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 NH2-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 out.
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 FKHR1L1 (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), FKHR1L1 (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 deprivation and inhibition of PI3-K activity cause FKHR1L1 dephosphorylation, induction of BIM expression, and FKHR1L1-dependent apoptosis (Dijkers et al., 2000, 2002; Stahl et al., 2002). In addition, overexpression of constitutively active FKHR1L1 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 FKHR1L1 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 FKHR1L1 (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 pharmacologic-
physical 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 \textit{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 (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 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 \textit{bim} gene.

FOXO transcription factors can induce \textit{bim} gene expression and promote a BIM-dependent death of sympathetic neurons

To investigate whether FOXO transcription factors can regulate \textit{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 \textit{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 \textit{bim} antisense strategy to inhibit BIM expression (Whitfield et al., 2001). We found that the \textit{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 \textit{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-

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antisense (AS) or missense (MS) oligonucleotides and an expression construct encoding GFP, and were sorted for GFP expression by FACSort after 24 h. BIM, BAX, BCL-2, BCL-X, 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 \textit{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 infected with missense oligonucleotides (P ≤ 0.05, t tests, FKHRL1(A3) + MS vs. vector + MS), and this was blocked in neurons injected with the \textit{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 FKHR1(A3)-induced death.

**NGF withdrawal, PI3-K inhibition, and FKHR1 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. 4A), 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. 4B), 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. 4C). 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. 4D) and increased in a dose-dependent manner in neurons expressing constitutively active FKHR1(A3) and, to a lesser extent, wild-type FKHR1 (Fig. 4E). Although FKHR1(A3) was found to be a potent activator at both high and low concentrations, wild-type FKHR1 did not significantly activate the reporter at the lower concentration, and only caused a modest activation, relative to FKHR1(A3), at the higher concentration. This is consistent with the ability of NGF to regulate the localization of wild-type FKHR1, but not FKHR1(A3) (Fig. 2 and text), with some FKHR1 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.
taining 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 FKHR1 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.

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 FKHR1 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 FKHR1(A3) fused to GST. As expected, GST-FKH bound to a control oligonucleotide (Fas ligand [FasL]) con-
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 FKHR1L1(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 FKHDDBD—the DBD of FKHR1L1(A3) with a FLAG epitope tag (Fig. 7 A). This region of FKHR1L1 can bind to FOXO sites in DNA (Fig. 5) and can act as a competitive inhibitor of FKHR1L1-mediated transcription (Dijkers et al., 2002). We found that FKHDDBD localizes to the nucleus in sympathetic neurons (Fig. 7 B) and is a specific inhibitor of FOXO transcriptional activity: FKHDDBD could inhibit FKHR1L1(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 FKHDDBD expression on the survival of sympathetic neurons for 3 d after NGF withdrawal (Fig. 7 D). Survival of neurons injected with the FKHDDBD 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 FKHDDBD compared to those injected with empty vector, and FKHDDBD 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
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 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).

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 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′-ACCTTGAGATCTGCTGAGAGG-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 215b9 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′-CAAGTCACTAGGTTACCCAGCCGGTGGC-3′ and 5′-GCCCCAACCCGGGTTGTCCATTGCATTG-3′ incorporating bim1 mutations (mut1). The bim-LUC(m2) reporter was generated using oligonucleotides 5′-GAGAAGCGAGTAAGTTCCTCGAATCCGCCC-3′ and 5′-GCCGGAATCTGACGAGTTACCTTCGTTCTCC-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 ~1,700 into pGL3-Basic, pGL3-Promoter was obtained from Promega. pCD-FKH(DBD) encoding NH2-terminal FLAG-tagged FKHDDBD was generated by PCR amplification of the D31 (amino acids 141–268) of FKHD1(A3) using primers 5′-ACTGGATCCGCTGGGCTGCCGCACCGGG-3′ and 5′-ACTGGATCCGCTGGGCTGCCGCACCGGG-3′ followed by cloning into BamHI and EcoRI-restricted pDNA1–FLAG (Vekrellis et al., 1997), pECE-FKHRL1, pECE-FHKL1(A3), both provided by M.E. Greenberg (Harvard Medical School, Boston, MA; pMEKK1, and pCD-FLAGΔHS 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 2.55 ng/ml NGF (Cedarlane) at 30 ng/ml and fluoro-N-sulfonylurea and uridine, each at 20 μM. Typically 4,600–8,000 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.
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 P3-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 medium containing 2% horse serum, 1% FCS, and 100 ng/ml 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 × 10⁵ cells per well and were transfected 24 h later with 4 μg of bim antisense or misense 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 × 10⁴ cells per well and were transfected with 0.5–1.5 μg of reporter and expression construct DNAs together with 50 ng of pRK-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-FKHR1L(Thr112); Upstate Biotechnology; anti-FKHR1L (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 (Serotec). Relative reading of the manuscript and for helpful discussions. We would also like to thank Susana Terzano and Jonathan Whitfield for critical reading of the manuscript and for helpful discussions. We would also

**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 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 quantitated 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'-CTACCAGATCCCCACCTTTTC-3' and 5'-GCCCTCCTCCTGATGAATCTC-3'; neurotDMat-1 (NF-M, 5'-AGCCTGCAACCTGGCGGCAA-3' and 5'-GCCAGCGCGTCCGCTTGTA-3'); luc-LUC hybrids, 5'-AGCAGTCT- GCCATACAGC-3' and 5'-CTATGTGTTCGTTGCTCC-3'; and Reportlucerase, 5'-TGATCCAGACAAAGGAAAAGG-3' and 5'-CTAGTGGGCCCATAACAAAGG-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 Axiosplan 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 FKHR1L(DBD) and HA-tagged FKHR1L was assessed 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-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 = 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 Axiosert 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 misense 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 (Lecens Scientific Limited). Firefly output was normalized to Renilla output to control for transfection efficiency.

**Expression and purification of GST-FKH**

The FKHR1L 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 glyceraldehyde (15%) and DTT (2 mM) and stored at − 80°C in aliquots. Purification 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 Klence polymerase and α-[32P]dTTP. The following pairs of oligonucleotides were used (FOXO sites underlined): FasLα 5′-CTA GAAATAAATAAAAT-3′ and FasLB 5′-CTATGTATTATTTATCTATT-3′; bimTA 5′-CTACTTCAGGAATACAGCGC-3′ and bimBL 5′-CTACGTCGTTGTACCCATCT-3′; and bim2A 5′-CTATGCGAATGGTTACCGTTC-3′. The mut1 and mut2 oligonucleotides were identical to bim1 and bim2 except that they contain the point mutation as shown in Fig. 4 A. 20-μl binding reactions contained 20 mM Hepes, pH 7.9, 50 mM KCl, 2 mM MgCl₂, 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 32P-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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