FOXO transcription factors directly activate \textit{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 \textit{bim} promoter via two conserved FOXO binding sites and that mutation of these sites abolishes \textit{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 \textit{bim} and \textit{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
though BIM induction in cerebellar granule neurons correlates with dephosphorylation of FKHR1 (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.

**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](image-url)

**Figure 2.** NGF regulates the phosphorylation and localization of FKHR1 in sympathetic neurons. (A) NGF regulates FKHR1 phosphorylation via PI3-K. Neurons were cultured with or without NGF (+NGF), or treated with 100 μM LY294002 (+LY) for 16 h. FKHR1 expression and phosphorylation was then assessed by immunoblotting using anti-FKHR1 and antiphospho (Thr32)-FKHR1 antibodies. Representative blots are shown (asterisk indicates the phospho-specific antibody detects a nonspecific band of this size). (B) NGF regulates FKHR1 localization. Neurons were injected with an expression construct encoding 0.1 mg/ml wild-type FKHR1 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 FKHR1. Representative images are shown. FKHR1 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.
FOXO transcription factors regulate bim in neurons

NGF regulates FKHR1 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 FKHR1 is expressed in sympathetic neurons and that phosphorylation at one of the critical phosphorylation sites (Thr32) 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 FKHR1 from the cytoplasm to the nucleus (Fig. 2 B), whereas a constitutively active human FKHR1 mutant, FKHR1(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 FKHR1 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 gene 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 FKHR1(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 FKHR1(A3) expression on neuronal survival could, therefore, be evaluated in the context of normal or inhibited BIM expression. Neurons were infected with an FKHR1(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 cojected with the missense oligonucleotides, we observed significantly increased death of neurons expressing FKHR1(A3) compared with the corresponding control neurons. Furthermore, this depended on BIM expression because coinjecting antisense (AS) or missense (MS) oligonucleotides and an expression construct encoding GFP, and were sorted for GFP expression by FACS® analysis. In immunoblotting experiments, we observed a sixfold increase in BIM 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).
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.

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**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).
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 (500X) of unlabeled FasL, bim1, or bim2 oligonucleotides (lanes 3–5). 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 FKHR1(A3) and NGF withdrawal

Next, we investigated whether the bim1 and bim2 FOXO binding sites are necessary for activation of the bim promoter by FKHR1(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 FKHR1(A3). Neuronal PC12 cells were transiently cotransfected with the bim-LUC reporters and an FKHR1(A3) expression construct or empty vector and luciferase activity determined 24 h later (Fig. 6 B). Activation of the bim-LUC reporter by FKHR1(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 FKHR1(A3). Modest activation of the bim-LUC(dm) reporter at the higher concentration of FKHR1(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 FKHR1(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.
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.

**Discussion**

We have found that FOXO transcription factors regulate transcription of the *bim* gene in sympathetic neurons. The transcriptional activity: FKH(DBD) could inhibit FKHL1(A3)-mediated activation of the *bim*-LUC reporter but did not block activation of the *c-jun*-LUC reporter by MEK kinase 1 (MEK1), 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.
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Inhibition of FOXO activity delays the NGF withdrawal–induced death of sympathetic neurons. (A) Structure of FKHR1 and FKH(DBD).

Key regulatory phosphorylation sites are shown for FKHR1. FKH(DBD) is the DNA-binding domain (DBD) of FKHR1 (amino acids 141–268) with the S253A mutation found in FKHR1(A3) and an NH₂-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). Representative 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 FKHR1(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. FKHR1(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 FKHR1(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).

The finding that FKHR1(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 FKHR1 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.

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′-ACCTGGATTCTGTGGTAGGG-3′. The rat bim promoter was isolated using the 5′ RACE product as a probe to screen the rat P1 artificial chromosome (PAC) library RPC11 (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 21b9 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′-CAAGTCACTAAGGTACCACGCCGGTCGACC-3′ and 5′-GCCACCCCCGGGTGGTATCCACTAGGTA-3′ incorporating bim1 mutations (mut1). The bim-LUC(m2) reporter was generated using oligonucleotides 5′-GAGAAACGCAAGGTAAGTT and 5′-ACCTTGCGATTCTGTCTGTAGG-3′ incorporating bim2 mutations (mut2). The bim-LUC(m1m2) 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-Basic-Promoter was obtained from Promega.

pCD-FKHDBD encoding NH2-terminal FLAG-tagged FKHDBD was generated by PCR amplification of the DDBI (amino acids 141–268) of FKHR(1 A3) using primers 5′-ACTGGATCCGCTGGGGCGGCCGGCATCCTAAGGGC-3′ and 5′-ACTAAGTCACTAAGGTACCACGCCGGTCGACC-3′ with pARE2A using 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-Basic-Promoter was obtained from Promega.

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 2.55 NGF (Cedrine) 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 105 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 containing 2% horse serum and 1% FCS, when FITC-tomycin and 100 ng/ml of NGF (Promega) to obtain a neuronal phenotype. Cells were transfected using Lipofectamine 2000 (Invitrogen). Naive 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 × 10^5 cells per well and were transfected with 0.5–1.5 μg of reporter and expression construct DNAs together with 50 ng of pBLC-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% bromophenol blue) by incubating at 100°C for 20 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 striping 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(Thr32); 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 (BD Transduction Labs); anti-β-galactosidase (Promega); and rat monoclonal antibulin (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 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-FKHRL1 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–goat 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-tomycin staining was clearly higher than background fluorescence in neighboring un.injected cells. Experiments were scored in a blinded manner whenever possible.

Localization of FLAG-tagged FKH(DD) 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 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 (Nake 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 concentrations) together with 5 mg/ml Texas red dextran M = 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 Axiosvert 100; Carl Zeiss MicroImaging, Inc.). Viable injected cells were recounted at 24, 48, and 72 h. Experiments 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 sample and the average taken. Primers used were as follows:

- **bim**
  - 5′-TCCTCCCATCCCTCCTTTCTT-3′ and 5′-GGCCCTCTGGGTTAAGCTTC-3′;
  - **neurofilament-M**
  - 5′-TCCTACAGACCTGCCTTTGGC-3′ and 5′-TCCTACAGACCTGCCTTTGGC-3′;
  - **FOXO transcription factors regulate bim in neurons | Gilley et al. 621**

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The FKHR1 DD was subcloned from pCD-FKH(DD) into pGEX-6P-2 (Amersham Biosciences), the fusion protein was mixed with glycerol (to 15%) and purified on glutathione Sepharose 4B (Amersham Biosciences). After purification of the fusion protein, the GST-FKH was used to pull down bim protein from an in vitro transcription assay. The GST-FKH and bim were then resolved by SDS-PAGE with Coomassie blue staining.
like to thank Jo Buddle for help with FACS®

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