Cellular/Molecular

Egr-1 Transactivates *Bim* Gene Expression to Promote Neuronal Apoptosis

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The proapoptotic BH3-only protein Bim is a crucial regulator of neuronal apoptosis. Previous studies have indicated the involvement of the c-Jun, FOXO1/3a, and B/C-Myb transcription factors in the regulation of Bim during neuronal apoptosis. However, the mechanism underlying the transcriptional regulation of Bim in activity deprivation-induced neuronal apoptosis has remained unclear. The present study demonstrates that early growth response 1 (Egr-1), rather than c-Jun, FOXO1/3a, or B/C-Myb, directly transactivates *Bim* gene expression to mediate apoptosis of rat cerebellar granule neurons. We showed that Egr-1 was sufficient and necessary for neuronal apoptosis. Suppression of Egr-1 activity using dominant-negative mutant or knockdown of Egr-1 using small interfering RNAs led to a decrease in Bim expression, whereas overexpression of Egr-1 resulted in induction of Bim. Deletion and site-directed mutagenesis of the Bim promoter revealed that *Bim* transcriptional activation depends primarily on a putative Egr-binding sequence between nucleotides −56 and −47 upstream of the start site. We also showed that Egr-1 binding to this sequence increased in response to activity deprivation *in vitro* and *in vivo*. Moreover, inhibition of Egr-1 binding to the *Bim* promoter, by mithramycin A and chromomycin A3, reduced the activity deprivation-induced increases in *Bim* promoter activity and mRNA and protein levels and protected neurons from apoptosis, further supporting the Egr-1-mediated transactivation of *Bim*. Additionally, Bim overcame the Egr-1 knockdown-mediated inhibition of apoptosis, whereas Bim knockdown impaired the increase in apoptosis induced by Egr-1. These findings establish *Bim* as an Egr-1 target gene in neurons, uncovering a novel Egr-1/Bim pathway by which activity deprivation induces neuronal apoptosis.

Introduction

The Bcl-2 homology 3 (BH3)-only members of the Bcl-2 protein family are key regulators of apoptosis (Youle and Strasser, 2008). Among the BH3-only proteins, Bim plays a critical role in a variety of neuronal death paradigms, including cerebellar granule neurons (CGNs) deprived of activity (Putcha et al., 2001; Shi et al., 2005), sympathetic neurons during removal of nerve growth factor (NGF) (Whitfield et al., 2001), and cortical neurons exposed to β-amyloid peptide (Biswas et al., 2007b; Yao et al., 2007). For example, CGNs and sympathetic neurons from *Bim* knock-out mice display a significant delay in apoptosis (Putcha et al., 2001; Coultas et al., 2007), demonstrating that Bim is a critical mediator of a proapoptotic signaling pathway in these neurons.

Transcriptional control is an important mechanism regulating *Bim* expression during neuronal apoptosis (Ham et al., 2005). Several studies have indicated that the c-Jun N-terminal protein kinase (JNK)/c-Jun pathway is involved in the regulation of *Bim* in sympathetic neurons and in CGNs (Harris and Johnson, 2001; Whitfield et al., 2001; Biswas et al., 2007a); however, in CGNs, we and others found that the JNK/c-Jun pathway has no effect on *Bim* upregulation (Shi et al., 2005; Ma et al., 2007; Hongisto et al., 2008). Other transcriptional regulators of *Bim* expression include the FOXO transcription factors in sympathetic neurons (Gilley et al., 2003; Biswas et al., 2007a) and in other cell types, such as lymphocytes (Dijkers et al., 2000), although a role for FOXO in CGNs after activity deprivation is less clear. This point is addressed further in Discussion. Although Bim is also a target of the Cdk4/E2F/B/C-Myb pathway in sympathetic neurons and PC12 cells (Biswas et al., 2005), it is not known whether B-Myb and C-Myb are involved in the regulation of *Bim* in CGNs.

Early growth response 1 (Egr-1) (also called NGFI-A, Zif268, or Krox24) is a zinc finger transcription factor implicated in a wide range of cell death paradigms (Thiel and Cibelli, 2002), including stress-induced apoptosis (Virolle et al., 2001), apoptosis in cancer cells (Muthukkumar et al., 1995; Chen et al., 2010), and inflammation-associated apoptosis (Lee et al., 2004). In the CNS, Egr-1 induction is observed in animal models of neuronal death-related diseases, such as Parkinson's disease (Smith et al., 1997) and ischemia (Tureyen et al., 2008). Although it has been reported that Egr-1 is upregulated (Catania et al., 1999) and plays a crucial role in the activity deprivation-induced apoptosis of CGNs (Levkovitz and Baraban, 2001), its direct targets in neuronal apoptosis are unknown.

Until now, there has been no evidence for a functional relationship between Egr-1 and Bim in neurons. In the present study,

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Bim induction in response to activity deprivation was shown to be independent of c-Jun, FOXO1/3a, or B/C-Myb transcription factors. We found that Egr-1 bound to the *Bim* promoter and directly transactivated *Bim* expression during apoptosis. Furthermore, Bim acted downstream of Egr-1 to mediate its proapoptotic effect. Together, this study has identified a proapoptotic Egr-1/Bim signaling mechanism underlying neuronal apoptosis.

Materials and Methods

Cell culture and transfection. Rat CGNs of either sex were prepared from 7- or 8-d-old Sprague Dawley rat pups as described previously (Ma et al., 2007; Yuan et al., 2009). Briefly, neurons were dissociated from freshly dissected cerebella by mechanical disruption in the presence of trypsin and DNase and then seeded at a density of 1.5×10^6 cells/ml in basal modified Eagle's medium containing 10% fetal bovine serum and potassium at concentrations that cause membrane depolarization (25 mM KCl). For immunofluorescence analysis, CGNs were seeded on coverslips (Thermo Fisher Scientific). Activity deprivation was performed as described previously (D'Mello et al., 1993; Shi et al., 2005; Yuan et al., 2009). After 7 d *in vitro* (DIV7), neurons maintained in serum containing 25 mM KCl were rinsed three times with serum-free media and incubated in serum-free media containing 25 or 5 mM KCl [25K (control) or 5K (activity deprivation)]. All experiments were performed using serum-free media, to avoid complications caused by the presence of survival factors in serum (Miller and Johnson, 1996; Song et al., 2010).

For the administration of inhibitors, DIV7 CGNs were incubated in 25K or 5K media in the presence or absence of the following reagents: SP600125 (anthra[1,9-cd]pyrazol-6(2*H*)-one) (Calbiochem), insulin-like growth factor-1 (IGF-1) (Invitrogen), mithramycin A (Biomol), and chromomycin A3 (Calbiochem). Cells that did not receive drugs received a control vehicle. To avoid toxicity, the final concentration of DMSO was <0.1%. Human embryonic kidney (HEK) 293 cells were cultured in DMEM with 10% fetal bovine serum. For transient gene transfection, DIV6 CGNs were transfected using the calcium phosphate transfection method described previously (Ma et al., 2007; Song et al., 2010). HEK 293 cells were transfected using Lipofectamine 2000.

Constructs and dual-luciferase reporter assays. Bim, *Egr-1*, and specificity protein 1 (*Sp1*) constructs were cloned from rat cDNA and incorporated into the pshuttle–Flag–IRES–hrGFP-1 expression vector. The dominant-negative (dn) Egr plasmid was generated by PCR amplification of the zinc finger region (amino acids 322–427) of mouse Egr-1 from pCMV–Flag–NLS–dnEgr (amino acids 322–533) as described previously (Zhang et al., 2003). Briefly, the PCR product (forward primer, 5′-GTTTAGTGAACCGTCAGAAT-3′ and reverse primer, 5′-AATTGATATCTTAGTCTGCTTTCTTGTCCTTCT-3′) was cloned into the HindIII and EcoRV sites of the pCMV–Flag–NLS expression vector. The pEBGN–dnSp1 plasmid, which encodes a fusion protein consisting of glutathione *S*-transferase and the Sp1 zinc finger DNA-binding domain, has been described (Petersohn and Thiel, 1996; Al-Sarraj et al., 2005). The pcDNA3–dnMyb expression plasmid (Santilli et al., 2005) and the Myb response reporter pMIM-1 (Sala et al., 1999) were gifts from Dr. Arturo Sala (University College London, London, United Kingdom). The p6MBS Myb response reporter was provided by Dr. Toru Nakano (Osaka University, Osaka, Japan) (Takahashi et al., 2000). The Egr-responsive reporter plasmid pEBS1^4 has been described previously (Thiel et al., 1994).

The *Bim*–luciferase (Luc) reporter, containing a 5.2 kb DNA fragment spanning nucleotides −2545 to +2658 of the *Bim* 5′-flanking region, relative to the transcription start site, and the *Bim*–Luc (dmFOXO) reporter, incorporating mutations of two FOXO binding sites, were kindly provided by Dr. Jonathon Ham (University College London, London, United Kingdom) and have been described previously (Gilley et al., 2003). A series of reporter constructs with the same 3′ terminus but different 5′ termini, were also constructed by PCR using *Bim*–Luc as the template and the common reverse primer 5′-CATTTGGTCTTTTTTTCTGCAAGGAAACCGGAGGG-3′. The forward primers were as follows: 5′-GATCCCAGTACAGCTCATGGCG-3′ for *Bim* (nucleotides −846 to +2658)–Luc, 5′-GGCGGCGCACGCAAACTGCAG-3′ for *Bim* (ΔMyb, nucleotides −187 to +2658)–Luc, 5′-GGACTTACCTTCCGGCTTGCC-3′ for *Bim* (nucleotides −90 to +2658)–Luc, and 5′-GCAGGCACTGGGTCAACAGC-3′ for *Bim* (nucleotides +36 to +2658)–Luc. All PCR products were subcloned as an XhoI/HindIII fragment into the plasmid pGL3-Basic (Promega). The mutant *Egr* site (GCGGGGGGCG to ACAAGAAACC) (Tremblay and Drouin, 1999) was introduced into the 5.2 kb *Bim*–Luc plasmid by overlap extension PCR. First, two DNA fragments with a 17 bp overlap (TGAACAAGAAACCGAGC) containing the mutant *Egr* site were amplified by PCR from the 5.2 kb *Bim*–Luc plasmid using the following primer pairs: forward, 5′-CTCAGATTCACAGAGACTTCG-3′ and reverse, 5′-GCTCGGTTTCTTGTTCAGTCCGGCCCGGGCAAGC-3′; and forward, 5′-TGAACAAGAAACCGAGCCCGCGGTGATTG-3′ and reverse, 5′-CATTTGGTCTTTTTTTCTGCAAGGAAACCGGAGGG-3′. The full-length *Bim* promoter (5.2 kb), containing the mutant Egr site, was obtained by mixing the two DNA fragments produced from the first-step PCR and then using them as the template in the second PCR reaction with the outermost primers. The subsequent PCR product was subcloned as a KpnI/HindIII fragment into pGL3-Basic. All constructs were confirmed by DNA sequencing.

For the dual-luciferase reporter assays, cells were transfected with 1 μg of a luciferase reporter plasmid and 200 ng of the pRL–CMV *Renilla* luciferase reporter plasmid (Promega). After transfection, neurons were kept in conditioned media for 12 or 24 h and then transferred to serum-free media containing 25K or 5K for 12 h. *Firefly* luciferase activity was normalized to *Renilla* luciferase activity as reported previously (Ma et al., 2007).

Western blotting and antibodies. Western blot analysis was performed as described previously (Ma et al., 2007; Yuan et al., 2009). Briefly, lysates were separated using SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane. Membranes were blocked in Tris-buffered saline with 5% milk and 0.05% Tween 20 and probed with primary antibodies at 4°C overnight. The following primary antibodies were used: anti-Bim, anti-phospho-c-Jun (Ser73), anti-phospho-FOXO1/3a (Thr24/32), and anti-phospho-Akt (Ser473) [all from Cell Signaling Technology (CST)]; anti-Egr-1 and anti-Sp1 (from Santa Cruz Biotechnology); and anti-Flag and anti-Tubulin antibodies (from Sigma-Aldrich). Appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) were used for detection with the ECL and ECL-plus systems (GE Healthcare).

Reverse transcription-PCR. Total RNA was extracted and isolated from CGNs using TRIzol reagent (Invitrogen) as described previously (Ma et al., 2007). Reverse transcription (RT) was performed using SuperscriptIII reverse transcriptase (Invitrogen) and oligo-dT primers. The following primer pairs were used: *bim*, forward, 5′-CTACCAGATCCCCACTTTTC-3′ and reverse, 5′-GCCCTCCTCGTGTAAGTCTC-3′; *c-jun*, forward, 5′-TGGGCACATCACCACTACAC-3′ and reverse, 5′-AGTTGCTGAGGTTGGCGTA-3′; and β-actin, forward, 5′-CAACTGGGACGATATGGAGAAG-3′ and reverse, 5′-TCTCCTTCTGCATCCTGTCAG-3′.

RNA interference. Two *Bim* small interfering (si) RNAs (siBima and siBimb) were designed to target rat *bim* mRNA (NM_171988) sequences: 5′-CAACCAUUAUCUCAGUGCA-3′ and 5′-GACAGAGAAGGUGGACAAUUG-3′, respectively. Two *Egr-1* siRNAs (siEgr-1a and siEgr-1b) were used to target the rat *egr-1* mRNA (NM_012551) sequences: 5′-GGACUUAAAGGCUCUUAAU-3′ and 5′-GGACAAGAAAGCAGACAAA-3′, respectively. A nontargeting siRNA was used as a negative control for all siRNA transfection experiments. All siRNAs were synthesized by Shanghai GenePharma. The efficacy and specificity of siRNAs were determined as described previously (Ma et al., 2007; Yuan et al., 2009). Briefly, siBima, siBimb, siEgr-1a, or siEgr-1b, together with rat Flag–*Egr-1* or rat Flag–*Bim* plasmids, were transfected into HEK 293A cells for 24 h. Cell lysates were harvested and subjected to Western blotting using a Flag antibody.

Immunofluorescence. CGNs were grown on coverslips and processed according to the immunofluorescence protocol as described previously (Yuan et al., 2009). CGNs were fixed using freshly prepared 4% paraformaldehyde, followed by permeabilization with 0.1% Triton X-100 in TBS and blocking in 3% donkey serum. The cells on the coverslips were incubated with the following primary antibodies: rabbit anti-FOXO1 (sc-11350; diluted 1:50) and rabbit anti-FOXO3a (sc-11351; diluted 1:100) (from Santa Cruz Biotechnology); rabbit anti-Bim monoclonal antibody (catalog #2933; diluted 1:100), rabbit anti-Egr-1 monoclonal

antibody (catalog #4154; diluted 1:500), and mouse anti-β-galactosidase (β-Gal) monoclonal antibody (catalog #2372; diluted 1:100) (all from CST). Rabbit primary antibodies were detected with an anti-rabbit secondary antibody conjugated to Alexa Fluor 555 (Invitrogen). Mouse primary antibodies were detected using an anti-mouse secondary antibody conjugated to Alexa Fluor 488 (Invitrogen). Fluorescent staining was visualized using the 40× (numerical aperture 1.3) oil-immersion objective on a confocal microscope (LSM 710 Meta; Carl Zeiss). Images were recorded with sequential acquisition settings at a resolution of 512 × 512 pixels and 12-bit depth. All samples were scanned using identical confocal settings. Images were processed using the LSM 710 software (Carl Zeiss) and Photoshop CS2 (Adobe Systems). Cells were counted in an unbiased manner (at least 300 transfected cells for each group) and were scored blindly without previous knowledge of their treatments.

Immunofluorescence analysis of Bim protein levels in transfected CGNs required an immunofluorescence-grade anti-Bim antibody that did not detect significant nonspecific bands on Western blots (Putcha et al., 2002). Western blotting was used to test the specificities of four Bim antibodies (CST catalog #2933; Santa Cruz Biotechnology sc-11425; Stressgen AAP-330; and Millipore Bioscience Research Reagents AB17003) (data not shown). Of the four antibodies, only one (catalog #2933; designated CST anti-Bim antibody in this paper) specifically recognized Bim and was therefore chosen for the detection of Bim by immunofluorescence in CGNs. The effectiveness and specificity of the CST anti-Bim antibody was further confirmed by examining Bim protein levels after siRNA-mediated Bim knockdown, and this antibody was used throughout the present study to detect Bim. In addition, all immunofluorescence-grade antibodies used in this study were tested to ensure that they did not detect significant nonspecific proteins on a Western blot (data not shown).

Gel mobility shift assays. Nuclear extracts were prepared as described previously (Li et al., 2001; Yuan et al., 2009). Consensus binding site probes for Sp1 or Egr transcription factors were synthesized according to TransCruz Gel Shift Oligonucleotides from Santa Cruz Biotechnology (Sp1 probe, sc-2502; Egr probe, sc-2529). The *Bim* probes contained dual putative Egr binding sites [GCGGGGGGCG from the *Bim* promoter (nucleotides −56 to −47 relative to the transcription initiation site; forward, 5′-GGATCCA**GCGGGGGGCG**A**GCGGGGGGCG**A-3′; reverse, 5′-T**CGCCCCCCGCTCGCCCCCCGC**TGGATCC-3′] as well as those bearing mutated putative Egr sites (forward, 5′-GGATCCA**ACAAGAAACC**AAC**AAGAAACC**A-3′; reverse, 5′- T**GGTTTCTTGTTGGTTTCTTGT**TGGATCC-3′). Synthetic oligonucleotides were annealed and labeled with [γ-^{32}P]ATP (PerkinElmer Life and Analytical Sciences) using T4 polynucleotide kinase (New England Biolabs). Binding reactions were performed for 20 min at 24°C in binding buffer [0.5 mM dithiothreitol, 0.5 mM $MgCl_2$, 0.5 mM $ZnSO_4$, 0.5 mM EDTA, 50 mM NaCl, 4% glycerol, 50 ng/ml poly(dI-dC), and 10 mM Tris-HCl, pH 7.5] with labeled oligomers (100,000 to 150,000 cpm) in a total volume of 20 μl. For supershift analysis, 2 μg of anti-Egr-1 (sc-189x) and anti-Sp1 antibodies (sc-59x) (both from Santa Cruz Biotechnology) or preimmune serum were added to nuclear extracts for 1 h at 4°C, followed by an additional 20 min at 24°C in the presence of labeled probes. For drug analysis, mithramycin A or chromomycin A3 were added to probes for 20 min at 24°C, followed by another 20 min at 24°C in the presence of nuclear extracts. The protein–DNA complexes were analyzed on a 4% nondenaturing polyacrylamide gel containing 3% glycerol and 0.25× Tris-borate-EDTA at 4°C and exposed to photography.

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP assay kit (Upstate Cell Signaling Solutions) according to the instructions of the manufacturer and as described previously (Ma et al., 2007). Two micrograms of Egr-1 antibody (sc-189x) were used for immunoprecipitation. Purified DNA was subjected to PCR amplification using primers spanning the *Bim* promoter Egr site (forward, 5′-ACCCTCCCCACTACCTG-3′; reverse, 5′-GTGACTGCAACGACAACG-3′).

Survival assays. CGN apoptosis was quantified as described previously (Ma et al., 2007). DIV6 CGNs were transfected with plasmids or siRNAs using the calcium phosphate coprecipitation method in addition to pCMV–GFP, which marks transfected cells. Twenty-four hours after transfection, neurons were incubated in media containing 25K or 5K. Neurons were stained with Hoechst 33258 (5 μg/ml) to visualize nuclear morphology and propidium iodide (PI) (0.25 μg/ml) to detect membrane damage. Apoptosis was quantified by scoring the number of PI-positive cells and/or cells with pyknotic nuclei relative to the total number of green fluorescent protein (GFP)-positive cells in the same visual field. Cells were counted in an unbiased manner (at least 800 transfected cells for each group) and were scored blindly without previous knowledge of their treatment. The overall transfection efficiency using the calcium phosphate method in CNG culture was ~ 1%.

Statistical analysis. All experiments were repeated at least three times using independent culture preparations. All measurements were performed blindly. The significance of difference between means was analyzed by the ANOVA and *post hoc* Bonferroni/Dunn tests (for multiple comparisons) and by the Student's *t* test (for single comparisons). The results are represented as means ± SEM. Statistical significance was determined by a value of $p < 0.05$ for all analyses.

Results

Bim is critical for apoptosis induced by activity deprivation in CGNs

To induce apoptosis by activity deprivation, DIV7 CGNs, cultured in 25K plus serum, were transferred to 5K serum-free medium (D'Mello et al., 1993). CGNs were also transferred to 25K serum-free medium as a control. This model has been used by us (Liu et al., 2009; Yuan et al., 2009) and others (Miller and Johnson, 1996; Watson et al., 1998; Morrison et al., 2006) to delineate mechanisms of activity deprivation-induced neuronal apoptosis (Contestabile, 2002).

In CGNs, increased Bim expression, in response to activity deprivation, has been reported by others (Harris and Johnson, 2001; Putcha et al., 2001) and later by us (Shi et al., 2005; Ma et al., 2007). In the current study, activity deprivation resulted in a dramatic increase in *bim* mRNA and protein levels (Fig. 1*A*). To confirm that the *Bim* gene is transcriptionally upregulated, CGNs were transfected with a 5.2 kb *Bim* promoter (nucleotides −2545 to +2658) based luciferase reporter. This *Bim*–Luc reporter was constructed and kindly provided by Dr. Ham's group (Gilley et al., 2003; Gilley and Ham, 2005) and has been used previously in our laboratory (Shi et al., 2005). Culturing CGNs in 5K media led to an approximately fourfold increment in *Bim* promoter activity (Fig. 1*A*, right), confirming that *Bim* is transcriptionally upregulated by activity deprivation.

To determine the role of Bim in CGN apoptosis, two siRNAs were designed to reduce rat Bim protein levels. These siRNAs completely abolished the expression of a transfected rat Flag–Bim construct in HEK 293 cells (Fig. 1*B*, left). In CGN cells, immunofluorescence analysis using the CST anti-Bim antibody (catalog #2933; described in Materials and Methods) revealed a significant increase in Bim protein levels after activity deprivation, which was abolished by the two Bim siRNAs (Fig. 1*B*, middle and right), demonstrating the efficacy of the siRNAs in CGNs. In addition, there was a significant decrease in neuronal apoptosis after the siRNA knockdown of Bim (Fig. 1*C*). These results are consistent with previous studies (Harris and Johnson, 2001; Putcha et al., 2001; Shi et al., 2005) and confirmed that Bim is induced by activity deprivation and is critical for neuronal apoptosis.

c-Jun, FOXO1/3a, and B/C-Myb are not involved in the regulation of Bim induction

We have reported previously that the JNK/c-Jun pathway is not involved in the transcriptional upregulation of Bim during apo-

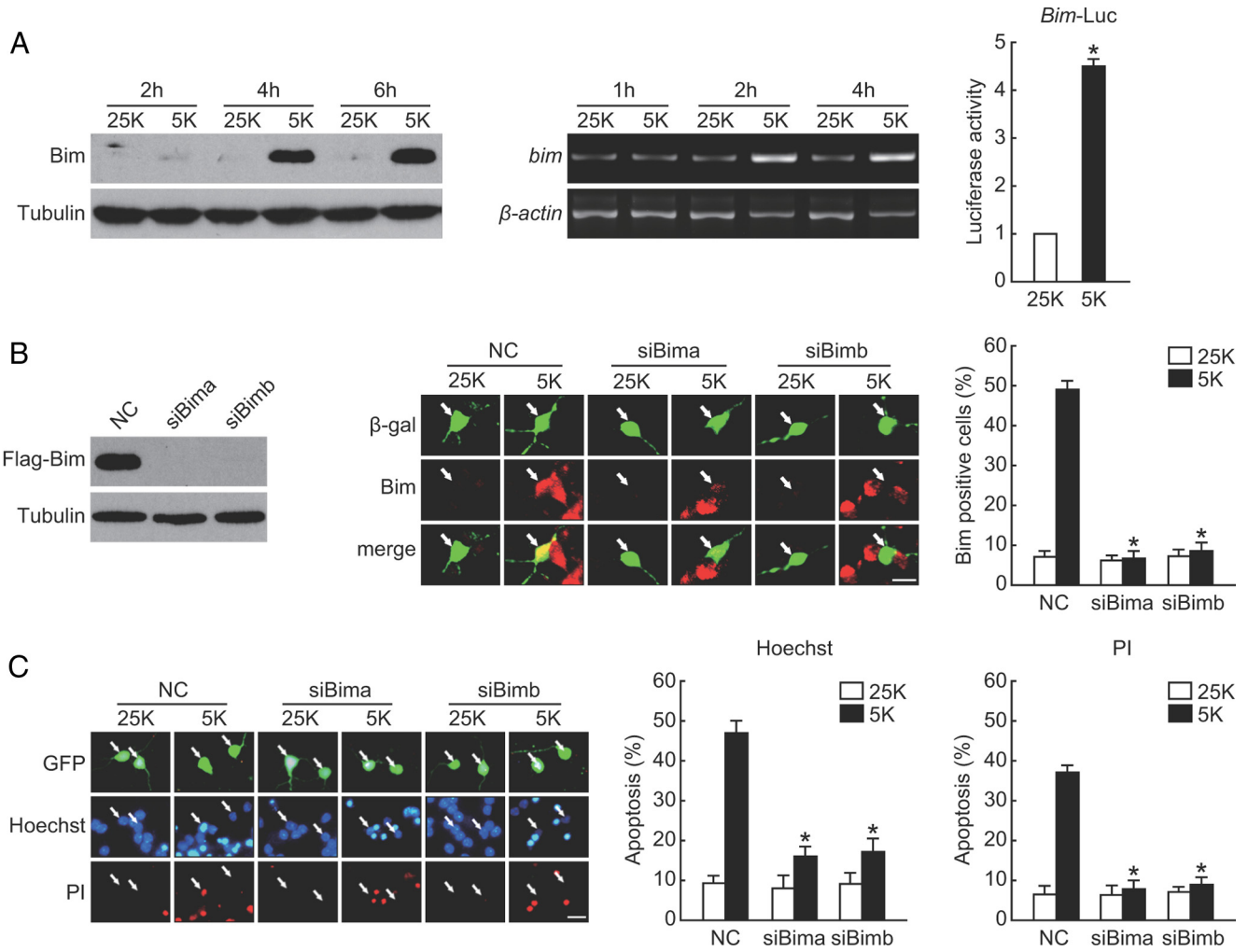

Figure 1. Bim is essential for activity deprivation-induced apoptosis in CGNs. ***A***, Activity deprivation-induced Bim expression. DIV7 CGNs were incubated in 25K (control) or 5K media for the times indicated. Cell lysates were subjected to Western blotting using Bim and β-Tubulin antibodies (left). RT-PCR was performed using *bim*- and β-*actin*-specific primers (middle panel). DIV6 neurons were transfected with 1 μg of *Bim*–Luc for 12 h. After incubation in 25K or 5K media for 12 h, dual-luciferase reporter assays were performed. The levels of luciferase activity were normalized to *Renilla* luciferase activity (right). ***B***, The efficacy and specificity of *Bim* siRNAs. HEK 293 cells were transfected with *Bim* siRNAs, or negative control RNAs (NC), together with a rat Flag–Bim plasmid, for 24 h. Cell lysates were harvested and subjected to Western blotting using a Flag antibody (left). DIV6 CGNs were transfected with Bim siRNAs, or NC, together with β-Gal, for 24 h. After incubation in 25K or 5K media for 6 h, neurons were stained for Bim (red) and β-Gal (green) (middle). Scale bar, 10 μm. The percentage of Bim-positive neurons was quantified (right). The percentage of Bim-positive neurons represents the proportion of transfected cells (as identified by staining for β-Gal) exhibiting higher levels of Bim staining compared with control cells in 25K media. ***C***, Knockdown of Bim suppresses CGN apoptosis. DIV6 CGNs were transfected with *Bim* siRNAs, or the NC, together with GFP for 24 h (left). After incubation in 25K or 5K media for 12 h, CGNs were double stained with Hoechst 33258 and PI. Apoptosis was determined by the percentage of GFP-positive cells that were also PI positive or had pyknotic nuclei (middle and right). Scale bar, 10 μm. All data in this figure represent the means ± SEM of three independent experiments. $^*p < 0.05$.

ptosis of CGNs (Shi et al., 2005; Ma et al., 2007), which is consistent with results from another study using the JNK inhibitor SP600125 (Hongisto et al., 2008). To further demonstrate that JNK does not regulate Bim transcriptionally, we showed that SP600125 had no effect on the degree of Bim expression in 5K media (Fig. 2*A*), although it completely abrogated activity deprivation-induced c-Jun phosphorylation, c-*jun* mRNA upregulation, and neuronal apoptosis (Fig. 2*A*), which is consistent with previous reports by us (Xie et al., 2004; Shi et al., 2005) and others (Cao et al., 2004; Xifró et al., 2006). Thus, these results clearly demonstrated that the JNK/c-Jun pathway is not involved in *Bim* expression and that the inhibition of JNK may block neuronal apoptosis through mechanisms independent of Bim transcription (see Discussion).

The FOXO transcription factors FOXO1 and FOXO3a undergo nucleocytoplasmic shuttling and modulate neuronal apoptosis after phosphorylation by Akt in response to IGF-1 signaling (Brunet et al., 1999; Tran et al., 2003). In this study, there was no difference in the phosphorylation of FOXO1 (Thr24), FOXO3a (Thr32), or its upstream kinase Akt (Ser473) in CGNs in either 25K or 5K media, although there was significant upregulation of *bim* mRNA and protein levels in 5K media compared with 25K media (Fig. 2*B*). Although IGF-1 stimulated the increased phosphorylation of FOXO1/3a and Akt (Fig. 2*B*) and sequestered FOXO1/3a in the cytoplasm (Fig. 2*C*) as reported previously (Brunet et al., 1999; Tran et al., 2003), it did not repress Bim induction, as determined by Western blotting and RT-PCR analysis (Fig. 2*B*, left and middle). In agreement with previous studies by us (Li et al., 2000) and others (D'Mello et al., 1993; Dudek et al., 1997), treatment with IGF-1 rescued CGNs from apoptosis in 5K media (Fig. 2*B*, right), suggesting that IGF-1 may promote neuronal survival by a Bim transcription-independent mechanism (see Discussion). No nucleocytoplasmic shuttling of FOXO1/3a occurred in either the 25K or 5K

medium (Fig. 2*C*), consistent with our recent study, which showed that phosphatidylinositol 3-kinase (PI3K)/Akt signaling remained constant in both 25K and 5K media (Song et al., 2010). In addition, a *Bim*–Luc reporter (dmFOXO), with two mutated FOXO binding sites (Gilley et al., 2003), remained fully responsive to activity deprivation (Fig. 2*D*), further confirming that FOXO1/3a does not regulate the activity deprivation-mediated induction of Bim in CGNs.

It has been reported that B-Myb and C-Myb are upregulated and are involved in the control of Bim during the apoptosis of sympathetic neurons and PC12 cells, induced by NGF deprivation (Liu and Greene, 2001; Biswas et al., 2005). Although we confirmed that the B-Myb and C-Myb antibodies (sc-13028 and sc-517, respectively; Santa Cruz Biotechnology) were able to recognize rat B-Myb and C-Myb overexpression in HEK 293 cells, no endogenous B-Myb or C-Myb expression was detected in CGNs (data not shown). To assess further whether activity deprivation stimulates Myb transactivation activity, two widely used and well-recognized Myb-responsive luciferase reporter plasmids, the pMIM-1 reporter (Sala et al., 1999) and the p6MBS reporter (Takahashi et al., 2000), were used. Activity deprivation did not induce luciferase activity from either pMIM-1 or p6MBS (Fig. 2*E*). Moreover, the transfection of dnMyb construct, which inhibits B/C-Myb-dependent gene expression (Santilli et al., 2005), failed to decrease *Bim*–Luc activity in 5K media (Fig. 2*E*). Furthermore, eliminating two previously identified Myb binding sites (Biswas et al., 2005) from the *Bim*–Luc construct (ΔMyb, nucleotides −187 to +2658) did not affect its response to activity deprivation (Fig. 2*E*). In conclusion, the data showed that the B/C-Myb proteins are not induced by activity deprivation and are not involved in the regulation of Bim expression in response to activity deprivation in CGNs.

Together, these results suggested that activity deprivation in CGNs induces *Bim* expression by a mechanism that does not require c-Jun, FOXO1/3a, or B/C-Myb.

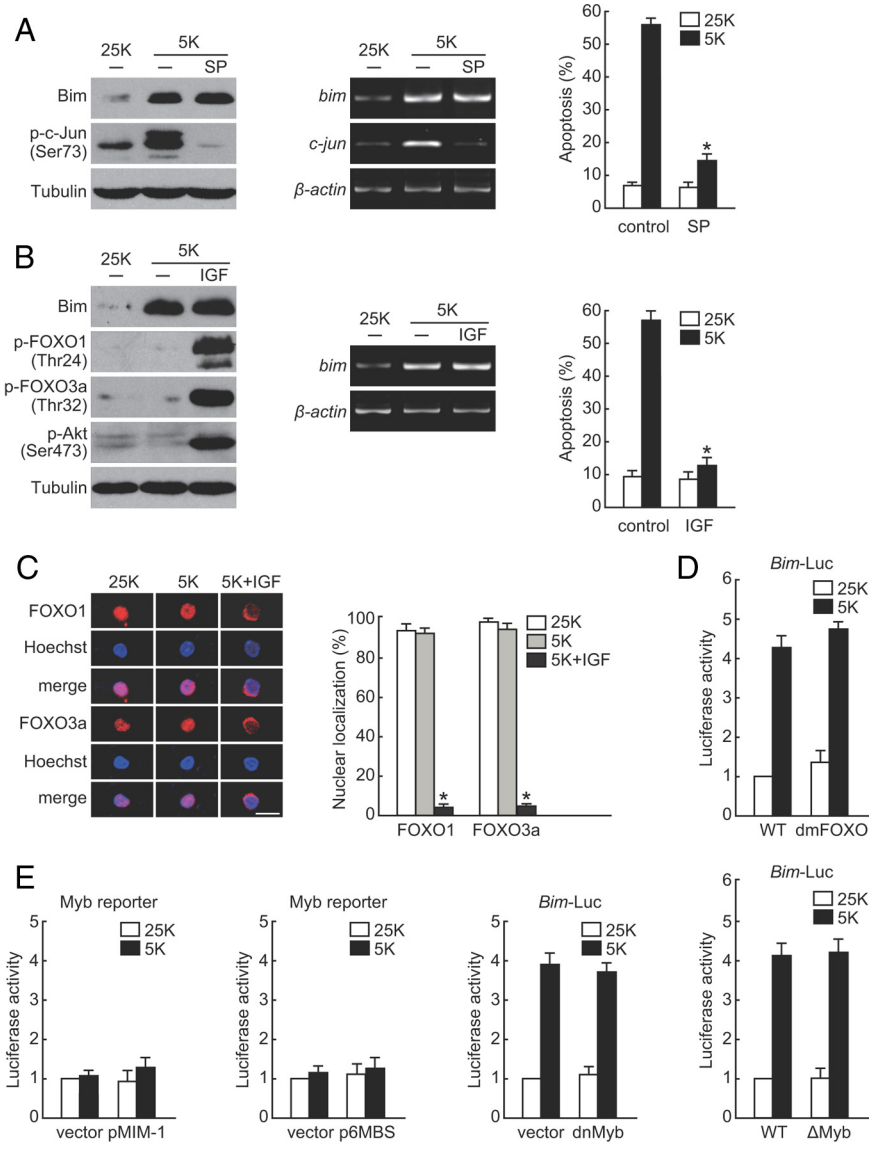

Figure 2. c-Jun, FOXO1/3a, and B/C-Myb are not involved in the regulation of Bim induction. ***A***, DIV7 CGNs, maintained in media containing 25K, 5K, or 5K plus 5 μM of the JNK inhibitor SP600125 (SP) for 4 h, were lysed and subjected to Western blotting with the antibodies indicated (left). Total mRNA was extracted and subjected to RT-PCR with primers specific to *bim*, *c-jun*, and *β-actin* (middle). Apoptosis assays of CGNs with the same treatments were performed and analyzed as described in Figure 1C (right). ***B***, DIV7 CGNs, maintained in media containing 25K, 5K, or 5K plus 100 ng/ml IGF-1 (IGF) for 4 h, were lysed and subjected to Western blotting with the antibodies indicated (left). RT-PCR was performed as described in ***A*** (middle). Apoptosis of CGNs with the same treatments was analyzed as described in Figure 1*C* (right). ***C***, DIV7 CGNs, maintained in media containing 25K, 5K, or 5K plus 100 ng/ml IGF-1 (IGF) for 4 h, were stained for FOXO1, FOXO3a, and Hoechst 33258 and were analyzed by fluorescence microscopy. Representative images are shown on the left, and the results are quantified on the right. Scale bar, 10 μm. ***D***, DIV6 CGNs were transfected with 1 μg of wild-type *Bim*–Luc (WT) or 1 μg of mutated *Bim*–Luc (dmFOXO), carrying two mutated FOXO binding sites on the *Bim* promoter. After incubation in 25K or 5K media for 12 h, dual-luciferase reporter assays were performed as described in Figure 1*A*. ***E***, DIV6 CGNs were transfected with 1 μg of pMIM-1, p6MBS, or control vector for 12 h (two panels on the left); DIV6 CGNs were transfected with 1 μg of *Bim*–Luc (WT) together with dnMyb, or control vector for 24 h, or 1 μg of truncated *Bim* (ΔMyb, nucleotides −187 to +2658)–Luc whose B/C-Myb binding sites were deleted, for 12 h (two panels on the right). After incubation in 25K or 5K media for 12 h, dual-luciferase reporter assays were performed. All data in this figure represent the means ± SEM of three independent experiments. *$p < 0.05$.

GC-rich elements in the *Bim* promoter are responsible for *Bim* expression

The organization of the Bim promoter was analyzed to search for other transcription factors that may mediate *Bim* expression during apoptosis. A previous report revealed that the *Bim* promoter immediately upstream of exon 1 is very GC rich and contains multiple putative Sp1 recognition sites (Bouillet et al., 2001). This was confirmed by our own analysis, which revealed several putative Sp1 and Egr-1 recognition sites within the GC-rich region. To test whether the GC-rich elements are involved in Bim induction, two inhibitors, mithramycin A and chromomycin A3, which repress transcription by selectively displacing GC-rich DNA binding transcription factors, such as Sp1 and Egr-1 (Ray et al., 1989; Chiang et al., 1996; Lombó et al., 2006), were used. Both inhibitors significantly attenuated the activity deprivation-induced increase in the *Bim*–Luc reporter activity in a dose-dependent manner (Fig. 3*A*). Consistent with this

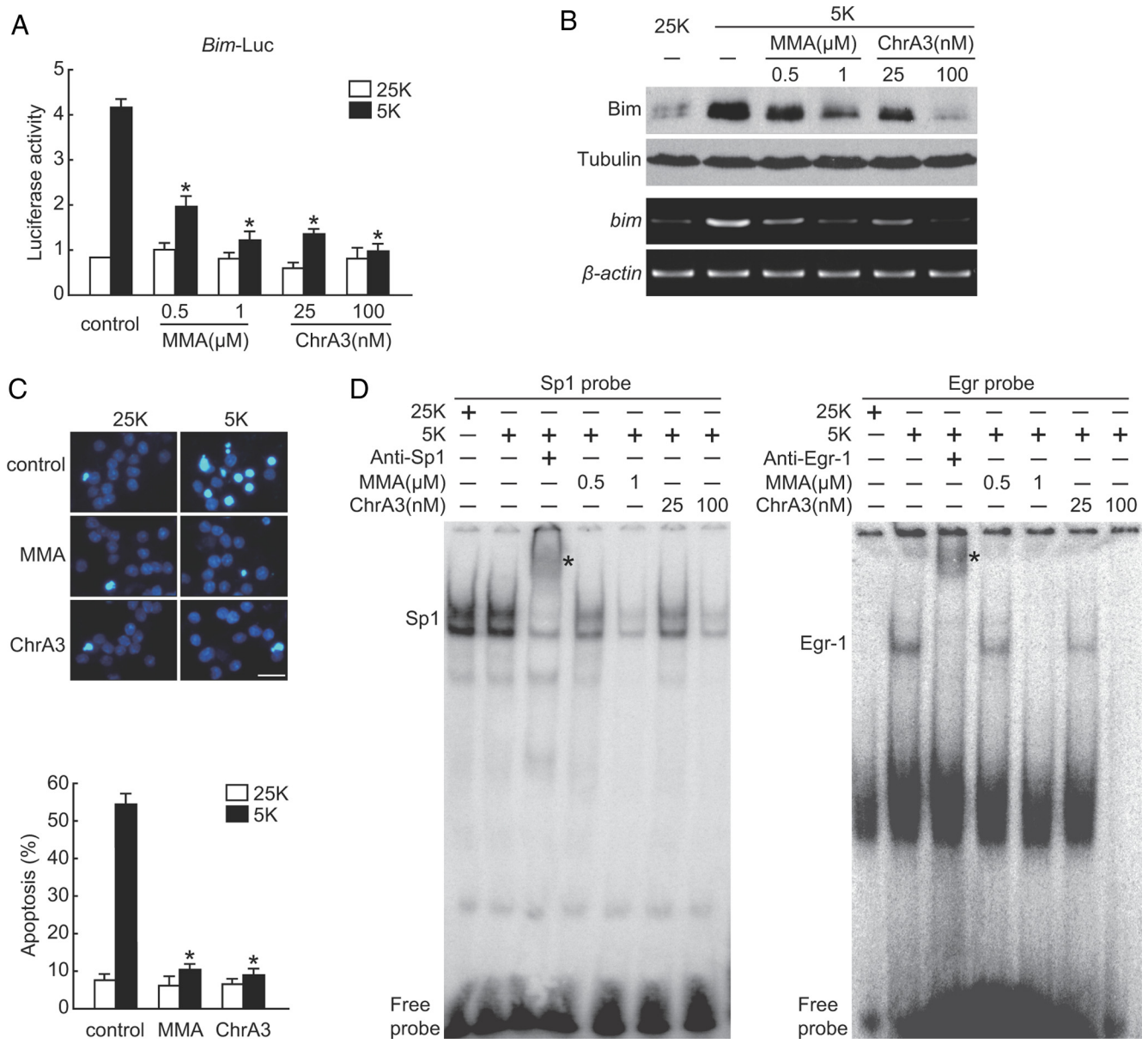

Figure 3. The GC-rich elements in the *Bim* promoter are responsible for *Bim* expression. ***A***, DIV6 neurons were transfected with 1 μg of *Bim*–Luc for 12 h. After incubation in 25K and 5K media, or 25K and 5K media plus either mithramycin A (MMA) or chromomycin A3 (ChrA3) for 12 h, dual-luciferase reporter assays were performed. Data represent the means ± SEM of three independent experiments. *$p < 0.05$. ***B***, DIV7 CGNs, cultured in 25K and 5K media, or 5K media with MMA or ChrA3 for 4 h, were lysed and subjected to Western blotting with the antibodies indicated (top). Total mRNA was extracted and subjected to RT-PCR with primers specific to *bim* and *β-actin* (bottom). ***C***, DIV7 CGNs were transferred to 25K or 5K media in the absence or presence of 1 μM MMA or 100 nM ChrA3. Twelve hours later, neurons were stained with Hoechst 33258. Representative imagines are shown (top). Neuronal apoptosis was quantified by scoring the percentage of total neurons with condensed or fragmented nuclei (bottom). Scale bar, 10 μm. Data represent the means ± SEM of three independent experiments. *$p < 0.05$. ***D***, Gel mobility shift assays using ^{32}P-labeled consensus binding site probes for Sp1 or Egr transcription factors with nuclear extracts prepared from DIV7 CGNs maintained in media containing 25 or 5 mM KCl for 2 h. Sp1 or Egr-1 antibodies were preincubated with nuclear extracts to allow formation of the DNA–protein complexes. MMA or ChrA3 were added to probes before incubation in the presence of nuclear extracts. *, band supershifted by Sp1 or Egr-1 antibody. The results are representative of three separate experiments.

result, both inhibitors also reduced the activity deprivation-induced increase in *bim* mRNA and protein levels in a dose-dependent manner (Fig. 3*B*), suggesting that GC-rich DNA sequences are involved in modulating the transcription of *Bim* during apoptosis. Furthermore, mithramycin A and chromomycin A3 produced a dramatic decrease in CGN apoptosis in 5K media (Fig. 3*C*), in agreement with previous studies, which showed that mithramycin A prevents CGN activity deprivation-induced apoptosis (Ferrante et al., 2004).

Although it is well documented that mithramycin A and chromomycin A3 repress transcription by displacing DNA-bound Sp1 (Ray et al., 1989; Chiang et al., 1996; Lombó et al., 2006), it is unclear whether they inhibit Egr-1 binding to DNA. To address this issue, gel mobility shift assays were performed using ^{32}P-labeled consensus binding site probes for Sp1 or Egr transcription factors with nuclear extracts prepared from CGNs. Adding either an Sp1 or Egr-1 antibody to the cell extracts led to a marked supershifting of the bands and concomitant reductions in the original DNA–protein complex. In contrast, the addition of mithramycin A or chromomycin A3 disrupted the formation of specific DNA–protein complexes (Fig. 3*D*), reflecting the inhibition of both Sp1 and Egr-1 binding to their

respective consensus sites. Together, the data suggest that activation of the GC-rich sequences in the *Bim* promoter is responsible for *Bim* expression.

Egr-1 mediates activity deprivation-induced neuronal apoptosis

It has been reported that Egr-1 is upregulated and plays a critical role in activity deprivation-induced CGN apoptosis, whereas Sp1 has no proapoptotic effects (Catania et al., 1999; Levkovitz and Baraban, 2001; Ryu et al., 2003). Consistent with these studies, Sp1 protein levels remained constant during activity deprivation, whereas Egr-1 levels increased within 1 h and continued increasing for up to 4 h (Fig. 4*A*). Furthermore, activity deprivation caused only a modest increase in activity from an Sp1-regulated reporter, pEBS6^4 (Al-Sarraj et al., 2005), which was overcome by a dnSp1 mutant that does not affect Egr activity (Petersohn and Thiel, 1996). In contrast, there was a robust response from the Egr-regulated pEBS1^4 reporter (Thiel et al., 1994), and this was repressed by the dnEgr mutant, which contains only the zinc finger DNA-binding domain (amino acids 322 to 427) (Fig. 4*B*) (Levkovitz and Baraban, 2001).

Next, the roles of Sp1 and Egr-1 in neuronal apoptosis were assessed. dnEgr, but not dnSp1, significantly attenuated apoptosis, whereas wild-type Egr-1 enhanced apoptosis in both 25K and 5K media (Fig. 4*C*,*D*). To identify the specific effect of Egr-1 in apoptosis, siRNAs that target rat *Egr-1* were developed and their efficacy verified by their ability to reduce the expression of rat Flag–*Egr-1* in HEK 293 cells (Fig. 4*E*, left). Immunofluorescence analysis revealed that *Egr-1* siRNAs abolished the activity deprivation-induced upregulation of Egr-1 (Fig. 4*E*, middle and right) and reduced the induction of pEBS1^4 reporter activity (Fig. 4*F*), demonstrating the efficacy of the *Egr-1* siRNAs in CGNs. Furthermore, *Egr-1* knockdowns dramatically counteracted apoptosis induced by 5K media treatment (Fig. 4*G*). Egr-1 expression and activity are thus required in activity deprivation-induced CGN apoptosis.

Egr-1 is necessary and sufficient for Bim induction

The fact that, after activity deprivation, the elevation and activation of Egr-1 precedes Bim induction raises the issue as to whether Egr-1 modulates *Bim* expression during neuronal apoptosis. To determine whether Sp1 or Egr-1 is involved in *Bim* regulation, CGNs were transfected with dnSp1 or dnEgr. Almost 75% of induced Bim expression was effectively blocked by dnEgr but not by dnSp1 (Fig. 5*A*), demonstrating that Egr-1 but not Sp1 is involved in *Bim* regulation. In support of this result, siRNA-mediated knockdown of Sp1 did not affect the induction of Bim in CGNs (data not shown). Furthermore, siRNAs specifically targeted against *Egr-1* also significantly suppressed Bim expression (Fig. 5*B*). To examine whether Egr-1 transcriptionally regulates *Bim*, the *Bim*–Luc assay, in conjunction with the manipulation of Egr-1 protein and activity, was performed in neurons. The results demonstrated that the increment in *Bim*–Luc activity was substantially diminished by dnEgr and by *Egr-1* siRNAs (Fig. 5*C*). Therefore, Egr-1 is essential for the transcriptional induction of Bim during CGN apoptosis.

To determine whether Egr-1 is sufficient to upregulate Bim expression, the effect of wild-type Egr-1 on the transcription and expression levels of Bim was examined. Transfection of CGNs with wild-type *Egr-1* significantly increased Bim expression and *Bim*–Luc activity (Fig. 5*D*). Thus, Egr-1 is necessary and sufficient for Bim induction.

A putative Egr binding site is required for activation of *Bim* promoter

To determine whether transcriptional regulation of the *Bim* gene is regulated directly by Egr-1, the core sequences and putative transcription factor binding sites responsible for *Bim* promoter activation were identified. A series of reporter constructs containing deletions of the *Bim* 5′-flanking region cloned upstream of the *firefly* luciferase gene were generated. All of the deletion *Bim* promoter constructs tested had a common 3′ end. The constructs were transfected into CGNs, and *firefly* luciferase activity was monitored after activity deprivation. One of the deletion constructs (nucleotides −90 to +2658) showed the same activity as the original construct (nucleotides −2545 to +2658) in 5K media, whereas another deletion construct (nucleotides +36 to +2658) almost completely abolished the promoter activity (Fig. 6*A*). Thus, the principal *Bim* transcriptional control element was narrowed down to a region spanning nucleotides −90 to +36, which encompasses the transcription start site.

Promoter analysis for transcription factor binding sites using PromoterInspector software (Genomatix Software GmbH) identified a potential Egr binding site between nucleotides −56 to −47 in the core region of the *Bim* promoter. This putative Egr site is highly conserved between human (GCGGGGGCG, nucleotides −121 to −113), rat (GCGGGGGGCG, −56 to −47), and mouse (ACGGGGGCG, −26 to −18) *Bim* genes, with only one to two mismatches. To evaluate the role of this putative Egr site in *Bim* promoter activation, mutations (GCGGGGGGCG to ACAAGAAACC) of the putative Egr site were designed according to a method described previously (Tremblay and Drouin, 1999) and introduced into the *Bim*–Luc construct. These mutations clearly disrupted activation of the *Bim* promoter in response to activity deprivation (Fig. 6*B*), confirming that nucleotides −56 to −47 of the putative Egr-1 binding site are crucial for *Bim* promoter activation.

Egr-1 binds directly to the Egr site in the *Bim* promoter

To determine whether Egr-1 regulates the transcription of Bim directly by binding to the putative Egr site at nucleotides −56 to −47 of the *Bim* promoter, gel mobility shift assays were performed using an end-labeled oligonucleotide containing either a wild-type or mutated putative Egr site (designated WT and Mut, respectively). A prominent DNA–protein complex was detected in nuclear extracts from CGNs deprived of potassium (5K) but not in extracts from CGNs maintained in 25K media (Fig. 7*A*, left, lanes 1 and 2). Furthermore, a strong supershifted band was generated by the addition of an Egr-1 antibody to the binding reactions with a concomitant reduction of the DNA–protein complex. No supershift was observed after the addition of nonimmune IgG (Fig. 7*A*, left, lanes 3 and 4). The major DNA–protein complex completely disappeared when the mutated Egr site probe was tested (Fig. 7*A*, left, lanes 5 and 6), suggesting that the complex formed was Egr-1 specific. These results demonstrated that Egr-1 binds directly to the Egr site identified in the *Bim* promoter and that Egr-1 is induced after activity deprivation in CGNs.

Because mithramycin A and chromomycin A3 have been shown to prevent Egr-1 binding to GC-rich sequences, gel mobility shift assays were performed to examine whether the drugs interfered with Egr-1 binding to the *Bim* probe in nuclear extracts from CGNs. Both inhibitors blocked the activity deprivation-induced enhancement of the specific *Bim*-probe/Egr-1 complex in a dose-dependent manner (Fig. 7*A*, right). These results also indicated that mithramycin A and chromomycin A3 prevented

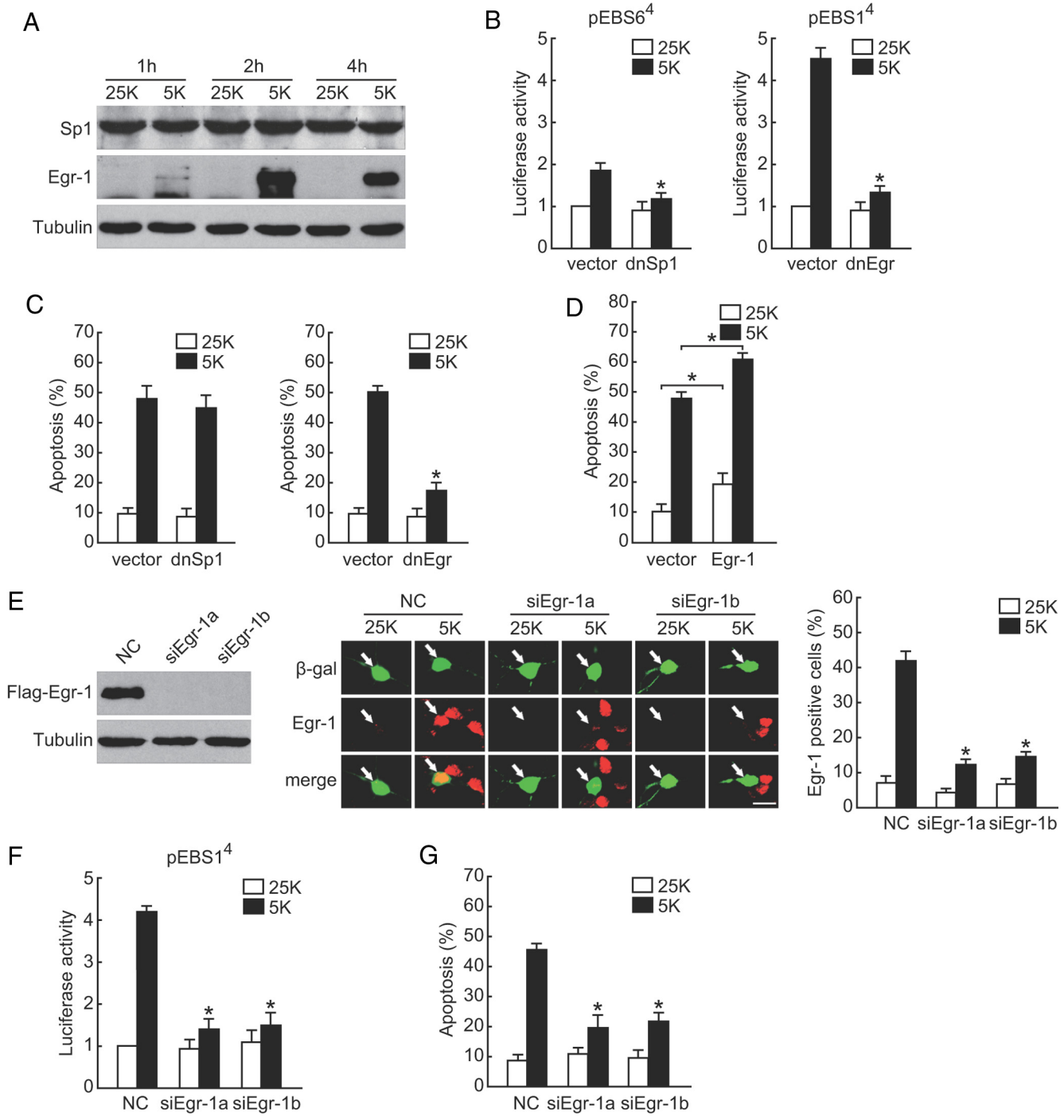

Figure 4. Egr-1 mediates activity deprivation-induced neuronal apoptosis. ***A***, DIV7 CGNs were incubated in 25K or 5K media for the times indicated and subjected to Western blotting with the antibodies indicated. ***B***, Activity deprivation triggers Egr and Sp1 transcriptional activities. DIV6 CGNs were transfected with 1 μg of pEBS6^4, together with dnSp1 or control vector, for 24 h (left). After incubation in 25K or 5K media for 12 h, dual-luciferase reporter assays were performed. The same assay was conducted using pEBS1^4 together with dnEgr (right). ***C***, The Egr family, rather than Sp1, contributes to apoptosis. DIV6 CGNs were transfected with dnSp1 (left), dnEgr (right), or their control vectors, together with GFP, and apoptosis was quantified as described in Materials and Methods. ***D***, Overexpression of Egr-1 promotes CGN apoptosis. DIV6 CGNs were transfected with Egr-1 or control vector, together with GFP, and the level of apoptosis was quantified. ***E***, The efficacy and specificity of Egr-1 siRNAs. HEK 293 cells were transfected with *Egr-1* siRNAs, or the negative control RNAs (NC), together with rat Flag–*Egr-1* for 24 h and were subjected to Western blotting using a Flag antibody (left). DIV6 CGNs were transfected with *Egr-1* siRNAs, or the NC, together with β-Gal for 24 h, and then incubated in 25K or 5K media for 2 h. Next, neurons were stained for Egr-1 (red) and β-Gal (green) (middle). Scale bar, 10 μm. The percentage of Egr-1-positive neurons was quantified as described in Figure 1*C* (right). ***F***, CGNs were transfected with pEBS1^4, together with *Egr-1* siRNAs, or the NC, and dual-luciferase reporter assays were performed. ***G***, DIV6 CGNs were transfected with *Egr-1* siRNAs, or the NC, together with GFP, and the level of apoptosis was quantified. All data in this figure represent the means ± SEM of three independent experiments. *$p < 0.05$.

the interaction of Egr-1 with the Egr binding site in the *Bim* promoter during neuronal apoptosis.

To confirm that Egr-1 and the *Bim* promoter interact *in vivo*, a ChIP assay was used with CGNs treated with 25K or 5K media for 2 h. Chromatin was sonicated into 0.5 kb fragments (Fig. 7*B*, left) and precipitated with a rabbit Egr-1 antibody or normal rabbit IgG as a control. Precipitated immunocomplexes were analyzed by Western blotting with an Egr-1 antibody. Egr-1 protein,

cross-linked to DNA, was readily detected in activity deprived CGNs but not in CGNs maintained in 25K media (Fig. 7*B*, middle). As a control, no Egr-1 protein was present when a nonimmune rabbit IgG was used for ChIP analysis. Precipitated DNA was assayed by PCR with primers designed to amplify a 256 bp fragment (nucleotides −150 to +106) of the *Bim* promoter flanking the Egr-1 binding site. The 256 bp DNA fragment was amplified strongly from activity-deprived CGN precipitates, in contrast to precipitates from 25K media, in which little or no DNA was observed (Fig. 7*B*, right). As a control, no discernable DNA was recovered when normal rabbit IgG was used for immunoprecipitation, and the PCR amplification of input DNA was constant (Fig. 7*B*, right). Thus, Egr-1 binds to the Egr site in the *Bim* promoter *in vivo*, and activity deprivation in CGNs leads to enhanced Egr-1 binding.

Bim acts downstream to mediate the proapoptotic effect of Egr-1

Based on the obvious importance of Egr-1 and Bim in CGN apoptosis and on the direct regulation of *Bim* by Egr-1, we hypothesized that Bim might mediate the proapoptotic effects of Egr-1. Manipulations of Egr-1 and Bim, using knockdown or overexpression approaches, were combined to investigate their functional relationship. The two *Egr-1* siRNAs, known to abolish Egr-1 protein levels in CGNs (Fig. 4*E*), significantly blocked CGN apoptosis in 5K media (Fig. 8*A*). In contrast, overexpression of wild-type *Bim*, combined with transfection of either of the two *Egr-1* siRNAs, removed the protection conferred by suppressing Egr-1 (Fig. 8*A*). Overexpression of wild-type *Egr-1* enhanced apoptosis, whereas the knockdown of endogenous *Bim*, by either of the two siRNAs, markedly reduced the level of neuronal death elicited by Egr-1 (Fig. 8*B*). These findings demonstrated that Bim lies downstream of the Egr-1-dependent proapoptotic signaling cascade.

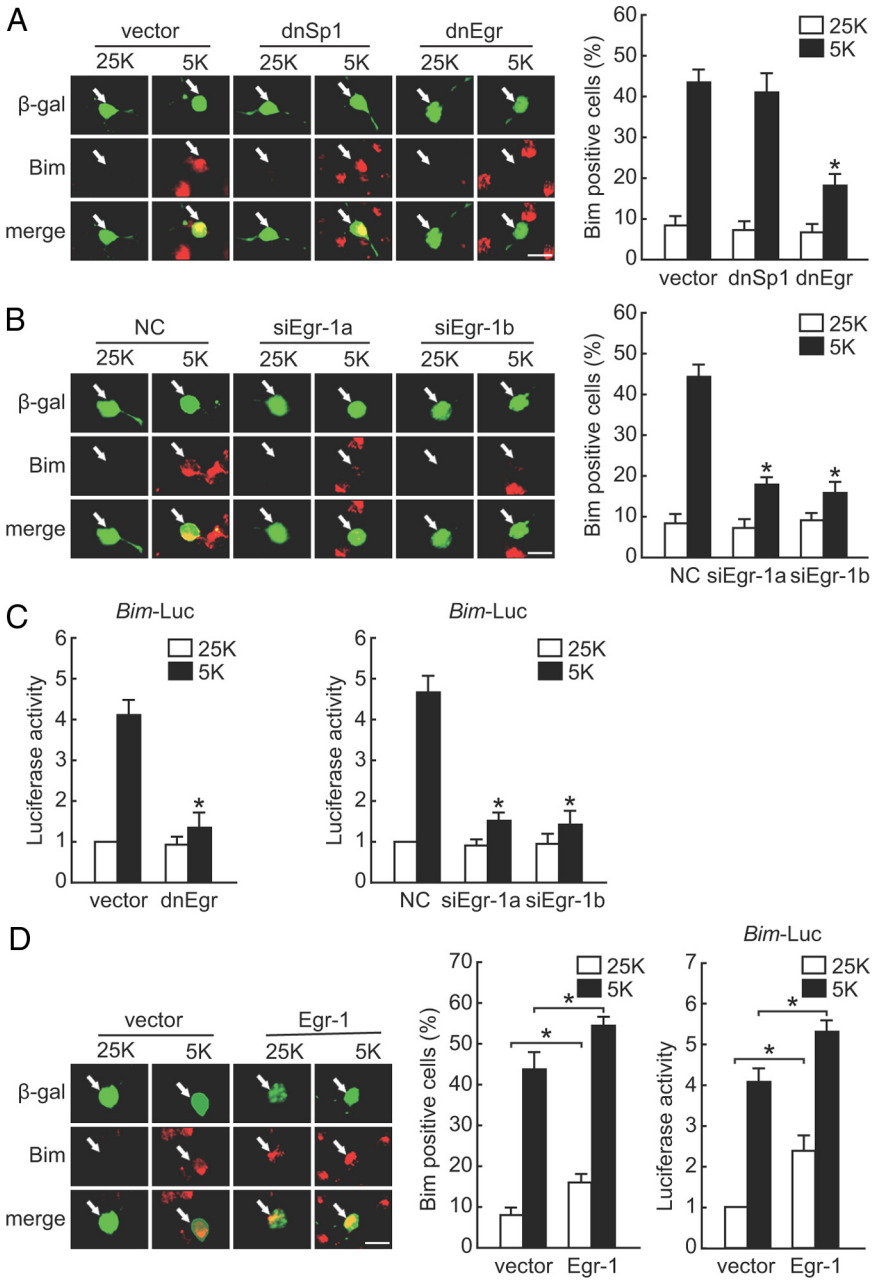

Figure 5. Egr-1 is necessary and sufficient for Bim induction. ***A***, Egr, but not Sp1, is necessary for increased Bim expression. DIV6 CGNs transfected with dnSp1, dnEgr, or control vectors, together with β-Gal as a marker for 24 h (left). After incubation in 25K or 5K media for 6 h, neurons were stained for Bim (red) and β-Gal (green). Scale bar, 10 μm. The percentages of Bim-positive neurons under the treatments indicated were quantified (right). ***B***, DIV6 CGNs were transfected with *Egr-1* siRNAs or the negative control RNAs (NC), together with β-Gal and quantified as described in ***A***. ***C***, DIV6 CGNs were transfected with 1 μg of *Bim*–Luc together with dnEgr or the control vector (left), or 1 μg of *Bim*–Luc together with the *Egr-1* siRNAs or the NC (right) for 24 h. After incubation in 25K or 5K media for 12 h, dual-luciferase reporter assays were performed. ***D***, DIV6 CGNs were transfected with wild-type *Egr-1* or the control vector, together with β-Gal (left), or with wild-type *Egr-1* or the control vector, together with 1 μg of *Bim*–Luc (right). Representative immunofluorescence images, quantification, and dual-luciferase reporter assays were performed as described above. The data represent the means ± SEM of three experiments. $^*p < 0.05$.

Discussion

This study has identified *Bim* as a *bona fide* target gene of Egr-1 in CGNs. Our findings suggest that Egr-1, rather than c-Jun, FOXO1/3a, or B/C-Myb transcription factors, directly induces *Bim* transcription to promote CGN apoptosis.

The JNK/c-Jun pathway is thought to modulate *Bim* expression in CGNs (Harris and Johnson, 2001; Putcha et al., 2001). However, we and others have reported that two JNK pathway inhibitors (SP600125 and CEP-11004) and a dominant-negative c-Jun mutant did not affect Bim induction (Shi et al., 2005; Ma et al., 2007; Hongisto et al., 2008). Furthermore, although a c-Jun-regulated AP-1 site (TGACTCA) at nucleotides −2447 to −2441 of the *Bim* promoter has been reported (Biswas et al., 2007a), our recent study showed that, in activity deprivation-induced CGN apoptosis, c-Jun predominantly heterodimerizes with ATF2, favoring an ATF site (TG/TACNTCA) rather than the AP-1 site, which was not activated by activity deprivation (Yuan et al., 2009).

In CGNs, the phosphorylation and nuclear sequestration of FOXO1 or FOXO3a has been shown to result in Bim expression

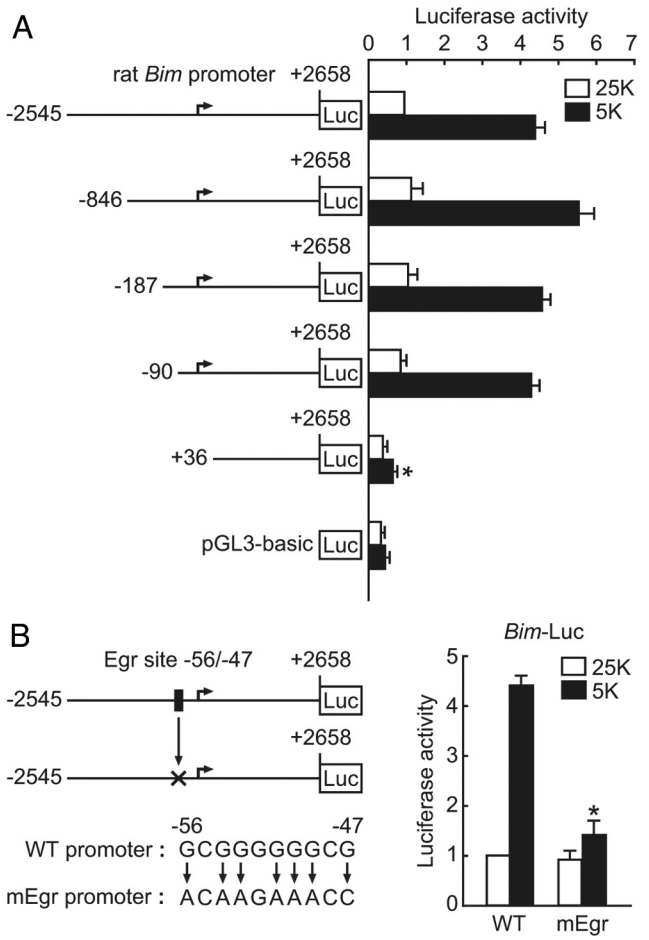

Figure 6. A putative Egr binding site located between nucleotides −56 to −47 of the *Bim* promoter is required for its activation. ***A***, *Bim* promoter 5′ sequential deletion constructs. Fragments of different lengths of the *Bim* promoter with the same 3′ end were cloned into pGL3-Basic. DIV6 CGNs were transfected with 1 μg of each of the *Bim* promoter-based reporters for 12 h. After incubation in 25K or 5K media for 12 h, dual-luciferase reporter assays were performed. ***B***, The wild-type putative Egr site, GCGGGGGGCG (WT), was mutated to ACAAGAAACC (mEgr), as represented by a cross. DIV6 neurons were transfected with 1 μg of *Bim*–Luc containing the WT or mEgr sites. The reporter assays were performed as described in ***A***. The data represent the means ± SEM of three experiments. *$p < 0.05$.

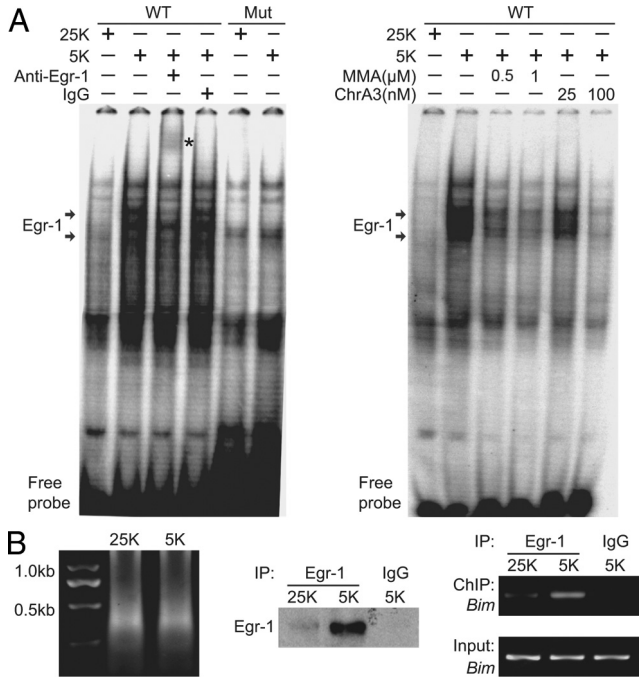

Figure 7. Egr-1 binds directly to the Egr site on the *Bim* promoter. ***A***, Gel mobility shift assay using ^{32}P-labeled oligonucleotides corresponding to the wild-type (WT) and mutant Egr site (Mut) sequence of the *Bim* promoter with nuclear extracts prepared from DIV7 CGNs maintained in media containing 25 or 5 mM KCl for 2 h. Egr-1 antibodies were preincubated with nuclear extracts to allow formation of the DNA–protein complex, and normal IgG was used as a control (left). For DNA-binding drug treatment, oligonucleotides were first preincubated with the indicated concentrations of mithramycin A (MMA) and chromomycin A3 (ChrA3) and subsequently used in gel mobility shift assays with CGN nuclear extracts (right). *, band supershifted by the Egr-1 antibody. ***B***, Cell lysates from DIV7 CGNs, maintained in 25K or 5K media for 2 h, were subjected to ChIP analysis using the Egr-1 antibody. Chromatin was sonicated into 0.5 kb fragments (left). Western blot analysis with the Egr-1 antibody demonstrates the immunoprecipitation (IP) specificity and efficiency (middle). DNA isolated and purified from immunoprecipitated material was amplified by PCR with primers designed to cover a 256 bp fragment of the *Bim* promoter spanning the Egr site. The amplified PCR fragments were analyzed on a 2% agarose gel (right). Equal amounts of total genomic DNA (Input) were used for the immunoprecipitations in each of the conditions (right). Results are representative of three separate experiments.

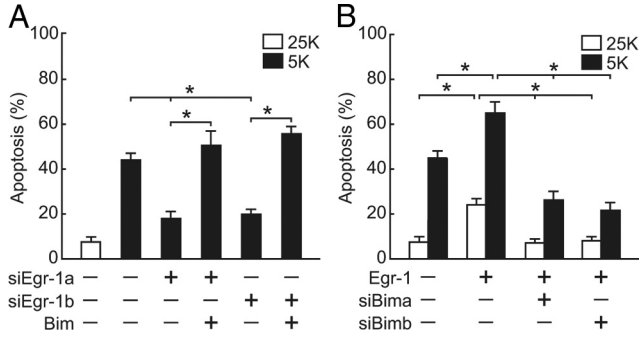

Figure 8. Bim acts downstream to mediate the proapoptotic effect of Egr-1. ***A***, DIV6 CGNs were cotransfected with or without *Bim* or the negative control siRNAs (NC) and the *Egr-1* siRNAs, as indicated, for 24 h. Then CGNs were transferred to 25K or 5K media for 12 h, and the level of apoptosis was quantified. ***B***, DIV5 CGNs were cotransfected with or without *Egr-1* or NC and the *Bim* siRNAs, as indicated on the graph, for 24 h. Then CGNs were switched to 25K or 5K media for 12 h, and the level of apoptosis was quantified. The data represent the means ± SEM of three experiments. *$p < 0.05$.

(Linseman et al., 2002; Yuan et al., 2008). However, as demonstrated in the present study and our recent report (Song et al., 2010), activity deprivation did not affect the phosphorylation and nucleocytoplasmic shuttling of FOXO1/3a. Furthermore, mutations of two FOXO binding sites in the *Bim* promoter failed to affect promoter activity, suggesting that FOXO1 and FOXO3a do not participate in the regulation of Bim, at least in our model; however, the use of different culture conditions may explain the discrepancy. Whereas Yuan et al. (2008) treated CGNs with serum-containing media, in our laboratory, activity deprivation was done in the absence of serum to eliminate the influence of potential survival-promoting factors in serum (Miller and Johnson, 1996).

In contrast to a previous report that Bim expression was blocked by IGF-1 in a PI3K/Akt-dependent manner (Linseman et al., 2002), in this study, IGF-1 did not reduce Bim expression. One possible explanation for this difference is that, in determining whether IGF-1 signaling regulates Bim, the protein band identified by Linseman et al. was not, in fact, Bim. The authors believed that their antibody detected only BimS (~15 kDa); however, the three alternatively spliced variants of Bim (BimEL, 23 kDa; BimL, 18 kDa; and BimS, 15 kDa) all contain the BimS coding region (O'Connor et al., 1998). Thus, a BimS antibody should also recognize BimEL and BimL. In a subsequent study by

the same group (Butts et al., 2005), another Bim variant (BimEL, ~23 kDa) was detected in the same CGN cell line; however, studies by Johnson et al. (Harris and Johnson, 2001; Putcha et al., 2001) and our group (Shi et al., 2005) have consistently shown that CGNs primarily express BimEL and only extremely low levels of BimS. To address this inconsistency, we investigated the specificity of the anti-Bim antibody (Linseman et al., 2002) and found that it did not recognize any of the Bim variants in CGN cells, thus casting doubt on *Bim* regulation by IGF-1 and FOXO3a in CGNs. In this study, the Bim expression described is BimEL (~23 kDa), and a pretested CST anti-Bim antibody was used to demonstrate that inhibition of FOXO1/3a did not regulate Bim expression in CGNs.

Although the transcriptional upregulation of Bim is independent of the JNK/c-Jun and PI3K/Akt/FOXO1/3a pathways, the inhibition of JNK by SP600125 or the activation of PI3K/Akt signaling by IGF-1 may prevent apoptosis through alternative mechanisms, for example, the posttranslational modification and inactivation of some components of the cell death machinery and the decreased transcription of pro-apoptosis genes. Inhibiting the JNK pathway may suppress the proapoptotic activity of Bim by reducing the JNK-mediated phosphorylation of Bim at Ser65 or Thr112 (Putcha et al., 2003; Becker et al., 2004; Hübner et al., 2008), or it may block the c-Jun-dependent upregulation of Dp5, another proapoptotic BH3-only protein (Ma et al., 2007; Towers et al., 2009). IGF-1 may increase the Akt-mediated phosphorylation of Bim at Ser87 to reduce its proapoptotic potency (Qi et al., 2006), or it may inactivate caspase 9 downstream of Bim (Cardone et al., 1998). Many other Akt substrates, including Bad, IKK, and GSK-3, may also mediate the prosurvival effects of IGF-1 (Datta et al., 1999). These observations suggest that activity deprivation-induced neuronal apoptosis is a coordinated process that requires the cooperation of multiple proapoptotic mechanisms with the Egr-1/Bim pathway.

In the NGF withdrawal-induced apoptosis of sympathetic neurons or PC12 cells, *Bim* has been identified as a target of the Cdk4-E2F-B/C-Myb pathway (Biswas et al., 2005). It is possible that B/C-Myb regulate Bim in sympathetic neurons but not in CGNs; however, we found that the cyclin-dependent kinase (Cdk) inhibitors, roscovitine and olomoucine, reduced the elevated transcription of *Bim* (data not shown) in CGNs, consistent with previous studies in which Cdk1 or Cdk4 were identified as regulators of Bim (Biswas et al., 2005; Yuan et al., 2008). An interesting question here is whether any of the Cdk proteins regulate *Egr-1* transcription level during apoptosis. This issue is currently under investigation in our laboratory.

Although previous studies have attributed the biological effects of GC-rich DNA binding drugs, such as mithramycin A and chromomycin A3, mainly to the inhibition of the Sp1 family from binding to DNA, our study revealed that these drugs also inhibit Egr-1 binding to DNA. This result highlights the necessity of treating such drugs with caution, because their ability to suppress other transcription factors beside the Sp1 proteins, for example, the Egr family, should also be considered. Their neuroprotective abilities have been demonstrated in the apoptosis of CGNs induced by activity deprivation (Ferrante et al., 2004), in oxidative stress-induced cortical neuron death (Chatterjee et al., 2001), and in dopaminergic neurotoxicity (Hagiwara et al., 2009). However, the neuroprotective mechanism remains unclear. In this study, mithramycin A or chromomycin A3 repressed *Bim* transcriptional upregulation and neuronal apoptosis by blocking Egr-1 binding to the *Bim* promoter. This not only supported the likelihood of Egr-1-mediated regulation of Bim in neuronal apoptosis but also suggested the proapoptotic Egr-1/Bim pathway as the means by which the GC-rich DNA binding drugs provide neuroprotection. Mithramycin A has been clinically approved by the U.S. Food and Drug Administration and has been demonstrated to improve motor symptoms and prolong life in a mouse model of Huntington's disease (Ferrante et al., 2004); therefore, additional exploration of its neuroprotective mechanism in disease models should help to make it a promising drug for the treatment of many neurological diseases.

Bim appears to play an important role in many neurological diseases characterized by deregulated neuronal death. The induction of Bim has been observed in neuronal apoptosis induced by the Alzheimer's disease-associated amyloid-β peptide (Biswas et al., 2007b), in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model and cellular model of Parkinson's disease (Liou et al., 2005; Perier et al., 2007), and in focal ischemia models (Okuno et al., 2004; Gao et al., 2005). Intriguingly, amyloid-β peptide resulted in enhanced DNA binding activity of Egr-1 (Giri et al., 2003), and the upregulation of Egr-1 has also been found in mouse brain after MPTP treatment (Smith et al., 1997) or transient focal ischemia (Tureyen et al., 2008). Thus, it will be interesting to examine whether Egr-1 transactivates Bim to promote neuronal death in the animal models of many neurological diseases such as Alzheimer's disease, Parkinson's disease, and stroke.

The present study has demonstrated that Egr-1 directly transactivates *Bim* gene expression to promote neuronal apoptosis. First, we have shown that the suppression of Egr-1 activity or the knockdown of Egr-1 blocked Bim induction, whereas the overexpression of Egr-1 elevated *Bim* transcription. Second, Egr-1 bound directly to an Egr-1-recognition site at nucleotides −56 to −47 of the *Bim* promoter *in vitro* and *in vivo*, which was further supported by a recent report of Egr-1-mediated upregulation of Bim in human cancer cells (Yamaguchi et al., 2010). Third, the prevention of Egr-1 binding to the *Bim* promoter by GC-rich DNA binding inhibitors decreased Bim induction and neuronal apoptosis. Finally, experiments using siRNA-mediated knockdown combined with overexpression analysis suggested that Egr-1 acts upstream of Bim to promote apoptosis. Together, our findings demonstrate that the Egr-1/Bim pathway acts as a general signaling mechanism in neuronal cell death.

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