCC

MyoD

-211 AGGGAGCAAG TCAGGCCGGG ACGTAGCGCC TGCGCGGGAC CCTCACTTGC CACCAAGACC

IRF1/IRF2

-151 CCCACAAACC CCGCCCCATC CTGCCTTACG CCCCGCCCCA AGGTCGTTCT CCCGACCCGG

SP1 NFκB

-91 GGTCCCGCCC CAAGGCCGTC CTCCCGCCCC GCCGCTTGGT GGCGGCCGCA TGCTGCCCGG

SP1 SP1

-31 ATATAAAGGG TCGGCCCCAC ATCCCAGGGA CCAGCGAGCG GCCTTGAGAG GCTCTGGCTC

TATA +1

+30 TTGCTTCTTA GGCGGCCCGA GGACGCCATG GCCGAGTGCC CGACACTCGG GGAGGCAGTC

SP1 SP1

+90 ACCGACCACC CGGACCGATG GCCGAGTGCC CGACACTCGG GGAGGCAGTC ACCGACCACC

+150 CGGACCGCCT GTGGGCCTGG GAGAAGTTCG TGTATTTGGA CGA

Reverse primer

C

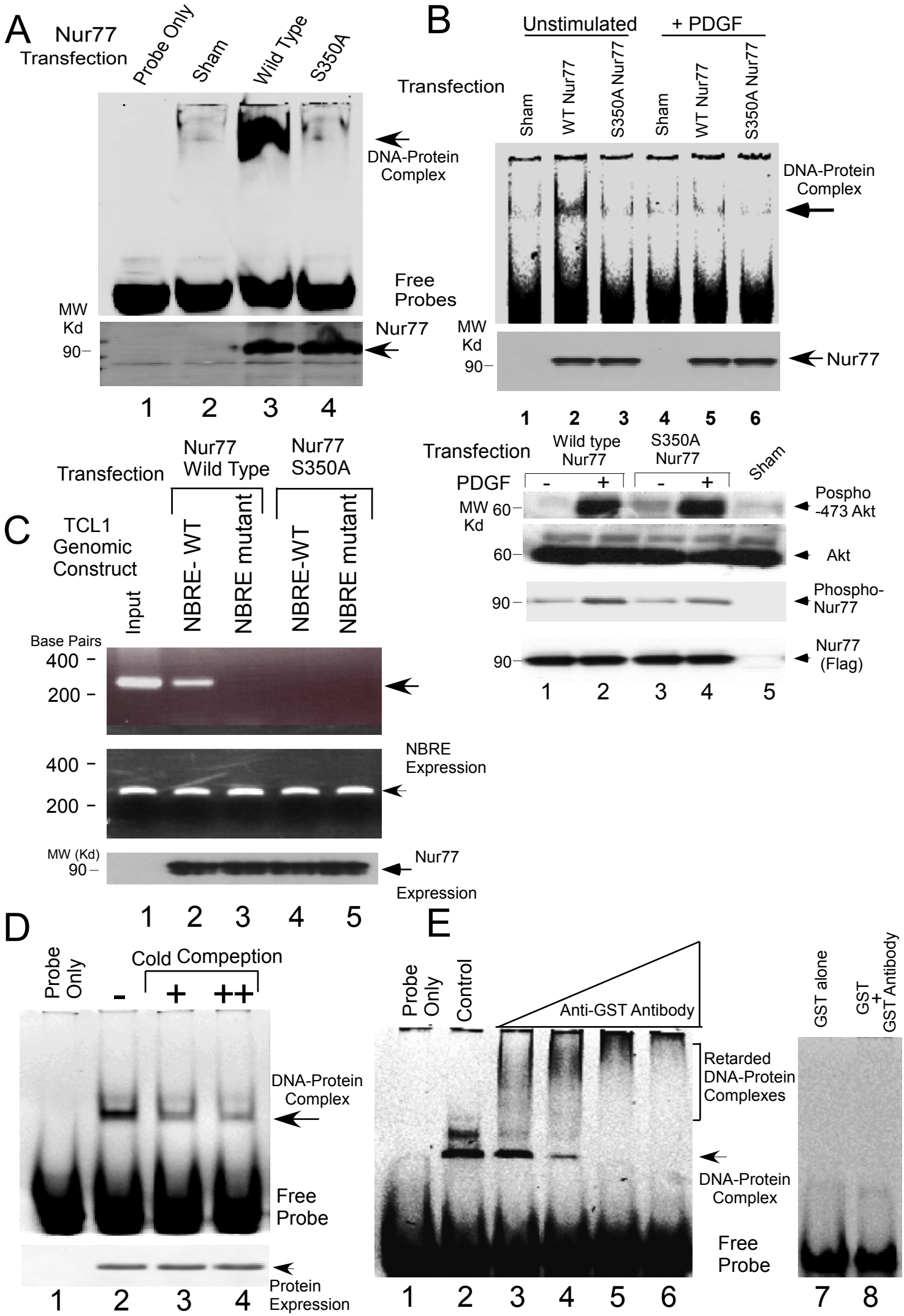
NBRE Consensus Sequence **AAAAGGTCA**

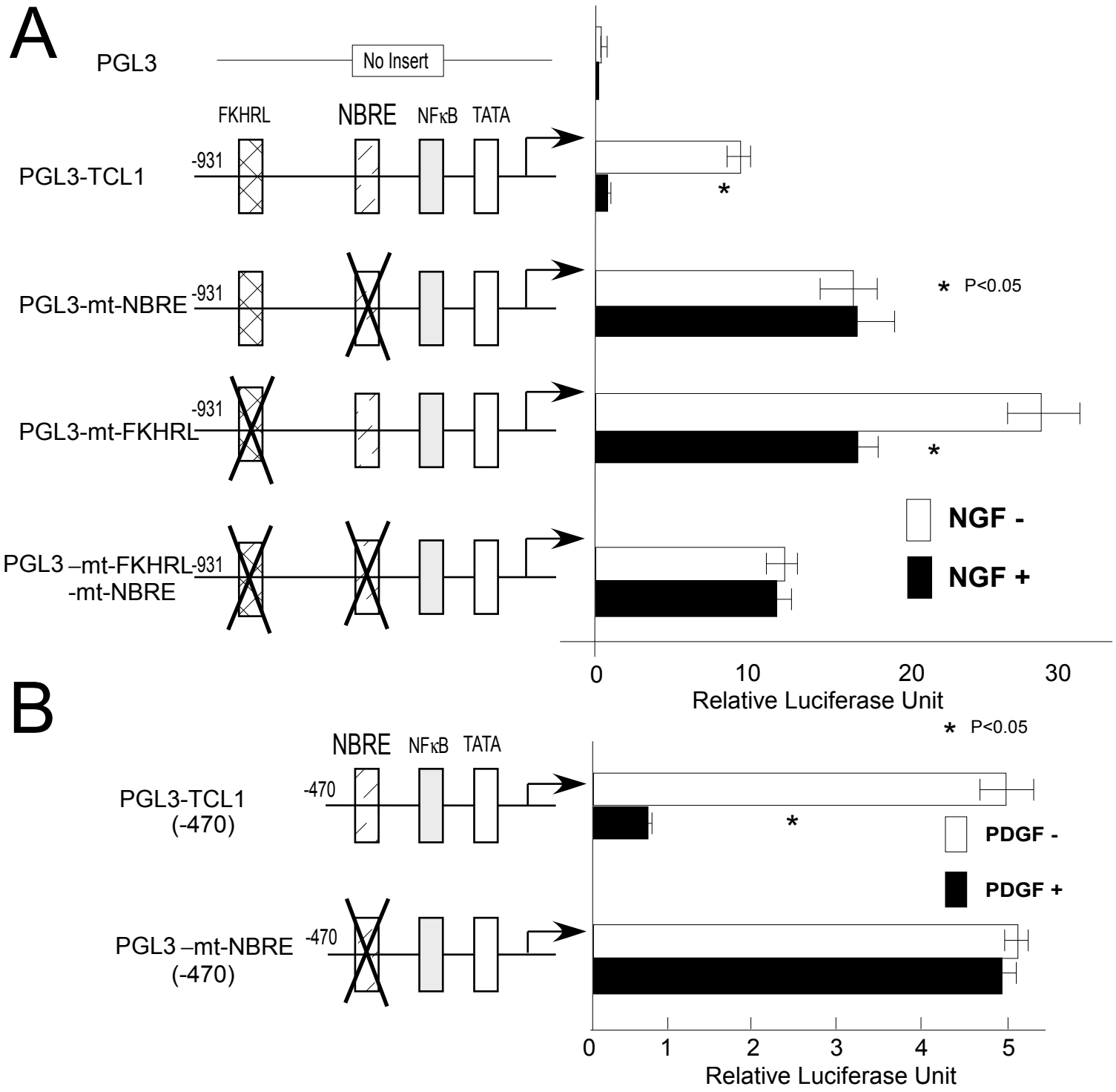
Human TCL1 NBRE sequence -396 CC**AAGGTCA** -388 (-340) (-332)

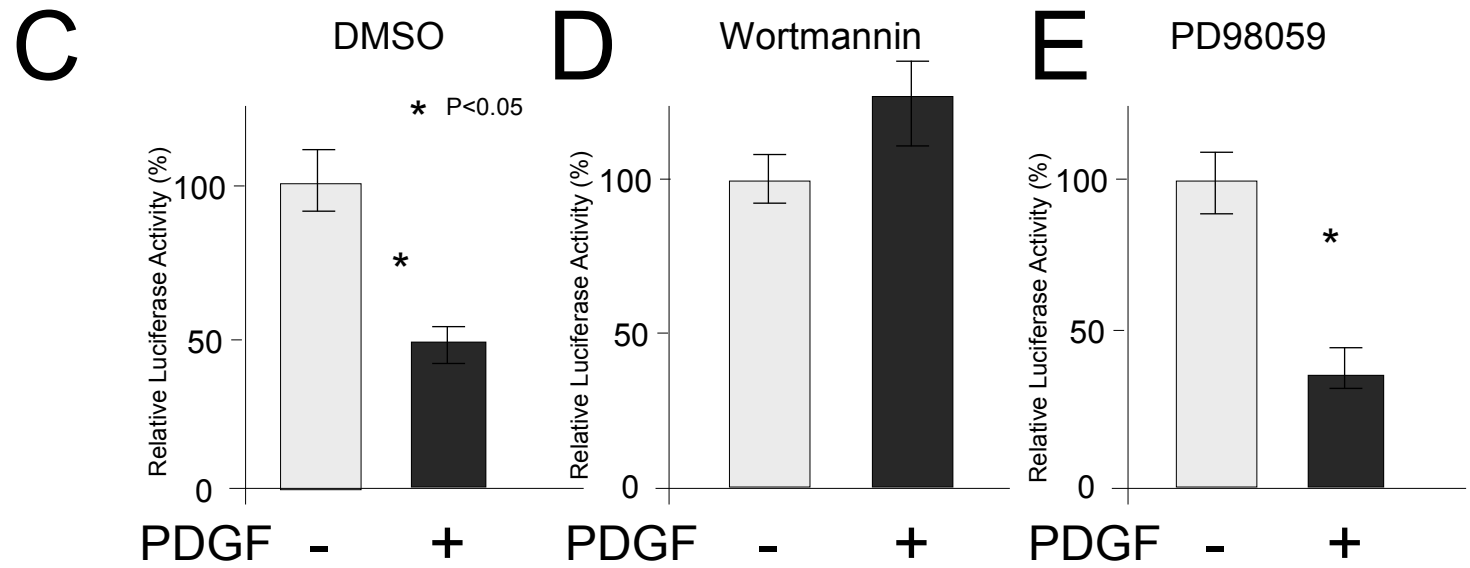
Mouse TCL1 NBRE sequence -336 **A**CC**TGGTCA** -328

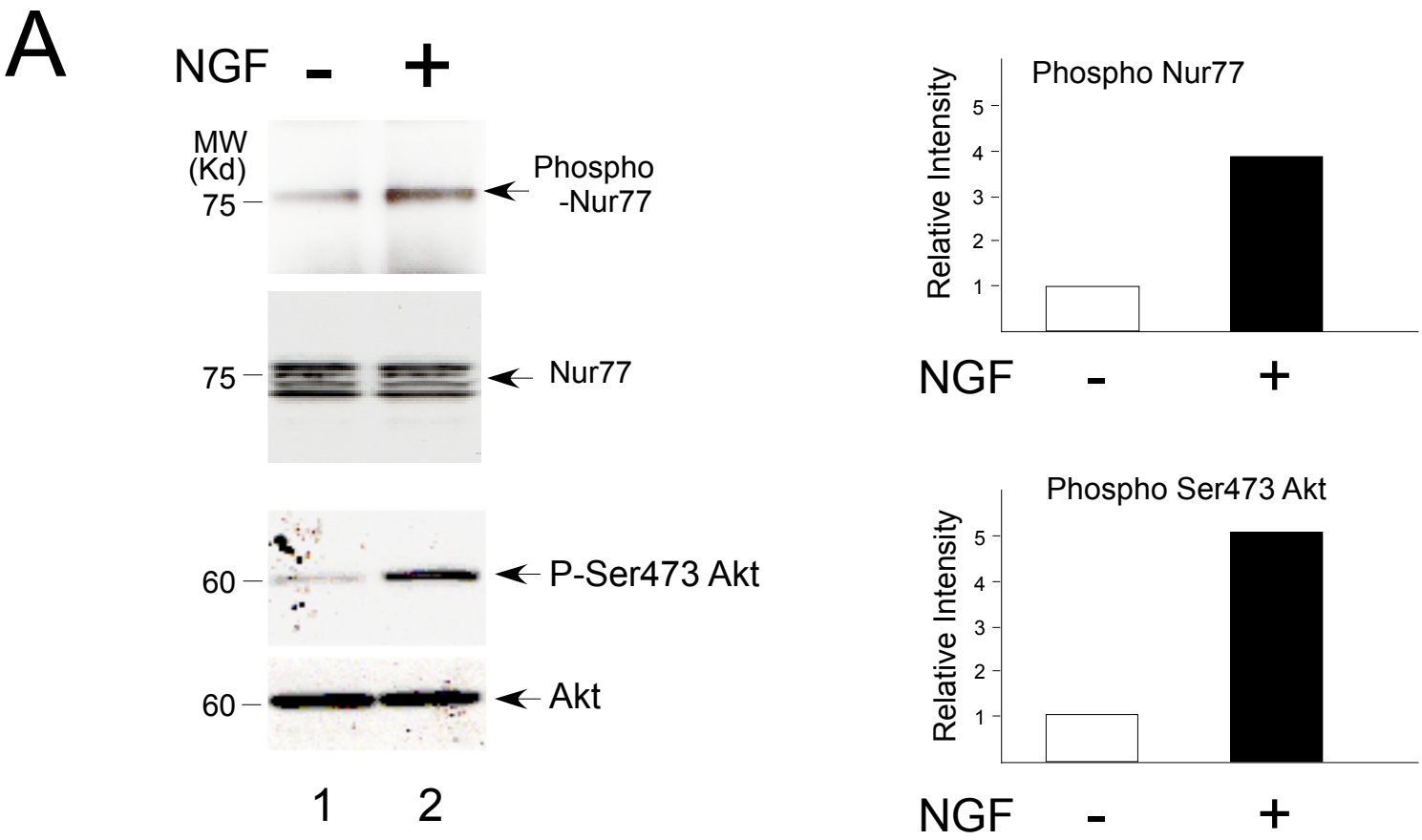
Rattus TCL1 NBRE sequence -402 **A**CG**AGGTCA** -394

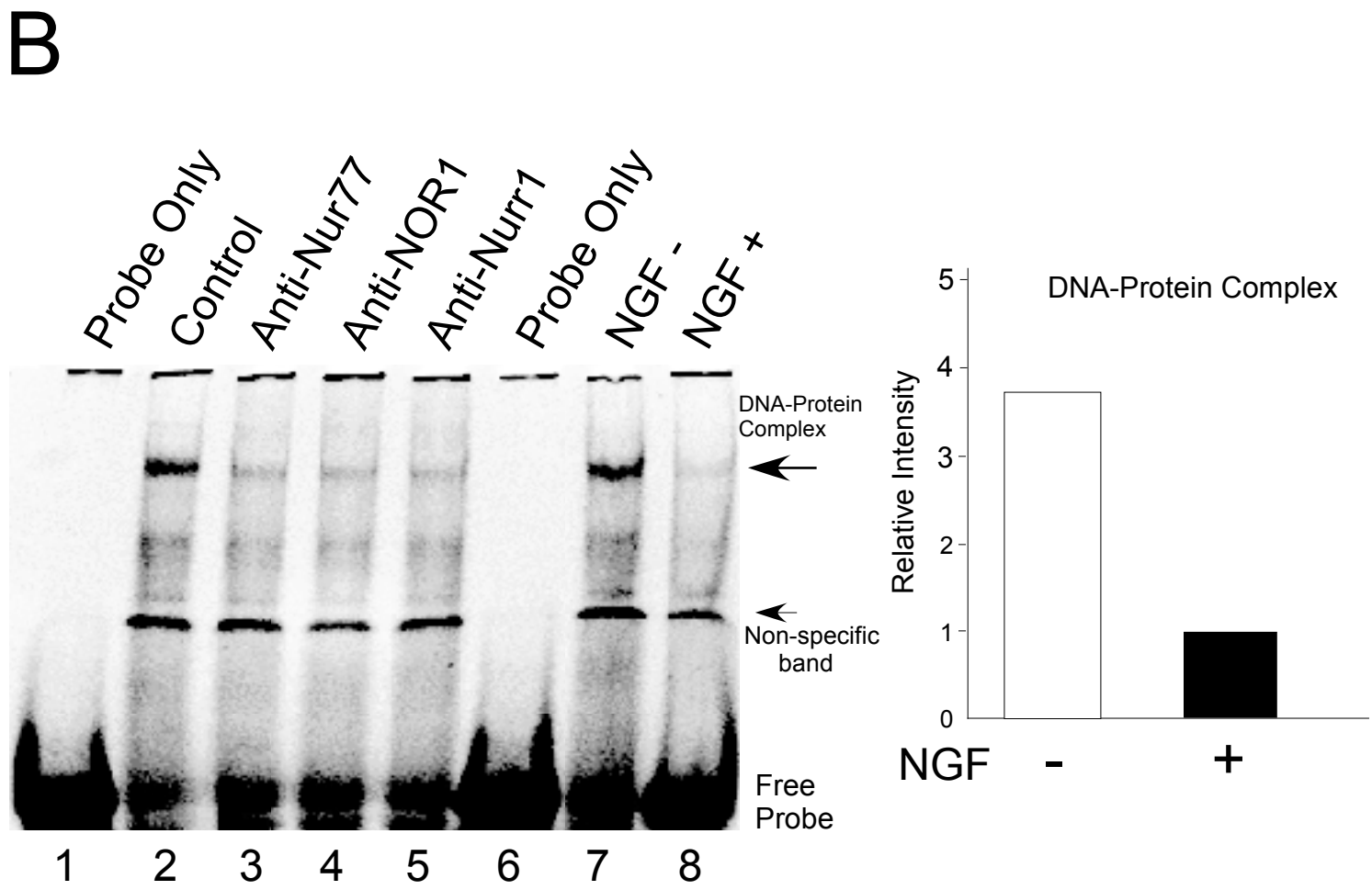
NBRE (NGFI/Nur77) Binding Responsive Element)

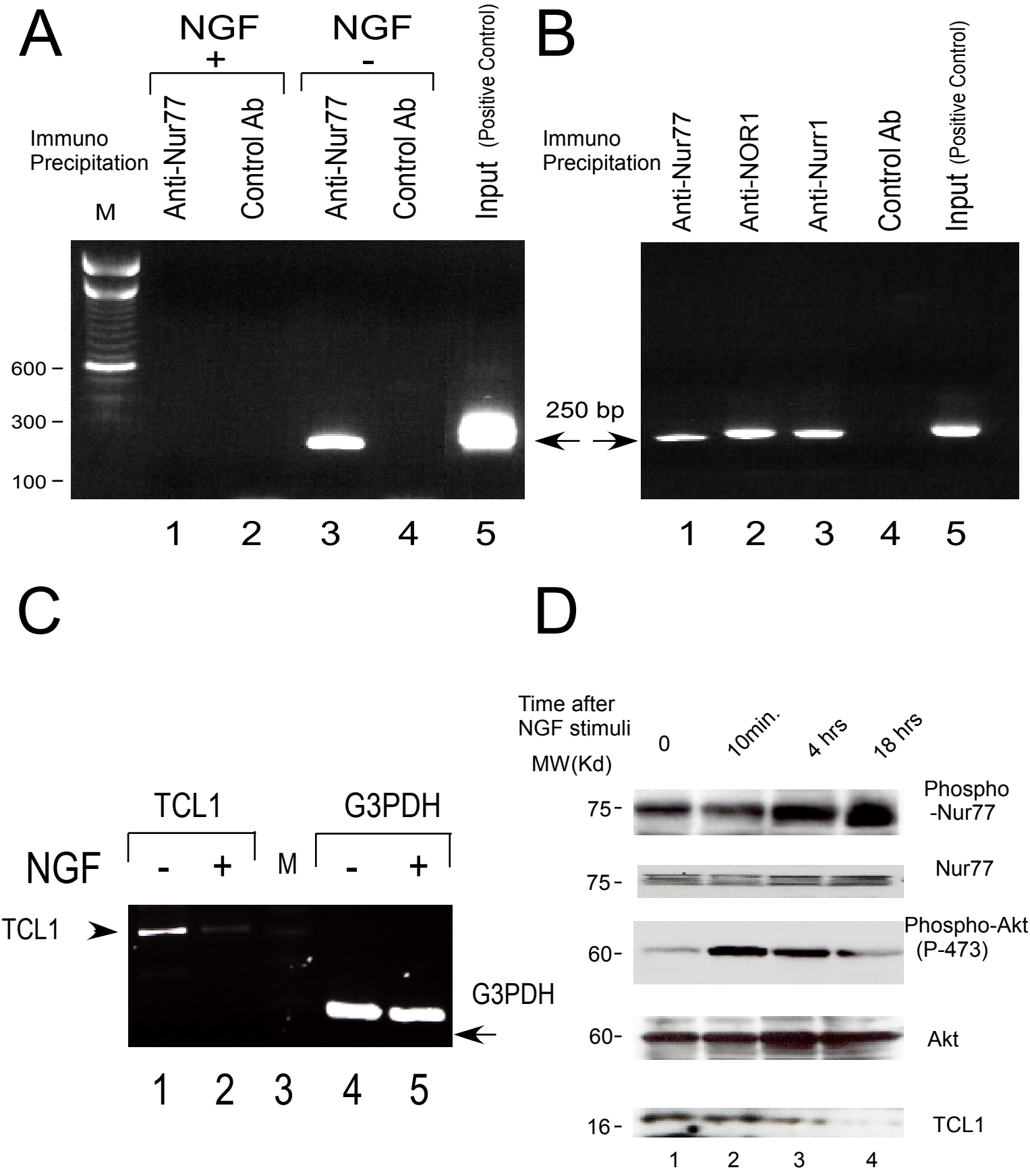

A
Nur77
Transfection
Probe Only
Sham
Wild Type
S350A
DNA-Protein Complex
Free Probes
MW Kd
90
Nur77
1 2 3 4
B
Unstimulated
+ PDGF
Transfection
Sham
WT Nur77
S350A Nur77
Sham
WT Nur77
S350A Nur77
DNA-Protein Complex
MW Kd
90
Nur77
1 2 3 4 5 6
C
TCL1 Genomic Construct
Transfection
Nur77 Wild Type
Nur77 S350A
Input
NBRE- WT
NBRE mutant
NBRE-WT
NBRE mutant
Base Pairs
400
200
NBRE Expression
MW (Kd)
90
Nur77 Expression
1 2 3 4 5
Transfection
Wild type Nur77
S350A Nur77
Sham
PDGF
- + - +
MW Kd
60
Pospho -473 Akt
Akt
Phospho-Nur77
Nur77 (Flag)
1 2 3 4 5
D
Probe Only
Cold Competion
-
+
++
DNA-Protein Complex
Free Probe
Protein Expression
1 2 3 4
E
Probe Only
Control
Anti-GST Antibody
Retarded DNA-Protein Complexes
DNA-Protein Complex
Free Probe
1 2 3 4 5 6
GST alone
GST + GST Antibody
7 8


A
PGL3
No Insert
FKHRL
NBRE
NFκB
TATA
PGL3-TCL1
-931
PGL3-mt-NBRE
PGL3-mt-FKHRL
PGL3 –mt-FKHRL
-mt-NBRE
* P<0.05
NGF -
NGF +
0
10
20
30
Relative Luciferase Unit
B
* P<0.05
PGL3-TCL1
(-470)
-470
PGL3 –mt-NBRE
(-470)
PDGF -
PDGF +
0
1
2
3
4
5
Relative Luciferase Unit


C
DMSO
* P<0.05
D
Wortmannin
E
PD98059
Relative Luciferase Activity (%)
100
50
0
PDGF - +


A
NGF - +
MW (Kd)
75
Phospho -Nur77
75
Nur77
60
P-Ser473 Akt
60
Akt
1 2
Phospho Nur77
Relative Intensity
5
4
3
2
1
NGF - +
Phospho Ser473 Akt
Relative Intensity
5
4
3
2
1
NGF - +


B
Probe Only
Control
Anti-Nur77
Anti-NOR1
Anti-Nurr1
Probe Only
NGF -
NGF +
DNA-Protein Complex
Non-specific band
Free Probe
1 2 3 4 5 6 7 8
DNA-Protein Complex
Relative Intensity
5
4
3
2
1
0
NGF - +


A
NGF
+
NGF
-
Immuno
Precipitation
M
Anti-Nur77
Control Ab
Anti-Nur77
Control Ab
Input (Positive Control)
600
300
100
1 2 3 4 5
B
Immuno
Precipitation
Anti-Nur77
Anti-NOR1
Anti-Nurr1
Control Ab
Input (Positive Control)
250 bp
1 2 3 4 5
C
TCL1
G3PDH
NGF
- + M - +
TCL1
G3PDH
1 2 3 4 5
D
Time after
NGF stimuli
MW(Kd)
0
10min.
4 hrs
18 hrs
75
Phospho
-Nur77
75
Nur77
60
Phospho-Akt
(P-473)
60
Akt
16
TCL1
1 2 3 4