Pro-apoptotic Bim Induction in Response to Nerve Growth Factor Deprivation Requires Simultaneous Activation of Three Different Death Signaling Pathways*

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Bim is a pro-apoptotic member of the Bcl-2 family that is induced and contributes to neuron death in response to nerve growth factor (NGF) deprivation. Past work has revealed that Bim is downstream of multiple independent transcriptional pathways in neurons, including those culminating in activation of the c-Jun, FoxO, and Myb transcription factors. This study addresses the issue of whether the three signaling pathways are redundant with respect to Bim induction or whether they act cooperatively. Examination of the proximal Bim promoter reveals binding sites for FoxO, Mybs, and, as shown here, c-Jun. We find that mutation of any one of these types of sites abolishes induction of a Bim promoter-driven reporter in response to NGF deprivation. Moreover, down-regulation of either c-Jun, FoxOs, or Mybs by short hairpin RNAs blocks induction of Bim promoter-reporter activity triggered by withdrawal of NGF. This was the case for reporters driven by either the proximal promoter or a promoter that also includes additional regulatory elements in the first intron of the Bim gene. Such short hairpin RNAs also suppressed the induction of endogenous Bim protein. These findings thus indicate that the Bim promoter acts as a coincidence detector that optimally responds to the simultaneous activation of three different pro-apoptotic transcriptional pathways. Such a mechanism provides a “fail-safe” that prevents neurons from dying by accidental activation of any single pathway. It also permits neurons to utilize individual pathways such as JNK signaling for other purposes without risk of demise.

The molecular mechanisms by which neurons die when deprived of trophic support are only partially understood. In many such cases there is evidence that neurons require transcription-dependent events in order to die (1, 2). In the case of nerve growth factor (NGF) deprivation, at least three different transcriptional pathways have been described that are required for death. These are the “JNK-c-Jun” pathway that includes activation of the c-Jun N-terminal kinase and phosphorylation/activation of the transcription factor c-Jun (3); the “cell cycle” pathway that includes events associated with the cell cycle in mitotic cells such as activation of cyclin-dependent kinase 4 (Cdk4), phosphorylation of retinoblastoma family members, and induction of the transcription factors B- and C-myb (4); and the “FoxO” pathway in which FoxO family transcription factors move from cytoplasm to nucleus as a consequence of reduced phosphorylation resulting from shut down of the kinase AKT (5).

An important question about these transcriptional pathways is whether they target different cell death genes or whether they share the same targets. At least in one case, a shared target has been recognized, the pro-apoptotic protein Bim (Bcl-2 interacting mediator of cell death or Bcl-2-like protein 11). Bim is a BH3-only member of the Bcl2 family that is induced in response to NGF deprivation and that participates in the ensuing death process (6, 7). Several types of evidence have shown it to be a transcriptional target of the JNK/c-Jun pathway in neuronal cells. For instance, dominant-negative c-Jun and a chemical inhibitor of the JNK pathway reduce Bim induction evoked by NGF withdrawal in sympathetic neurons and neuronal PC12 cells (6, 8, 9). Moreover, Bim induction after NGF withdrawal is reduced in sympathetic neurons of mice carrying a mutant c-Jun gene that lacks activating Ser-63/Ser-73 phosphorylation sites (10). Likewise for the FoxO pathway, Gilley et al. (16) demonstrated that overexpression of FoxO transcription factors can induce Bim (as well as Bim-dependent apoptotic death) in sympathetic neurons and that FoxO3a can directly bind and activate the Bim promoter. Moreover, mutation of the FoxO binding sites abolished activation of the promoter in response to NGF withdrawal. We recently reported that Bim is also a direct target of a cell cycle pathway in which NGF deprivation leads to activation of Cdk4, phosphorylation of the retinoblastoma family member p130 and consequent loss of p130-Cdk4 complexes, de-repression of the transcription factors B- and C-Myb, and induction of Bim (4, 11, 12). In addition, we identified two Myb binding sites in the promoter region of the Bim gene that are required for Bim induction in response to activation of the cell cycle pathway and NGF deprivation (11). Chemical inhibition of Cdk4 or shRNA-mediated depletion of Cdk4 or Mybs also abolished NGF withdrawal-promoted Bim induction.

Identification of Bim as a common target of three different transcriptional pathways in turn raises the question of whether...
such regulation occurs independently and in a redundant fashion or whether this occurs by a cooperative or additive mechanism. The answer to this issue has been unclear. Overexpression of activators of each of the pathways can promote neuron death, whereas independent blockade of any one of the pathways is sufficient to suppress neuron death in full or in part. This finding also raises the question of why multiple pathways are involved in the death process and in Bim regulation.

In the findings presented here, we have addressed these issues and find that maximal induction of Bim in response to NGF deprivation requires simultaneous occupancy of the Bim promoter by Mybs, c-Jun, and FoxOs. Such findings thus indicate that the Bim promoter functions as a coincidence detector that is maximally driven only when all three death pathways are activated. Such a mechanism provides a fail-safe for prevention of accidental neuron death and permits neurons to employ single pathways such as JNK signaling for other purposes.

EXPERIMENTAL PROCEDURES

Materials—Platinum TaqDNA polymerase, anti-V5 antibody, and Lipofectamine 2000 were from Invitrogen, anti-human NGF antiserum was from Sigma, anti-FKHR1 (FoxO3A) antibody was from Cell Signaling, anti-Zs-Green antibody was from Clontech, anti-c-Jun antibody was from Cell Signaling, anti-ERK1, anti-GFP, and anti-Myc antibodies were from Santa Cruz Biotechnology Inc., and the Bim antibody was from StressGen. pSIREN vector was from BD Biosciences. Human recombinant NGF was a kind gift from Genentech.

Cell Culture—PC12 cells were cultured on collagen-coated dishes with RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum as described previously (13). Neuronal differentiation was induced with NGF (100 ng/ml) in RPMI 1640 medium supplemented with 1% horse serum. For NGF deprivation, cultures pretreated with recombinant NGF were a kind gift from Dr. D. Accili (Columbia University).

Transfections—DNA was prepared with plasmid maxi kits (Qiagen). For reporter assays, neuronal PC12 cells were co-transfected with 0.5 μg of reporter plasmid, 0.1 μg of Renilla vector, and 0.5 μg of control plasmid or plasmid expressing c-Jun, shRNA-c-Jun, shRNA-FoxO, or shRNA-Cdk4 in 500 μl of serum-free medium/well in 24-well dishes using Lipofectamine 2000. 6 h later, medium with Lipofectamine 2000 was replaced with fresh complete medium. 293 cells were transfected as previously described (17).

Chromatin Immunoprecipitation (ChIP)—ChIP assays were performed using a kit (Upstate Biotechnology, Inc.) according to the manufacturer’s protocol with the following exceptions. 5–10 × 10^6 PC12 cells were used after treatment ± NGF for each sample. Rabbit polyclonal c-Jun antibody was used at a final concentration of 1 μg/ml. The primers used for PCR amplification of the rat Bim promoter were 5′-GGGATCCCTGATGAGAAGAGCAGCACG-3′ and 5′-AAGGATCCATGAGCAGCAGACTG-3′ (sequence from Ref. 19). PCR was performed for 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s. PCR products were then analyzed on a 2% agarose gel.

Preparation of shRNA—Cdk4 shRNA and B-Myb shRNA were described previously (11). Two c-Jun shRNAs were prepared in the pSIREN vector by using BD™ Knock-out RNAi systems according to the manufacturer’s instructions (BD Biosciences) based on the sequences: 5′-CTGGGCACTAGATCCACAC-3′ and 5′-AACCTCAAGCTTCTCAACAC-3′ (sequence from Ref. 18). FoxO shRNA was prepared in the pSIREN vector by using BD™ Knock-out RNAi systems according to the manufacturer’s instructions (BD Biosciences) based on the sequence 5′-AAGGATAGGGGCCAGACGAC-3′ (19). shRNAs were transfected using Lipofectamine 2000 at a final level of 0.5 μg/24-well culture.

Western Immunoblotting—Neuronal PC12 cells were lysed and protein was analyzed by Western immunoblotting as described previously (9). Immunostaining—Neuronal PC12 cells were transfected as described above with appropriate constructs of shRNA. 48 h later, cells were subjected to NGF withdrawal for 18 h and then immunostained as described by Angelastro et al. (20). Briefly, PC12 cells were fixed with 4% paraformaldehyde for 10 min. After three washes in PBS, cells were blocked in 3% non-immune goat serum for 2 h. The cultures were immunolabeled with rabbit anti-Bim (1:500; Stressgen) antibody, rabbit c-Jun antibody, and verified by sequencing. Myc-FoxO3a was a kind gift from Dr. D. Accili (Columbia University).

Induction of Bim Requires Activation of Multiple Pathways

The Bim promoter-reporter constructs BimP-luc and Bim-luc(Myb) have been described previously (9). The other mutant Bim promoter-reporters were intron-containing Bim-luc reporter that has been reported previously (16). The other mutant Bim promoter-reporters were based on site-directed mutagenesis using PfuTurbo DNA polymerase (Stratagene) according to the manufacturer’s protocol and were verified by sequencing. The Bim-luc(AP1m) was generated using oligonucleotides 5′-GATCTTACCGCCGCGCCGCGCCGCAGCTGTCCGCTGTCGAGACGACGAAGATC-3′. The Bim-luc(FoxOm) was generated using oligonucleotides 5′-CAGATCTGAGGTAAACAGCAGCAAC-3′ and 5′-CACCCGGGGTGGTTACACCTTGAATGTTGATGTTGAGACGAGTAC-3′. The

such regulation occurs independently and in a redundant fashion or whether this occurs by a cooperative or additive mechanism. The answer to this issue has been unclear. Overexpression of activators of each of the pathways can promote neuron death, whereas independent blockade of any one of the pathways is sufficient to suppress neuron death in full or in part. This finding also raises the question of why multiple pathways are involved in the death process and in Bim regulation.

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Induction of Bim Requires Activation of Multiple Pathways

The Bim promoter-reporter constructs BimP-luc and Bim-luc(Myb) have been described earlier (11). The BimP-luc construct is driven by the neuronal differentiation promoter of the rat Bim promoter (sequence from Ref. 18). FoxO shRNA was prepared in the pSIREN vector by using BD™ Knock-out RNAi systems according to the manufacturer’s instructions (BD Biosciences) based on the sequence 5′-AAGGATAGGGGCCAGACGAC-3′ (19). shRNAs were transfected using Lipofectamine 2000 at a final level of 0.5 μg/24-well culture.
Induction of Bim Requires Activation of Multiple Pathways

**A. Bim-luc-w**

- Relative luciferase activity
- **Control** vs **C-Jun**

**B. Bim-luc-(AP1m)**

- Relative luciferase activity
- **Control** vs **C-Jun**

**C. Luciferase Assay**—Neuronal PC12 cells were transfected as described above with the appropriate luciferase reporter and pcDNA-LacZ/pCMS-EGFP with or without other DNAs. 24 or 48 h later, cells were washed with cold phosphate-buffered saline, triturated off the plates, and pelleted in microcentrifuge tubes. Cell pellets were lysed in buffer provided in the Promega luciferase system. Dual luciferase assays were carried out according to the manufacturer’s instructions, using a TD-20/20 Luminometer (Turner Designs). Relative luciferase activities were obtained by normalizing the luciferase activity against Renilla luciferase activity. Data are presented in the text and figures as mean ± S.E. (n = 3–6).

**Statistics**—The statistical significance of differences between means was evaluated by Student’s t test and was performed as unpaired, two-tailed distribution of arrays and presented as p values.

**RESULTS**

**A. Bim-luc-w**

- Relative luciferase activity
- **Control** vs **C-Jun**

**B. Bim-luc-(AP1m)**

- Relative luciferase activity
- **Control** vs **C-Jun**

**C. Bim-luc-(AP1m)**

- Relative luciferase activity
- **NGF** vs **Anti-NGF**

**D. Bim-luc-(FOXOm)**

- Relative luciferase activity
- **NGF** vs **Anti-NGF**

**FIGURE 1. c-Jun induces Bim promoter-driven luciferase activity via an AP1 site, and its occupancy of the promoter is elevated by NGF withdrawal.** A, Bim promoter-driven luciferase activity is induced by c-Jun. Neuronal PC12 cells were co-transfected with 0.5 μg of Bim-luc and 0.1 μg of the Renilla luciferase expression construct pRL-CMV vector (Control) or vector expressing c-Jun and maintained for 24 h, after which cells were harvested and luciferase assays were performed. The data are reported as relative luciferase activity normalized to Renilla luciferase activity and represent mean ± S.E. of four experiments. Asterisk denotes statistically significant differences from Control; *, p < 0.001. B, c-Jun induces Bim promoter-reporter activity through its AP1 binding site. Experiments were carried out as in panel A except using Bim-luc(AP1m) in which the AP1 site was mutated. Data were normalized as in panel A and represent mean ± S.E. for three experiments. C, NGF withdrawal enhances occupancy of the Bim promoter by c-Jun. PC12 cells were treated with NGF for 6 days and then maintained for an additional day ± NGF. Equal numbers of cells from each condition were subjected to ChIP as described under “Experimental Procedures” using anti c-Jun. The immunoprecipitated material was subjected to semi-quantitative PCR using Bim primers that flanked the c-Jun binding site. PCR products were verified by agarose gel electrophoresis. Templates were DNA derived from cells before ChIP (Input) and DNA in immunoprecipitates derived by ChIP with anti-c-Jun or no antibody.

**RESULTS**

**A. Bim-luc-w**

- Relative luciferase activity
- **Control** vs **C-Jun**

**B. Bim-luc-(AP1m)**

- Relative luciferase activity
- **Control** vs **C-Jun**

**C. Bim-luc-(AP1m)**

- Relative luciferase activity
- **NGF** vs **Anti-NGF**

**D. Bim-luc-(FOXOm)**

- Relative luciferase activity
- **NGF** vs **Anti-NGF**

**FIGURE 2. c-Jun, Myb, and FoxO binding sites all are required to activate Bim promoter-reporter activity in response to NGF deprivation.** A–D, mutation of either Myb, AP1, or FoxO sites abolishes Bim promoter-reporter activity induced by NGF withdrawal. Neuronal PC12 cells were transfected with 0.5 μg of Bim-luc (A) or mutant reporter Bim-luc(mybm) (B), Bim-luc(AP1m) (C), or Bim-luc(FoxOm) (D) and 0.1 μg of the Renilla luciferase expression construct pRL-CMV and were maintained with or without NGF for 18 h, after which cells were harvested and luciferase assays were performed. Data were normalized as in Fig. 1A and represent mean ± S.E. for three experiments. Asterisk denotes statistically significant difference from cultures maintained with NGF; *, p < 0.01.

has not been clear whether c-Jun is directly involved in Bim regulation. Examination of the proximal rat Bim promoter (−2774/+155) that has been employed in past studies of Bim regulation (11) revealed a single putative AP1 (c-Jun binding) site that matches the previously described AP1 consensus sequence TGA CTCA (21) at position −2447/−2441 relative to the transcription start site. A luciferase reporter driven by this promoter showed significant activation when co-expressed with c-Jun in neuronal (NGF-treated) PC12 cells (Fig. 1A). In contrast, there was no activation when c-Jun was co-expressed with a reporter in which the AP1 site was mutated to CAA CT TG (mutated bases are underlined) (Fig. 1B). We used neuronal PC12 cells for this and subsequent assays because these undergo apoptotic death in response to NGF withdrawal and appear to do so by mechanisms similar to sympathetic neurons (3, 11, 17, 22). These observations thus indicate that c-Jun can activate the Bim promoter and that the identified AP1 site is required for Bim induction by c-Jun.

To determine whether c-Jun actually occupies the endogenous Bim promoter in living cells and whether such occupancy is enhanced in response to NGF withdrawal, we performed ChIP assays. Accordingly, neuronal PC12 cells were maintained with or without NGF for 24 h and cross-linked chromatin was subjected to ChIP with anti-c-Jun, followed by PCR amplification of a unique portion of the Bim promoter containing the identified AP1 site. The identity of the PCR product as the predicted portion of the Bim promoter was verified by sequencing.
Induction of Bim Requires Activation of Multiple Pathways

Binding Sites in the Bim Promoter for c-Jun, Myb, and FoxOs All Are Required to Induce Bim Promoter-Reporter Activity in Response to NGF Deprivation—We previously reported that the proximal Bim promoter contains binding sites for Mybs and that B- and C-Myb are induced upon NGF withdrawal by a mechanism reliant on activation of Cdk4 and consequent gene derepression (11). Moreover, our findings indicated that there is increased occupancy of the Bim promoter by C-Myb in response to NGF deprivation and that Myb binding sites are required for activation of a Bim promoter-reporter by co-expressed Mybs (11, 23). Gilley et al. (16) also identified binding sites for FoxO in a Bim promoter and found that these mediate activation of a promoter-reporter construct by the co-expressed FoxO family member FoxO3a.

The proximal Bim promoter that we have employed contains one of the two FoxO binding sites identified by Gilley et al. (16) that were reported to be sufficient to promote reporter activation. Moreover, a similar proximal human Bim promoter has been shown to be activated by paclitaxel and requires the same FoxO binding site for activation (24). A comparable proximal mouse Bim promoter has also been shown to be induced by E2F1 (25). As described above, the proximal promoter also possesses a binding site for c-Jun that mediates activation of a promoter-reporter by this transcription factor. Given these findings, we wished to determine whether Bim expression is independently and redundantly regulated by each of the transcription factors in response to NGF withdrawal or whether they act in some type of cooperative fashion. To achieve this, we generated mutant Bim promoters lacking binding sites for either c-Jun, FoxOs, or Mybs and compared their responses to NGF deprivation with that of the wild-type promoter-reporter.

As we previously reported, 18 h of NGF withdrawal elicited an approximate 3-fold elevation in activity of the wild-type reporter (Fig. 2A), whereas loss of the Myb sites resulted in complete loss of responsiveness (Fig. 2B). Significantly, mutation of either the API site or the FoxO binding site (see also Ref. 16) also fully suppressed activation of the reporter by NGF deprivation (Fig. 2, C and D). These findings thus suggest that...
induction of Bim in response to NGF withdrawal requires occupation of the Bim promoter by all three of the transcription factors in question.

shRNAs Targeted to c-Jun or FoxOs Block Induction of the Proximal Bim Promoter to NGF Deprivation—As an additional means to further examine the roles of the multiple transcription factors in regulating Bim expression after NGF withdrawal, we turned to shRNAs to suppress their expression. We have already shown that shRNA targeted to Mybs or to their upstream regulator Cdk4 fully blocks activation of our Bim promoter-reporter in response to NGF deprivation (11). To knock down c-Jun and FoxOs we employed constructs that have been either previously reported to target these transcription factors or that we designed and tested ourselves. shJun targets c-Jun exclusively (18). shFoxO targets FoxO1a and FoxO3a and possibly targets FoxO6 (19). We have also used a second c-Jun shRNA (see “Experimental Procedures” for target sequence) and a second FoxO shRNA that has been reported to be unique to FoxO3a (26), although this targets a very similar area of the gene as our other FoxO shRNA. These constructs substantially disrupt expression of the corresponding endogenously and exogenously expressed genes (Fig. 3 and data not shown). As we observed with shRNA directed to Mybs (11), co-transfection with shRNAs directed toward c-Jun or FoxOs (but not to a control shRNA) each abolished the response of the Bim promoter-reporter to NGF withdrawal (Fig. 4A). Such findings thus confirm the interpretation that all three transcription factors are required for Bim induction after NGF deprivation.

Knock Down of c-Jun, FoxOs, or Mybs Suppresses Induction of an Intron-1-containing Bim Promoter-Reporter to NGF Withdrawal—In addition to the proximal Bim promoter used in the above experiments, another has been described that also contains non-coding regions of exons 1 and 2 and the first intron of the rat Bim gene (16). It was found that the first intron contains additional regulatory sequences that respond to NGF deprivation and that include a FoxO binding site (16, 27). Examination of this intron also reveals putative binding sites for Mybs.3 To assess whether our observations with the proximal promoter-reporter also hold for the more extended regulatory region, we made use of a reporter driven by the more extensive intron-containing sequence. To assure that regulation by each of the transcription factors was inhibited irrespective of potential binding sites, we again turned to shRNAs to impair their expression in reporter assays. As for the proximal Bim promoter-reporter construct, knock down of either c-Jun, FoxOs, B-Myb, or Cdk4 (which is upstream of Mybs) repressed activation of the extended Bim promoter-reporter in response to NGF deprivation (Fig. 4B).

Knock Down of FoxOs and c-Jun Blocks the Induction of Endogenous Bim in Response to NGF Withdrawal—The above-described promoter-reporter assays support a model in which simultaneous activation of c-Jun, FoxOs, and Mybs is required for Bim induction after NGF withdrawal. We next wished to extend these observations to determine whether all three transcription factors are essential for induction of endogenous Bim protein. Neuronal PC12 cells were transfected with shRNAs targeted to either a control protein (firefly luciferase) or to one of the transcription factors and then assessed by immunocytochemistry for Bim expression with or without removal of NGF for 1 day. We previously used this approach to show that knock down of either Mybs or Cdk4 blocks up-regulation of Bim after NGF deprivation (11). Similarly, transfection with shRNAs directed against c-Jun or FoxOs also suppressed up-regulation of Bim (26, 27).

3 S. C. Biswas, unpublished findings.
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of endogenous Bim in PC12 cells deprived of NGF (Fig. 5). Quantification (Fig. 5B) showed that the effects of knocking down c-Jun and FoxOs were similar to one another as well as to that of knocking down Mybs (11).

Lastly, to confirm whether our findings were also applicable to primary neurons, we repeated the above experiment with cultures of newborn rat sympathetic neurons. As in the case of neuronal PC12 cells, knock down of either c-Jun or FoxOs was sufficient to fully repress induction of endogenous Bim protein after NGF deprivation (Fig. 6).

DISCUSSION

The pro-apoptotic BH3-only protein Bim is induced and participates in neuronal death brought about by NGF deprivation (6, 7, 9). Three different apoptotic transcriptional pathways have been implicated in regulation of Bim protein expression under such circumstances. These are the JNK/c-Jun, AKT/FoxO, and cell cycle (Cdk4/E2F/Myb) pathways. The involvement of multiple pathways has raised the questions of whether these act redundantly and are each sufficient for Bim induction or whether each pathway is necessary, but not sufficient, for this action.

Although a number of studies support involvement of JNK/c-Jun signaling in Bim induction after NGF deprivation, it was unclear whether this was due to direct interaction of c-Jun with the Bim promoter. We identified an AP1 binding site in the Bim promoter and found that NGF deprivation leads to enhanced occupancy of the endogenous Bim promoter by endogenous c-Jun. This finding complements prior observations that the Bim promoter contains binding sites for Mybs and for FoxO and that the occupancy of these sites is enhanced by NGF deprivation. Thus, there are binding sites on the Bim promoter for the transcription factors that are activated as an end result of each pathway, and each shows elevated occupancy after NGF withdrawal.

To test the hypothesis that activation of all three pathways is required for optimal Bim induction by NGF withdrawal, we examined expression of both Bim promoter-driven luciferase reporters and of endogenous Bim. We found that in contrast to an intact wild-type construct, Bim promoter-reporters separately mutated to abolish binding sites for either Mybs, FoxOs, or c-Jun failed to show induction in response to NGF deprivation. Similar results were achieved with the wild-type promoter-reporter when we individually knocked down expression of any of the three transcription factors with shRNAs. Comparable findings were made using a Bim promoter-reporter that contained additional sequence, including the first intron of the rat Bim gene (which possesses additional regulatory sites). Finally, shRNAs to the individual transcription factors each suppressed induction of endogenous Bim in NGF-deprived cells.

Our findings support a model in which the Bim promoter acts as a coincidence detector that does not yield induction of the gene unless all three transcriptional pathways (JNK/c-Jun, AKT/FoxO, and Cdk4/E2F/Myb) are activated in response to NGF deprivation. That is, induction does not occur unless the promoter is simultaneously occupied by c-Jun, FoxOs, and Mybs. Although it is possible to induce Bim by overexpression of individual transcription factors (11, 16), our findings indicate that this does not happen under physiologic conditions. What purpose might be served by a mechanism requiring three different signaling pathways to drive Bim expression and death? One might be as part of a fail-safe system to ensure that neurons do not accidentally commit suicide by launching any single pathway. Additionally, requiring all three pathways would permit neurons to use each separately for other physiologic functions. For example, JNK signaling appears to play required roles...
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in neurite outgrowth and synaptic remodeling as well as in neuronal repair (28), and c-Jun is an immediate early gene for NGF signaling that appears to be necessary for neuronal differentiation (29). In addition, it is reported that c-Myb is expressed in healthy rat brain neurons (30). Finally, such a mechanism would permit selective responses to different apoptotic stimuli. For instance, although neuron death evoked by DNA damage requires Myb induction (23), in this case Bim is not required for neuron death and alternative signaling pathways appear to cooperate with Mybs to cause apoptosis4 (31).

Although the present study has focused on NGF deprivation, the same mechanism could pertain to other paradigms of neuron death involving Bim induction. For example, Bim is elevated in entorhinal neurons of Alzheimer disease patients and in culture is both induced by β amyloid and required for death evoked by this peptide (32). This induction correlates with activation of the cell cycle pathway in Alzheimer disease neurons and requires activation of this pathway in cellular Alzheimer disease models (32). Death under such circumstances also requires activation of the JNK/c-Jun pathway (33), and there is evidence that JNK activity is required for β amyloid-promoted Bim induction (34). In addition, it is reported that intraneuronal expression of β amyloid represses AKT signaling (35) and that there is reduced hippocampal phospho-AKT in transgenic mice that overexpress β amyloid (36). Such actions would favor nuclear translocation of FoxOs. Thus, it may well be that all three of the pathways considered here are active and required for Bim induction in Alzheimer models as well as in Alzheimer disease itself.

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4 S. C. Biswas and L. A. Greene, unpublished observations.