

# NF-Y is essential for expression of the proapoptotic *bim* gene in sympathetic neurons

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Neuronal apoptosis has a major role during development and aberrant apoptosis contributes to the pathology of certain neurological conditions. Studies with nerve growth factor (NGF)-dependent sympathetic neurons have provided important insights into the molecular mechanisms of neuronal apoptosis and the signalling pathways that regulate the cell death programme in neurons. The BH3-only protein Bim is a critical mediator of apoptosis in many cell types and in sympathetic neurons is required for NGF withdrawal-induced death. However, regulation of *bim* expression is complex and remains incompletely understood. We report that a conserved inverted CCAAT box (ICB) in the rat *bim* promoter is bound by the heterotrimeric transcription factor NF-Y. Interestingly, NF-Y is required for *bim* promoter activity and its induction following NGF withdrawal. We demonstrate that NF-Y activity is essential for endogenous Bim expression and contributes to NGF withdrawal-induced death. Furthermore, we find that the transcriptional coactivators CBP and p300 interact with NF-Y and FOXO3a and bind to this region of the *bim* promoter. The amount of CBP/p300 bound to *bim* increases after NGF deprivation and inhibition of CBP/p300 activity reduces *bim* induction. Our results indicate that NF-Y cooperates with FOXO3a to recruit CBP/p300 to the *bim* promoter to form a stable multi-protein/DNA complex that activates *bim* transcription after survival factor withdrawal.

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The intrinsic (mitochondrial) pathway of apoptosis hinges on the balance of activity between proapoptotic and antiapoptotic members of the Bcl-2 superfamily. Bim is a BH3-only Bcl-2 family protein whose proapoptotic function is critical for apoptosis via the intrinsic pathway and Bim is one of just three BH3-only members that can bind to all five of their prosurvival relatives.<sup>1</sup> In addition, there is mounting evidence that Bim can induce mitochondrial outer membrane permeabilisation by binding to and directly activating the multidomain proapoptotic protein Bax.<sup>2</sup>

During development, sympathetic neurons depend on target-derived nerve growth factor (NGF) for their survival, and in its absence the cells die by apoptosis, usually within 24–48 h.<sup>3</sup> Following NGF withdrawal, *bim* RNA and Bim protein levels increase rapidly and peak at around 16 h later.<sup>4</sup> The overexpression of Bim<sub>EL</sub> in sympathetic neurons is sufficient to induce the release of cytochrome *c* and apoptosis in the presence of NGF and sympathetic and sensory neurons isolated from *bim*<sup>-/-</sup> knockout mice are significantly protected from trophic factor withdrawal-induced death.<sup>4,5</sup>

Because Bim is a potent inducer of apoptosis in many cell types, its expression is tightly regulated. In models of neuronal cell death, in which the cells depend on NGF for survival, three transcriptional pathways have been described. Activation of the MLK-JNK-c-Jun pathway is a key event for *bim* upregulation in sympathetic neurons: the overexpression of a dominant

negative c-Jun protein reduces the increase in *bim* mRNA and protein level that occurs after NGF withdrawal<sup>4</sup> and the *jun*<sup>AA</sup> knock-in mutation, which eliminates the two major JNK phosphorylation sites in c-Jun, reduces the increase in Bim protein level after NGF withdrawal.<sup>6</sup> Furthermore, *bim* transcription is negatively regulated by PI3-K-Akt signalling: active Akt phosphorylates FOXO3a, which is sequestered in the cytoplasm by 14-3-3 protein and following NGF withdrawal FOXO3a translocates into the nucleus of sympathetic neurons and activates *bim* expression via two conserved FOXO sites.<sup>7</sup> Moreover, FOXO activity contributes to the NGF deprivation-induced death of sympathetic neurons.<sup>7</sup> Additionally, *bim* is activated by a Cdk4-E2F-Myb pathway following NGF withdrawal in neuronally differentiated PC12 cells and this requires Myb-binding sites in the *bim* promoter.<sup>8</sup> Previous studies have therefore identified important transcriptional mechanisms by which *bim* is regulated following growth factor withdrawal, however, nothing is currently known about the *bim* core promoter.

The DNA region that contains the core promoter is critical for the recruitment and assembly of the RNA polymerase II transcription initiation complex.<sup>9</sup> It is important to fully understand the *bim* core promoter architecture, as the regulatory pathways that activate or repress *bim* transcription must ultimately act upon this region. Here, we use NGF-dependent sympathetic neurons to investigate how the DNA-binding

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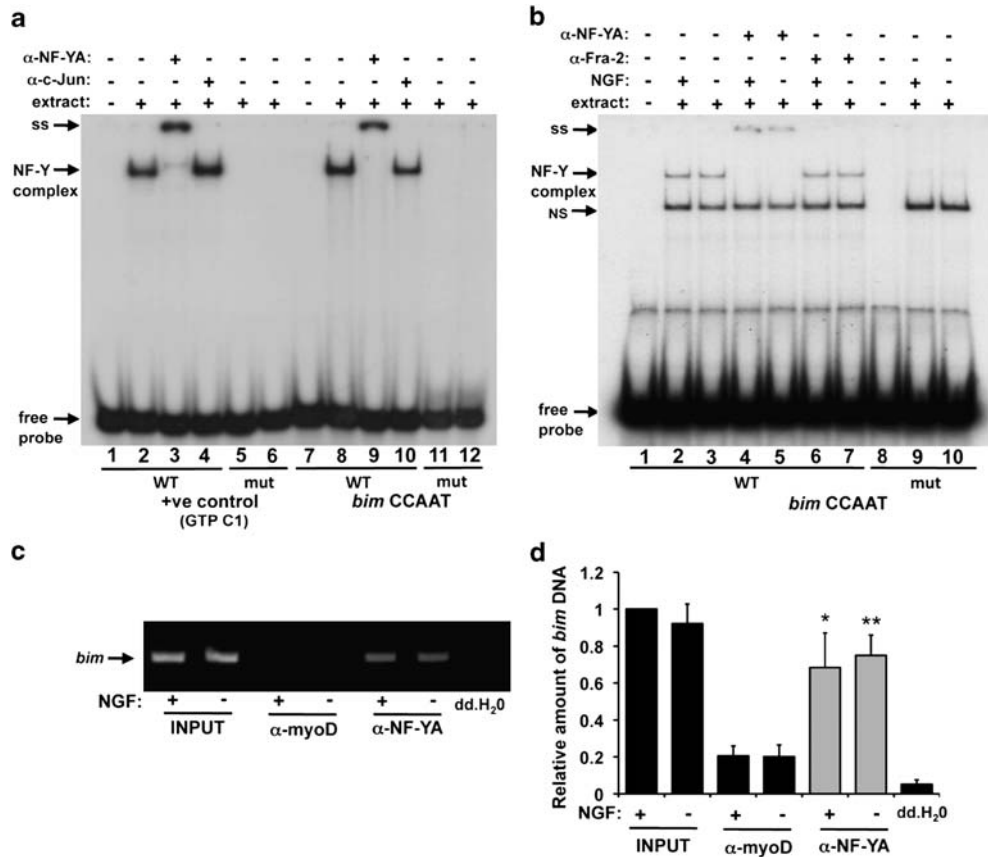
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**Abbreviations:** NGF, nerve growth factor; ICB, inverted CCAAT box; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; GTP C1, GTP cyclohydrolase I; co-IP, co-immunoprecipitation; ss, supershift; gp IgG, guinea pig IgG; TRD, Texas red dextran; CGN, cerebellar granule neuron

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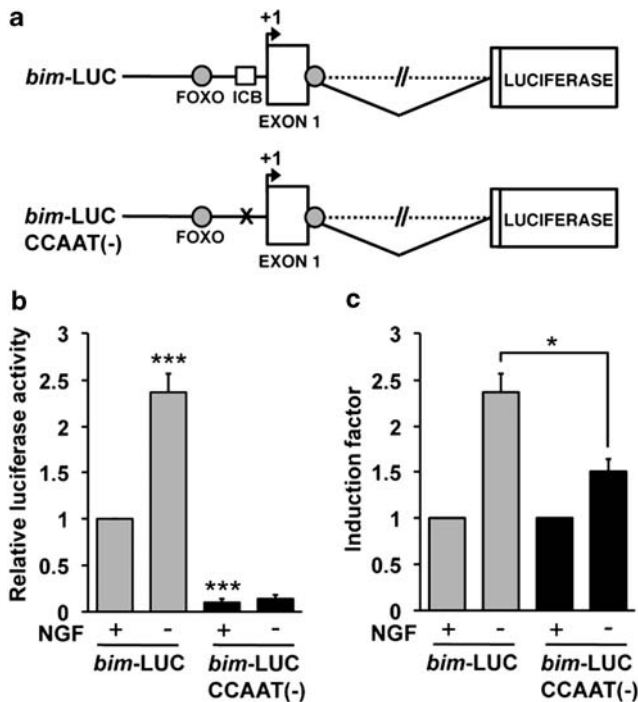
**Figure 2** NF-Y binds to the *bim* ICB in the presence of NGF and following NGF withdrawal. (a) EMSA comparing *bim* CCAAT probes to a positive control probe (for rat GTP C1), using nuclear extracts from PC6-3 cells differentiated in the presence of NGF for 7 days. An NF-YA antibody (H-209) was used to supershift the NF-Y/CCAAT box complex and a c-Jun antibody (H-79) was used as a negative control. (b) EMSA with *bim* CCAAT probes and whole cell extracts from sympathetic neurons maintained in the presence of NGF (+NGF) or withdrawn from NGF (-NGF) for 16 h. An NF-YA antibody (H-209) was used to supershift the NF-Y/CCAAT box complex and a Fra-2 (L-15) antibody was used as a negative control. NS indicates a non-specific band. Representative images of several experiments are shown in each case. (c) PC6-3 cells were differentiated into a neuronal-like phenotype and then either maintained in the presence of NGF (+NGF) or withdrawn from NGF (-NGF) for 16 h. ChIPs were performed using an NF-YA antibody (H-209), or a MyoD antibody (M-318) as a negative control. The equivalent of 1% of the +NGF or -NGF chromatin used in each ChIP assay was also run on each gel (input) and PCR was also carried out with ddH<sub>2</sub>O as a no-template negative control. ChIP samples were analysed by semi-quantitative PCR using primers that flank the *bim* ICB and a representative image is shown. (d) PCR was quantitated at 25 cycles and the average amount of *bim* promoter DNA, precipitated by each antibody, was calculated. The data are presented as the mean ± S.E., *n* = 4. There is a significant increase in the amount of *bim* promoter DNA precipitated by the NF-YA antibody in both +NGF (*P* = 0.03) and -NGF (*P* = 0.009) conditions, compared with the negative control antibody (\*\**P* < 0.01, \**P* < 0.05).

cells were stained with an anti-NF-YA antibody. NF-YA immunostaining was considerably brighter in the nuclei of injected cells (Figure 4a) and over 90% of the injected neurons expressed high levels of NF-YA. Sympathetic neurons were then co-microinjected with YA13 m29, or the empty vector pSG5, along with the *bim*-LUC construct (Figure 4b). Expression of YA13 m29 completely inhibited the induction of *bim*-LUC following NGF withdrawal and also caused a significant decrease in the basal level of *bim*-LUC.

We then studied the effect of YA13 m29 on endogenous Bim protein levels (Figures 4c and d). Sympathetic neurons were microinjected with the YA13 m29 construct, or pSG5, and then cultured ± NGF for 16 h, after which time the cells were fixed and stained with an anti-Bim antibody. Bim is present in the cytoplasm at low levels in the presence of NGF, but strongly increases in level following NGF withdrawal (Figure 4c). Following NGF deprivation, almost 60% of the cells injected with pSG5 expressed high Bim levels

(Figure 4d). However, when the cells were injected with the YA13 m29 construct the percentage of cells expressing high levels of Bim was significantly reduced, to 10% (Figure 4d).

To investigate the role of NF-Y in NGF withdrawal-induced death, sympathetic neurons were microinjected with the YA13 m29 construct, or pSG5, together with Texas red dextran (TRD) as a marker. The neurons were then maintained in the presence or absence of NGF and the number of viable injected cells was counted at 0, 24, 48 and 72 h. Viable neurons were morphologically normal and retained TRD in the nucleus (Figure 5a). At 72 h following NGF withdrawal, the number of viable cells injected with the pSG5 control was below 20% (Figure 5b), whereas cells injected with YA13 m29 were partially protected from cell death (39% were viable) (Figure 5b). The injected neurons not protected by YA13 m29 died by apoptosis following NGF withdrawal (Supplementary Figure S1). These results indicate that NF-Y activity is necessary for cell death in NGF-deprived sympathetic neurons.



**Figure 3** The ICB is critical for basal promoter function and contributes to the induction of *bim* transcription following NGF withdrawal. (a) Structure of the *bim-LUC* and *bim-LUC CCAAT (-)* reporter constructs. *Bim-LUC* contains 2.5 kb of the rat *bim* promoter, the non-coding exon 1 and the 2.5 kb intron upstream of the firefly luciferase gene. The location of the conserved FOXO-binding sites and the ICB are shown. The *bim-LUC CCAAT (-)* construct contains four point mutations within the ICB consensus that were introduced by site-directed mutagenesis. (b) Sympathetic neurons were microinjected with *bim-LUC* or *bim-LUC CCAAT (-)* (both at 10 ng/ $\mu$ l) along with pRL-TK at 5 ng/ $\mu$ l (to control for injection efficiency). The cells were maintained in medium containing NGF (+ NGF) or withdrawn from NGF (-NGF) for 16 h, after which time luciferase activity was measured. The data are presented as the mean  $\pm$  S.E.,  $n = 5$ . *Bim-LUC* is activated significantly following NGF withdrawal ( $P = 0.0003$ ). Mutation of the ICB significantly decreases the basal promoter level of *bim-LUC* ( $P = 0.0009$ ). (c) The basal levels of *bim-LUC* and *bim-LUC CCAAT (-)* were normalised to one and the induction factors of the two constructs were compared. Mutation of the ICB significantly decreases the induction of *bim-LUC* following NGF withdrawal ( $P = 0.018$ ). (\*\*\*) $P < 0.001$ , \* $P < 0.05$ )

**CBP and p300 are important for activation of the *bim* promoter following NGF withdrawal.** The ICB and NF-Y activity are necessary for *bim* promoter activation following NGF withdrawal, but the mechanism by which NF-Y functions has yet to be elucidated, since NF-Y is bound to the ICB in both the presence and absence of NGF. CBP and p300 are two distinct but highly homologous transcriptional coactivators. They can activate the transcription of a multitude of target genes and can interact with over 350 viral and mammalian proteins<sup>16</sup> including c-Jun, FOXO3a, Myb and NF-Y, which activate *bim* transcription in sympathetic neurons.<sup>4,7,8</sup> We therefore investigated whether CBP/p300 are important for *bim* promoter activation following NGF withdrawal. In immunocytochemistry experiments, we confirmed that CBP and p300 localise to the nucleus of sympathetic neurons, with ~95% of sympathetic neurons expressing high levels of CBP and p300 in the presence and absence of NGF (Supplementary Figure S2). To study whether CBP and p300 are required for *bim* promoter activation, antibodies

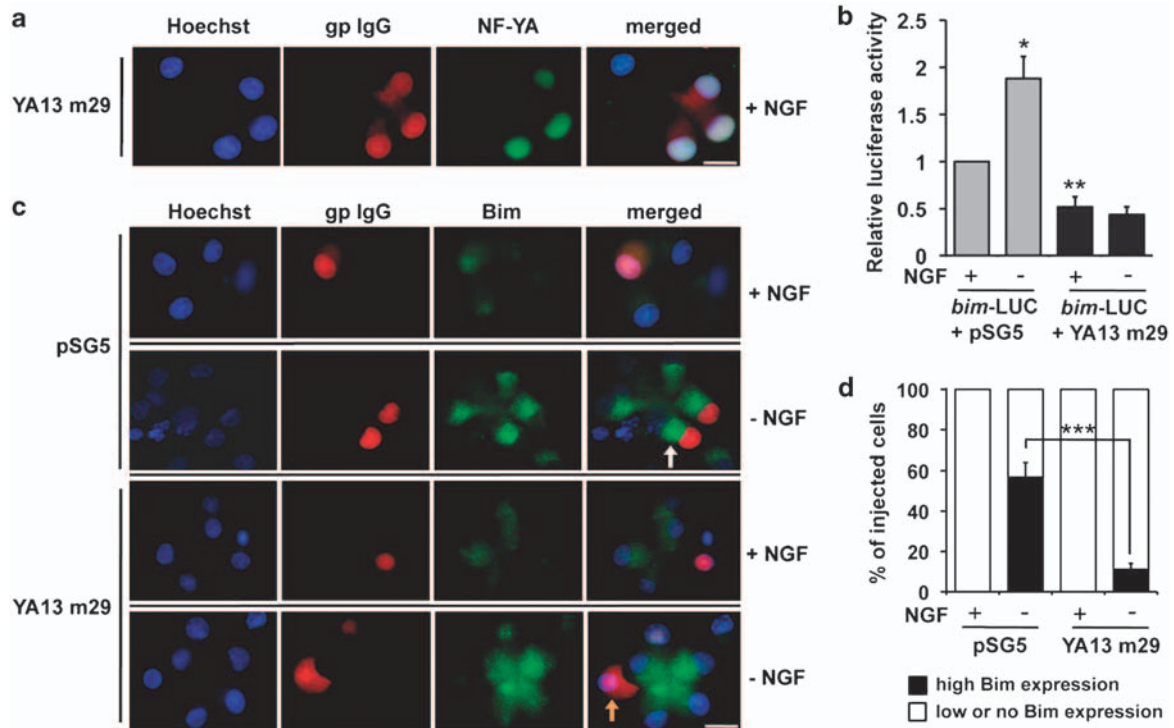
specific for CBP or p300 were co-injected into sympathetic neurons together with the *bim-LUC* construct (Figure 6a). The microinjection of specific antibodies can be used as a suitable alternative to conventional protein knockdown techniques in these cells.<sup>17-19</sup> The *bim-LUC* construct was not significantly activated following NGF withdrawal when antibodies against CBP or p300 were co-injected, compared with the control rabbit IgG (Figure 6a). We also performed a positive control antibody co-injection experiment using a CREB reporter construct pCRE-LUC (Figure 6b), which requires CBP and p300 for its activation by protein kinase A and CREB. Sympathetic neurons were co-injected with pCRE-LUC together with the CBP or p300 antibodies or rabbit IgG. Following injection the cells were treated with CPTcAMP, a membrane permeable cAMP analogue. The pCRE-LUC construct was significantly activated by CPTcAMP treatment when co-injected with IgG compared with the control construct without CRE sites, p-LUC. Importantly, injection of the CBP or p300 antibodies significantly decreased this induction.

Studies with p300 and CBP knockout mice indicate that CBP and p300 are present in cells at limiting concentrations.<sup>20,21</sup> Therefore, overexpression of p300 or CBP should activate the *bim* promoter in the presence of NGF. Sympathetic neurons were co-microinjected with pRc/RSV-mCBP or the control empty vector pcDNA3.1, together with the *bim-LUC* reporter construct (Figure 6c). *Bim-LUC* was significantly activated by overexpression of either CBP or p300. Interestingly, overexpression of p300 did not activate *bim-LUC CCAAT (-)* (Figure 6d) suggesting that the ICB must be intact for activation of the *bim* promoter by p300.

**The amount of CBP/p300 bound to the *bim* promoter is regulated by NGF withdrawal.** To confirm that p300 and CBP can bind to the endogenous *bim* promoter, we performed ChIP assays using differentiated PC6-3 cells and the p300 and CBP antibodies, along with antibodies against MyoD and Bim as negative controls (Figures 7a and b). The binding of p300 or CBP to the *bim* core promoter was studied by PCR using the primers that were previously used to study the binding of NF-Y to the ICB. In the presence of NGF, there was some binding of p300 and CBP to the *bim* promoter but this was significantly enriched following NGF withdrawal (Figures 7a and b). Therefore, p300 and CBP are bound to the same region of the *bim* promoter as NF-Y and the amount of p300 and CBP bound to the promoter increases ~2.1-fold following NGF withdrawal.

**CBP/p300 interact with NF-Y in the presence of NGF and following NGF withdrawal.** To investigate whether NF-Y and CBP/p300 interact in extracts from NGF-dependent cells, co-immunoprecipitation (co-IP) experiments were carried out (Figure 7c and Supplementary Figure S3a). Nuclear extracts were prepared from differentiated PC6-3 cells cultured  $\pm$  NGF for 16 h. The nuclear extracts were immunoprecipitated with antibodies against p300, CBP, NF-YA or rabbit IgG. Immunoblotting was then performed using an NF-YA antibody (Figure 7c). Importantly, NF-YA was precipitated with antibodies against CBP and p300 but not the control rabbit IgG. In addition, the NF-Y antibody





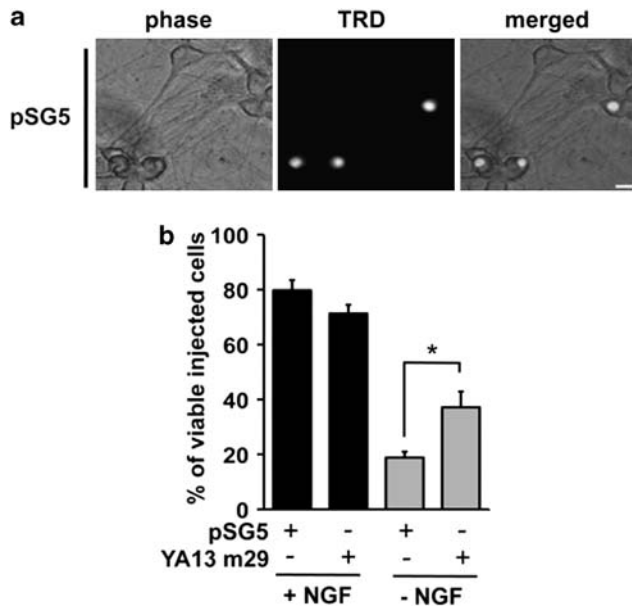
**Figure 4** NF-Y activity is critical for endogenous Bim expression in sympathetic neurons. (a) The NF-YA dominant negative mutant, YA13 m29, localises to the nucleus in microinjected sympathetic neurons. Cells were injected with the YA13 m29 expression vector at 20 ng/ $\mu$ l along with 2.5  $\mu$ g/ $\mu$ l of gp IgG. After 24 h, neurons were fixed and then stained with Hoechst dye, to visualise the nuclei, and antibodies to gp IgG (to identify injected cells) and NF-YA (H-209) to detect YA13 m29. Representative images of several experiments are shown. The scale bar represents 20  $\mu$ m. (b) Sympathetic neurons were co-microinjected with *bim*-LUC at 10 ng/ $\mu$ l along with pRL-TK (5 ng/ $\mu$ l), plus YA13 m29 or the control empty vector pSG5 at 20 ng/ $\mu$ l. The cells were maintained in medium containing NGF (+ NGF) or withdrawn from NGF (-NGF) for 16 h, after which time luciferase activity was measured. The data are presented as the mean  $\pm$  S.E.,  $n = 4$ . *Bim*-LUC is activated significantly following NGF withdrawal when co-injected with pSG5 ( $P = 0.015$ ). When *bim*-LUC is co-injected with YA13 m29, there is a significant decrease in basal promoter level ( $P = 0.003$ ) and the induction of *bim*-LUC, following NGF withdrawal, is abolished. (c) YA13 m29 reduces endogenous Bim expression in microinjected sympathetic neurons. Cells were injected with the YA13 m29 construct or the control empty vector pSG5 at 20 ng/ $\mu$ l along with 2.5  $\mu$ g/ $\mu$ l of gp IgG. The cells were maintained in medium containing NGF (+ NGF) or withdrawn from NGF (-NGF) for 16 h. Following NGF withdrawal, neurons were fixed and then stained with Hoechst dye and antibodies to gp IgG and Bim (#2819). In the presence of NGF, cells either expressed no Bim protein or low levels of Bim protein, whereas following NGF withdrawal many cells expressed high levels of Bim protein. Therefore, cells were scored as no/low Bim expression (comparable to + NGF conditions) or high Bim expression. The white arrow indicates a sympathetic neuron injected with pSG5 that is expressing high levels of Bim protein. The orange arrow indicates a sympathetic neuron injected with YA13 m29 that is expressing low levels of Bim protein. Representative images are shown. The bar represents 20  $\mu$ m. (d) Quantitation of immunocytochemistry in (c). Data are presented as the mean  $\pm$  S.E.,  $n = 6$ . White bars indicate no or low Bim staining and black bars indicate high Bim staining. Microinjection of the YA13 m29 construct significantly decreases Bim expression following NGF withdrawal compared with the control pSG5 ( $P = 0.002$ ). (\*\* $P < 0.01$ , \* $P < 0.05$ )

immunoprecipitated p300 and CBP (Supplementary Figure S3a). These results demonstrate that NF-Y interacts with CBP and p300 in the nucleus of PC6-3 cells cultured in the presence or absence of NGF.

**Following NGF withdrawal FOXO3a binds to the *bim* promoter and interacts with CBP/p300.** Since FOXO3a contributes to the induction of *bim* transcription after NGF withdrawal<sup>7</sup> we investigated whether, like NF-Y, FOXO3a bound to CBP and p300, as reported in other systems.<sup>22</sup> First, we performed CHIP assays on differentiated PC6-3 cells using a FOXO3a antibody and MyoD and Bim antibodies as negative controls (Figures 7e and f and Supplementary Figures S3c and d). The binding of FOXO3a to FOXO site 1 (located upstream of the ICB) and FOXO site 2 (located at the exon 1/intron junction) was studied by PCR. Following NGF withdrawal there was a large increase ( $\sim 4.5$  fold) in the amount of FOXO3a bound to each site (Figures 7e and f and Supplementary Figures S3c and d).

We then investigated in co-IP experiments whether FOXO3a can interact with CBP/p300 in nuclear extracts prepared from differentiated PC6-3 cells cultured  $\pm$  NGF (Figure 7d and Supplementary Figure S3b). The lysates were immunoprecipitated with antibodies against p300, CBP, FOXO3a or rabbit IgG. Immunoblotting was then performed using a FOXO3a antibody (Figure 7d). In -NGF extracts, FOXO3a was precipitated with antibodies against CBP and p300, but this was not the case in the presence of NGF, or in either condition for the control rabbit IgG. In the reverse experiment, CBP and p300 were precipitated with the FOXO3a antibody, but again only after NGF withdrawal (Supplementary Figure S3b).

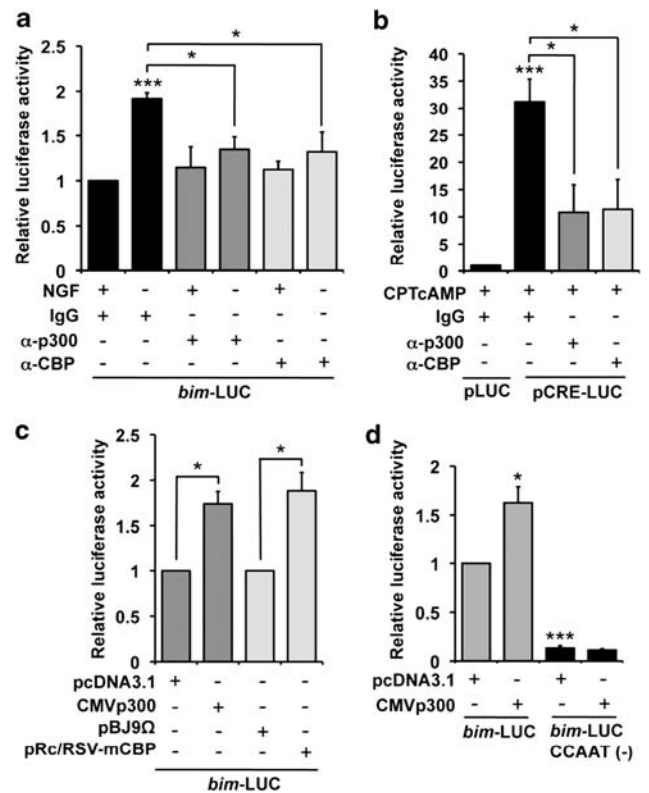
**The *bim* promoter is not activated in response to NGF withdrawal following the combined loss of NF-Y and FOXO binding sites.** To assess the relative contribution of the FOXO and NF-Y binding sites in the *bim* promoter, we mutated both FOXO sites and the ICB within *bim*-LUC



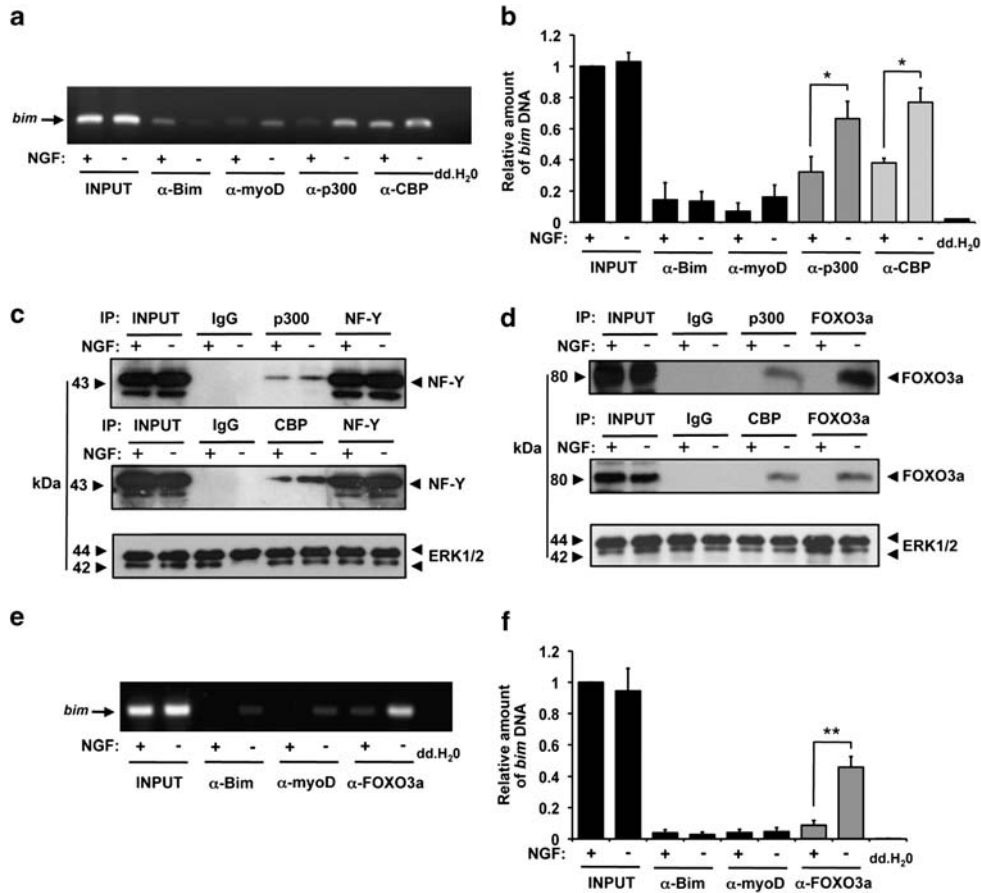
**Figure 5** NF-Y activity contributes to cell death following NGF withdrawal in sympathetic neurons. Sympathetic neurons were injected with the YA13 m29 construct or the control empty vector pSG5 at 200 ng/ $\mu$ l, along with TRD at 5  $\mu$ g/ $\mu$ l as a marker for injected cells. The cells were either maintained in medium containing NGF (+NGF) or withdrawn from NGF (-NGF) and the number of viable, injected cells was determined at 0, 24, 48 and 72 h. Viable cells were counted in a blinded manner. (a) Example images of sympathetic neurons injected with pSG5 and TRD. Neurons were considered viable if they were morphologically normal and retained TRD in their nucleus. The scale bar represents 20  $\mu$ m. (b) The number of viable cells, injected with YA13 m29 or pSG5, at 72 h following NGF withdrawal. The data are presented as the mean  $\pm$  S.E.,  $n = 4$ . Microinjection of the YA13 m29 construct significantly increases the percentage of viable cells at 72 h -NGF compared with the control pSG5 ( $P = 0.023$ ). (\* $P < 0.05$ )

(Figure 8a). We compared *bim*-LUC CCAAT(-) FOXO(dm) with the wild type *bim*-LUC in microinjection experiments with sympathetic neurons (Figure 8b). Loss of both FOXO sites, together with the ICB, significantly reduced the basal promoter level, as shown previously for *bim*-LUC CCAAT(-). Interestingly, loss of all three sites completely abolished the induction of *bim*-LUC following NGF withdrawal. This indicates that the two FOXO sites are required for the residual induction that is seen with *bim*-LUC CCAAT(-) after NGF deprivation.

We also investigated the effect on cell survival after NGF withdrawal of inhibiting both NF-Y and FOXO activity. YA13 m29 was co-expressed with the FKH (DBD), and TRD was used as a marker. The FKH (DBD) construct expresses the DNA-binding domain (DBD) of FOXO3a, which is a specific inhibitor of FOXO transcriptional activity.<sup>7</sup> Following injection, the neurons were withdrawn from NGF for 72 h and the number of viable injected cells was determined (Figure 8c). In accordance with Figure 5b, at 72 h following NGF withdrawal, the number of viable cells injected with the control (pSG5+pcDNA.1) was ~15%, whereas cells that were injected with YA13 m29 were partially protected from cell death (~40% of cells were viable at 72 h -NGF). As demonstrated previously by Gilley *et al.*,<sup>7</sup> neurons injected with the FKH (DBD) construct were better protected (58% of cells were viable at 72 h -NGF). Co-expression of both YA13



**Figure 6** CBP and p300 are important for activation of the *bim* promoter in sympathetic neurons. (a) Co-microinjection of antibodies specific to p300 or CBP with *bim*-LUC. Sympathetic neurons were microinjected with *bim*-LUC at 10 ng/ $\mu$ l and pRL-TK at 5 ng/ $\mu$ l, together with the p300 (C-20:585) or CBP (C-20:583) antibodies, or rabbit IgG as a control, each at 1  $\mu$ g/ $\mu$ l. The cells were maintained in medium containing NGF (+NGF) or withdrawn from NGF (-NGF) for 16 h, after which time luciferase activity was measured. The data are presented as the mean  $\pm$  S.E.,  $n = 6$ . *Bim*-LUC is activated significantly following NGF withdrawal when co-injected with rabbit IgG ( $P = 0.0001$ ). *Bim*-LUC is not activated significantly when co-microinjected with the p300 and CBP antibodies. There is a significant decrease in the induction of *bim*-LUC following NGF withdrawal when co-injected with the p300 antibody ( $P = 0.034$ ) and the CBP antibody ( $P = 0.013$ ) compared with the control rabbit IgG. (b) Sympathetic neurons were microinjected with pCRE-LUC or pLUC (as a control), each at 20 ng/ $\mu$ l, and pRL-TK at 5 ng/ $\mu$ l, together with the p300 (C-20:585) or CBP (C-20:583) antibodies or rabbit IgG, each at 1  $\mu$ g/ $\mu$ l. After injection, the cells were treated with 500  $\mu$ M CPTcAMP for 16 h, after which time luciferase activity was measured. The data are presented as the mean  $\pm$  S.E.,  $n = 5$ . The pCRE-LUC is activated significantly following addition of CPTcAMP, in comparison with pLUC ( $P = 0.0002$ ). There is a significant decrease in the activation of pCRE-LUC following addition of CPTcAMP when co-injected with the p300 antibody ( $P = 0.019$ ) and the CBP antibody ( $P = 0.028$ ) compared with the control rabbit IgG. (c) Co-microinjection of the p300 expression vector, CMVp300, or the CBP expression vector, pRc/RSV-mCBP, with *bim*-LUC. Sympathetic neurons were microinjected with *bim*-LUC at 10 ng/ $\mu$ l and pRL-TK at 5 ng/ $\mu$ l, along with CMVp300 or the control empty vector pcDNA3.1 at 50 ng/ $\mu$ l, or pRc/RSV-mCBP or the control empty vector pBJ9 $\Omega$  at 50 ng/ $\mu$ l. Following injection the cells were maintained in medium containing NGF for 16 h, after which time luciferase activity was measured. The data are presented as the mean  $\pm$  S.E.,  $n = 4$ . Co-microinjection of CMVp300 or pRc/RSV-mCBP induces a significant activation of *bim*-LUC ( $P = 0.016$  and  $P = 0.049$ , respectively). (d) The ICB is required for *bim* activation by p300. Sympathetic neurons were microinjected with *bim*-LUC or *bim*-LUC CCAAT(-) at 10 ng/ $\mu$ l and pRL-TK at 5 ng/ $\mu$ l, along with CMVp300 or the control empty vector pcDNA3.1 at 50 ng/ $\mu$ l. Following injection, the cells were maintained in medium containing NGF for 16 h, after which time luciferase activity was measured. The data are presented as the mean  $\pm$  S.E.,  $n = 4$ . Co-microinjection of CMVp300 induces a significant activation of *bim*-LUC ( $P = 0.013$ ). Co-microinjection of CMVp300 does not activate *bim*-LUC CCAAT(-). (\*\* $P < 0.001$ , \* $P < 0.05$ )



**Figure 7** The amount of CBP/p300 and FOXO3a bound to the *bim* gene increases following NGF withdrawal and CBP/p300 can bind to both FOXO3a and NF-Y. (a) PC6-3 cells were differentiated into a neuronal-like phenotype and then either maintained in the presence of NGF (+ NGF) or withdrawn from NGF (–NGF) for 16 h. ChIP assays were performed using antibodies specific to p300 (C-20:585) or CBP (C-20:583). MyoD (M-318) and Bim (# 2819) antibodies were used as negative controls. The equivalent of 1% of the + NGF or –NGF chromatin used in each ChIP assay was also run on each gel (input) and PCR was also carried out with ddH<sub>2</sub>O as a no-template negative control. ChIP samples were analysed by semi-quantitative PCR and a representative image is shown. (b) PCR was quantitated at 30 cycles and the average amount of *bim* promoter DNA, precipitated by each antibody, was calculated. The data are presented as the mean  $\pm$  S.E.,  $n = 3$ . There is a significant increase in the amount of *bim* promoter DNA precipitated by the p300 ( $P = 0.05$ ) and the CBP ( $P = 0.014$ ) antibodies following NGF withdrawal (–NGF) compared with the + NGF control. (c) Co-immunoprecipitation of NF-Y with antibodies against p300 or CBP. Nuclear extracts were prepared from PC6-3 cells differentiated in the presence of NGF for 7 days and then either maintained in the presence of NGF (+ NGF) or withdrawn from NGF (–NGF) for 16 h. The nuclear extracts were immunoprecipitated with antibodies against p300 (C-20:585), CBP (C-20:583), NF-YA (H-209), or rabbit IgG as a control. Immunoblots were then carried out on the precipitated samples using an NF-YA antibody. Total ERK1/2 (# 9102) was used as a loading control. (d) Co-immunoprecipitation of FOXO3a with antibodies against p300 or CBP. Nuclear extracts (as above) were immunoprecipitated with antibodies against p300, CBP, FOXO3a (#2497) or rabbit IgG. Immunoblots were then carried out on the precipitated samples using an antibody against p300 or CBP. Total ERK1/2 was used as a loading control. Representative images of several experiments are shown in each case. (e) FOXO3a binds to FOXO site 2 (exon 1/intron site) following NGF withdrawal. ChIPs were performed as described above, using an antibody specific to FOXO3a (H-144:Sc-11351 X). ChIP samples were analysed by semi-quantitative PCR and a representative image is shown. (f) PCR was quantitated at 30 cycles and the average amount of *bim* promoter DNA, precipitated by each antibody, was calculated. The data are presented as the mean  $\pm$  S.E.,  $n = 4$ . There is a significant increase in the amount of *bim* promoter DNA precipitated by the FOXO3a ( $P = 0.006$ ) antibody following NGF withdrawal (–NGF) compared with the + NGF control. (\*\* $P < 0.01$ , \* $P < 0.05$ )

m29 and FKH (DBD) did not significantly increase cell survival compared with injection of the FKH (DBD) construct alone (Figure 8c). This is probably because the FKH (DBD) construct alone already gives the maximum protection observed in this microinjection assay, similar to that conferred by Bcl-2, dominant negative c-Jun or the JNK binding domain of JIP-1.<sup>23,24</sup>

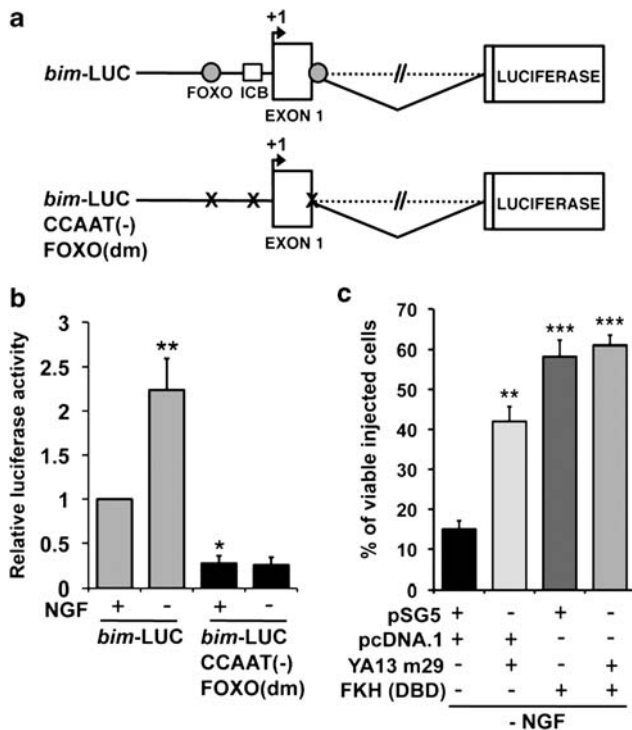
## Discussion

We have studied the role of a conserved inverted CCAAT box in the *bim* core promoter that is bound by NF-Y. This is the first

*bim* promoter element that has been shown to be important for basal promoter function and as the *bim* promoter does not contain a TATA box, it is likely that the NF-Y-binding-ICB has an important role in pre-initiation complex assembly as reported for other TATA-less promoters.<sup>25</sup> In addition, the ICB is critical for *bim* promoter activation following NGF withdrawal.

We used the YA13 m29 dominant negative mutant<sup>15,26,27</sup> to establish that NF-Y activity is required for *bim* promoter function and expression of the endogenous Bim protein. Importantly, we also demonstrated that expression of YA13 m29 partially protects sympathetic neurons from NGF





**Figure 8** The ICB and FOXO sites are critical for the induction of *bim* transcription following NGF withdrawal. (a) Structure of the *bim*-LUC and *bim*-LUC CCAAT (-) FOXO (dm) reporter constructs. The location of the two conserved FOXO-binding sites and the ICB are shown. The *bim*-LUC CCAAT (-) FOXO (dm) construct contains point mutations within the ICB and FOXO consensus sequences, that were introduced by site-directed mutagenesis. (b) Sympathetic neurons were microinjected with *bim*-LUC or *bim*-LUC CCAAT (-) FOXO (dm) (both at 10 ng/ $\mu$ l) along with pRL-TK at 5 ng/ $\mu$ l. The cells were maintained in medium containing NGF (+NGF) or withdrawn from NGF (-NGF) for 16 h, after which time luciferase activity was measured. The data are presented as the mean  $\pm$  S.E.,  $n=5$ . *Bim*-LUC is activated significantly following NGF withdrawal ( $P=0.01$ ). Mutation of the ICB and FOXO sites significantly decreases the basal promoter level of *bim*-LUC ( $P=0.015$ ), and the induction of *bim*-LUC, following NGF withdrawal, is abolished. (c) NF-Y and FOXO activity contribute to cell death following NGF withdrawal in sympathetic neurons. Sympathetic neurons were injected with the YA13 m29 construct or the control empty vector pSG5 at 200 ng/ $\mu$ l, together with the FKH (DBD) construct or the control empty vector pcDNA1 at 50 ng/ $\mu$ l. TRD was added to the injection mix at 5  $\mu$ g/ $\mu$ l as a marker for injected cells. The cells were withdrawn from NGF and the number of viable, injected cells was determined at 72 h. Viable cells were counted in a blinded manner. The data are presented as the mean  $\pm$  S.E.,  $n=4$ . Microinjection of the YA13 m29 construct ( $P=0.002$ ) or FKH(DBD) ( $P=0.0007$ ) or YA13 m29 + FKH(DBD) ( $P=0.0002$ ) significantly increases the percentage of viable cells at 72 h -NGF compared with the control pSG5 + pcDNA1. Microinjection of FKH(DBD) together with YA13 m29 does not increase the percentage of viable cells at 72 h -NGF compared with injection of FKH(DBD) alone. (\*\* $P<0.01$ , \*\*\* $P<0.001$ , \* $P<0.05$ )

withdrawal-induced death (~40% of the cells are viable at 72 h -NGF). This level of protection is similar to the effect of a complete *bim*<sup>-/-</sup> knockout on neuronal survival.<sup>4,5</sup> This partial protection is likely to be because the other BH3-only proteins that increase in level after NGF withdrawal, Puma and Dp5, can partially compensate for the loss of Bim.<sup>28-30</sup>

NF-Y can recruit the transcriptional cofactors CBP and p300 to DNA to potentiate transcription.<sup>31</sup> As the amount of NF-Y bound to the *bim* promoter does not increase after NGF withdrawal, we hypothesised that the recruitment of transcriptional cofactors to the *bim* promoter may be regulated by NGF

withdrawal. We found that co-injection of antibodies against CBP or p300 inhibits the induction of *bim*-LUC after NGF withdrawal and that CBP and p300 bind to the region of the *bim* promoter that is bound by NF-Y, and that the amount of CBP/p300 associated with *bim* significantly increases after NGF withdrawal.

Since CBP and p300 do not bind DNA directly,<sup>32</sup> they must interact with proteins that are already bound to the *bim* promoter. We have shown that mutation of the ICB abolishes the activation of the *bim*-LUC reporter by p300 and that NF-Y interacts with CBP and p300 in extracts from neuronal cells. This suggests that CBP/p300 may activate the *bim* promoter through protein-protein interactions with NF-Y bound to the ICB. Furthermore, studies in other systems have demonstrated that CBP/p300 can interact with the other regulatory factors, c-Jun, FOXO and Myb,<sup>22,33,34</sup> which activate *bim* transcription in neurons. We have shown that FOXO3a binds to two conserved FOXO sites in the *bim* promoter following NGF withdrawal. In addition, we have demonstrated that FOXO3a interacts with CBP and p300 in extracts from neuronal cells, in the absence of NGF.

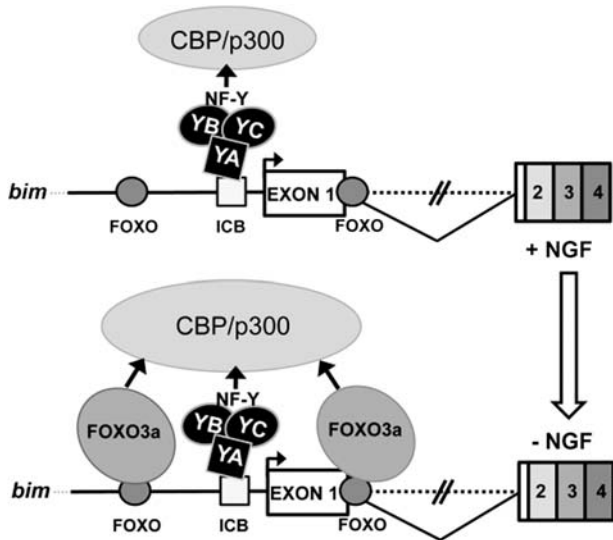
CBP and p300 can act as a 'bridge' to connect sequence-specific transcription factors to the basal transcription machinery<sup>32</sup> and their large size allows multiple proteins to bind to them simultaneously. It is possible that a multi-protein enhanceosome-like structure<sup>35</sup> may form on the *bim* promoter following NGF withdrawal, since we have shown that CBP/p300 can bind to NF-Y and FOXO3a, and NF-Y is known to recruit the basal transcription machinery and RNA polymerase II to the promoters of CCAAT box-containing genes.<sup>25</sup> It will be interesting to establish whether this mechanism is also critical in other cell types as the ICB is common to the rat, mouse and human *bim* genes and NF-Y, FOXO3a and CBP/p300 are widely expressed. Preliminary experiments using mouse cerebellar granule neurons (CGNs) suggest that the ICB may be important for *bim* transcription in these cells (Supplementary Figure S4).

In summary, our results support a model (Figure 9) in which NF-Y is bound to the ICB in the presence of NGF and recruits some CBP/p300 to the *bim* promoter. In sympathetic neurons deprived of NGF, FOXO3a translocates from the cytoplasm into the nucleus and binds to the two conserved FOXO sites that flank the ICB.<sup>7</sup> CBP/p300 binding to the *bim* promoter increases significantly after NGF withdrawal because CBP/p300 can be recruited by FOXO3a as well as NF-Y. Therefore, following NGF withdrawal, NF-Y and FOXO3a (and potentially other factors such as c-Jun and Myb) recruit CBP/p300 to form a stable multi-protein complex that increases the rate of *bim* transcription (Figure 9). Such protein-protein interactions would therefore integrate the different signalling pathways that cooperatively activate the *bim* promoter following survival factor withdrawal.

## Materials and Methods

**Plasmid constructs.** The *bim*-LUC reporter consists of a 5.2 kb fragment, containing the region immediately 5' to the rat *bim* initiator codon, sub-cloned into pGL3-basic.<sup>7</sup> The *bim*-LUC CCAAT(-) construct was generated using the oligonucleotides 5'-GAGCCCGCGGTGAGCTCGCGCCGGAGCGGG-3' and 5'-CCCGCTCCGGCGGAGCTCACCGCGGGCTC-3'. The altered bases (underlined) create four point mutations in the ICB. The *bim*-LUC CCAAT (-) FOXO(dm)





**Figure 9** Hypothetical model showing how transcription factors bind to the *bim* promoter in sympathetic neurons in the presence of NGF and following NGF withdrawal. In the presence of NGF, NF-Y is bound to the ICB and can recruit some CBP/p300 to the *bim* promoter. In sympathetic neurons deprived of NGF, FOXO3a translocates from the cytoplasm into the nucleus and binds to the two conserved FOXO sites in the *bim* promoter that flank the ICB. CBP/p300 binding to the *bim* promoter increases significantly after NGF withdrawal because CBP/p300 can interact with FOXO3a as well as NF-Y. Following NGF withdrawal, NF-Y and FOXO3a may recruit CBP/p300 to form a stable multi-protein complex that increases the rate of transcription of *bim*

construct was generated by incorporating point mutations into FOXO site 1 and FOXO site 2 within *bim*-LUC CCAAT (-) using oligonucleotides described previously.<sup>7</sup> Mutagenesis was carried out using the QuikChange II XL site-directed mutagenesis kit (Stratagene, Agilent, Santa Clara, CA, USA). YA13 m29 is a dominant negative mutant of the NF-YA subunit<sup>15</sup> cloned into the expression vector pSG5 (Stratagene). The expression vector for p300 was supplied by Upstate (Millipore, Billerica, MA, USA). It consists of the human p300 cDNA cloned into pCMV $\beta$  (CMVp300). The expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA), which contains the CMV promoter, was used as an empty vector control. The expression vector for CBP was kindly provided by Professor Tony Kouzarides (The Gurdon Institute, Cambridge, UK). It contains the mouse CBP cDNA cloned into pRc/RSV (pRc/RSV-mCBP). The expression vector pBJ9 $\Omega$ , which is also under the control of the RSV promoter, was used as an empty vector control (also supplied by T Kouzarides). The CREB reporter construct pCRE-LUC and the control without CRE sites, pLUC, were supplied by Stratagene. FKH (DBD)<sup>7</sup> is the DNA-binding domain of FOXO3a cloned into the expression vector pcDNA1 (Invitrogen).

**Cell culture.** Sympathetic neurons were isolated from the superior cervical ganglia (SCG) of 1-day-old Sprague Dawley rats and cultured as described previously.<sup>36</sup> Cells were maintained in SCG medium supplemented with 2.5S NGF (Cedarlane, Burlington, Canada) at 50 ng/ml, and fluorodeoxyuridine and uridine (both from Sigma-Aldrich, St Louis, MO, USA), each at 20  $\mu$ M. PC6-3 cells, a subline of the PC12 cell line,<sup>37</sup> were cultured as described previously.<sup>19</sup> For differentiation, PC6-3 cells were maintained in medium containing 2% horse serum, 1% FCS and NGF (Promega, Fitchburg, WI, USA) at 100 ng/ml, for 5–7 days. In NGF withdrawal experiments, cells were rinsed twice with medium (without NGF) and then refed with medium containing an anti-NGF antibody at 100 ng/ml (Chemicon, Millipore). For experiments with pCRE-LUC and pLUC, sympathetic neurons were refed with + NGF medium containing 500  $\mu$ M CPTcAMP (Sigma-Aldrich).

Cerebellar granule neurons were prepared from 7-day-old mice and maintained in medium containing 25 mM KCl, as described previously.<sup>38,39</sup> In KCl deprivation experiments, neurons were washed once and then incubated in serum-free BME supplemented with L-Gln, HEPES, antibiotics and 1  $\mu$ M of the NMDA antagonist (+) MK-801, and 5 mM KCl (K5 medium) to induce apoptosis.

**Microinjection.** Sympathetic neurons were cultured *in vitro* for 5–7 days and then microinjected as described previously.<sup>4</sup> Approximately 150 cells were injected per coverslip and each injection experiment was repeated at least three times. Plasmids were injected into the nucleus of each cell and over 90% of cells survived injection.

Dual luciferase assays were carried out on injected cells using the Dual luciferase reporter assay system (Promega). Cells were harvested for luciferase assays at 16 h following NGF withdrawal.

For antibody coinjection experiments, the Santa Cruz (Santa Cruz, CA, USA) p300 (C-20:Sc-583 X) and CBP (C-20:Sc-583 X) antibodies were diluted in PBS (-) and centrifuged in Microcon YM-3 centrifugal filters (Millipore) to remove sodium azide. Antibodies were injected at a final concentration of 1  $\mu$ g/ $\mu$ l and purified rabbit immunoglobulin in PBS (Jackson ImmunoResearch, West Grove, PA, USA) was used as a control.

In cell survival assays, neurons were injected with the YA13 m29 expression vector or the control empty vector (pSG5) at 200 ng/ $\mu$ l, along with 5  $\mu$ g/ $\mu$ l of TRD (70 000 MW; Molecular Probes, Invitrogen, Carlsbad, CA, USA).

**Transfection of CGNs.** CGNs cultured in 24-well plates were transfected at 5 days *in vitro* with 0.5  $\mu$ g of pRL-TK together with 1.5  $\mu$ g of either the *bim*-LUC or *bim*-LUC CCAAT(-) constructs, using calcium phosphate precipitation as described previously.<sup>39</sup> After 24 h the cells were either left untreated (K25) or deprived of KCl (K5). Four hours after deprivation, transfected neurons were harvested for luciferase assays using the Dual luciferase reporter assay system.

**Immunocytochemistry.** Neurons were injected with the YA13 m29 expression vector or the control empty vector (pSG5) at 20 ng/ $\mu$ l along with 2.5  $\mu$ g/ $\mu$ l of purified gp IgG (Jackson ImmunoResearch) and fixed and stained as described before.<sup>7</sup> In brief, cells were fixed in 4% paraformaldehyde, permeabilised in 0.5% Triton-X-100 and blocked in 50% goat serum (Sigma-Aldrich). After washing, cells were incubated in a primary antibody to detect NF-YA (Santa Cruz Sc-10779:H-209) or Bim (# 2819; Cell Signalling, Danvers, MA, USA). Secondary antibodies were a FITC-conjugated anti-rabbit IgG, and a rhodamine anti-gp IgG if neurons were microinjected (both from Jackson ImmunoResearch). Secondary-only controls were carried out to confirm the specificity of primary antibodies and cells were scored in a blinded manner whenever possible. TUNEL analysis was carried out using an *in situ* cell death detection kit (Roche, Basel, Switzerland). All slides were viewed using a Zeiss Axioplan 2 fluorescence microscope.

**Electrophoretic mobility shift assay.** EMSA experiments were performed on nuclear lysates from differentiated PC6-3 cells maintained in medium containing NGF or withdrawn from NGF for 16 h, and whole cell lysates from sympathetic neurons maintained in medium containing NGF or withdrawn from NGF for 16 h. Nuclear lysates were prepared from PC6-3 cells and whole cell extracts were prepared from sympathetic neurons as described previously.<sup>19,40</sup> Double-stranded oligonucleotides with 5' overhangs were labelled by filling in with Klenow polymerase (Roche) and ( $\alpha$ -<sup>32</sup>P) dCTP (3000 Ci/mmol; PerkinElmer, Wellesley, MA, USA). Oligonucleotide pairs used for EMSA experiments were as follows: *bim* CCAAT wt 5'-CTAGCCCGCGGTGATTGGGCGCCGGAG-3' and 5'-CTAGCTCCGGCGCCCAATCACCGCGGG-3' and *bim* CCAAT mut 5'-CTAGCC CGCGGTGAGCTCGCGCGGAG-3' and 5'-CTAGCTCCGGCGCGAGTCACCGG-3' (the mutated bases of the ICB are underlined). Oligonucleotides containing a CCAAT box from the rat GTP C1 gene were used as a positive control: GTP C1 wt 5'-CTAGGCTCGGCCAATGAGAACGCCT-3' and 5'-CTAGAGGCGTTCTCATTG GCCCGAGC-3' and GTP C1 mut 5'-CTAGCTCGGGGAGCTGAGAACGCCT-3' and 5'-CTAGAGGCGTTCTCAGCTCCCCGAGC-3'.<sup>12</sup> Binding reactions were set up in a 20  $\mu$ l volume as described previously,<sup>12</sup> with 5  $\mu$ g of nuclear extract from PC6-3 cells and 10  $\mu$ g of whole cell extract from sympathetic neurons. Supershift experiments were carried out by incubating samples on ice with antibodies (4  $\mu$ g) for 2 h. The Santa Cruz NF-YA (H-209:Sc-10779 X) antibody was used in EMSA experiments along with c-Jun (H-79:Sc-1694 X) or Fra-2 (L-15:Sc-171 X) antibodies as negative controls (Santa Cruz). Samples were incubated with 0.4 ng of <sup>32</sup>P-labelled oligonucleotide for 15 min at room temperature and run on a 5% polyacrylamide gel. After electrophoresis, gels were fixed in 10% acetic acid/10% methanol, dried and bands were visualised by exposing the gel to MXB X-ray film (Kodak, Rochester, NY, USA).

**Chromatin immunoprecipitation.** Differentiated PC6-3 cells ( $\sim 4 \times 10^6$  cells per 9 cm dish) were either maintained in the presence of NGF, or withdrawn from NGF for 16 h. The cells were fixed in 1% formaldehyde for 3 h at room

temperature and ChIP was performed using a ChIP assay kit from Upstate (Millipore) with some modifications, as described previously.<sup>19</sup> The following antibodies from Santa Cruz were used for ChIP: NF-YA (H-209:Sc-10779 X), p300 (C-20:Sc-585 X), CBP (C-20:Sc-583 X) and FOXO3a (H-144:Sc-11351 X). Millipore Bim (AB17003) and Santa Cruz MyoD (M-318:Sc-760 X) antibodies were used as negative controls.

ChIP samples were analysed by semi-quantitative PCR. To detect binding of NF-Y to the ICB in the rat *bim* promoter the following primer pair was used: 5'-CAGGCCAAGTCACTAGGGTAAACAC-3' and 5'-GAATCCGGTGACTGCAAC GACAA-3'. The same primer pair was used to detect binding of p300 and CBP to this region. The following primer pairs were used to detect binding of FOXO3a to FOXO site 1: 5'-CTAAGTTCGGCTCTGCGAGGT-3' and 5'-TGCAGGCTGCGAC AGGTAGT-3', and FOXO3a to FOXO site 2: 5'-GATTCACACCACCTCGCT-3' and 5'-GCCTGTCTTATCCATCCC-3'. Samples were analysed between 25 and 30 cycles and the average amount of *bim* promoter DNA, precipitated by each antibody, was calculated using ImageQuant image analysis software (GE Healthcare, Waukesha, WI, USA).

**Co-immunoprecipitation.** The co-immunoprecipitation (co-IP) experiments were performed on nuclear lysates from differentiated PC6-3 cells maintained in + NGF medium or withdrawn from NGF for 16 h. A 50% slurry of protein A/G-agarose (Santa Cruz) was prepared in PBS (–) and added to a 1.5-ml microfuge tube containing 2 µg of antibody to immunoprecipitate either FOXO3a (Cell Signaling; #2497), p300, CBP or NF-Y (NF-Y, p300 and CBP antibodies were the same for co-IP and ChIP), and resuspended in 500 µl of ice-cold PBS (–). This was also repeated for the non-specific control, purified rabbit immunoglobulin in PBS (Jackson ImmunoResearch). The mixtures were incubated at 4°C for 90 s and the beads were collected by centrifugation at 15 700 × g for 2 s at 4°C. The supernatant containing the unbound antibody was removed and the beads were resuspended in 1 ml of non-denaturing lysis buffer (1% v/v Triton-X100, 50 mM Tris-HCl pH 7.4, 300 mM NaCl, 5 mM EDTA, 0.02% v/v Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub> pH 7.4, 1 mM PMSF). The tubes were inverted four times to mix and the beads were collected by centrifugation as previously. The beads were then washed in 1 ml of non-denaturing lysis buffer and collected by centrifugation. Around 80 µg of nuclear lysate was added to each tube containing specific or control antibody-bound beads. The mixture was incubated at 4°C for 2 h with rotation. The beads were then collected by centrifugation and the supernatant was removed. The beads were resuspended in 1 ml of ice-cold wash buffer, the tubes inverted four times to mix, and the beads were collected by centrifugation as previously. This wash step was repeated three times and the final supernatant was removed to leave the beads in ~15 µl of wash buffer (containing the immune complexes). The beads were resuspended in 30 µl of SDS gel sample buffer, boiled for 5 min at 100°C and analysed by immunoblotting as described previously.<sup>7</sup> The NF-YA, FOXO3a, p300 and CBP antibodies used for immunoprecipitation were also used for immunoblots and an ERK1/2 antibody (Cell Signaling; # 9102) was used to check protein loading.

To prepare nuclear extracts for co-IP, neuronally differentiated PC6-3 cells were washed twice in ice-cold PBS (–) and then scraped off the dishes in 2 ml of PBS (per 9 cm dish). Cells were pelleted by centrifugation at 400 × g for 5 min at 4°C and resuspended in 200 µl of buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) before incubation on ice for 15 min. NP40 (10%) was added to each lysate (to a final concentration of 1%) and the samples were vortexed for 15 s before incubation for 15 min at 4°C. The lysates were pelleted by centrifugation at 13 000 × g for 30 s, resuspended in 200 µl of ice-cold buffer C (20 mM Hepes pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 25% v/v glycerol) and incubated at 4°C with shaking. After 1 h, lysates were vortexed for 30 s and pelleted by centrifugation for 10 min at 15 700 × g at 4°C. The supernatant was removed and this was the nuclear extract.

**Statistical analysis.** In each set of experiments, data are normalised to a control sample (for example, *bim*-LUC + NGF is set to one). For microinjection, the relative induction of a DNA construct is calculated by dividing the normalised luciferase activity in the absence of NGF by the normalised luciferase activity in the presence of NGF. All data are presented as the mean ± S.E. of multiple experiments.

The statistical significance of differences between means was evaluated by performing an unpaired Student's *t*-test (for two-tailed distributions), followed by a Bonferroni correction. To compare normalised data with the control sample, which has no error associated to it (for example, when comparing data to *bim*-LUC + NGF, which is set to one), the log<sub>10</sub> values of the data were taken and a one

sample *t*-test was used (for two-tailed distributions). Statistical significance is presented as *P*-values in the Figures.

### Conflict of interest

The authors declare no conflict of interest.

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