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The proapoptotic *dp5* gene is a direct target of the MLK-JNK-c-Jun pathway in sympathetic neurons

Emily Towers, Jonathan Gilley, Rebecca Randall, Rosie Hughes, Mark Kristiansen and Jonathan Ham*

Molecular Haematology and Cancer Biology Unit, Camelia Botnar Laboratories, UCL Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK

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ABSTRACT

The death of sympathetic neurons after nerve growth factor (NGF) withdrawal requires *de novo* gene expression. *Dp5* was one of the first NGF withdrawal-induced genes to be identified and it encodes a proapoptotic BH3-only member of the Bcl-2 family. To study how *dp5* transcription is regulated by NGF withdrawal we cloned the regulatory regions of the rat *dp5* gene and constructed a series of *dp5*-luciferase reporter plasmids. In microinjection experiments with sympathetic neurons we found that three regions of *dp5* contribute to its induction after NGF withdrawal: the promoter, a conserved region in the single intron, and sequences in the 3′ untranslated region of the *dp5* mRNA. A construct containing all three regions is efficiently activated by NGF withdrawal and, like the endogenous *dp5*, its induction requires mixed-lineage kinase (MLK) and c-Jun N-terminal kinase (JNK) activity. JNKs phosphorylate the AP-1 transcription factor c-Jun, and thereby increase its activity. We identified a conserved ATF site in the *dp5* promoter that binds c-Jun and ATF2, which is critical for *dp5* promoter induction after NGF withdrawal. These results suggest that part of the mechanism by which the MLK-JNK-c-Jun pathway promotes neuronal apoptosis is by activating the transcription of the *dp5* gene.

INTRODUCTION

Apoptosis occurs extensively during the normal development of the mammalian nervous system, and is important for establishing neuronal populations of the correct size and for eliminating neurons that have made inappropriate connections (1,2). Developing sympathetic neurons depend on nerve growth factor, synthesized by their target tissues, for survival. In the absence of nerve growth factor (NGF), these cells die by apoptosis and their death requires *de novo* gene expression (3). Sympathetic neurons have been widely used for *in vitro* studies of the molecular mechanisms of neuronal apoptosis and a considerable amount has been learned about the signalling pathways that regulate the cell death programme (4,5). Following NGF withdrawal, the stress-responsive mixed-lineage kinase (MLK) and c-Jun N-terminal kinase (JNK) protein kinase cascade is activated and JNKs phosphorylate the AP-1 transcription factor c-Jun, which increases c-Jun activity and c-Jun expression (6–10). The MLK-JNK-c-Jun pathway is required for normal NGF withdrawal-induced death and promotes the release of mitochondrial cytochrome *c* and caspase activation (11–15).

The release of cytochrome *c* and other proapoptotic proteins from mitochondria is regulated by the Bcl-2 protein family (16). In sympathetic neurons, the multi-domain proapoptotic Bcl-2 family member Bax is essential for cytochrome *c* release and cell death after NGF deprivation (17). In contrast, the antiapoptotic proteins Bcl-2 and Bcl-x_L, which can form heterodimers with Bax, inhibit cytochrome *c* release and protect against NGF withdrawal-induced death (14,18–20). Finally, several proapoptotic BH3-only Bcl-2 family members are expressed in sympathetic neurons and three of these are regulated by NGF withdrawal: the *dp5*, *bim* and *puma* mRNAs and proteins increase in level after NGF deprivation, in all cases before the cell death commitment point (14,21–24). These BH3-only proteins may promote sympathetic neuron apoptosis by binding to the antiapoptotic members of the Bcl-2 family, which would then be unable to interact with Bax,

*To whom correspondence should be addressed. Tel: +44 20 7905 2294; Fax: +44 20 7813 8100; Email: j.ham@ich.ucl.ac.uk
Present addresses:
Emily Towers, Centre for Auditory Research, UCL Ear Institute, 332-336 Gray's Inn Road, London WC1X 8EE, UK
Jonathan Gilley, Laboratory of Neuronal Development and Survival, The Babraham Institute, Cambridge CB2 4AT, UK
Rebecca Randall, Developmental Signalling Laboratory, Cancer Research UK, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

or possibly by directly binding to and activating Bax (25).

The BH3-only proteins that increase in level after NGF withdrawal are downstream targets of the MLK-JNK-c-Jun pathway. Expression of a c-Jun dominant negative mutant (JunΔ169) or the *JunAA* knock-in mutation in mice, which eliminates the two major JNK phosphorylation sites in c-Jun, reduce the increase in *bim* RNA and protein levels after NGF withdrawal (14,24). In addition, the MLK inhibitor CEP-1347, which prevents JNK activation, also reduces the increase in *bim* and *dp5* mRNA levels after NGF deprivation (22,23). To understand in general how JNKs and AP-1 transcription factors promote neuronal apoptosis it is important to determine the molecular mechanisms by which these proteins regulate Bim and Dp5 expression and NGF-dependent sympathetic neurons have been a useful model for these studies (26,27). Here, we use a *dp5* reporter gene assay, expression vectors for JNK and AP-1 inhibitor proteins, specific chemical inhibitors, and site-directed mutagenesis to investigate how NGF withdrawal activates *dp5* transcription in sympathetic neurons. We show that an ATF-binding site in the *dp5* promoter, closely related in sequence to the jun1 and jun2 TRE elements in the *c-jun* promoter, can bind c-Jun and ATF2 *in vitro* and in chromatin, and is critical for *dp5* promoter activity in sympathetic neurons and for promoter induction following NGF withdrawal.

MATERIALS AND METHODS

5′ RACE and cloning of the *dp5* promoter

5′ rapid amplification of cDNA ends (RACE) was performed on rat brain mRNA using the Marathon™ cDNA amplification kit (Clontech Laboratories Inc.) with the *dp5*-specific primer 5′-CTGCAGCCGCAGCGC GGTCACC-3′. To isolate the *dp5* promoter, the 5′ RACE product was used as a probe to screen the rat P1 artifical chromosome (PAC) library RPCI31 (generated by P.Y. Woon and P. de Jong, UK Human Genome Mapping Project Resource Centre, Cambridge, UK). A 4-kb fragment from upstream of exon 1 was cloned and the remaining promoter sequence between the start codon and upstream 4 kb fragment was generated by PCR. These sequences were ligated at an EcoRI restriction site to generate a 4.5 kb fragment in pGEM®-T Easy (Promega UK Ltd, Southampton, UK).

Plasmid constructs

The *dp5* reporter construct *dp5*-LUC was generated by subcloning a 1017 bp fragment containing the *dp5* promoter sequence from −980 to +37, relative to the transcriptional start site, into pGL3-basic (Promega UK Ltd.), upstream of the luciferase gene. To obtain a reporter construct also containing part of the *dp5* intron (*dp5*-LUC + I 400), a 395 bp region of the intron that is highly conserved was subcloned into *dp5*-LUC downstream of the SV40 polyadenylation termination sequence. The construct *dp5*-LUC + 3′ UTR was made by subcloning three fragments (a 645 bp fragment containing the *dp5* stop codon, a 2.36 kb fragment and a 2.02 kb fragment) that spanned the entire *dp5* 3′ UTR into *dp5*-LUC downstream of the luciferase gene. *dp5*-LUC + ALL was generated by subcloning the 395 bp intron fragment into *dp5*-LUC + 3′ UTR. For all constructs, fragment orientation and positioning was confirmed by restriction enzyme analysis and DNA sequencing.

Point mutations within the ATF-binding site in the *dp5* promoter were introduced into *dp5*-LUC + ALL using the QuikChange™ II XL site-directed mutagenesis kit (Stratagene Europe, Amsterdam, The Netherlands) and the oligonucleotides 5′-CCCGGGCCGGATAACG TCTCCCCCTCCCCGCG-3′ and 5′-CGCGGGGAGGG GGAGACGTTATCCGGCCCGGG-3′ containing four point mutations. Incorporation of the correct mutations was confirmed by DNA sequencing.

The expression vectors for the JIP-1 JNK-binding domain (JBD) and c-Jun (ala) are described in (12) and (28).

Cell culture

Sympathetic neurons were prepared from the superior cervical ganglia (SCG) of 1-day-old Sprague Dawley rats (supplied by the Biological Services Unit, University College London). Neurons were isolated and cultured as described previously (29), in DMEM (Sigma-Aldrich, Poole, UK) containing 10% foetal calf serum, 2 mM glutamine (Invitrogen Ltd, Paisley, UK) and penicillin-streptomycin (SCG medium). Unless stated otherwise, SCG medium was supplemented immediately prior to use with 2.5 S NGF (Cedarlane Laboratories Ltd., Hornby, Ontario) at 50 ng/ml and the antimitotic agents fluorodeoxyuridine and uridine (both from Sigma-Aldrich) each at 20 μM. Sympathetic neurons were plated on 13 mm diameter glass coverslips coated with poly-L-lysine and laminin placed in 3.5 cm dishes. Cells were maintained in 2 ml of medium at 37°C in 10% CO_2 for 5–7 days before being used for experiments. In NGF withdrawal experiments, the growth medium was removed and the cells were gently rinsed twice with SCG medium lacking NGF and antimitotic agents. The neurons were then refed with fresh medium containing NGF or a neutralising anti-NGF antibody (Chemicon Europe Ltd, Chandlers Ford, UK) at 100 ng/ml. The MLK3 inhibitor CEP-11004 (provided by Cephalon, Inc., West Chester, PA) was dissolved in DMSO and used at a final concentration of 400 nM.

The PC6-3 subline of the PC12 cell line (30) was cultured in RPMI 1640 medium (Invitrogen Ltd.) supplemented with 10% horse serum, 5% FCS, 2 mM glutamine and penicillin/streptomycin. Cells were grown on collagen-coated tissue culture dishes at 37°C in 5% CO_2 and passaged once a week. For differentiation, cells were plated at a density of 1×10^6 cells per 9 cm dish and maintained for 7 days in RPMI 1640 medium containing 2% horse serum, 1% FCS, penicillin/streptomycin and NGF (Promega UK Ltd.) at 100 ng/ml. In NGF withdrawal experiments, the neuronally differentiated PC6-3 cells were rinsed twice with differentiation medium lacking NGF and then refed with medium containing NGF or anti-NGF antibody, as required.

Microinjection and dual luciferase assay

Sympathetic neurons were microinjected as described previously (14). The different reporter constructs and expression vectors were injected at the concentrations indicated (see Results). The injection mix contained the DNA to be tested in 0.5× PBS ($-Ca^{2+}$, $-Mg^{2+}$) (Sigma-Aldrich) plus 5–10 ng/μl of the *Renilla* luciferase construct pRL-TK (Promega) to control for variations in the volume of DNA or number of cells injected per coverslip. The DNA was injected directly into the nucleus and at least 120 neurons were injected for each condition tested. Typically 50–80% of the neurons survived injection.

At 16–24 h after injection, the neurons were harvested and luciferase activity was determined using the Dual-Luciferase reporter assay system (Promega). Neurons were rinsed off the coverslips in ice-cold PBS and were collected by centrifugation before being lysed in 25 μl of passive lysis buffer. The luciferase assay was then performed using a Lumat LB 9507 luminometer following the protocol provided with the assay system. The output for firefly luciferase was normalized to the *Renilla* luciferase output (firefly luciferase output divided by *Renilla* luciferase output). The relative induction of each construct was then calculated (for example, normalized luciferase activity in the absence of NGF divided by normalized luciferase activity in the presence of NGF). Each experiment was performed at least three times using different neuron preparations and the standard error of the mean was calculated.

For antibody co-injection experiments, the c-Jun (H-79) X and ATF2 (C-19) X rabbit polyclonal antibodies (Santa Cruz Biotechnology Inc.) were diluted in PBS ($-Ca^{2+}$, $-Mg^{2+}$) and centrifuged in Microcon YM-3 centrifugal filters (Millipore Corporation, Bedford, MA) to remove sodium azide. The final antibody concentration was adjusted to 2 μg/μl. Purified rabbit immunoglobulin in PBS (Jackson ImmunoResearch Laboratories, Inc.) was used as a control. Neurons were microinjected with *dp5*-LUC + ALL or pLuc-MCS or pCRE-Luc (Stratagene) all at 20 ng/μl, pRL-TK (10 ng/μl) and antibody (1 μg/μl). After injection, in the case of *dp5*-LUC + ALL, the cells were rinsed twice with SCG medium and refed with medium containing NGF or anti-NGF antibody. For pLuc-MCS and pCRE-Luc the injected cells were refed with + NGF medium containing 500 μM CPTcAMP (Sigma-Aldrich). Twenty hours later, the cells were harvested and a dual luciferase assay performed.

RT–PCR

RNA was isolated from sympathetic neurons using an RNeasy kit (QIAGEN Ltd.). Total RNA was eluted in 30 μl and, after treatment with amplification grade DNase I (Invitrogen Ltd) to remove any contaminating DNA, 10 μl of the purified RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen Ltd). Fifty microliter PCR reactions were prepared using 1–4 μl of cDNA, 0.2 mM dNTPs, 0.1 μg of oligonucleotide primers and 0.05 U/μl of REDTaq DNA polymerase in 1× RedTaq PCR reaction buffer (Sigma-Aldrich). Cycling parameters were 94°C for 30 s, 58°C for 20 s and 72°C for 60 s and PCR cycles were performed a sufficient number of times for a product to be detected for each sample in a set, typically 30–38 cycles. The amplified products were resolved on 2.5% agarose gels containing ethidium bromide at 0.5 μg/ml. Images were captured using a UVIdoc gel documentation system (UVItec Ltd, Cambridge, UK) and quantified using ImageMaster TotalLab imaging software (GE Healthcare UK Ltd, Chalfont St. Giles, UK). Experiments were performed four times and the average of three PCR reactions and SEM were calculated for each cDNA sample. The following primers were used:

dp5, 5′-AGACCCAGCCCGGACCGAGCAA-3′ and 5′-ATAGCACTGAGGTGGCTATC-3′;
neurofilament (*nf-m*), 5′-ACGCTGGACTCGCTGGGCA A-3′ and 5′-GCGAGCGCGCTGCGCTTGTA-3′.

In vitro transcription and translation

ATF2, c-Fos, c-Jun, c-JunΔ169 and Δ169m0 were produced *in vitro* using the TNT T7 or T3 Coupled Reticulocyte Lysate system (Promega). All plasmids used contained the β-globin RNA leader sequence, which increases translation efficiency. The plasmid pBAT ATF2 (31) was used with the T3 coupled system as a template for the synthesis of ATF2. All other proteins were translated using the T7 coupled system. T7 c-Fos was provided by Curt Pfarr and Moshe Yaniv (Institut Pasteur, Paris). The plasmid pCDc-Jun and pCDFLAGΔ169, the expression vector for a c-Jun dominant negative mutant, are described in (7). Δ169m0 protein was made using the plasmid pCDFLAGΔ169m0, in which the m0 leucine zipper mutation (31) had been transferred into the c-Jun sequences in pCDFLAGΔ169 by subcloning. After coupled transcription/translation at 30°C for 60 min in a volume of 50 μl, an equal volume of 2x dialysis buffer (40 mM Hepes pH 7.9, 50 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 20% glycerol) was added to each translation. The translated proteins were divided into aliquots, frozen on dry ice, and stored at −80°C.

In some experiments, the *in vitro* translated proteins were labelled with ^{35}S-methionine and run on a 12% SDS polyacrylamide gel. The gel was fixed, treated with Amplify™ solution (GE Healthcare UK Ltd.) and dried using a BioRad gel dryer (model 583) before being exposed to Hyperfilm™ X-ray film (GE Healthcare UK Ltd).

Electrophoretic mobility shift assays

Double-stranded oligonucleotides were labelled with [α-^{32}P]dCTP (3000 Ci/mmol; PerkinElmer) using Klenow polymerase (Roche Diagnostics Ltd, Lewes, UK) to fill in 5′ overhangs. The following pairs of oligonucleotides were used (binding sites are underlined): collagenase TRE, 5′-C TAGAGCATGAGTCAGACAC-3′ and 5′-CTAGGTGT CTGACTCATGCT-3′; jun2 TRE, 5′-CTAGAGCATT ACCTCATCCC-3′ and 5′-GTACGGGATGAGGTAA TGCT-3′; *dp5* ATF, 5′-CTAGGCCGGATGATGTAAC CCCCT-3′ and 5′-CTAGAGGGGGTTACATCATCCG

GC-3′; *dp5* ATF mutant, 5′-CTAGGCCGGATAACGTC TCCCCCT-3′ and 5′-CTAGAGGGGGAGACGTTATC CGGC-3′.

Binding reactions were prepared with 40 mM KCl, 20 mM Hepes pH 7.9, 5 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 10% glycerol, 0.5 μg/μl BSA, 1 μg poly(dI-dC) and 4 μl of *in vitro* translated protein/unprogrammed rabbit reticulocyte lysate to a final volume of 19 μl. The reaction was prepared without the radiolabelled oligonucleotide, and incubated at room temperature for 15 min. One microlitre containing 0.4 ng of ^{32}P-labelled probe was then added before a further 15 min incubation at room temperature. The binding reactions were loaded onto a 5% polyacrylamide/0.25× TBE native protein gel. Following electrophoresis at 180 V for ~2 h at room temperature, the gel was fixed for 15 min in 10% acetic acid, 10% methanol and dried at 80°C under vacuum. The bands were visualised by exposing the dried gel to Kodak MXB X-ray film (Kodak Ltd, Hemel Hempstead, UK) or a phosphorimager screen overnight. The exposed phosphorimager screen was scanned using a Typhoon™ 8600 phosphorimager and the image analysed using ImageQuant software and saved as a TIFF file. Exposed X-ray films were scanned using an Epson photo scanner (model 4990) and the resulting images were saved as TIFF files.

Sympathetic neuron whole-cell extracts for EMSA experiments were prepared as follows. The SCG neurons isolated from 30 1-day-old rats were plated in four 3.5 cm dishes coated with poly-L-lysine and laminin. After 7 days *in vitro*, the medium was removed, the cells were rinsed twice with SCG medium, and two dishes were refed with medium containing NGF and two with medium containing anti-NGF antibody. Sixteen hours later, the dishes were placed on ice and the medium was removed. The neurons were then rinsed off using 1 ml of ice-cold PBS per dish and transferred to 1.5 ml microfuge tubes on ice. The cells were pelleted by centrifugation for 5 min at 4°C and the cells from each pair of dishes were pooled and resuspended in 1 ml of ice-cold PBS. The cells were spun again for 5 min at 4°C and then suspended in 30 μl of whole cell extract buffer (0.1% NP-40, 250 mM KCl, 50 mM Hepes pH 7.9, 10% glycerol, 0.2 mM EDTA, 0.2 mM EGTA, containing the following inhibitors added just before use, 4 mM NaF, 4 mM Na_3VO_4, 1 mM DTT, 0.5 mM PMSF, 2% v/v Sigma mammalian protease inhibitor cocktail). The lysates were incubated on ice for 30 min and were gently pipetted up and down from time to time. Cell debris was removed by centrifugation for 30 min at 16 000 × *g* in a microfuge at 4°C, and the supernatants were frozen in aliquots on dry ice and stored at −80°C. Protein concentration was determined using the Bio-Rad protein assay.

EMSA experiments with sympathetic neuron extracts were carried out as described above, except that the volume of the binding reaction was 25 μl and 4 μl of whole cell extract buffer was included. From 4 to 8 μg of whole cell extract was used per binding reaction and for supershift assays 2–4 μl of antibody was added. The antibodies were c-Jun (H-79) X, ATF2 (C-19) X, phospho-c-Jun (KM-1) X (all from Santa Cruz Biotechnology, Inc.), phospho-ATF2 (Thr71) (Cell Signaling Technology) and, as a control, Bim AB17003 (Chemicon). Binding reactions were for 2 h at 4°C, after which 0.4 ng of the ^{32}P-labelled double-stranded oligo was added. The samples were incubated for 15 min at room temperature, and then electrophoresed on a 5% polyacrylamide, 0.25× TBE gel.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described (32) with a number of modifications. Most of the buffers used were from an Upstate ChIP assay kit (Upstate Ltd, Dundee, UK). PC6-3 cells were plated at a density of 1×10^6 cells per 9 cm dish and differentiated for 7 days. After 7 days, the cell density had increased to $\sim 4 \times 10^6$ cells per 9 cm dish. The differentiated cells were rinsed twice and cultured in medium containing NGF or anti-NGF antibody for 16 h. Proteins and DNA were cross linked by adding 37% formaldehyde to the culture medium to a final concentration of 1% and incubating at room temperature for 3 h. Two hundred microlitre of aliquots of fragmented chromatin ($\sim 8 \times 10^6$ nuclei) were used per ChiP, and the c-Jun (H-79) X and ATF2 (C-19) X antibodies were diluted 1:1000 in ChIP dilution buffer in a volume of 2 ml. The phospho-c-Jun (serine 63) KM-1 X antibody was diluted 1:100. For preclearing and for recovery of the immune complexes 50% protein A/G-agarose (without DNA, Santa Cruz Biotechnology, Inc.) in ChIP dilution buffer containing BSA (10 μg/μl) was used.

After immunoprecipitation, washing and DNA purification, ChIP samples were analysed by PCR using *Taq* DNA polymerase and CoralLoad PCR buffer (QIAGEN Ltd.). To detect binding of c-Jun and ATF2 to the jun1 and jun2 TREs in the rat *c-jun* promoter the following primer pair was used: 5′-TGGAGAAAGAAGGGCCCA ACTGTAG-3′ and 5′-GTGCAACTCTGAGTCCTTATC CAGC-3′.PCR conditions were: 5 min at 94°C followed by 30–35 cycles of 30 s at 94°C, 45 s at 52°C, 1 min at 72°C, followed by 10 min at 72°C. For the *dp5* ATF site the following primers were used: 5′-AAGTTACCTCTCGG CTTTTTCTCC-3′ and 5′-CTGGACCCCAAGTTTCGC TC-3′. PCR conditions were the same as those used for *c-jun* except that annealing was for 45 s at 62°C and Q-Solution (Qiagen Ltd, Crawley, UK) was added to the PCR reactions to facilitate amplification of the GC-rich *dp5* promoter fragment. The PCR products were run on non-denaturing 8% polyacrylamide/1× TBE gels and then stained with SYBR Green 1 (Sigma-Aldrich). Images were captured using an UVIdoc gel documentation system (UVItec Ltd) and saved as TIFF files.

Adenovirus infection

Recombinant adenoviruses that express the FLAG-tagged c-Jun dominant negative mutant FLAGΔ169 (AdvJunΔ169) or *Escherichia coli* β-galactosidase (AdvlacZ) under the control of the CMV promoter were previously described (14). Purified adenovirus preparations were titred by $TCID_{50}$ assay using HEK 293A cells, and by infecting sympathetic neurons and performing immunocytochemistry with the FLAG-specific M2 monoclonal antibody (Sigma-Aldrich) or a monoclonal antibody against β-galactosidase (Promega) as described (14).

For infection experiments, sympathetic neurons growing on glass coverslips were transferred into the wells of 24-well plates with 1 ml of SCG medium per well. At 5 days *in vitro*, the medium was replaced with 0.5 ml of medium containing recombinant adenovirus particles at the lowest multiplicity of infection (MOI) that would lead to the expression of the FLAGΔ169 or β-galactosidase protein in ~50% of infected neurons (14). After overnight infection at 37°C, the virus-containing medium was removed and replaced with 1 ml of fresh SCG medium containing NGF. After 24–36 h, the cells were gently rinsed twice with SCG medium lacking NGF and antimitotic agents and then refed with 1 ml of SCG medium containing NGF or anti-NGF antibody, as appropriate. After a further 16–24 h, the cells were harvested for protein analysis or RNA analysis, as described in the immunoblotting and RT–PCR sections, respectively.

Immunoblotting

Neurons were washed off the coverslips into microfuge tubes using ice cold PBS. After centrifugation, the neurons were lysed in sample buffer (2% SDS, 2 mM β-mercaptoethanol, 60 mM Tris, pH 6.8, 0.01% bromophenol blue) by incubating at 100°C for 15 min. Proteins were separated on 12% SDS polyacrylamide gels and transferred to Immobilon-P (Millipore) using the Bio-Rad Mini-PROTEAN III transfer system. Protein detection was performed as described previously (14) or following protocols supplied with the primary antibodies. The following primary antibodies were used: the M2 anti-FLAG epitope mouse monoclonal antibody (Sigma-Aldrich), a mouse monoclonal anti-β-galactosidase antibody (Promega), a rat monoclonal anti-α-tubulin antibody (Serotec Ltd, Kidlington, UK), a mouse monoclonal anti-c-Jun antibody (BD Transduction Laboratories), a mouse monoclonal phospho-c-Jun (serine 63) antibody (KM-1; Santa Cruz Biotechnology, Inc.), a rabbit polyclonal phospho-c-Jun (serine 73) antibody (9164; Cell Signalling Technology), a rabbit polyclonal anti-ATF2 antibody (C-19; Santa Cruz Biotechnology, Inc.), a rabbit polyclonal phospho-ATF2 (threonine 71) antibody (9221; Cell Signalling Technology), a rabbit polyclonal ERK1/2 antibody (Cell Signalling Technology).

RESULTS

The *dp5* promoter, intron and 3′ UTR contain sequences that respond to NGF withdrawal in sympathetic neurons

The rat *dp5* gene contains two exons divided by an 18.9 kb intron, of which only a short region towards the 5′ end is conserved between the rat, mouse and human DNA sequences. The Dp5 open reading frame (ORF) is located in exon 1, which also contains part of the 3′ UTR, the remainder of which is contained within exon 2 (Figure 1a).

As a first step in studying how *dp5* expression is regulated, we used the 5′ RACE technique to map the major *dp5* RNA start site in rat brain mRNA. This is 37 bp upstream of the start of the Dp5 ORF and we designated it as +1 (Figures 1a and 3). We then cloned different

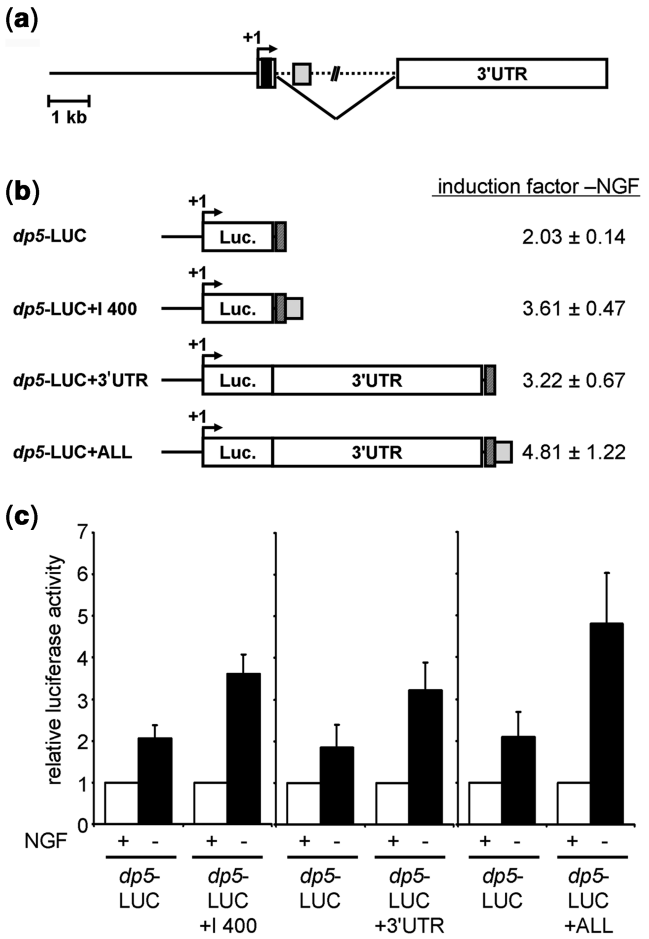

Figure 1. The *dp5* promoter, intron and 3′ UTR contain sequences that respond to NGF withdrawal in sympathetic neurons. (**a**) Structure of the rat *dp5* gene. The rat *dp5* gene consists of two exons and an 18.9 kb intron. The transcriptional start site mapped by 5′ RACE is indicated as +1 (see Figure 3 for DNA sequence). Exon 1 contains the Dp5 ORF (black box) as well as a small region of the 3′ UTR, the remainder of which is in exon 2. A short region (<400 bp long) towards the 5′ end of the intron is conserved between the rat, mouse and human *dp5* genes (grey box). (**b**) *dp5* reporter gene structure and induction levels in sympathetic neurons following NGF deprivation. The construct *dp5*-LUC contains the *dp5* promoter sequence from –980 to +37 cloned upstream of the firefly luciferase gene (*Luc*) and the SV40 poly-A termination signal (striped box). In addition to these sequences, other constructs contained either ~400 bp from the conserved region of the *dp5* intron inserted downstream of the poly-A termination signal (*dp5*-LUC + I 400), the *dp5* 3′ UTR inserted downstream of luciferase (*dp5*-LUC + 3′ UTR), or both the intron fragment and 3′ UTR inserted as above (*dp5*-LUC + ALL). The induction factor for each reporter construct in sympathetic neurons following NGF withdrawal is shown. (**c**) Relative luciferase activity of *dp5* reporter constructs in sympathetic neurons maintained in the presence and absence of NGF. Sympathetic neurons were microinjected with *dp5*-LUC (5 ng/μl), *dp5*-LUC + I 400 (5 ng/μl), *dp5*-LUC + 3′ UTR (15 ng/μl) or *dp5*-LUC + ALL (10 ng/μl) together with pRL-TK (5 ng/μl). Following microinjection, cells were rinsed twice with medium lacking NGF and were refed either with medium supplemented with NGF or medium containing neutralising anti-NGF antibody. After 20–24 h luciferase activity was measured. Firefly luciferase activity was normalized as outlined in the Materials and Methods and luciferase induction –NGF (black bars) was calculated relative to +NGF (white bars), which was set as 1. The mean of at least four independent experiments ±SEM is shown.

regions of the rat *dp5* gene and constructed a series of *dp5*-luciferase reporter plasmids (Figure 1b). These contained 1 kb of promoter sequence including the *dp5* transcriptional start site upstream of the firefly luciferase gene, either alone or with the entire *dp5* 3′ UTR and/or ~400 bp of the conserved intron sequence (Figure 1b). We microinjected the reporter constructs, together with the control *Renilla* luciferase construct pRL-TK, into the nuclei of sympathetic neurons cultured *in vitro*. After injection, the cells were maintained for 16–24 h in the presence of NGF or a neutralising anti-NGF antibody, after which time relative luciferase activity was determined by dual luciferase assay (33,34). In each case, the different *dp5* reporter constructs were compared to the construct containing only 1 kb of the promoter sequence (*dp5*-LUC). In microinjected neurons, this construct has a much higher level of luciferase activity, i.e. promoter activity, than the promoterless vector pGL3-Basic (data not shown) and, after NGF withdrawal, it is induced 2.03-fold (±0.14) on average (Figure 1b). This indicates that the 1 kb *dp5* promoter fragment contains sequences that respond to NGF withdrawal. Following NGF deprivation, both constructs containing the 1 kb *dp5* promoter and either the conserved intron region or the 3′ UTR sequence showed higher levels of luciferase activity than the construct with the promoter alone, with induction factors of 3.61- and 3.23-fold, respectively (Figure 1c). This suggests that these two regions also contain elements that contribute to the increase in *dp5* expression after NGF withdrawal. The construct *dp5*-LUC + ALL, which contained 1 kb of *dp5* promoter sequence, the conserved region of the intron, and the 3′ UTR was induced the most after NGF deprivation, 4.81-fold (Figure 1c). Since *dp5*-LUC + ALL was efficiently activated by NGF withdrawal it was used as the standard *dp5* reporter construct in all subsequent experiments.

Inhibition of the MLK-JNK pathway reduces the increase in endogenous *dp5* mRNA level and *dp5* reporter activity after NGF deprivation

The MLK-JNK protein kinase cascade is activated by NGF withdrawal in sympathetic neurons (9,10,11). Mixed lineage kinases (MLKs) phosphorylate and activate the JNK kinases MKK4 and MKK7, which in turn phosphorylate and activate JNKs (35). MLK activity can be directly inhibited by the compound CEP-1347 (36). Treatment of sympathetic neurons with CEP-1347 blocks NGF withdrawal-induced death (37) and reduces *dp5* mRNA induction following NGF deprivation by ~75% (22). In addition, it has been reported that CEP-1347 reduces the increase in *dp5* RNA level in cortical neurons undergoing amyloid-beta-induced apoptosis (38).

To further study the role of the MLK-JNK pathway in regulating *dp5* expression we used CEP-11004, a MLK inhibitor closely related to CEP-1347, which also inhibits NGF withdrawal-induced death (39). We investigated the effect of CEP-11004 (at 400 nM) on the level of c-Jun and ATF2 and c-Jun and ATF2 phosphorylation in sympathetic neurons cultured in the presence or absence of NGF for 16 hours (Figure 2a). As previously described (7), c-Jun protein levels and c-Jun N-terminal phosphorylation increased after NGF withdrawal. The increased phosphorylation was associated with a decrease in the mobility of c-Jun in SDS PAGE. These changes were reversed by CEP-11004. Similarly, phosphorylation of c-Jun at serine 63 and serine 73 was significantly increased after NGF withdrawal and this increase was inhibited by CEP-11004. In contrast, ATF2 protein levels did not change after NGF withdrawal and phosphorylation of ATF2 at threonine 71 was only marginally increased at 16 h after the removal of NGF, as previously reported (12). To determine whether CEP-11004 had the same effect on *dp5* expression as CEP-1347, we treated sympathetic neurons with CEP-11004 at 400 nM in the presence or absence of NGF for 16 h and then isolated RNA and measured *dp5* mRNA levels by semi-quantitative RT–PCR (Figure 2b). Following NGF deprivation, the *dp5* mRNA increased in level by 4.27-fold in untreated control cells but this induction was reduced to 2.28-fold in cells treated with 400 nM CEP-11004 (Figure 2b and c). We then investigated the effect of CEP-11004 on the activity of the *dp5*-LUC + ALL reporter construct in microinjection experiments. The *dp5* reporter construct was induced 4.53-fold after NGF withdrawal and this was reduced to a relative induction of 1.76-fold following treatment with 400 nM CEP-11004 (Figure 2d). This result suggests that, like the endogenous *dp5* gene, induction of the *dp5* reporter construct depends on MLK activity in sympathetic neurons.

We also investigated the effect of directly inhibiting JNK activity by using the JBD of the scaffold protein JNK interacting protein 1 (JIP-1), which binds to and specifically inhibits JNKs but not other MAP kinases (35). It has previously been shown that expression of the JBD in sympathetic neurons inhibits JNK activity and promotes cell survival following NGF withdrawal (12,13). We microinjected an expression vector for the JBD or the empty vector pcDNA3 into sympathetic neurons together with *dp5*-LUC + ALL and luciferase activity was measured 20–24 h after NGF withdrawal (Figure 2e). Expression of the JBD strongly reduced the induction of the *dp5*-LUC + ALL reporter construct after NGF deprivation from 4.31-fold with pcDNA3 to 1.51-fold. This result indicates that JNK activity is required for normal induction of the *dp5* reporter construct following NGF withdrawal.

A conserved ATF site in the *dp5* promoter binds c-Jun and ATF2 and contributes to *dp5* basal promoter activity and induction after NGF withdrawal

The AP-1 family of basic/leucine zipper transcription factors includes the Jun and ATF subfamilies. Both c-Jun and activating transcription factor 2 (ATF2) are phosphorylated by JNKs after NGF deprivation in sympathetic neurons (9,10,12), although, in the case of ATF2, the increase in phosphorylation (at threonine 71) is more transient (12). Importantly, microinjection of a neutralising c-Jun antibody, expression of a c-Jun dominant negative mutant or conditional knockout of the *c-jun* gene in

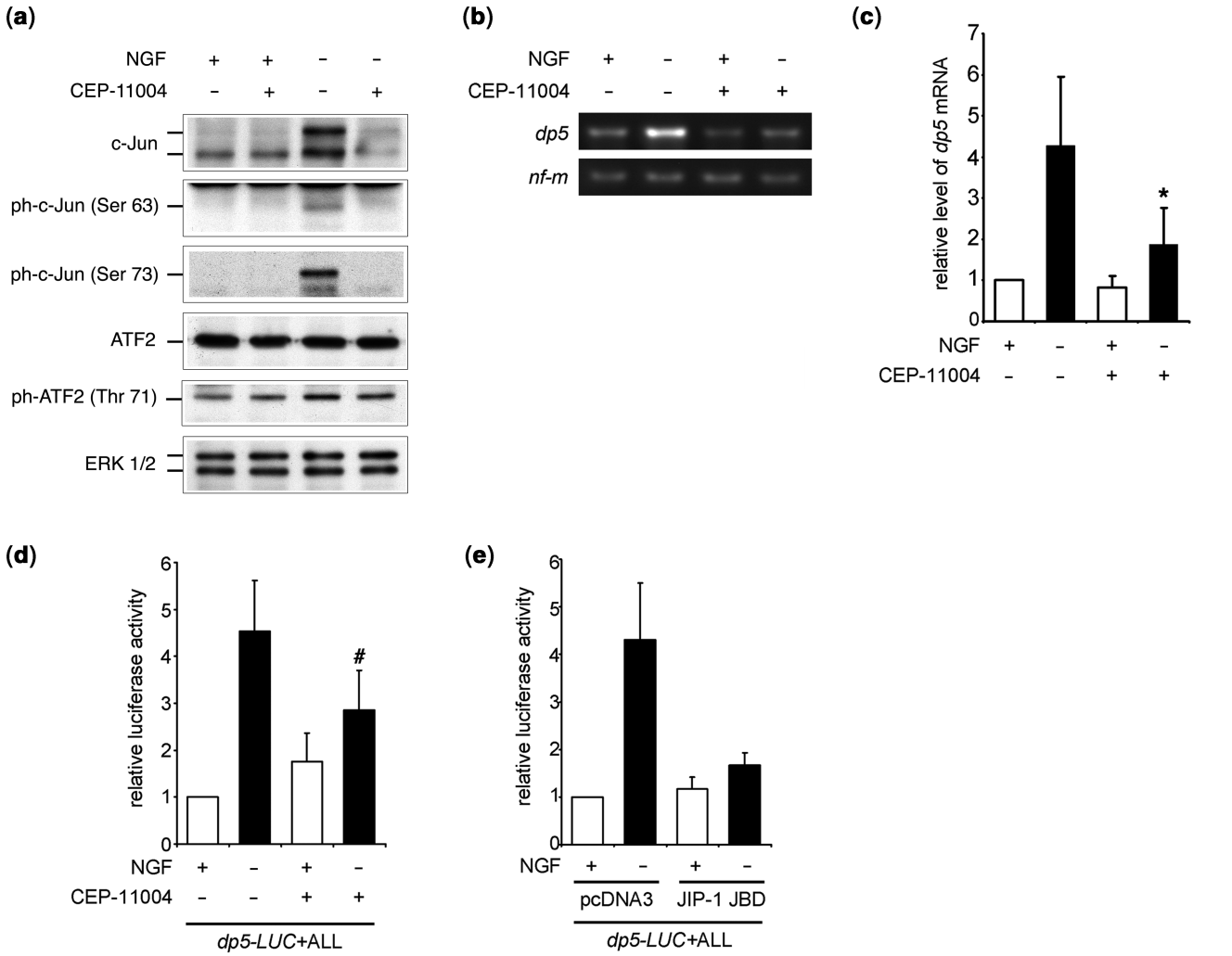

Figure 2. Inhibition of the MLK-JNK pathway reduces the increase in endogenous *dp5* mRNA level and the activation of a *dp5* reporter construct following NGF withdrawal. (**a**) CEP-11004 inhibits the increase in c-Jun protein level and c-Jun N-terminal phosphorylation that occurs after NGF withdrawal. Sympathetic neurons were maintained in the presence or absence of NGF and were treated with either DMSO or CEP-11004 at 400 nM for 16 h. The neurons were then harvested, lysed in SDS gel sample buffer and immunoblotting was performed with the antibodies indicated. The level of ERK1/2 was used as a loading control. Three independent experiments were performed and representative immunoblots are shown. (**b**) CEP-11004 reduces endogenous *dp5* mRNA expression after NGF deprivation. Sympathetic neurons were maintained in the presence or absence of NGF and were treated with either DMSO or CEP-11004 at 400 nM for 16 h. RNA was extracted and RT–PCR analysis performed using *dp5* and neurofilament (*nf-m*) specific primers. Representative images of agarose gels are shown. (**c**) Relative levels of *dp5* mRNA in sympathetic neurons treated with CEP-11004 after NGF withdrawal. Following RT–PCR, band intensity was measured and the normalized *dp5* level in each condition was calculated relative to the level in cells treated with NGF and DMSO, which was set as 1. The mean of four independent experiments ±SEM is shown. At 400 nM, CEP-11004 significantly reduced the increase in *dp5* mRNA level after NGF withdrawal (−NGF + CEP-11004 compared to −NGF). $^{*}P < 0.05$, Student's *t*-test. (**d**) CEP-11004 reduces induction of a *dp5* reporter construct after NGF withdrawal in sympathetic neurons. Sympathetic neurons were microinjected with *dp5*-LUC + ALL (10 ng/μl) and pRL-TK (5 ng/μl). Cells were maintained in the presence or absence of NGF and were treated with either DMSO or 400 nM CEP-11004 for 16 h. Normalized luciferase activity was calculated relative to the level + NGF treated with DMSO, which was set as 1. The mean of three independent experiments ±SEM is shown. At 400 nM, CEP-11004 significantly reduced the increase in the level of luciferase activity for *dp5*-LUC + ALL after NGF withdrawal (−NGF + CEP-11004 compared to −NGF). $^{\#}P < 0.02$, Student's *t*-test. (**e**) Expression of the JIP-1 JBD reduces induction of a *dp5* reporter construct after NGF withdrawal. Sympathetic neurons were microinjected with *dp5*-LUC + ALL (20 ng/μl), pRL-TK (10 ng/μl), and pcDNA3.1 or pCD JIP-1 JBD (100 ng/μl). Cells were maintained + NGF or –NGF for 20 h and then luciferase activity was measured. Luciferase activity was calculated relative to the level + NGF injected with pcDNA3.1, which was set as 1. The mean of six independent experiments ±SEM is shown.

sympathetic neurons in culture protects the cells from NGF withdrawal-induced death (6,7,15). AP-1 family members form homo- and heterodimers with each other, which depending on the particular dimer, specifically bind to the AP-1 site/TPA responsive element (TRE) (5′-TGA G/C TCA-3′) or the ATF site/cAMP response element (CRE) (5′-TGA CG TCA-3′) (35). The *c-jun* promoter itself contains two ATF sites, the jun1 and jun2 TREs, that bind heterodimers of c-Jun and ATF2 or ATF2 homodimers, and which are required for *c-jun* promoter activation following NGF withdrawal in sympathetic neurons (10).

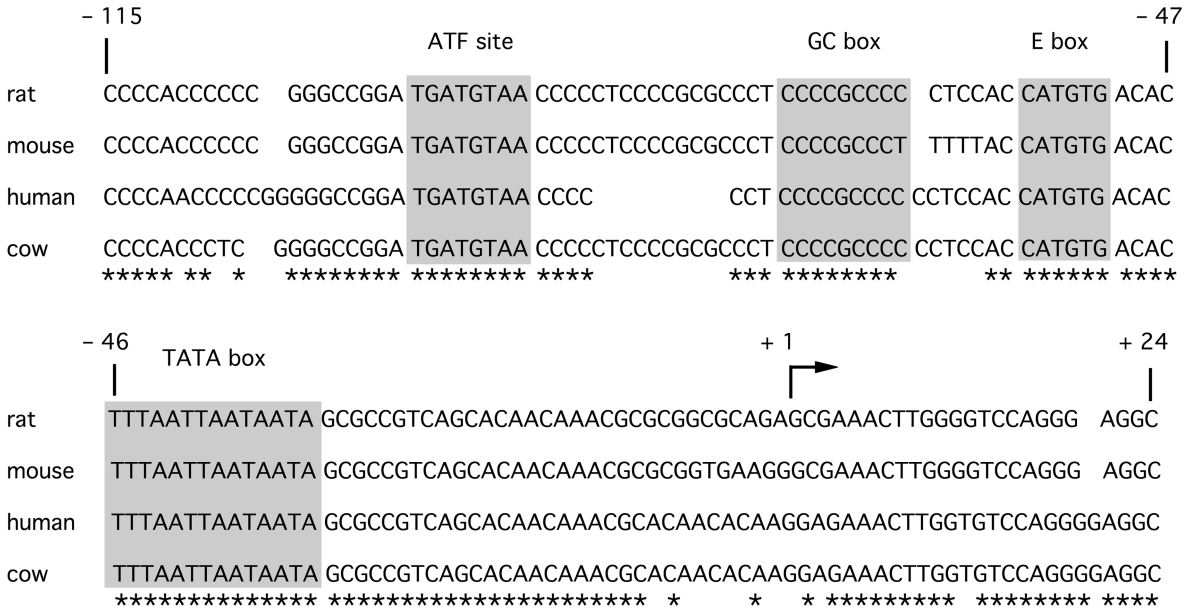

Figure 3. Alignment of the promoter sequences for the rat, mouse, human and cow *dp5* genes. Shaded regions indicate a conserved ATF site, a GC box, an E box and a TATA box. Asterisks represent bases conserved in all four species. Overall, 80% of the nucleotides are conserved. The transcriptional start site of the rat *dp5* gene determined by 5′ RACE is indicated as +1, together with the direction of transcription.

To identify conserved, potential transcription factor binding sites in the *dp5* promoter, we aligned the DNA sequence of the rat *dp5* promoter from −115 to +24, in relation to the transcriptional start site, with the corresponding regions of the mouse, human and cow *dp5* genes (Figure 3). Interestingly, we identified a potential ATF-binding site (5′-TGA TG TAA-3′) at positions −96 to −89 in the rat *dp5* promoter, which was conserved between the rat, mouse, human and cow sequences (Figure 3). Closer analysis of this site indicated that it only differs from the ATF/CRE consensus site by 2 bases and it is only 1 base different from the jun2 TRE site in the *c-jun* promoter (Figure 4a).

We tested the ability of representative AP-1 family members to bind to the *dp5* ATF site *in vitro* in an electrophoretic mobility shift assay (EMSA). We translated c-Fos, c-Jun and ATF2 *in vitro* (Figure 4b). As a control, we tested the binding of these AP-1 proteins to two well characterized binding sites, the *collagenase* TRE (a consensus AP-1 site) and the jun2 TRE (an ATF site) (Figure 4c). It has been reported that c-Jun homodimers have a very low affinity for both of these sites whereas c-Jun/c-Fos heterodimers bind with high affinity to the *collagenase* TRE, and c-Jun/ATF2 heterodimers bind with high affinity to the jun2 TRE (31). In agreement with these results, we observed a c-Jun/c-Fos/DNA complex, but no binding of c-Jun or c-Fos alone, with the *collagenase* TRE (lanes 1–4, Figure 4c), and a c-Jun/ATF2/DNA complex, but no binding of c-Jun alone, with the jun 2 TRE (lanes 5–8, Figure 4c). We then tested the binding of the AP-1 proteins to the *dp5* ATF site (lanes 9–14, Figure 4c). Neither c-Jun nor c-Fos alone bound to the *dp5* sequence but c-Jun/c-Fos heterodimers bound with a low affinity (compare lane 11 with lane 3). In contrast, c-Jun/ATF2 heterodimers and ATF2 homodimers bound the *dp5* site with an affinity similar to the affinity of c-Jun/ATF2 for the jun2 TRE (compare lanes 12 and 13 with lane 7, Figure 4c). Mutation of the *dp5* ATF site by the introduction of four base changes (Figure 4a) abolished the binding of all of the AP1 proteins (Figure 4d, lanes 8–14).

To investigate whether c-Jun and ATF2 in sympathetic neuron extracts can bind to the *dp5* ATF site we prepared extracts from neurons that had been cultured in the presence or absence of NGF for 16 h and performed an EMSA experiment (Figure 5a). Extracts from neurons maintained in the presence of NGF contained proteins that bound to the *dp5* ATF site (lane 2) and this binding (marked AP-1) was abolished by point mutations in the ATF site (lanes 5 and 6). After NGF withdrawal, a similar amount of specific AP-1-binding activity was observed but the pattern of bands was slightly different (compare lanes 2 and 3). To determine whether the specific protein complexes contained c-Jun and ATF2 we added antibodies specific for c-Jun or ATF2 or a Bim antibody, as a negative control, to the binding reactions. The Bim antibody did not alter the binding pattern observed with the –NGF extract (compare lanes 7 and 8) whereas the c-Jun antibody supershifted some of the AP-1 complexes (lane 9). Phosphorimaging and quantitation revealed that the c-Jun antibody supershifted 45% of the AP-1/*dp5* complexes. Addition of the ATF2 antibody did not cause a clear supershift but did displace 42% of the AP-1/*dp5* complexes (compare lanes 10 and 7) and addition of both the c-Jun and ATF2 antibodies together supershifted 71% of the specific protein complexes bound to the *dp5* ATF site (compare lanes 11 and 7). We also performed an EMSA experiment in which we tested the effect of

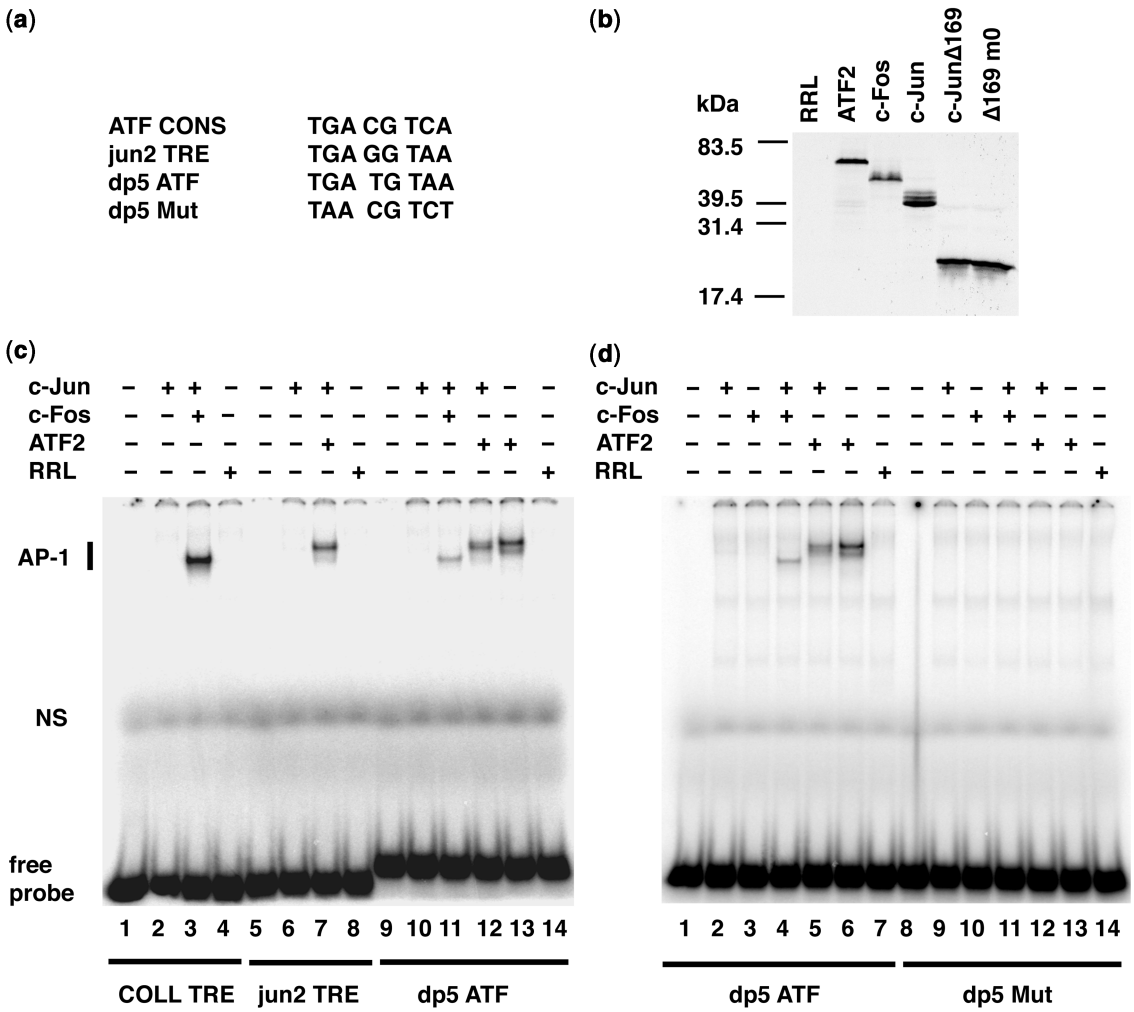

Figure 4. The *dp5* promoter contains an ATF site that binds AP-1 proteins *in vitro*. (**a**) Sequences of ATF sites. The ATF consensus sequence is shown together with the jun2 TRE sequence, the *dp5* ATF site and a mutant *dp5* ATF site. (**b**) *In vitro* translated proteins used for EMSA experiments. ATF2, c-Fos, c-Jun, c-JunΔ169 and Δ169m0 were transcribed and translated in a rabbit reticulocyte lysate. Unprogrammed lysate (RRL) was used as a negative control. The *in vitro* translated proteins were labelled with ^{35}S-methionine and separated on a 12% SDS polyacrylamide gel. The size of molecular weight markers run on the same gel is shown. (**c**) ATF2 homodimers, c-Jun/ATF2 and c-Jun/c-Fos can bind to the *dp5* ATF site. ^{32}P-labelled, double-stranded oligonucleotides containing either the collagenase TRE, the jun2 TRE, or the *dp5* ATF site were incubated with *in vitro* translated c-Jun, c-Fos, ATF2, or unprogrammed lysate (RRL), as indicated, and separated on a 5% polyacrylamide, 0.25× TBE gel. Several experiments were performed and a representative result is shown. Free probe, a non-specific complex (NS) and the AP-1 complexes are indicated. (**d**) Point mutations in the *dp5* ATF site abolish the binding of AP-1 proteins *in vitro*. Oligonucleotides containing either the wild type *dp5* ATF site or a mutant site, in which four nucleotides have been changed (a) were used for EMSA experiments with *in vitro* translated c-Jun, c-Fos, ATF2 or unprogrammed lysate (RRL), as indicated. Several experiments were performed and a representative result is shown.

phospho-c-Jun (serine 63) and phospho-ATF2 (threonine 71) antibodies (Figure 5b). With the –NGF extract the phospho-c-Jun antibody supershifted the majority of the AP-1 proteins bound to the *dp5* ATF site (compare lane 15 to lanes 14 and 13). We confirmed that this was a supershift by measuring the distribution of peaks in a vertical line through the –NGF lanes in a phosphorimage of the gel shown in Figure 5b. The AP-1 proteins were clearly displaced towards the top of the gel in lane 15 compared to lanes 14 and 13. In contrast, the phospho-ATF2 antibody only supershifted a small fraction of the *dp5*/AP-1 complex (compare lane 16 to lanes 14 and 13). These results demonstrate that after NGF withdrawal the c-Jun in sympathetic neuron extracts that binds to the *dp5* ATF site *in vitro* is phosphorylated at serine 63. In the case of ATF2, a smaller fraction of the AP-1 complex bound to the *dp5* ATF site is phosphorylated at threonine 71. We then compared the amount of phospho-c-Jun supershifted in DNA-binding assays performed with +NGF and –NGF extracts (Figure 5c). Phosphorimaging and quantitation of the supershifted complex in lanes 19 and 20 revealed that the amount of c-Jun phosphorylated at serine 63 was 2-fold greater at 16 h after NGF withdrawal.

To confirm that c-Jun and ATF2 can bind to the region of the *dp5* promoter that contains the ATF site in living cells, we performed ChIP assays using the Bim, c-Jun and ATF2 antibodies. Since large numbers of cells are required

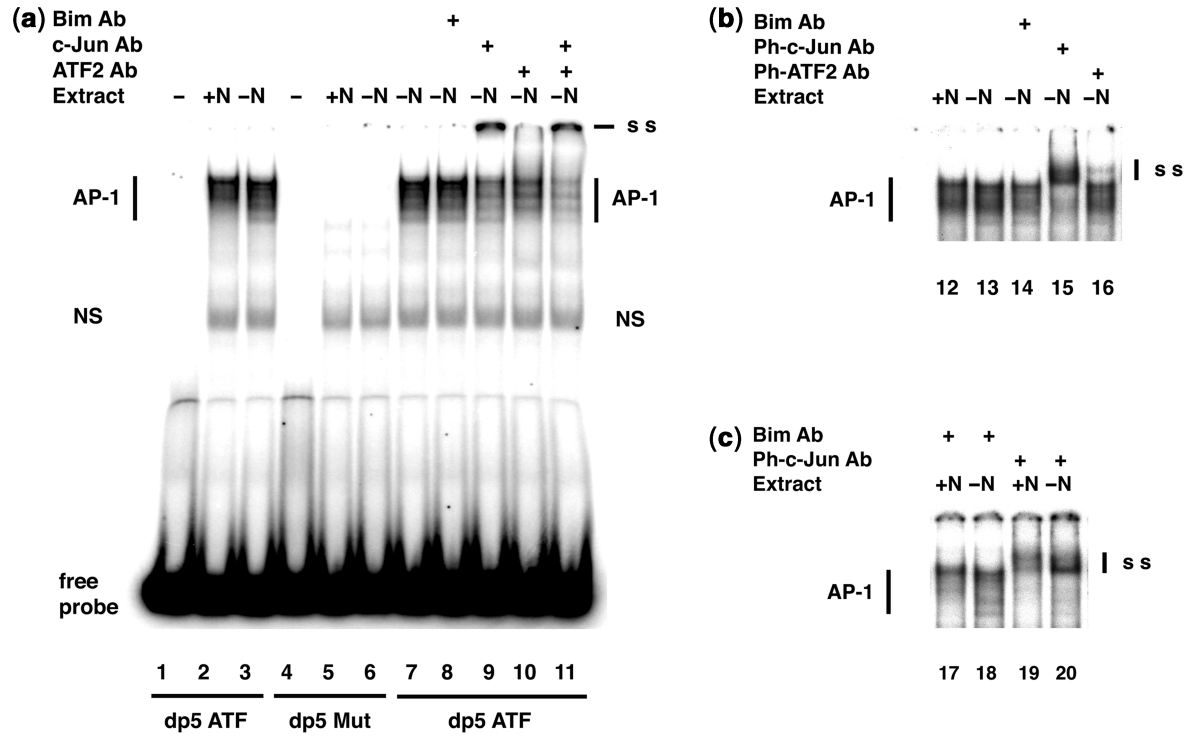

Figure 5. c-Jun and ATF2 in sympathetic neuron extracts bind to the *dp5* ATF site *in vitro*. Sympathetic neurons were cultured for 7 days and then refed with medium containing NGF (+N) or anti-NGF antibody (−N). Sixteen hours later, whole cell extracts were prepared. (**a**) EMSA experiments were performed using oligonucleotides containing either the wild type *dp5* ATF site or mutant site, in which four nucleotides have been changed (Figure 4a). Eight micrograms of +N or –N whole cell extract was used per binding reaction, as indicated. A control antibody (against Bim) or c-Jun or ATF2 antibodies were added as shown. The binding reactions were separated on a 5% polyacrylamide, 0.25× TBE gel. Several experiments were performed and a representative result is shown. Free probe, a non-specific complex (NS) and the AP-1 complexes are indicated. The four point mutations inhibited the binding of AP-1 proteins to the *dp5* ATF site. The c-Jun antibody caused a supershift (s s) whereas the ATF2 antibody partially reduced binding of the AP-1 complexes. (**b**) Effect of phospho-c-Jun (Serine 63) and phospho-ATF2 (Threonine 71) antibodies on the binding of AP-1 proteins to the *dp5* ATF site. Only the top of the gel is shown. The positions of the AP-1 complexes and supershifted complexes (s s) are indicated. (**c**) The amount of c-Jun phosphorylated at serine 63 bound to the *dp5* ATF site increases after NGF withdrawal. Only the top of the gel is shown. The positions of the AP-1 complexes and supershifted complexes (s s) are indicated.

for conventional ChIP assays we used neuronally differentiated PC12 cells that had been cultured in the presence or absence of NGF for 16 h, rather than sympathetic neurons. The binding of c-Jun or ATF2 to the *c-jun* and *dp5* promoters was studied by PCR using primers that flank the jun1 and jun2 TREs (a positive control) or the *dp5* ATF site, respectively (Figure 6a). The control antibody (Bim) did not immunoprecipitate either the *c-jun* or *dp5* promoter (lanes 3 and 4), whereas both the c-Jun and ATF2 antibodies precipitated the region of the *c-jun* promoter that contains the jun1 and jun2 TREs and the *dp5* promoter region that contains the ATF site (lanes 5–8). These results indicate that c-Jun and ATF2 can bind to the *dp5* ATF site in living cells. In the case of both *c-jun* and *dp5* the amount of c-Jun or ATF2 bound to the promoter had not increased significantly at 16 h after NGF withdrawal. These results are in agreement with previous studies that showed that the jun1 and jun2 TREs in the *c-jun* promoter are already bound by c-Jun and ATF2-containing complexes in a variety of unstimulated cells in culture and that the protein-DNA contacts are unchanged during gene activation by TPA and UV (40). Using an antibody specific for c-Jun phosphorylated at serine 63 we also investigated whether the amount of phospho-c-Jun associated with the *c-jun* and *dp5* promoters increased after NGF withdrawal (Figure 6b). We found that the c-Jun bound to the *c-jun* promoter was phosphorylated at serine 63 and that this increased in level after NGF withdrawal (lanes 14 and 15). Similarly, we also observed that there was increase in the amount of phospho-c-Jun associated with the *dp5* promoter at 16 h after NGF deprivation. In the case of both promoters, we found that phosphorylation of ATF2 at threonine 71 had not increased at 16 h after NGF withdrawal (data not shown), consistent with the results of the immunoblotting experiments with sympathetic neuron extracts (Figure 2a).

We then investigated the effect of introducing the four point mutations shown to abolish AP-1 binding into the construct *dp5*-LUC + ALL. The wild-type and mutant constructs were microinjected into sympathetic neurons and luciferase activity was measured after 20–24 h in the presence or absence of NGF (Figure 7). The mutations in the ATF site in the *dp5* promoter completely obliterated its induction after NGF withdrawal by (i) decreasing the basal activity of the construct by 86% (Figure 7a), and (ii) by reducing the induction factor after NGF withdrawal from 7.08- to 3.05-fold (Figure 7b). This demonstrates that abolishing the binding of c-Jun and ATF2

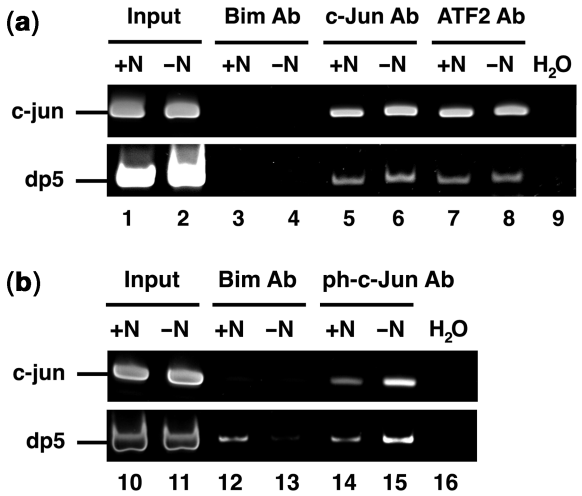

Figure 6. c-Jun and ATF2 bind to the *dp5* ATF site in the chromatin of neuronally differentiated PC12 cells. The PC6-3 subline of PC12 cells was treated with NGF at 100 ng/ml for 7 days. The neuronally differentiated cells were then refed with fresh differentiation medium containing NGF (+N) or anti-NGF antibody (−N). After 16 h, the cells were cross-linked with 1% formaldehyde and chromatin immunoprecipitations were performed using (**a**) the c-Jun(H-79) or ATF2(C-19) antibodies or a Bim antibody as a negative control or (**b**) the phospho-c-Jun (serine 63) (KM-1) or Bim antibodies. As a positive control, PCR was performed using primers that flank the jun1 and jun2 TREs in the *c-jun* promoter, which have previously been shown to bind c-Jun and ATF2 in chromatin. Binding of c-Jun and ATF2 to the *dp5* promoter was detected by performing PCR using primers that flank the *dp5* ATF site. The ChIP experiments were performed several times and representative gel images are shown. The positions of the *c-jun* and *dp5* PCR products are indicated. The equivalent of 1% of the +N and –N chromatin used for each ChIP assay was also run on each gel (input lanes). As a negative control a PCR reaction without chromatin was performed (H_2O).

to the *dp5* ATF site not only reduces the induction of *dp5* expression following NGF withdrawal but also decreases basal promoter activity. Finally, we investigated the effect of the MLK inhibitor CEP-11004 (at 400 nM) on the activity of the mutant construct (Figure 7c). CEP-11004 did not significantly reduce the 3-fold increase in the activity of the reporter with mutated ATF site that occurs after NGF withdrawal (Figure 7c). This is different to the effect of CEP-11004 on the wild type *dp5*-LUC + ALL construct (Figure 2d) and suggests that the 3-fold induction observed in the absence of the ATF site is independent of the MLK-JNK pathway.

Dominant negative c-Jun reduces the increase in *dp5* reporter activity and the increase in the level of endogenous *dp5* RNA after NGF withdrawal

Expression of a c-Jun dominant negative mutant (JunΔ169) in sympathetic neurons protects the cells against NGF withdrawal-induced death (7,14) and reduces the increase in the level of the c-Jun and Bim_{EL} proteins that normally occurs after the removal of NGF (10,14). We therefore investigated the effect of dominant negative c-Jun on *dp5* reporter activity and the endogenous *dp5* gene in sympathetic neurons.

First, using *in vitro* translated proteins we studied how JunΔ169 binds to the *dp5* ATF site in an EMSA experiment (Figure 8a). Like c-Jun, JunΔ169 was unable to bind to the *dp5* ATF site on its own, whereas ATF2 homodimers were able to do so (lanes 1–5, Figure 8a). When c-Jun was mixed with ATF2, c-Jun/ATF2 heterodimers formed a protein/DNA complex that ran just below the ATF2/DNA complex (compare lanes 6 and 5). JunΔ169 also formed heterodimers with ATF2 that could bind to the

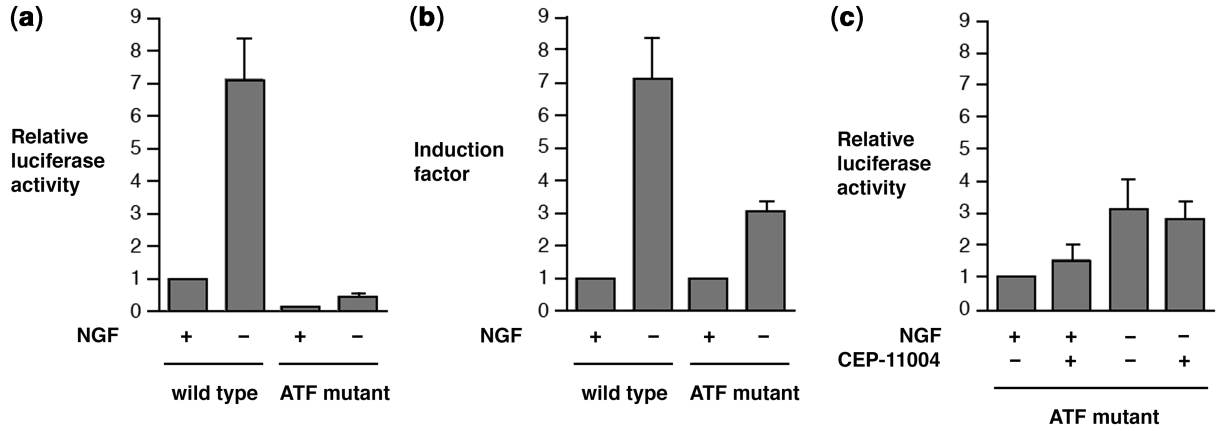

Figure 7. Mutation of an ATF-binding site in the *dp5* promoter reduces basal promoter activity and induction after NGF withdrawal. Sympathetic neurons were microinjected with *dp5*-LUC + ALL or a reporter construct in which the ATF-binding site in the *dp5* promoter had been mutated (*dp5*-LUC + ALLmut) (both at 10 ng/μl) as well as pRL-TK (5 ng/μl). Cells were maintained in medium containing or lacking NGF for 20 h, after which time luciferase activity was measured. (**a**) Normalized firefly luciferase activity was calculated relative to the level +NGF microinjected with the wild-type construct, which was set as 1. The mean of six independent experiments ±SEM is shown. (**b**) The induction factor for the same series of experiments was calculated for each reporter construct = (normalized firefly luciferase activity –NGF)/(normalized firefly luciferase activity +NGF). (**c**) CEP-11004 does not reduce the activity of the reporter with mutated ATF site after NGF withdrawal. The reporter construct in which the ATF-binding site in the *dp5* promoter had been mutated (*dp5*-LUC + ALLmut) (10 ng/μl) was microinjected into sympathetic neurons together with pRL-TK (5 ng/μl). Cells were maintained in medium containing or lacking NGF with either DMSO or 400 nM CEP-11004 as indicated for 20 h, after which time luciferase activity was measured. Normalized firefly luciferase activity was calculated relative to the level +NGF, which was set as 1. The mean of six independent experiments ± SEM is shown.

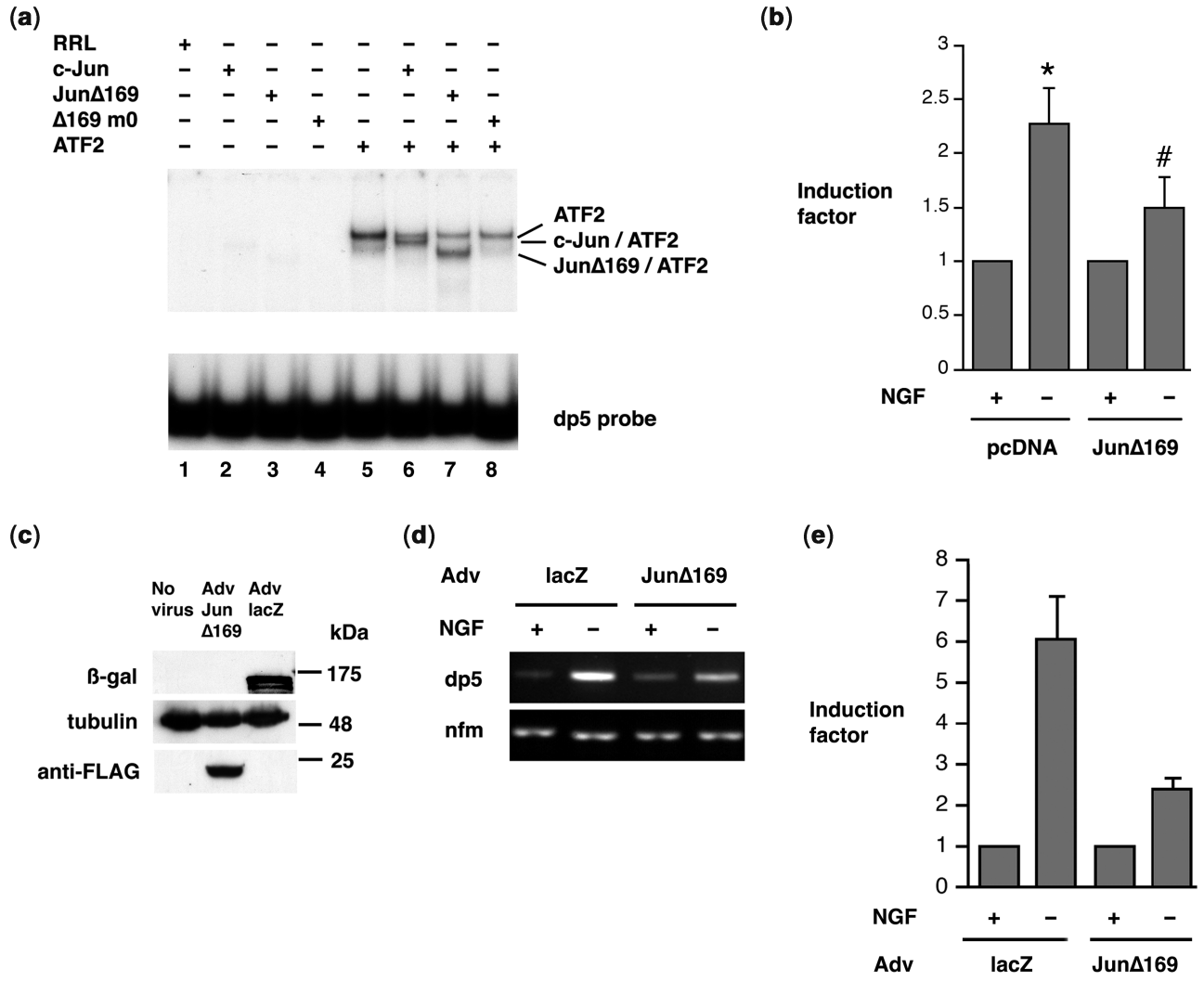

Figure 8. Expression of dominant negative c-Jun reduces the induction of a *dp5* reporter construct and the endogenous *dp5* RNA after NGF withdrawal. (**a**) c-JunΔ169 binds to the *dp5* ATF site as a heterodimer with ATF2. An oligonucleotide containing the *dp5* ATF site was incubated with *in vitro* translated c-Jun, c-JunΔ169, Δ169m0, ATF2 or unprogrammed rabbit reticulocyte lysate (RRL) as indicated, and an EMSA experiment was performed. Only the regions of the gel that contain the protein/DNA complexes and the unbound probe are shown, to make the figure more compact. (**b**) Co-injection of an expression vector for dominant negative c-Jun (JunΔ169) reduces induction of a *dp5* reporter construct after NGF withdrawal. Sympathetic neurons were microinjected with *dp5*-LUC + ALL (20 ng/μl), pRL-TK (10 ng/μl) and pcDNA1 or pCDJunΔ169 (100 ng/μl). Cells were maintained + NGF or –NGF for ~20 h and then luciferase activity was measured. Firefly luciferase levels were normalized and luciferase activity was calculated relative to the level + NGF, which was set as 1, for either pcDNA1 or pCDFLAGΔ169. The mean of nine independent experiments ±SEM is shown. In the case of empty vector the increase in reporter gene activity after NGF withdrawal was significant: $^{*}P < 0.002$, Student's *t*-test. For pCDJunΔ169 the increase in reporter activity after NGF deprivation was not significant: $^{\#}P > 0.1$, Student's *t*-test. (**c**) Sympathetic neurons infected with AdvJunΔ169 or AdvlacZ express recombinant proteins of the predicted size. Sympathetic neurons were cultured for 5 days *in vitro* and then infected with recombinant adenoviruses as indicated. After overnight infection, the cells were refed with fresh SCG medium containing NGF. Forty eight hours later, protein extracts were prepared and immunoblots performed with antibodies to β-galactosidase, α-tubulin (a loading control) and the FLAG epitope. The positions of protein molecular weight markers run on the same 12% gel are shown. (**d**) RT–PCR analysis of *dp5* and *nfm* mRNA levels in adenovirus-infected neurons expressing β-galactosidase or the JunΔ169 protein. Sympathetic neurons were cultured for 5 days *in vitro* and then infected with the recombinant adenoviruses indicated. After overnight infection, the cells were refed with fresh SCG medium containing NGF (+NGF) or anti-NGF antibody (-NGF). Sixteen hours later, RNA was isolated and semi-quantitative RT–PCR performed with primers specific for *dp5* or *nfm*. Thirty-five cycles of PCR were performed and the products were run on a 2.5% agarose gel. (**e**) AdvJunΔ169 reduces the increase in *dp5* RNA levels after NGF withdrawal. RT–PCR analysis of *dp5* and *nfm* mRNA levels in adenovirus-infected neurons was carried out as described in (d). *dp5* mRNA levels were normalized to *nfm* RNA levels to control for any differences in the amount of cDNA used for each RT–PCR reaction. The induction factor (the normalized *dp5* RNA level –NGF/+NGF) was then calculated for each virus. The average of five experiments ± SEM is shown.

dp5 ATF site (lane 7). However, when the m0 leucine zipper mutation (31), which blocks binding to ATF2, was present in JunΔ169 (Δ169m0), the dominant negative c-Jun/ATF2/DNA complex was no longer observed (lane 8). Thus, JunΔ169 can only bind to the *dp5* ATF site as a heterodimer with ATF2. We then investigated the effect of dominant negative c-Jun on the *dp5*-LUC + ALL reporter construct in a co-microinjection experiment (Figure 8b). We injected an expression vector for JunΔ169 or the empty vector pcDNA1 into sympathetic

neurons together with *dp5*-LUC + ALL and luciferase activity was measured 20–24 h after NGF withdrawal. Expression of JunΔ169 reduced the induction of the *dp5*-LUC + ALL reporter construct after NGF withdrawal from 2.275 ± 0.322-fold with pcDNA1 ($P < 0.002$ when –NGF is compared to + NGF) to 1.492 ± 0.29-fold, which was not a significant induction ($P > 0.1$) when –NGF was compared to + NGF. This result suggests that AP-1 activity is required for normal induction of the *dp5* reporter construct following NGF deprivation.

To determine whether AP-1 activity contributes to the induction of the endogenous *dp5* mRNA after NGF withdrawal we used a recombinant adenovirus that expresses dominant negative c-Jun (AdvJunΔ169) (14) and, as a control, an adenovirus that expresses *E. coli* β-galactosidase (AdvlacZ). In an immunoblotting experiment with protein extracts prepared from uninfected sympathetic neurons and adenovirus-infected cells we confirmed that AdvJunΔ169 and AdvlacZ express recombinant proteins with the predicted molecular weights (Figure 8c). We then tested the effect of the two adenoviruses on the level of *dp5* mRNA (Figure 8d and e). Sympathetic neurons were cultured for 5 days *in vitro* and then infected overnight with the JunΔ169 or β-galactosidase adenovirus at MOIs that led to expression of the recombinant proteins in ~50% of the infected neurons (14). After infection, the cells were refed with + NGF medium and ~24 h later were rinsed and maintained in medium containing NGF or a neutralising anti-NGF antibody for 16 h. RNA was then isolated and semi-quantitative RT–PCR was performed to measure *dp5* and *nfm* mRNA levels (Figure 8d). Expression of JunΔ169 did not alter the level of the *nfm* mRNA (Figure 8d). In the case of sympathetic neurons infected with AdvlacZ, the *dp5* mRNA increased 6.02-fold after NGF withdrawal, but in cells infected with AdvJunΔ169 the induction was reduced to 2.34-fold (Figure 8e). This result suggests that in sympathetic neurons AP-1 activity contributes to the induction of the endogenous *dp5* mRNA following NGF withdrawal.

Microinjection of an antibody against c-Jun, but not Jun B or Jun D antibodies or control IgG, has been shown to inhibit the NGF withdrawal-induced death of sympathetic neurons (6). To study the individual contributions of c-Jun and ATF2 to the activation of *dp5*-LUC + ALL after NGF withdrawal we therefore carried out an antibody co-injection experiment (Figure 9a). The *dp5* reporter construct was injected into the nuclei of sympathetic neurons together with control rabbit immunoglobulin or the c-Jun or ATF2 antibodies that had been used for chromatin immunoprecipitation. With the control antibody, *dp5*-LUC + ALL was activated 5.3-fold on average after NGF withdrawal whereas co-injection of the c-Jun or ATF2 antibodies reduced the induction factor to 2.83- or 2.47-fold, respectively. At the concentration tested, neither of these antibodies affected basal promoter activity in the presence of NGF. Importantly, the c-Jun and ATF2 antibodies did not affect the activation of pCRE-Luc, a CREB reporter construct, when CPTcAMP, a membrane permeable cAMP analogue, was added to the cells to activate protein kinase A and CREB (Figure 9b).

Finally, we investigated the effect of co-injecting an expression vector for c-Jun (ala) with *dp5*-LUC + ALL (Figure 9c). c-Jun (ala) is a mutant of c-Jun in which all of the potential JNK phosphorylation sites (serines 63 and 73 and threonines 91 and 93) have been mutated to alanine (28). Expression of c-Jun (ala) significantly reduced the induction factor after NGF withdrawal from 5.47 ± 1.35-fold with pcDNA3 to 2.87 ± 0.72-fold. These results suggest that following NGF withdrawal both c-Jun and ATF2 contribute to the activation of the *dp5* promoter in *dp5*-LUC + ALL and that the JNK phosphorylation sites in c-Jun are necessary for full induction of the *dp5* promoter.

DISCUSSION

In sympathetic neurons the level of the *dp5* RNA increases substantially after NGF withdrawal, and this depends on the MLK-JNK pathway (21,22). Here, we have investigated the mechanism by which this protein kinase cascade regulates *dp5* expression, and propose a model by which this occurs (Figure 10). To identify regulatory sequences in the *dp5* gene we made reporter constructs containing different regions of *dp5* linked to luciferase. In microinjection experiments with sympathetic neurons, we found that constructs containing 1 kb of promoter sequence alone or with a short sequence from the intron and/or the whole 3′ UTR were all activated following NGF deprivation. The highest level of induction was observed when all three regions were present together in the *dp5*-LUC + ALL construct. The increase in *dp5* RNA level after NGF deprivation was reduced by treating cells with the MLK inhibitor CEP-11004, confirming the results of Harris and Johnson (22), who used the related compound CEP-1347. Not only did CEP-11004 decrease *dp5* expression after NGF withdrawal, but it also reduced the induction of a *dp5* luciferase reporter plasmid, suggesting that this construct behaves in a similar manner to the endogenous *dp5* gene. We also co-injected sympathetic neurons with the *dp5* reporter construct and an expression vector for the JIP-1 JBD. This JNK inhibitor protein strongly reduced the induction of *dp5*-LUC + ALL after NGF withdrawal, suggesting that the activation of *dp5* transcription by MLKs is mediated by JNKs.

We identified a conserved ATF-binding site in the *dp5* promoter that is similar to the jun2 TRE in the *c-jun* promoter. We showed that this site is able to bind c-Jun and ATF2 *in vitro* and in chromatin, and to study its role in the *dp5* promoter we mutated it in a *dp5* reporter construct so as to abolish AP-1 binding. This reduced the induction of the reporter plasmid after NGF withdrawal and significantly reduced basal promoter activity suggesting that this site is an important *dp5* promoter element. In sympathetic neurons cultured in the presence of NGF, the *dp5* ATF site may contribute to basal promoter activity by binding c-Jun/ATF2 heterodimers. After NGF withdrawal, the level of active, phosphorylated c-Jun present in the nucleus increases (9,10) and this would lead to an increase in the activity of c-Jun/ATF2 heterodimers,

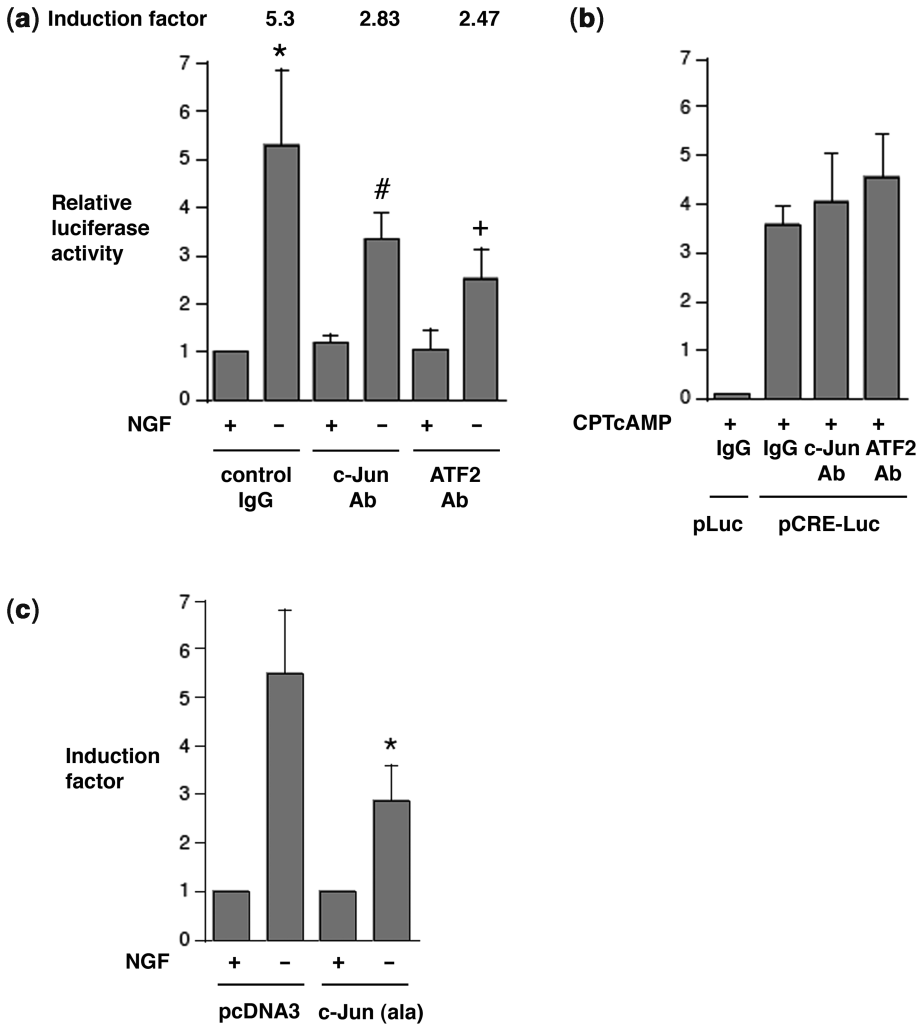

Figure 9. Co-injection of antibodies specific for c-Jun or ATF2 reduces the induction of a *dp5* reporter gene after NGF withdrawal. (**a**) Sympathetic neurons were cultured for 6 days *in vitro* and then microinjected with *dp5*-LUC + ALL (20 ng/μl), pRL-TK (10 ng/μl) and the c-Jun (H-79) or ATF2 (C-19) antibodies or rabbit immunoglobulin as a control (each at 1 μg/μl) as indicated. After injection, the cells were maintained in medium containing NGF (+) or anti-NGF antibody (−) for 20 h before luciferase activity was measured. For each experiment the level of normalized luciferase activity (firefly output/Renilla output) obtained for neurons injected with *dp5*-LUC + ALL + control IgG and maintained in medium containing NGF was set as 1 and other values were calculated relative to this (relative luciferase activity). The mean ± SEM for five independent experiments is shown, and induction factors (−NGF/+NGF) are given above the graph. Student's *t*-test was used to determine whether inductions were significant: $^{*}P < 0.05$; $^{\#}P < 0.02$; $^{+}P > 0.1$. (**b**) Sympathetic neurons were microinjected with pLuc-MCS or pCRE-Luc at 20 ng/μl, pRL-TK (10 ng/μl) and the indicated antibodies (1 μg/μl). After injection the cells were treated with 500 μM CPTcAMP to activate PKA and CREB. Normalized luciferase activity was calculated relative to pLuc-MCS, which lacks CRE sites and which was set as 0.1. The mean ± SEM for four independent experiments is shown. (**c**) Expression of c-Jun (ala) significantly reduces the induction of the *dp5* promoter after NGF withdrawal. *Dp5*-LUC + ALL (20 ng/μl), pRL-TK (10 ng/μl) and pcDNA3 or CMV c-Jun (ala) (100 ng/μl) were microinjected into sympathetic neurons as indicated. The cells were then maintained in medium containing NGF (+) or anti-NGF antibody (−) for 20 h before luciferase activity was measured. Firefly luciferase levels were normalized and luciferase activity was calculated relative to the level +NGF, which was set as 1, for either pcDNA3 or CMV c-Jun (ala). The mean of seven independent experiments ± SEM is shown. $^{*}P < 0.05$ when *Dp5*-LUC + ALL + CMV c-Jun (ala) –NGF is compared to *Dp5*-LUC + ALL + pcDNA3 –NGF in Student's *t*-test.

which could contribute to the induction of *dp5* transcription.

To further study the role of AP-1 in the induction of *dp5* transcription we tested the effect of overexpressing a c-Jun dominant negative mutant, JunΔ169, that can form heterodimers with ATF2 and bind to ATF sites, but which lacks the first 168 amino acids of c-Jun including the N-terminal transactivation domain, the JNK docking site and the JNK phosphorylation sites. We found that JunΔ169 reduced the induction of a *dp5* reporter construct and also decreased endogenous *dp5* RNA and protein levels after NGF withdrawal suggesting that *dp5* is an AP-1 target gene and that the MLK-JNK pathway activates *dp5* transcription via the ATF site (Figure 10). In addition, co-microinjection of either a c-Jun or ATF2 antibody together with *dp5*-LUC + ALL reduced the activation of the reporter construct after NGF withdrawal suggesting that both

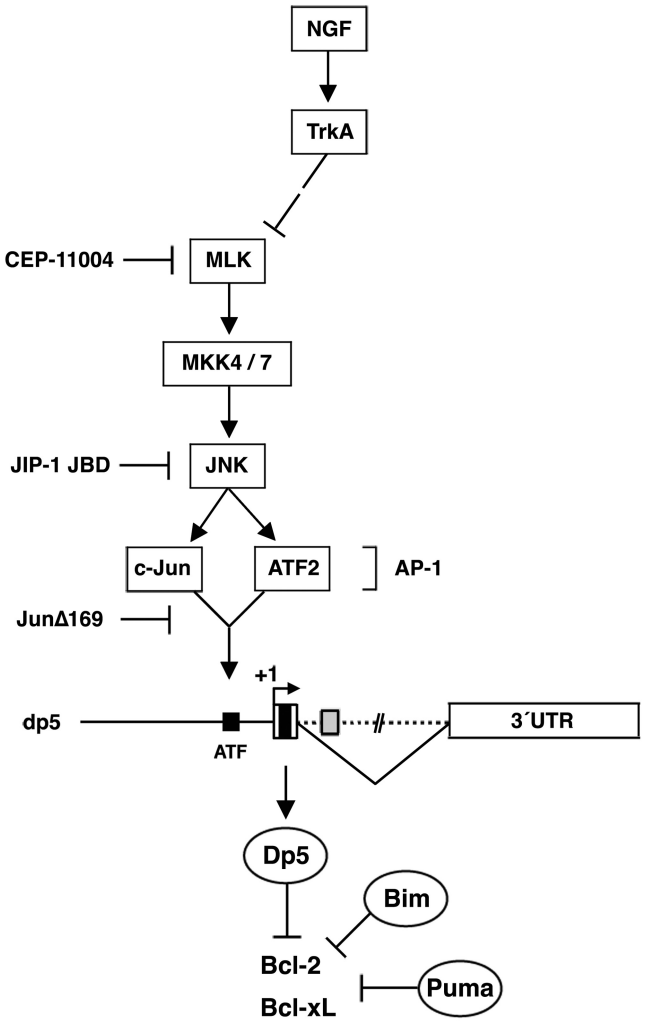

Figure 10. Proposed model showing the mechanism by which the JNK pathway contributes to increased expression of Dp5 after NGF withdrawal. Following NGF withdrawal, the MLK-MKK4/7-JNK pathway is activated leading to an increase in JNK activity and increased phosphorylation and increased expression of the AP-1 transcription factor c-Jun, which can form heterodimers with ATF2 (6–13). The *dp5* promoter contains a conserved ATF-binding site (5′-TGATGTAA-3′), which is bound by c-Jun and ATF2 *in vitro* and in chromatin. These AP-1 proteins contribute to increased transcription of the *dp5* promoter after NGF withdrawal, which depends on JNK and AP-1 activity. Mutation of the ATF site, expression of JunΔ169 or the JIP1-JBD, or treatment of sympathetic neurons with the MLK inhibitor CEP-11004 all reduce induction of the *dp5* promoter after NGF withdrawal. Together with the other BH3-only proteins induced by NGF withdrawal (Bim_{EL} and Puma) the Dp5 protein may promote cell death by binding to antiapoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-x_L (25,26).

c-Jun and ATF2 contribute to the activation of the *dp5* promoter (Figure 9).

The expression of Dp5 increases in a range of neural cell types following injury, stress or survival factor withdrawal, and several studies suggest that JNK may regulate Dp5 levels in response to a variety of apoptotic stimuli. *dp5* mRNA levels increase in cultured rat cortical neurons following treatment with β-amyloid peptide (41) and this increase can be blocked by CEP-1347 (38). Work carried out with oligodendrocytes also suggests that the β-amyloid-induced upregulation of *dp5* and cell death is mediated by the JNK pathway since treatment with β-amyloid peptide increased JNK phosphorylation and AP-1 DNA-binding activity (42). Recently, Ma *et al.* (43) reported that expression of shRNAs against *dp5* can partially protect rat cerebellar granule neurons (CGNs) against apoptosis induced by KCl deprivation. In addition, these authors also demonstrated that the ATF site in the *dp5* promoter is bound by c-Jun in CGNs and important for *dp5* promoter activation following survival signal withdrawal, in agreement with the results reported here.

Since the discovery that NGF withdrawal-induced death requires *de novo* gene expression (3) our understanding of the events that occur in sympathetic neurons following NGF deprivation has greatly increased and a number of the genes involved in this process have been identified. The MLK-JNK-c-Jun pathway is one of the important proapoptotic signalling pathways activated after NGF withdrawal (Figure 10). Activation of the MLK-JNK pathway leads to the phosphorylation of c-Jun bound to the *c-jun* promoter, which increases the rate of transcription of the *c-jun* gene and increases the level of c-Jun, an important JNK substrate which is required for the normal NGF withdrawal-induced death of sympathetic neurons (15). c-Jun contributes to the increased expression of the BH3-only proteins Bim_{EL} (14,24) and Dp5 (43, and this study), which together with Puma, bind to antiapoptotic Bcl-2 family members and promote mitochondrial outer membrane permeabilisation. In addition, JNKs can regulate Bim_{EL} at the post-translational level by phosphorylating serine 65 and potentiating its proapoptotic activity (44). Therefore in the future it will be important to identify other transcriptional targets of the JNK pathway as well as further investigate the post-translational effects of JNK phosphorylation.

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