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FOXO transcription factors directly activate *bim* gene expression and promote apoptosis in sympathetic neurons

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Developing sympathetic neurons die by apoptosis when deprived of NGF. BIM, a BH3-only member of the BCL-2 family, is induced after NGF withdrawal in these cells and contributes to NGF withdrawal–induced death. Here, we have investigated the involvement of the Forkhead box, class O (FOXO) subfamily of Forkhead transcription factors in the regulation of BIM expression by NGF. We find that overexpression of FOXO transcription factors induces BIM expression and promotes death of sympathetic neurons in a BIM-dependent manner. In addition, we find that FKHRL1 (FOXO3a) directly activates the *bim* promoter via two conserved FOXO binding sites and that mutation of these sites abolishes *bim* promoter activation after NGF withdrawal. Finally, we show that FOXO activity contributes to the NGF deprivation–induced death of sympathetic neurons.

Introduction

Programmed cell death (PCD) accounts for the death of approximately half of all neurons generated during embryogenesis and is essential for the correct innervation of target tissues and formation of neuronal networks during neural development (Oppenheim, 1991; Yuan and Yankner, 2000). A well-studied model of neuronal PCD is provided by sympathetic neurons, which depend on NGF for survival during early postnatal life. Developing sympathetic neurons undergo apoptosis when deprived of NGF in culture, providing a useful in vitro system for studying the molecular mechanisms of neuronal PCD (Deshmukh and Johnson, 1997).

NGF deprivation activates the intrinsic pathway of apoptosis in sympathetic neurons (Putcha et al., 2002). This involves the release of cytochrome *c* from the mitochondria into the cytosol which is regulated by members of the BCL-2 family through their ability to influence mitochondrial integrity (Hengartner, 2000). NGF withdrawal–induced apoptosis of sympathetic neurons can be blocked by inhibitors of RNA and protein synthesis (Martin et al., 1988), suggesting that increased expression of specific genes is necessary for this death, and it has been shown that expression of the proapoptotic BH3–only BCL-2 family members DP5/HRK and BIM increases after NGF withdrawal (Imaizumi et al., 1997; Putcha et al., 2001; Whitfield et al., 2001). Overexpression of each protein can induce cytochrome *c* release, and apoptosis in the presence of NGF and BIM is required for normal NGF withdrawal–induced death (a complementary requirement for DP5 has not yet been reported). In addition, the c-Jun NH_2-terminal kinase (JNK)–c-Jun pathway has been shown to contribute to the up-regulation of both BIM and DP5 after NGF withdrawal (Harris and Johnson, 2001; Whitfield et al., 2001). Coordinate up-regulation of the *bim* and *dp5/hrk* genes, therefore, provides a link between activation of the JNK–c-Jun pathway, which is a critical early consequence of NGF deprivation, and cytochrome *c* release (Estus et al., 1994; Ham et al., 1995; Eilers et al., 1998; Whitfield et al., 2001).

Although the JNK–c-Jun pathway appears to play a significant role in BIM induction after NGF withdrawal in sympathetic neurons, its precise contribution is still unclear and the involvement of other signaling pathways cannot be ruled

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Abbreviations used in this paper: DBD, DNA-binding domain; FasL, Fas ligand; FOXO, Forkhead box, class O; JNK, c-Jun NH_2-terminal kinase; PAC, P1 artificial chromosome; PCD, programmed cell death; PI3-K, phosphatidylinositol 3-kinase; SCG, superior cervical ganglia; SGK, serum and glucocorticoid–induced kinase.

out. Therefore, it is of note that JNK–c-Jun activation contributes to BIM induction in cerebellar granule neurons deprived of serum and depolarizing potassium, but the ability of insulin-like growth factor-1 to block this induction is not a result of JNK–c-Jun inhibition (Linseman et al., 2002). Instead, the insulin-like growth factor-1–mediated regulation of BIM expression correlates with the phosphorylation status of FKHRL1 (FOXO3a), a Forkhead transcription factor of the Forkhead box, class O (FOXO) subfamily, which has previously been implicated in the induction of BIM expression in hematopoietic cells deprived of their trophic support (Dijkers et al., 2000, 2002; Stahl et al., 2002).

The FOXO transcription factors, FKHR (FOXO1), FKHRL1 (FOXO3a), and AFX (FOXO4), share DNA-binding specificity to a core consensus site (Furuyama et al., 2000) and are targets of phosphatidylinositol 3-kinase (PI3-K) signaling, which regulates their activity via phosphorylation mediated by protein kinase B/Akt and serum and glucocorticoid–induced kinase (SGK; Burgering and Kops, 2002). In pro-B and T cells, trophic factor deprivation and inhibition of PI3-K activity cause FKHRL1 dephosphorylation, induction of BIM expression, and FKHRL1-dependent apoptosis (Dijkers et al., 2000, 2002; Stahl et al., 2002). In addition, overexpression of constitutively active FKHRL1 can induce BIM expression and apoptosis in the presence of survival signaling in these cells (Dijkers et al., 2000; Stahl et al., 2002). In neurons, the contribution of FOXO transcription factors to BIM induction and apoptosis is less clear. Constitutively active FKHRL1 can induce apoptosis in cerebellar granule neurons, but this appears to be Fas dependent (Brunet et al., 1999). Furthermore, although BIM induction in cerebellar granule neurons correlates with dephosphorylation of FKHRL1 (Linseman et al., 2002), a direct requirement for FOXO transcription factors has not been demonstrated.

Here, we have investigated the role of FOXO transcription factors in NGF-dependent sympathetic neurons. We report that FOXO transcription factors can directly activate the *bim* promoter and that this contributes to the induction of *bim* gene expression after NGF withdrawal. In addition, we find that FOXO activity is required for normal NGF withdrawal–induced apoptosis in these cells.

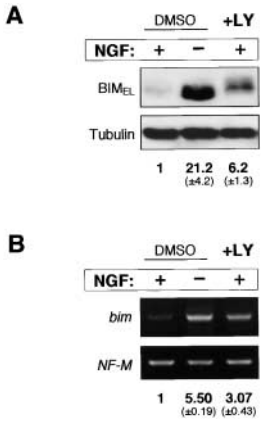

Figure 1. **PI3-K signaling regulates *bim* gene expression in sympathetic neurons.** (A) PI3-K inhibition by LY294002 induces BIM protein levels. Neurons cultured with (+) or without (−) NGF, were treated with 50 μM LY294002 (+LY) or vehicle (DMSO) for 16 h and BIM expression assessed by immunoblotting. A representative blot is shown. Changes in BIM protein levels were determined by densitometry after normalization to tubulin to control for loading. Values represent ±SEM of three experiments. (B) PI3-K inhibition induces *bim* mRNA levels. Neurons were treated as in A and *bim* and *neurofilament-M* (*NF-M*) mRNA levels were assessed by RT-PCR. Representative gel images are shown. Changes in *bim* mRNA levels were determined as changes in band intensity on ethidium bromide–stained 2.5% agarose gels after normalization to *NF-M* to control for sample input. Values represent ±SEM of five experiments.

Results

The *bim* gene is regulated by the PI3-K pathway in sympathetic neurons

To determine whether the PI3-K pathway regulates BIM expression in sympathetic neurons, we used the pharmacologi-

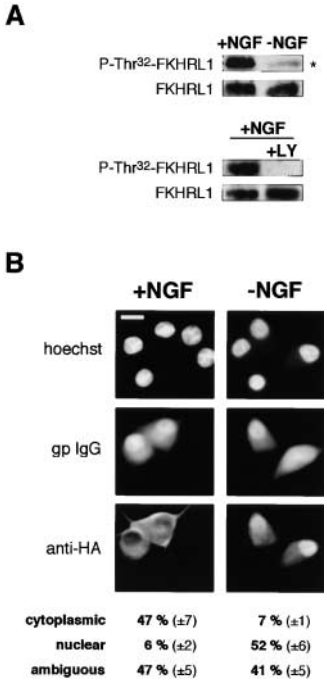

Figure 2. **NGF regulates the phosphorylation and localization of FKHRL1 in sympathetic neurons.** (A) NGF regulates FKHRL1 phosphorylation via PI3-K. Neurons were cultured with or without NGF (±NGF), or treated with 100 μM LY294002 (+LY) for 16 h. FKHRL1 expression and phosphorylation was then assessed by immunoblotting using anti-FKHRL1 and antiphospho (Thr^{32})-FKHRL1 antibodies. Representative blots are shown (asterisk indicates the phospho-specific antibody detects a nonspecific band of this size). (B) NGF regulates FKHRL1 localization. Neurons were injected with an expression construct encoding 0.1 mg/ml wild-type FKHRL1 together with guinea pig (gp) IgG as a marker and were cultured with or without NGF (±NGF) for 16 h. Neurons were then fixed and stained with Hoechst dye to visualize nuclei, and antibodies to gp IgG and the HA epitope to identify injected cells and detect HA-tagged FKHRL1. Representative images are shown. FKHRL1 localization was classified as cytoplasmic when nuclei showed no significant signal with anti-HA staining, and nuclear when the signal was greater in the nucleus than cytoplasm. In many neurons the staining pattern was ambiguous. Percentages representing ±SEM of three experiments are shown. Bar, 10 μm.

cal inhibitor LY294002 to inhibit PI3-K activity. In immunoblotting experiments, we observed a sixfold increase in BIM_{EL} protein levels in sympathetic neurons treated with 50 μM LY294002 to a level that is ~30% of the induction (21-fold) seen after NGF deprivation (Fig. 1 A). In addition, using reverse transcription PCR (RT-PCR), we found that the same treatment resulted in a more robust induction of *bim* mRNA to a level that is ~60% of that seen for NGF withdrawal (Fig. 1 B).

NGF regulates FKHRL1 phosphorylation and localization in sympathetic neurons

Several trophic factors, including NGF in PC12 cells (Zheng et al., 2002), regulate the activity of FOXO transcription factors via PI3-K signaling and Akt/SGK-mediated phosphorylation at three critical regulatory sites (Burgering and Kops, 2002). This promotes nuclear export of these factors and blocks their ability to transactivate their target genes. In immunoblotting experiments, we found that FKHRL1 is expressed in sympathetic neurons and that phosphorylation at one of the critical phosphorylation sites (Thr^{32}) substantially decreases after NGF withdrawal (Fig. 2 A). Furthermore, this phosphorylation requires PI3-K activity because it was inhibited by LY294002 (Fig. 2 A). We also found that NGF withdrawal promoted the translocation of ectopically expressed HA-tagged human FKHRL1 from the cytoplasm to the nucleus (Fig. 2 B), whereas a constitutively active human FKHRL1 mutant, FKHRL1(A3), containing mutations in its regulatory phosphorylation sites (Brunet et al., 1999), localized to the nucleus even in the presence of NGF (not depicted). Therefore, NGF withdrawal promotes changes in FKHRL1 phosphorylation and localization in sympathetic neurons that would be consistent with a role in the transcriptional up-regulation of the *bim* gene.

FOXO transcription factors can induce BIM expression and promote a BIM-dependent death of sympathetic neurons

To investigate whether FOXO transcription factors can regulate *bim* gene expression in sympathetic neurons, we infected the cells with an adenovirus expressing a constitutively active murine FKHR mutant, FKHR(ADA) (Nakae et al., 2001). We found that FKHR(ADA), which localized to the nucleus as expected (not depicted), consistently induced BIM protein and *bim* mRNA levels compared with neurons infected with a control LacZ adenovirus (Fig. 3 A).

Because FKHR(ADA) was found to induce expression of the proapoptotic BIM protein, we next investigated whether expression of a constitutively active FOXO transcription factor, in this case FKHRL1(A3), could induce sympathetic neurons to die in the presence of NGF, and whether this was dependent upon BIM expression. In this analysis, we used a *bim* antisense strategy to inhibit BIM expression (Whitfield et al., 2001). We found that the *bim* antisense oligonucleotides greatly reduced BIM protein levels in transfected PC12 cells, compared with the corresponding missense oligonucleotides, whereas levels of other BCL-2 family members (both pro- and antiapoptotic) and procaspase-3 were relatively unaffected (Fig. 3 B). The effect of FKHRL1(A3) expression on neuronal survival could, therefore, be evaluated in the context of normal or inhibited BIM expression. Neurons were injected with an FKHRL1(A3) expression construct or empty vector, together with a mixture of the *bim* antisense oligonucleotides or the corresponding scrambled missense oligonucleotides, and their survival was tracked over a 3-d period (Fig. 3 C). When coinjected with the missense oligonucleotides, we observed significantly increased death of neurons expressing FKHRL1(A3) compared with the corresponding control neurons. Furthermore, this depended on BIM expression because coinjec-

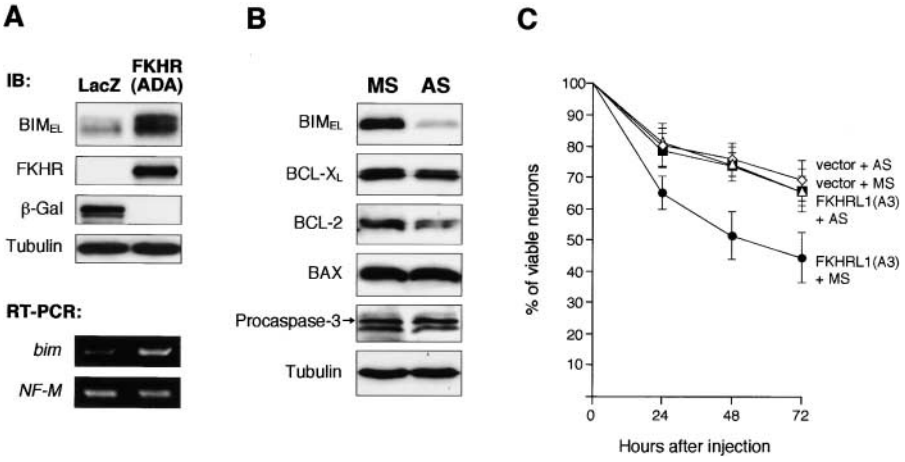

Figure 3. **FOXO transcription factors induce *bim* gene expression and promote a BIM-dependent death of sympathetic neurons.** (A) FKHR(ADA) expression increases endogenous BIM protein and *bim* mRNA levels. Neurons were infected with a control adenovirus (LacZ) encoding β-galactosidase (β-Gal) or an adenovirus encoding FKHR(ADA). BIM, FKHR, and β-Gal expression was assessed by immunoblotting (IB), and *bim* mRNA levels were assessed by RT-PCR. Tubulin and *NF-M* are loading and sample input controls. Images are representative of at least three experiments. (B) BIM expression can be selectively inhibited by *bim* antisense oligonucleotides. Naïve PC12 cells were cotransfected with *bim* antisense (AS) or missense (MS) oligonucleotides and an expression construct encoding GFP, and were sorted for GFP expression by FACS® after 24 h. BIM, BAX, BCL-2, BCL-X_L, and procaspase-3 protein levels in GFP-positive cells were then assessed by immunoblotting. Tubulin represents a loading control. Blots are representative of three experiments. (C) Neurons were injected with 0.2 mg/ml of an FKHRL1(A3) expression construct or empty vector together with 0.03 mg/ml of *bim* antisense (AS) or missense (MS) oligonucleotide mixtures, and Texas red dextran as a marker. Cells were left to recover for 4–6 h (time 0) and survival of the injected neurons were determined as the percentage of viable cells at each time point relative to time 0. Values represent ± SEM of four experiments. FKHRL1(A3) expression significantly increased death of neurons injected with missense oligonucleotides ($P \leq 0.05$, *t* tests, FKHRL1(A3) + MS vs. vector + MS), and this was blocked in neurons injected with the *bim* antisense oligonucleotides (FKHRL1(A3) + AS). The antisense oligonucleotides had no significant independent effect upon neuronal survival (vector + AS).

tion of the *bim* antisense oligonucleotides prevented the FKHRL1(A3)-induced death.

NGF withdrawal, PI3-K inhibition, and FKHRL1 activate the *bim* promoter

To further investigate the mechanisms involved in regulation of the *bim* gene in sympathetic neurons, we cloned the 5′ end of the rat *bim* gene (including a 2.5-kb region upstream of the major transcription start site, the noncoding exon 1, the 2.4-kb first intron, and the noncoding region of exon 2) upstream of the Firefly luciferase coding region to generate a *bim*-LUC reporter (Fig. 4 A), which has promoter activity in transiently transfected neuronal PC12 cells (not depicted). The effect of NGF withdrawal on *bim* promoter activity was assessed by luciferase immunostaining in cultured sympathetic neurons injected with the *bim*-LUC reporter (Fig. 4 B), which provided us with an indicator of reporter activity in individual injected cells. We observed a significant increase in the percentage of injected neurons expressing luciferase after NGF withdrawal. In addition, reporter activation after NGF withdrawal was confirmed by assaying for luciferase activity in injected neurons (Fig. 4 C). These data indicate that the *bim* promoter is activated after NGF withdrawal in sympathetic neurons.

Next, we tested whether inhibition of PI3-K activity by LY294002 or overexpression of FOXO transcription factors could also activate the *bim* promoter in sympathetic neurons in the presence of NGF. We found that *bim*-LUC reporter activity (as assessed by luciferase immunostaining) increased significantly in injected neurons treated with LY294002 (Fig. 4 D) and increased in a dose-dependent manner in neurons expressing constitutively active FKHRL1(A3) and, to a lesser extent, wild-type FKHRL1 (Fig. 4 E). Although FKHRL1(A3) was found to be a potent activator at both high and low concentrations, wild-type FKHRL1 did not significantly activate the reporter at the lower concentration, and only caused a modest activation, relative to FKHRL1(A3), at the higher concentration. This is consistent with the ability of NGF to regulate the localization of wild-type FKHRL1, but not FKHRL1(A3) (Fig. 2 and text), with some FKHRL1 escaping regulation by NGF when expressed at high levels.

These results indicated that the *bim*-LUC reporter behaves in a similar manner to the endogenous *bim* gene with respect to regulation by NGF, the PI3-K pathway, and FOXO transcription factors. The *bim*-LUC reporter could, therefore, be used to further investigate the involvement of FOXO transcription factors in *bim* promoter activation after NGF withdrawal.

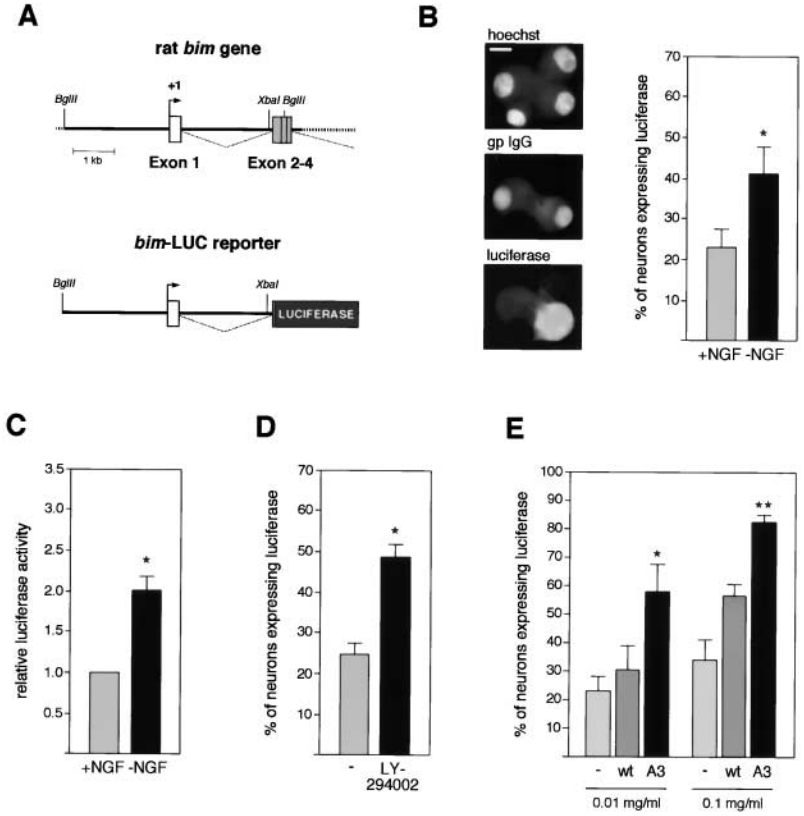

Figure 4. **The *bim* promoter is activated by NGF withdrawal, PI3-K inhibition, and FKHRL1(A3) expression in sympathetic neurons.** (A) Structure of the 5′ end of the rat *bim* gene and the *bim*-LUC reporter. Exons are shown as boxes; clear regions represent 5′ untranslated region and shaded regions coding sequence. An arrow indicates the major transcription start site (+1), as determined by 5′ RACE. The *bim*-LUC reporter consists of the 5.2-kb region 5′ to the *bim* initiator codon in pGL3-Basic. (B) Luciferase immunostaining reveals that the *bim* promoter is activated after NGF withdrawal in neurons injected with the *bim*-LUC reporter. Neurons were injected with 0.01 mg/ml of the *bim*-LUC reporter and gp IgG as a marker and were cultured with or without NGF (±NGF) for 20–24 h. Cells were then fixed and stained with Hoechst dye to visualize nuclei, and antibodies to gp IgG and luciferase to identify injected cells and to assess luciferase expression. Typical images are shown. Bar, 10 μm. Neurons were scored as expressing luciferase when staining was significantly greater than background staining in uninjected neurons. The data represent ±SEM of six experiments (*, $P < 0.05$, *t* test, +NGF vs. −NGF). Bar, 10 μm. (C) Assaying for luciferase activity confirms *bim* promoter activation after NGF withdrawal. Neurons were injected with 0.01 mg/ml of the *bim*-LUC reporter and 0.005 mg/ml of the *Renilla* luciferase expression construct pRL-TK, to control for injection, and were cultured with or without NGF (±NGF) for 18–20 h after which luciferase assays were performed. The relative fold change in Firefly activity was determined after normalization to *Renilla* activity. The data represent ±SEM of four experiments. (*, $P < 0.002$, *t* test, +NGF vs. −NGF). (D) The *bim* promoter is activated by LY294002. Neurons injected as in B were treated with 50 μM LY294002 or DMSO (−) for 20–24 h in the presence of NGF and the percentage of injected cells expressing luciferase determined as in (B). The data represent ±SEM of five experiments (*, $P < 0.001$, *t* test, LY294002 vs. DMSO). (E) The *bim* promoter is activated by FKHRL1(A3). Neurons were injected with 0.01 mg/ml of the *bim*-LUC reporter, gp IgG, and either 0.01 or 0.1 mg/ml of wild-type (wt) FKHRL1 or FKHRL1(A3) expression construct or equimolar amounts of empty vector (−) and were maintained in the presence of NGF. After 20–24 h, the percentage of injected cells expressing luciferase was determined as in B. The data represent ±SEM of at least three experiments (*, $P < 0.02$ and **, $P < 0.005$, *t* tests, FKHRL1(A3) vs. vector).

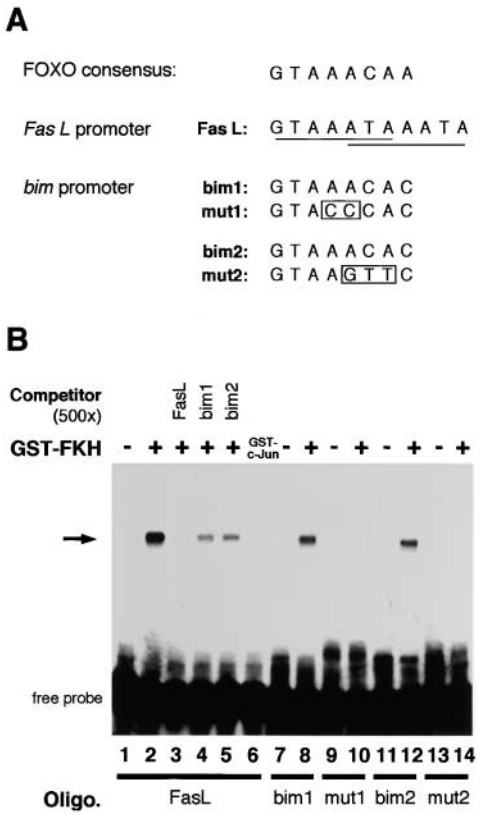

Figure 5. **FKHRL1 binds to FOXO binding sites located close to the *bim* promoter.** (A) The consensus binding site for the FOXO subfamily of Forkhead transcription factors and a known FOXO binding site in the Fas ligand promoter (FasL) are compared with the conserved bim1 and bim2 sites located close to the *bim* promoter. Deviation from the consensus at positions 1, 6, and 8 is not uncommon. Overlapping FasL FOXO sites are underlined. Mutated versions of the bim1 and bim2 sites (mut1 and mut2) are also shown (mutations are boxed). The bim2 site spans the *bim* exon 1/intron 1 splice site so the mut2 mutations were designed to minimize the chance of disrupting splicing (mut2 fits the GTRAGT donor splice consensus). (B) Purified GST-FKH protein was tested for binding to double-stranded ^{32}P-labeled oligonucleotides containing the FasL site and the wild-type and mutant bim sites listed in A by electrophoretic mobility shift assay. A representative gel is shown. An arrow indicates the mobility of specific complexes between GST-FKH and the labeled oligonucleotides. GST-c-Jun was incubated with the FasL oligonucleotide (lane 6) as a negative control. GST-FKH binding to the FasL oligonucleotide was competed with a 500-fold excess (500×) of unlabeled FasL, bim1, or bim2 oligonucleotide (lanes 3–5).

FOXO binding sites are located at the 5′ end of the *bim* gene

A search of the 5′ end of the *bim* gene identified four potential FOXO binding sites that closely match a consensus GTAAACAA described previously (Furuyama et al., 2000), two of which—one at position −204 relative to the transcription start site (bim1) and one at the boundary between exon 1 and the first intron (bim2; Fig. 5 A)—are conserved between rodents and humans. The ability of FKHRL1 to bind to these sites was, therefore, tested in an electrophoretic mobility shift assay using a purified fusion protein (GST-FKH) consisting of the DNA-binding domain (DBD) of FKHRL1(A3) fused to GST. As expected, GST-FKH bound to a control oligonucleotide (Fas ligand [FasL]) containing a known FOXO site from the FasL promoter (Brunet et al., 1999), whereas an unrelated GST fusion protein (GST-c-Jun) did not (Fig. 5 A; and Fig. 5 B, lanes 2 and 6). GST-FKH also bound to oligonucleotides containing the bim1 and bim2 sites but did not bind to mutated versions (mut1 and mut2) of these sites (Fig. 5 A; and Fig. 5 B, lanes 7–14). A 500-fold molar excess of unlabeled FasL oligonucleotide prevented formation of the GST-FKH–FasL complex and complex formation was also significantly reduced by unlabeled bim1 and bim2 oligonucleotides (Fig. 5 B, lanes 3–5). The FasL oligonucleotide competed more effectively than the bim1 and bim2 oligonucleotides perhaps because it contains two overlapping FOXO sites (Fig. 5 A). These data indicate that the bim1 and bim2 sites are FOXO binding sites.

FOXO binding sites are required for activation of the *bim* promoter by FKHRL1 and NGF withdrawal

Next, we investigated whether the bim1 and bim2 FOXO binding sites are necessary for activation of the *bim* promoter by FKHRL1(A3) and by NGF withdrawal using *bim*-LUC reporter constructs with mutations in these sites. The mutations that prevented GST-FKH binding to these sites (Fig. 5) were introduced into the reporter by site-directed mutagenesis to generate the single mutants, *bim*-LUC(m1) and *bim*-LUC(m2), and a double mutant, *bim*-LUC(dm) (Fig. 6 A).

The mutant *bim*-LUC reporters were initially tested for their ability to be activated by FKHRL1(A3). Neuronal PC12 cells were transiently cotransfected with the *bim*-LUC reporters and an FKHRL1(A3) expression construct or empty vector and luciferase activity determined 24 h later (Fig. 6 B). Activation of the *bim*-LUC reporter by FKHRL1(A3) was found to be dose dependent and the single FOXO site mutations in the *bim*-LUC(m1) and *bim*-LUC(m2) reporters only slightly diminished this activation. In contrast, activation of the *bim*-LUC(dm) reporter was greatly reduced compared with the wild-type reporter and this was especially apparent at the lower concentration of FKHRL1(A3). Modest activation of the *bim*-LUC(dm) reporter at the higher concentration of FKHRL1(A3) construct may be mediated by the two nonconserved FOXO binding sites more distal to the *bim* promoter. These results indicate that *bim* promoter activation by FKHRL1(A3) can be mediated by either the bim1 or the bim2 FOXO sites and that mutation of both is necessary to substantially reduce this activation.

Next, we tested whether the bim1 and bim2 FOXO sites are also required for activation of the *bim* promoter after NGF withdrawal in sympathetic neurons. Neurons were injected with the *bim*-LUC and *bim*-LUC(dm) reporters and their ability to be activated by NGF withdrawal was determined by luciferase immunostaining (Fig. 6 C). Whereas the wild-type *bim*-LUC reporter was activated as before, there was no apparent activation of the *bim*-LUC(dm) double mutant reporter because no significant increase in the number of injected neurons expressing luciferase was observed after NGF withdrawal. The ability of NGF withdrawal to activate the reporters was additionally investigated

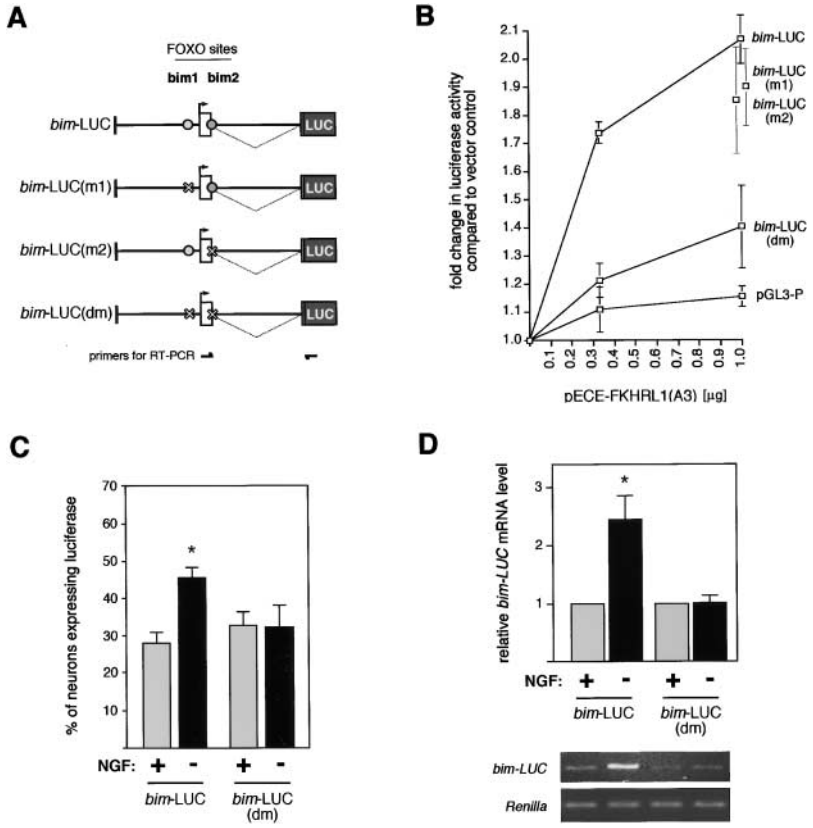

Figure 6. **FOXO binding sites are required for *bim* promoter activation by FKHRL1(A3) and NGF withdrawal.** (A) Structure of *bim*-LUC reporters containing mutations in the bim1 and bim2 FOXO sites. Reporters are represented as in Fig. 4 A. Wild-type sites are represented by shaded circles and mutated sites by crosses. The location of primers used for RT-PCR (D) is also shown. (B) Mutation of the bim1 and bim2 sites substantially reduces *bim* promoter activation by FKHRL1(A3). Neuronal PC12 cells were transiently transfected with the indicated reporters together with varying concentrations of the FKHRL1(A3) expression construct pECE-FKHRL1(A3) or empty vector and the *Renilla* luciferase expression construct pRL-TK to control for transfection efficiency. Luciferase activity was determined after 24 h and Firefly activity was normalized to *Renilla* activity. The fold change in normalized Firefly luciferase activity of cells transfected with pECE-FKHRL1(A3) relative to empty vector is plotted. The data represent ±SEM of at least three experiments. pGL3-Promoter (pGL3-P), an SV40-LUC reporter, is included for reference. Activation of *bim*-LUC(dm) was significantly reduced at both concentrations compared to *bim*-LUC ($P < 0.02$ at 0.33 μg and $P < 0.002$ at 1 μg, *t* tests). (C) The bim1 and bim2 mutations abolish *bim* promoter activation after NGF withdrawal. Sympathetic neurons were injected with 0.01 mg/ml of the *bim*-LUC or *bim*-LUC(dm) reporters together with gp IgG and were cultured with (+) or without (−) NGF for 20–22 h. Reporter activities were then determined by luciferase immunostaining as in Fig. 4 B. The data represent the mean of six experiments ± SEM (*, $P < 0.002$, *t* test, *bim*-LUC +NGF vs. −NGF). (D) Sympathetic neurons were injected with 0.02 mg/ml of the *bim*-LUC or *bim*-LUC(dm) reporters together with 0.01 mg/ml of pRL-TK to act as an injection control. Cells were then cultured with (+) or without (−) NGF for 16 h and *bim-LUC* and *Renilla luciferase* (*Renilla*) mRNA levels were determined by RT-PCR. A *bim*-specific and *luciferase*-specific primer spanning *bim* intron 1 (A) were used for *bim-LUC* amplification to prevent amplification from endogenous *bim* transcripts and to confirm that mutation of the bim2 site did not affect splicing. Relative changes in *bim-LUC* levels after NGF withdrawal were then calculated as changes in band intensities on ethidium-stained 2.5% agarose gels after normalization to *Renilla* levels. The data represent the mean of three experiments ± SEM (*, $P < 0.025$, *t* test, wild-type *bim*-LUC mRNA levels +NGF vs. −NGF). Representative gel images are also shown.

at the mRNA level by RT-PCR (Fig. 6 D). Activation of the wild-type *bim*-LUC reporter was again observed, this time as an increase in normalized *bim-LUC* mRNA levels after NGF withdrawal, but there was no apparent activation of the *bim*-LUC(dm) reporter. Therefore, the bim1 and bim2 FOXO binding sites appear to be required for *bim* promoter activation after NGF withdrawal in sympathetic neurons, at least in the context of the *bim*-LUC reporter.

FOXO activity contributes to NGF withdrawal–induced death

Because BIM expression contributes to NGF withdrawal–induced death (Putcha et al., 2001; Whitfield et al., 2001) and the FKHRL1(A3)-induced death of sympathetic neurons (Fig. 3 C), we investigated whether FOXO transcriptional activity contributes to NGF withdrawal–induced death. To inhibit FOXO activity, we generated an expression construct encoding FKH(DBD)—the DBD of FKHRL1(A3) with a FLAG epitope tag (Fig. 7 A). This region of FKHRL1 can bind to FOXO sites in DNA (Fig. 5) and can act as a competitive inhibitor of FKHRL1-mediated transcription (Dijkers et al., 2002). We found that FKH(DBD) localizes to the nucleus in sympathetic neurons (Fig. 7 B) and is a specific inhibitor of FOXO transcriptional activity: FKH(DBD) could inhibit FKHRL1(A3)-mediated activation of the *bim*-LUC reporter but did not block activation of the *c-jun*-LUC reporter by MEK kinase 1 (MEKK1), which involves activation of the JNK–c-Jun pathway and is not dependent on FOXO activity (Fig. 7 C).

Next, we investigated the effect of FKH(DBD) expression on the survival of sympathetic neurons for 3 d after NGF withdrawal (Fig. 7 D). Survival of neurons injected with the FKH(DBD) expression construct was compared directly to neurons injected with either empty vector or an expression construct encoding FLAGΔ169, a dominant negative c-Jun mutant, which delays NGF withdrawal–induced death as effectively as Bcl-2 (Ham et al., 1995). A significantly higher percentage of survival was seen at all time points for cells expressing FKH(DBD) compared with those injected with empty vector, and FKH(DBD) expression protected cells from death as effectively as FLAGΔ169. This indicates that FOXO activity contributes significantly to the NGF deprivation–induced death of sympathetic neurons.

Discussion

We have found that FOXO transcription factors regulate transcription of the *bim* gene in sympathetic neurons. The

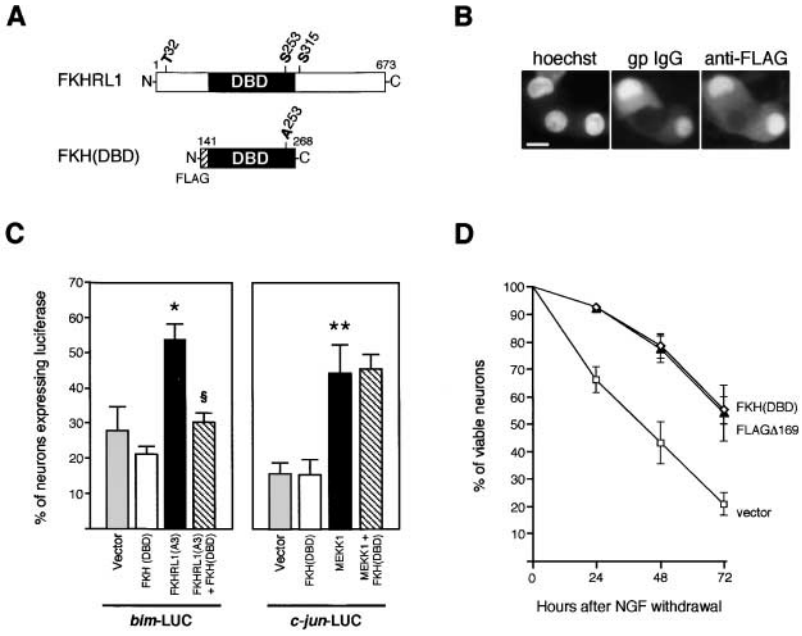

Figure 7. **Inhibition of FOXO activity delays the NGF withdrawal–induced death of sympathetic neurons.** (A) Structure of FKHRL1 and FKH(DBD). Key regulatory phosphorylation sites are shown for FKHRL1. FKH(DBD) is the DNA-binding domain (DBD) of FKHRL1 (amino acids 141–268) with the S253A mutation found in FKHRL1(A3) and an NH_2-terminal FLAG epitope tag. (B) FKH(DBD) localizes to the nucleus. Neurons were injected with 0.05 mg/ml of the FKH(DBD) expression construct and gp IgG. After 24 h, neurons were fixed and stained with Hoechst dye, to visualize nuclei, and antibodies to gp IgG and the FLAG epitope to identify injected cells and detect FLAG-tagged FKH(DBD). Representatives images are shown. Bar, 10 μm. (C) FKH(DBD) is a specific inhibitor of FOXO transcriptional activity. Neurons were injected with either 0.01 mg/ml of the *bim*-LUC reporter or 0.001 mg/ml of the *c-jun*-LUC reporter together with expression vectors (or the corresponding empty vectors) for 0.01 mg/ml of FKHRL1(A3), 0.1 mg/ml of MEKK1, and 0.05 mg/ml of FKH(DBD) as indicated, and gp IgG as a marker. 20–24 h later the percentage of cells expressing luciferase was determined (as in Fig. 1). The averages of at least three experiments ±SEM are shown. FKHRL1(A3) and MEKK1 significantly increased luciferase expression in cells injected with the *bim*-LUC and *c-jun*-LUC reporters respectively (*, $P < 0.02$, **, $P < 0.05$, *t* test), but FKH(DBD) only inhibited activation of the *bim*-LUC reporter by FKHRL1(A3) (§ indicates $P < 0.005$, *t* test). (D) Sympathetic neurons were injected with expression constructs encoding FKH(DBD) or FLAGΔ169 (a dominant-negative c-Jun mutant), or empty vector (all at 0.05 mg/ml) together with Texas red dextran as a marker. Cells were allowed to recover overnight and were then deprived of NGF. The number of viable injected neurons was determined at 0, 24, 48, and 72 h after NGF withdrawal. Survival is expressed as a percentage of the number of viable injected neurons at time 0. Experiments were performed in a blinded manner and the average of three experiments ±SEM is shown. Survival was significantly increased by FKH(DBD) compared with empty vector ($P < 0.005$ at 24 h, $P < 0.02$ at 48 h, $P < 0.01$ at 72 h, *t* tests).

observations that FKHRL1 is expressed in sympathetic neurons and that its PI3-K–dependent phosphorylation and localization are regulated by NGF in these cells is consistent with this (Fig. 2). In addition, our finding that expression of constitutively active FKHRL1 can activate the *bim* promoter in these cells (Fig. 4), and that this is largely abolished when two conserved FOXO binding sites located close to the *bim* promoter are mutated to prevent FOXO binding (Figs. 5 and 6), demonstrate that the *bim* gene is a direct FOXO target. This had previously only been implied based on a correlation between BIM expression levels and FKHRL1 phosphorylation status (Dijkers et al., 2000; Linseman et al., 2002; Stahl et al., 2002). More importantly, these FOXO binding sites appear to be required for *bim* promoter activation after NGF withdrawal in sympathetic neurons. Although these results were obtained using synthetic *bim*-LUC reporter constructs, we have found that the wild-type reporter behaves in a similar manner to the endogenous gene when subjected to a variety of stimuli. Therefore, we can predict that FOXO transcription factors contribute to the up-regulation of the *bim* gene in sympathetic neurons deprived of NGF.

We have also demonstrated an important role for FOXO transcription factors in the induction of apoptosis in sympathetic neurons. As well as showing that expression of constitutively active FKHRL1 can induce death (Fig. 3), we have also found that inhibition of FOXO activity can protect sympathetic neurons against NGF withdrawal–induced death (Fig. 7). Most of the FKHRL1(A3)-induced death occurred during the first 48 h, after which the rate of death was comparable to that of control neurons. Although this may simply reflect the fact that FKHRL1(A3) is expressed transiently in these assays, it is also possible that a subpopulation of the neurons are resistant to this death. Similarly, the observation that inhibition of FOXO activity does not block NGF withdrawal–induced death indefinitely might be due to the transient nature of the assay, although it is also possible that the eventual death of these neurons is the result of the NGF withdrawal–induced activation of FOXO-independent pathways, such as the JNK pathway.

The finding that FKHRL1(A3)-mediated death of sympathetic neurons is dependent on BIM expression (Fig. 3) suggests that the *bim* gene is a critical proapoptotic target of FOXO transcription factors in these cells. Given that *bim* deletion and BIM inhibition confer transient protection against NGF withdrawal–induced apoptosis (Putcha et al., 2001; Whitfield et al., 2001), inhibition of *bim* transcription probably contributes to the delay in NGF withdrawal–induced death when FOXO activity is inhibited. However, other FOXO-regulated genes could also be involved in this apoptosis. One possible candidate is the *FasL* gene which can be activated by FKHRL1 and which is modestly induced at the RNA level in NGF-deprived sympathetic neurons (Putcha et al., 2002), as well as in neuronal PC12 cells and cerebellar granule neurons deprived of survival signals (Brunet et al., 1999; Le-Niculescu et al., 1999). However, an analysis of sympathetic neurons with inactivating mutations in their *FasL* or *Fas* genes suggested that the Fas pathway does not contribute to NGF withdrawal–induced death (Putcha et al., 2002). Other potential candidates include the

genes encoding transforming growth factor-β2, the BH3-only protein NIP3, and the cysteine protease legumain, which have all been identified as FOXO targets (Samatar et al., 2002; Tran et al., 2002). One way to test whether other FOXO-regulated genes might contribute to this death would be to assess whether inhibition of FOXO activity confers additional protection against NGF withdrawal–induced death in *bim* deficient neurons.

Use of the pharmacological inhibitor LY294002 in this work enabled us to assess the role of the PI3-K signaling pathway in the regulation of the *bim* gene. However, in addition to its ability to inhibit FOXO activity, PI3-K signaling can also inhibit the JNK pathway in some cell types via Akt-mediated phosphorylation of mixed lineage kinase 3 (Barthwal et al., 2003). Therefore, the demonstration that c-Jun phosphorylation increases in sympathetic neurons treated with LY294002 (Tsui-Pierchala et al., 2000; Putcha et al., 2001) suggests that cross-talk between these pathways also occurs in these cells. However, despite the fact that PI3-K inhibition can result in activation of c-Jun and the FOXO transcription factors—which both appear to be important regulators of *bim* transcription in these cells—the LY294002-induced increase in *bim* mRNA is still only ∼60% of that observed after NGF withdrawal (Fig. 1 B). Although this may simply reflect the fact that PI3-K inhibition may not activate these factors to the same extent as NGF withdrawal, it is also possible that regulation of *bim* gene transcription by NGF in sympathetic neurons might involve additional signaling pathways and transcription factors. Furthermore, because the LY294002-induced increase of *bim* mRNA levels is not fully reflected at the protein level (induced BIM protein levels are only ∼30% of that seen after NGF withdrawal; Fig. 1 A), it is possible that NGF-regulated pathways also modulate BIM expression posttranscriptionally.

A hypothetical model of *bim* gene regulation by NGF in sympathetic neurons is outlined in Fig. 8. Based on current knowledge, it now appears that this is significantly more complex than the comparable situation in hematopoietic cells where the PI3-K–mediated regulation of FOXO transcription factors alone might be responsible for full BIM induction after growth factor withdrawal (Dijkers et al., 2000, 2002; Stahl et al., 2002). Because neurons cannot be replaced, additional regulatory complexity may have evolved to prevent unnecessary apoptosis resulting from inappropriate BIM expression (Sanchez and Yuan, 2001). Therefore, further investigation may help elucidate the relative contributions that the JNK–c-Jun and PI3-K–FOXO signaling pathways make to regulation of *bim* gene expression and apoptosis in NGF-dependent sympathetic neurons and other neuronal populations, and may additionally lead to the identification of other pathways that contribute to this regulation.

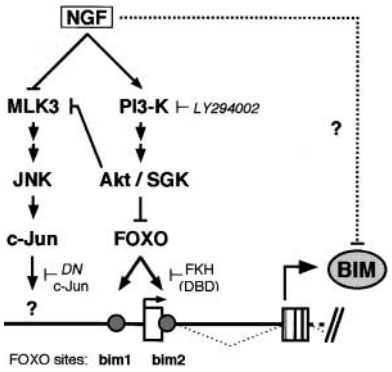

Figure 8. **Regulation of *bim* gene expression by NGF in sympathetic neurons.** FOXO transcriptional activity is inhibited by NGF via PI3-K signaling and Akt/SGK-mediated phosphorylation. NGF withdrawal and LY294002 inhibit PI3-K signaling, resulting in FOXO activation and subsequent activation of the *bim* promoter via the bim1 and bim2 FOXO binding sites. The JNK pathway is repressed in the presence of NGF. PI3-K signaling could account for some of this repression via Akt-mediated inhibition of MLK activity but PI3-K–independent pathways may also be involved. NGF withdrawal and LY294002 activate the JNK pathway via mixed lineage kinase 3 (MLK3), resulting in c-Jun activation. Dominant-negative c-Jun (*DN* c-Jun) can partially inhibit BIM induction after NGF withdrawal, which suggests that c-Jun/AP-1 may also directly regulate the *bim* promoter. Furthermore, our results do not rule out the possibility that other transcription factors/signaling pathways might also be involved in regulation of *bim* promoter activity or that NGF might regulate BIM expression post transcriptionally.

Materials and methods

5′ RACE and library screening

5′ RACE was performed on rat brain mRNA using the Marathon™ cDNA Amplification kit (CLONTECH Laboratories, Inc.) with the *bim*-specific primer 5′-ACCTTGCGATTCTGTCTGTAGG-3′. The rat *bim* promoter was isolated using the 5′ RACE product as a probe to screen the rat P1 artificial chromosome (PAC) library RPCI31 (generated by P.Y. Woon and P. de Jong, UK Human Genome Mapping Project Resource Centre, Cambridge, UK). Restriction fragments from PAC clones 62g18 and 215h9 were subcloned and sequenced.

Plasmid constructs

The *bim*-LUC reporter was constructed by subcloning a 5.2-kb fragment containing the region 5′ to the *bim* initiator codon into pGL3-Basic (Promega). The integrity of the construct was confirmed by sequencing. Mutations in the bim1 and bim2 sites were incorporated into the *bim*-LUC reporter using the QuikChange™ XL Site-Directed Mutagenesis kit (Stratagene). The *bim*-LUC(m1) reporter was generated using oligonucleotides 5′-CAAGTCACTAGGGTACCCACGCCGGGGTGGGC-3′ and 5′-GCCCACCCCGGCGTGGGTACCCTAGTGACTTG-3′ incorporating bim1 mutations (mut1). The *bim*-LUC(m2) reporter was generated using oligonucleotides 5′-GAGAAACGCAAGGTAAGTTCTCCTCGACTCCCGG-3′ and 5′-CCGGGAGTCGAGGAGAACTTACCTTGCGTTTCTC-3′ incorporating bim2 mutations (mut2). The *bim*-LUC(dm) reporter was generated by replacing the wild-type bim1 site from *bim*-LUC(m2) with the mutated bim1 site from *bim*-LUC(m1) by standard cloning procedures. Sequencing confirmed that mutations had been incorporated correctly. The *c-jun*-LUC reporter was generated by inserting the human *c-jun* promoter (−1,600 to +170) into pGL3-Basic. pGL3-Promoter was obtained from Promega.

pCD-FKH(DBD) encoding NH_2-terminal FLAG-tagged FKH(DBD) was generated by PCR amplification of the DBD (amino acids 141–268) of FKHRL1(A3) using primers 5′-ACTGGATCCGCTGGGGGCTCCGGGCAGCCG-3′ and 5′-ACTGAATTCCTAGGCTGCGCGGCCACGGCTC-3′ followed by cloning into BamHI and EcoRI-restricted pcDNA1-FLAG (Vekrellis et al., 1997). pECE-FKHRL1, pECE-FKHRL1(A3), both provided by M.E. Greenberg (Harvard Medical School, Boston, MA) pMEKK1, and pCD-FLAGΔ169 have been described previously (Ham et al., 1995; Eilers et al., 1998; Brunet et al., 1999).

Cell culture and transient transfection

Sympathetic neurons were isolated from the superior cervical ganglia (SCG) of 1-d-old Sprague Dawley rats and cultured as described previously (Ham et al., 1995; Eilers et al., 1998). SCG medium was supplemented with 2.5S NGF (Cedarlane) at 50 ng/ml and fluorodeoxyuridine and uridine, each at 20 μM. Typically 6,400–8,000 neurons were plated on 13-mm-diam glass coverslips coated with poly-L-lysine and laminin or 10^5 neurons were plated on 3.5-cm poly-L-lysine and laminin-coated tissue culture dishes. Cells were used for experiments after 5–7 d in vitro. In

NGF-withdrawal experiments, neurons were rinsed twice with medium (without NGF) and were re-fed with medium containing 100 ng/ml anti-NGF antibody. The PI3-K inhibitor LY294002 was typically used at a concentration of 50 μM.

The PC6-3 subline of the PC12 cell line was maintained as described previously (Pittman et al., 1993). Cells were differentiated for 5–7 d in RPMI 1640 medium containing 2% horse serum, 1% FCS, penicillin/streptomycin and 100 ng/ml of NGF (Promega) to obtain a neuronal phenotype. Cells were transfected using Lipofectamine 2000 (Invitrogen). Naïve PC12 cells were seeded in 6-well plates at 4×10^5 cells per well and were transfected 24 h later with 4 μg of *bim* antisense or missense oligonucleotide mixtures together with 1 μg of GFP expression construct. After 24 h, GFP expressing cells were collected by FACS® and processed for immunoblotting. For *bim*-LUC reporter activity assays, neuronal PC12 cells were cultured in 24-well plates at 4×10^4 cells per well and were transfected with 0.5–1.5 μg of reporter and expression construct DNAs together with 50 ng of pRL-TK (Promega). A transfection efficiency of 10–20% was achieved.

Immunoblotting

Neurons were treated as described in Results, washed in ice-cold PBS, and lysed in sample buffer (2% SDS, 2 mM β-mercaptoethanol, 60 mM Tris, pH 6.8, and 0.01% bromphenol blue) by incubating at 100°C for 15 min. Proteins were separated on 8–12% SDS polyacrylamide gels and transferred to Immobilon-*P* membrane (Millipore) using the Mini-PROTEAN III transfer system (Bio-Rad Laboratories). Protein detection membrane stripping was performed as described previously (Whitfield et al., 2001) or following protocols supplied with the primary antibodies. The following primary antibodies were used: rabbit polyclonal antiphospho-FKHRL1(Thr^{32}; Upstate Biotechnology); anti-FKHRL1 (Upstate Biotechnology); anti–caspase-3 (Upstate Biotechnology); anti-BAX (Vekrellis et al., 1997); anti-BIM (CHEMICON International, Inc.); sheep polyclonal anti-FKHR (Upstate Biotechnology); mouse monoclonal BCL-2 and anti–BCL-X_L (BD Transduction Labs); anti–β-galactosidase (Promega); and rat monoclonal antitubulin (Serotec). Appropriate HRP-conjugated secondary antibodies (Amersham Biosciences and Santa Cruz Biotechnology, Inc.) were used for detection using the ECL and ECL-plus systems (Amersham Biosciences). Relative band intensities were determined using a GS-800 calibrated densitometer (Bio-Rad Laboratories) and Quantity One software.

RT-PCR

Neurons were treated as described in Results and RNA was isolated using the RNeasy kit (QIAGEN). One third of the RNA were reverse transcribed using Superscript II reverse transcriptase (Invitrogen) as instructed. Reactions were made up to 200 μl with water and 10 μl was used in 50-μl PCR reactions containing 200 μM dNTPs, oligonucleotide primers at 0.5 μM, and 0.05 U/ μl of REDTaq DNA polymerase in 1X REDTaq PCR buffer (Sigma-Aldrich). Typically 28–36 cycles of 94°C for 30 s, 59 or 60°C for 30 s, and 72°C for 30 or 60 s were performed. PCR products were resolved on 2.5% agarose gels and visualized by ethidium bromide staining. Images were captured using a UVItec Gel-DOC system and bands were quantified using ImageMaster TotalLab imaging software (Amersham Biosciences). Reactions were analyzed over a range of cycles to confirm that the PCR amplification dynamics were in the linear range. Experiments were performed at least three times and PCR was performed twice for each cDNA sample and the average taken. Primers used were as follows: *bim*, 5′-CTACCAGATCCCCACTTTTC-3′ and 5′-GCCCTCCTCGTGTAAGTCTC-3′; *neurofilament-M* (*NF-M*), 5′-ACGCTGGACTCGCTGGGCAA-3′ and 5′-GCGAGCGCGCTGCGCTTGTA-3′; *bim-LUC* hybrids, 5′-AGGCACTGGGTCAACAGC-3′ and 5′-CTTTATGTTTTTGGCGTCTTCC-3′; and *Renilla luciferase*, 5′-TGATCCAGAACAAAGGAAACGG-3′ and 5′-CCTGATTTGCCCATACCAATAAGG-3′.

Microinjection and immunocytochemistry

Neurons were microinjected as described previously (Whitfield et al., 2001). Experiments were performed at least three times with 200 neurons injected per injection mix. Neurons were injected with plasmids at the concentrations indicated.

For luciferase immunostaining, neurons were coinjected with 2.5 mg/ml purified guinea pig IgG (Sigma-Aldrich) to act as a marker and were treated as described in Results. After 20–24 h, neurons were fixed with 4% PFA (20 min), rinsed in PBS, and blocked with 50% horse serum in PBS (30 min). After rinsing in PBS, neurons were incubated for 1 h with a goat anti-luciferase polyclonal antibody (Promega) diluted 1:100 in PBS containing 10% horse serum. Neurons were again rinsed in PBS and incubated for 1 h with FITC-conjugated anti–goat IgG and rhodamine-conjugated anti-gp IgG antibodies (Jackson ImmunoResearch Laboratories), and diluted 1:100 in PBS containing 10% horse serum. After rinsing in PBS, nuclei were then stained with Hoechst dye at 10 μg/ml in water. After a final rinse in water, coverslips were mounted on slides in Citifluor. Slides were examined using a fluorescence microscope (model Axioplan 2; Carl Zeiss MicroImaging, Inc.) and images captured using a digital camera (Photometrix Quantix) and SmartCapture VP software. Injected neurons were scored as positive for luciferase expression when FITC staining was clearly greater than background fluorescence in neighboring uninjected cells. Experiments were scored in a blinded manner whenever possible.

Localization of FLAG-tagged FKH(DBD) and HA-tagged FKHRL1 and FKHRL1(A3) was determined by immunostaining using the anti-FLAG M2 (Sigma-Aldrich) and anti-HA 12CA5 (Roche) mAbs as described previously (Ham et al., 1995; Eilers et al., 1998).

Adenoviral infection

Sympathetic neurons were cultured for 4–5 d and were infected overnight with the required adenoviral multiplicity of infection by re-feeding with a minimal volume of SCG medium containing recombinant adenoviruses. Neurons were then re-fed with virus-free SCG medium and incubated for 48 h. The FKHR(ADA) adenovirus (Ad-ADA-Foxo1; provided by D. Accili, Columbia University, New York, NY) and the LacZ adenovirus have been described previously (Nakae et al., 2001; Whitfield et al., 2001). We used each adenovirus at the lowest multiplicity of infection that resulted in infection of >70% of neurons as determined by immunostaining for expression of the encoded proteins.

Survival assays

In survival assays, neurons were injected with expression vectors and/or oligonucleotides (at the indicated concentrations) together with 5 mg/ml Texas red dextran $M_r = 70{,}000$ (Molecular Probes) as a marker. After treatment (as described in Results), number of viable, morphologically normal injected neurons was determined using an inverted fluorescence microscope (model Axiovert 100; Carl Zeiss MicroImaging, Inc.). Viable injected cells were recounted at 24, 48, and 72 h. Experiments were scored in a blinded manner whenever possible. The *bim* antisense and missense oligonucleotides have been described previously (Whitfield et al., 2001).

Luciferase assays

Luciferase assays were performed using the Dual-Luciferase reporter assay system (Promega) and a Turner TD-20e luminometer (Jencons Scientific Limited). Firefly output was normalized to *Renilla* output to control for transfection efficiency.

Expression and purification of GST-FKH

The FKHRL1 DBD was subcloned from pCD-FKH(DBD) into pGEX-6P-2 and transformed into *Escherichia coli* DH5α. Expression and purification of GST-FKH was performed according to standard procedures (Smith and Corcoran, 1994). After purification on glutathione Sepharose 4B (Amersham Biosciences), the fusion protein was mixed with glycerol (to 15%) and DTT (to 2 mM) and stored at −80°C in aliquots. Purity was assessed by SDS-PAGE with Coomassie blue staining.

Electrophoretic mobility shift assay

Double stranded oligonucleotides with 5′ overhangs were labeled by filling in with Klenow polymerase and α-[^{32}P]dCTP. The following pairs of oligonucleotides were used (FOXO sites underlined): FasLA 5′-CTAGAATAAGTAAATAAATAA-3′ and FasLB 5′-CTAGTTATTTATTTACTTATT-3′; bim1A 5′-CTAGCTAGGGTAAACACGCCG-3′ and bim1B 5′-CTAGCGGCGTGTTTACCCTAG-3′; and bim2A 5′-CTAGGCAAGGTAAACACTCCT-3′ and bim2B 5′-CTAGAGGAGTGTTTACCTTGC-3′. The mut1 and mut2 oligonucleotides were identical to bim1 and bim2 except that they contained the point mutations shown in Fig. 4 A. 20-μl binding reactions contained 20 mM Hepes, pH 7.9, 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 2 mM DTT, 10% glycerol, 0.5 mg/ml BSA, 2 μg of poly (dI dC), and cold competitor oligonucleotide as required. 1 μl of purified GST FKH was added and the samples were incubated at room temperature for 15 min. Finally the samples were incubated with 0.4 ng of ^{32}P-labeled oligonucleotide for 15 min and loaded onto a 6%-nondenaturing polyacrylamide gel containing 25 mM Tris, 192 mM glycine, 1 mM EDTA, and 5% glycerol. After electrophoresis at 4°C, the gel was fixed, dried, and exposed in a phosphorimager cassette (Amersham Biosciences). The screen was scanned in a Typhoon phosphorimager (Amersham Biosciences).

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