Published in final edited form as:

Apoptosis. 2008 June ; 13(6): 803–810.

p38 MAP kinase mediates arsenite-induced apoptosis through FOXO3a activation and induction of Bim transcription

Beibei Cai and Zhengui Xia

Toxicology Program, Department of Environmental and Occupational Health Sciences, University of Washington, Box 357234, Seattle, Washington 98195-7234, USA

Abstract

Sodium arsenite induces apoptosis in PC12 cells by activating the stress-activated p38 MAP kinase and the pro-apoptotic Bcl-2 family protein Bim_{EL}. However, the relationship between p38 and Bim_{EL} in this apoptosis has not been fully defined. Here, we report that sodium arsenite stimulates the protein expression and promoter activity of Bim_{EL} in a p38-dependent manner. Sodium arsenite also caused nuclear translocation of FOXO3a, indicative of FOXO3a activation. Addition of a p38 inhibitor prevented FOXO3a nuclear translocation. RNAi knock down of FOXO3a inhibited Bim promoter activity, Bim_{EL} protein expression, and arsenite-induced apoptosis. Our data identify p38 activation of FOXO3a and subsequent induction of Bim_{EL} expression as a novel apoptotic mechanism. Together with our previous finding that Bim_{EL} is phosphorylated and activated by p38, these results demonstrate that p38 induces apoptosis by regulating Bim_{EL} at both the transcriptional and post-translational levels.

Keywords

Apoptosis; p38; Bim; FOXO3a; Arsenite

Introduction

Arsenicals are environmental toxicants. They have also been utilized clinically to treat various human diseases including psoriasis, syphilis, and rheumatosis. Recent clinical use of trivalent arsenic as an anticancer drug, especially in the treatment of acute promyelocytic leukemia [1-3], has stimulated intense interest in elucidating the underlying mechanisms of arsenic-induced apoptosis.

p38 is a member of the stress-activated MAP kinases and its activation has been implicated in various forms of apoptosis. For example, several chemotherapeutic agents induce apoptosis through p38 [4,5], and p38 may be a tumor suppressor [6]. In addition, sodium arsenite-induced apoptosis also requires p38 activation [7-9].

Apoptosis induced by cellular stress, including treatment with arsenite, is mediated by the mitochondrial-initiated cell death pathway [7,8,10]. The Bcl-2 family proteins regulate this form of cell death by modulating mitochondrial membrane potential [11]. Interestingly, the function of both pro- and anti-apoptotic Bcl-2 family proteins is regulated by MAP kinases including p38 [12-16]. For example, sodium arsenite induces PC12 cell apoptosis by stimulating p38 which phosphorylates and activates the pro-apoptotic Bim_{EL} [9]. Bim_{EL} is one of the three isoforms generated from the single Bim gene locus through alternative splicing [17,18], and is the most abundant Bim isoform in neurons and in PC12 cells [15,19,20].

In addition to regulation by phosphorylation, induction of Bim expression has also been implicated in some forms of apoptosis including that induced by trophic withdrawal [16]. However, relatively less is known regarding mechanisms governing Bim transcriptional regulation during apoptosis. The Bim promoter contains two putative binding sites for FOXO3a, a forkhead family transcription factor. Furthermore, FOXO3a can directly activate the Bim promoter and has been implicated in several forms of apoptosis [21-23].

The objective of this study was to determine if arsenite-induced apoptosis depends on induction of Bim_{EL} and to investigate the underlying regulatory mechanisms. Our data implicate p38 and FOXO3a in regulation of Bim_{EL} expression.

Materials and methods

Plasmids

The following plasmids have been described: constitutive active and dominant negative pRC/RSV-Flag MKK3, wild-type p38 α [24]; human U6-RNA pol III promoter driven shRNA constructs of Bim and the empty vector [14]; shRNA of a scrambled phosphodiesterase 2 (PDE2) [9]; P3.6 Bim promoter-luciferase [18]; and HA-FKHRL1 wt (FOXO3a) [25].

Reagents

Sodium arsenite (Sigma-Aldrich, Inc., Saint Louis, MO) was dissolved in DMEM (Mediatech, Inc., Herndon, VA) as 1000X stock. Z-VAD-FMK (R&D Systems, Inc., Minneapolis, MN), actinomycin D (Sigma), SB 203580 and SB 202190 (Sigma) were dissolved in dimethyl sulfoxide (DMSO) as 1000X stock. Equal volume of DMSO was used as vehicle control, which did not cause any measurable toxicity. The following antibodies were purchased commercially: anti-phospho-p38, anti-p38, anti-FOXO3a (for immunocytochemistry) (Cell Signaling Technology, Beverly, MA); anti-total Bim (Stressgen Biotechnologies, Inc., San Diego, CA); anti-FKHRL1(N-15) (for Western) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). *siGENOME SMART* pool interfering RNAs (siRNA) to rat MAPK14 (p38 α) (Dharmacon RNA technologies, Chicago, IL), to FOXO3a (also known as FKHRL1; Santa Cruz), a nonspecific double-stranded RNA oligomer (si-NS) and Oligofectamine (Invitrogen Inc., Carlsbad, CA) were also purchased commercially. Lipofectamine2000 reagent was from Invitrogen.

Cell culture and transient transfection

Rat pheochromocytoma (PC) 12 cells were cultured and transfected as described [9]. One day before transient transfection or sodium arsenite treatment, cells were plated onto poly-D-lysine (Sigma)-coated plates. Plasmid transfection was performed using Lipofectamine2000 in regular growth media. eGFP was co-transfected to mark transfected cells. siRNA transfection was performed using the Oligofectamine reagent per manufacturer's protocol. Briefly, cells were seeded a day before transfection at 30-50% confluence and placed into DMEM free of antibiotics, serum, and glutamine. siRNA (100 or 200 pmol per well to make final concentrations of 50 or 100 nM, respectively) was mixed with Oligofectamine and Opti-MEM (Invitrogen). The mixture was then added to cells which were incubated at 37°C, 7.5% CO₂ for 4 h. Equal volumes of DMEM containing 20% horse serum, 10% fetal bovine serum (FBS), and 2% glutamine was added to the plates and cells were harvested 2 days after transfection.

Western analysis

Cell lysates were prepared as described [26]. Thirty or eighty micrograms of protein were used for Western analysis for p38, Bim, or FOXO3a.

Apoptosis assay

Apoptosis was determined by nuclear condensation and/or fragmentation after staining with the DNA dye Hoechst 33258 (bis-benzimide) [27]. To obtain unbiased counting, slides were coded and cells were scored blind without prior knowledge of treatment. At least 2,000 total or 1,000 transfected cells were counted for each data point.

Luciferase assay

The p3.6 Bim promoter-driven luciferase reporter (0.5 μ g) and EF-lacZ (0.25 μ g) plasmids were transfected into 0.5×10^6 cells/well of a 24 well plate. Twenty-four hours later, cells were pre-treated with p38 inhibitors SB 203580, SB 202190, or vehicle control for 60 min, followed by treatment with 15 μ M sodium arsenite for 16 h. The Bim promoter-driven luciferase reporter activity was normalized to the co-transfected beta-galactosidase (EF-lacZ) activity (or protein concentration). Each experiment was performed in triplicate.

Immunocytochemistry and microscopy

PC12 cells were fixed with 4% paraformaldehyde for 20 min, permeabilized in cold methanol for 15 min at -20°C , and blocked with 10% goat serum in phosphate buffered saline with 0.1% Triton (PBST) for 1 h. Cells were then incubated with anti-FOXO3a (1:200) antibody in PBST followed by incubation with secondary antibody anti-rabbit Alexa Fluor 488 (1:200, Molecular Probes, Eugene, OR). For nuclear staining, cells were incubated in Hoechst 33258 for 15 min. FOXO3a cellular localization was scored using an inverted fluorescence microscope (Leitz DMIRB; Leica, Heidelberg, Germany) with a 100 $\times$ objective oil lens. The images of FOXO3a immunostaining were taken under a Zeiss Axiovert 200M deconvolution microscope (63 $\times$ oil lens) incorporated with a Marianas imaging system from Intelligent Imaging Innovations, Inc.

Data analysis

Data were from at least three independent experiments. Statistical analysis of data was performed using one tail Student's *t*-test. Error bars represent standard error of means (SEM). ***, $p < 0.001$.

Results

Sodium arsenite-induced apoptosis in PC12 cells requires p38 and Bim_{EL}

Sodium arsenite treatment of PC12 cells induced apoptosis in a dose and time dependent manner (Fig. 1a). RNAi knock down of p38 (Fig. 1b) or Bim_{EL} (Fig. 1c) both protected PC12 cells from arsenite-induced apoptosis. These data validate our previous finding that both p38 and Bim_{EL} are required for arsenite to induce apoptosis in PC12 cells [9].

Sodium arsenite treatment stimulates Bim_{EL} expression, a process that requires p38 activity

Western analysis was performed to determine if sodium arsenite treatment increases Bim_{EL} protein expression. Indeed, the protein level of Bim_{EL} began to increase at 4 h after arsenite treatment and continued to increase for 24 h (Fig. 2a). siRNA to p38 (Fig. 2b) attenuated arsenite induction of Bim_{EL} protein. These data suggest that p38 activity is required for arsenite induction of Bim_{EL} protein.

Next, we investigated if induction of Bim_{EL} protein occurs at the transcriptional level. PC12 cells were treated with a non-toxic low dose of actinomycin D (100 ng/ml), a transcription inhibitor. Actinomycin D significantly inhibited arsenite-induced apoptosis (Fig. 3a), suggesting that this apoptosis requires de novo gene expression. Interestingly, sodium arsenite treatment also stimulated Bim promoter-driven luciferase activity (Fig. 3b). Treatment with actinomycin D abolished arsenite induction of Bim promoter activity (Fig. 3c) and Bim_{EL}

protein expression (Fig. 3d). Thus, Bim_{EL} protein induction is mediated through increased transcription.

The effect of p38 inhibition on Bim promoter activity was also investigated. Inhibition of p38 activity was accomplished by treatment with SB 203580, SB 202190 (Fig. 4a), or transient expression of dominant negative (dn) MKK3 (Fig. 4b), an upstream activating kinase for p38. Both treatments significantly reduced Bim promoter activity upon arsenite treatment.

PC12 cells were also transfected with constitutive active (ca) MKK3 along with wild type p38 α to ectopically activate p38 in transfected cells. Co-expression of caMKK3 and p38 was sufficient to induce Bim promoter activity in a manner that was sensitive to SB203580 (Fig. 5), indicating that p38 activation is sufficient to stimulate Bim transcription. Data in Figs. 4 and 5 suggest that p38 is both necessary and sufficient to mediate arsenite induction of Bim_{EL} transcription and protein expression.

FOXO3a mediates sodium arsenite-induced Bim expression

The Bim promoter contains two putative FOXO3a binding sites and FOXO3a has been implicated in regulating Bim expression in several other cell types [21-23]. Furthermore, other members of the MAP kinase family, such as JNK and ERK5, have been reported to directly or indirectly regulate FOXO3a expression [28,29]. To determine if FOXO3a plays a role in arsenite induction of Bim_{EL} expression, we examined if FOXO3a is activated upon arsenite treatment. FOXO3a is normally cytosolic but translocates to the nucleus upon activation where it can induce target gene expression. Indeed, immunostaining studies showed that nuclei are mostly devoid of FOXO3a in resting PC12 cells (Fig. 6a). However, FOXO3a translocated to the nucleus upon arsenite treatment. Importantly, treatment with the p38 inhibitor SB 203580 blocked FOXO3a's nuclear translocation (Fig. 6b). These data suggest that FOXO3a is activated upon arsenite treatment and that this activation is regulated by p38.

To determine if FOXO3a regulates Bim_{EL} expression in PC12 cells, we co-transfected cells with a Bim-luciferase reporter and a FOXO3a expression vector. Expression of FOXO3a stimulated Bim-luciferase activity 4-fold on its own; it also synergized with arsenite treatment to further activate the Bim promoter (Fig. 7a). The synergistic effect suggests that FOXO3a may function in the signaling pathway in which arsenite stimulates Bim_{EL} expression.

To examine the effect of blocking FOXO3a function in Bim_{EL} expression and apoptosis, we utilized RNA interference (RNAi) technology to knock down the expression of endogenous FOXO3a. PC12 cells were transiently transfected with FOXO3a specific siRNAs (si-FOXO3a) or a control non-silencing siRNA (si-NS). Transient expression of the FOXO3a siRNA greatly reduced FOXO3a expression 48 h later (Fig. 7b). In addition, transfection of FOXO3a siRNA almost completely inhibited arsenite induction of Bim_{EL} protein expression and Bim promoter activity (Fig. 7b, c). Finally, siRNA knockdown of FOXO3a protected PC12 cells against arsenite-induced apoptosis (Fig. 8). Together, these data demonstrate a critical role for FOXO3a in arsenite-induced Bim_{EL} expression and apoptosis.

Discussion

The objective of this study was to determine if transcriptional regulation of Bim_{EL} contributes to sodium arsenite-induced apoptosis and to identify key signaling molecules responsible for Bim_{EL} induction. We have published evidence that arsenite-induced apoptosis requires p38 and Bim_{EL} [9]. Here we discovered that Bim_{EL} protein levels and promoter activity are both increased upon arsenite treatment. Furthermore, p38 is both necessary and sufficient to mediate Bim_{EL} induction. The effect of p38 on Bim_{EL} induction is mediated through FOXO3a. Since increases in Bim_{EL} expression are sufficient to induce apoptosis in PC12 cells [9], we conclude

that arsenite-induced apoptosis depends on activation of p38 and FOXO3 which increases Bim_{EL} expression.

Bim has been implicated in many forms of apoptosis and may function as a tumor suppressor [30]. Loss of Bim accelerates tumorigenesis [31]. Thus, it is important to understand mechanisms regulating the activity and expression of Bim under various apoptotic conditions. Bim's pro-apoptotic activity is stimulated by phosphorylation through pro-apoptotic JNK and p38 MAP kinases and inhibited by phosphorylation through pro-survival ERK [9,16,32-36]. Besides phosphorylation, increased Bim protein expression is also associated with some forms of apoptosis [16,37]. In this study, we discovered that sodium arsenite-induced apoptosis requires induction of Bim_{EL} transcription and protein expression. Our data is the first to demonstrate transcriptional regulation of Bim_{EL} expression by the p38 MAP kinase signaling pathway.

FOXO3a is a transcription factor that has been implicated in stimulation of apoptosis. Two of its known targets implicated in apoptosis are Fas ligand [25] and Bim [21-23]. FOXO3a directly binds to the Bim promoter and activates Bim transcription. Several signaling pathways have been implicated in regulating the activity of FOXO3a including the pro-survival Akt kinase which phosphorylates and inhibits FOXO3a [25]. Activated ERK1/2 also phosphorylates FOXO3a, leading to the degradation and subsequent inhibition of FOXO3a [38]. ERK5, another member of the MAP kinases that is often pro-survival [39,40], is also a negative regulator of FOXO3a and fibroblasts from ERK5 and MEK5 null mice display increased FOXO3a activity [28]. JNK regulates FOXO3a nuclear translocation in breast cancer cells after paclitaxel treatment [29]. We discovered that arsenite-induced FOXO3a nuclear translocation is blocked by p38 inhibition, identifying p38 as a novel upstream regulator for FOXO3a.

It is interesting that although both ERK1/2 and p38 are proline-directed MAP kinases and share the same consensus phosphorylation site (PX₁₋₂SP/T), they exert opposite effects on FOXO3a. ERK1/2 directly phosphorylates FOXO3a at Ser 294, Ser 344 and Ser 425, targeting it for degradation via an MDM2-mediated ubiquitin-proteasome pathway, and promoting cell survival [38]. However, we report here that p38 activation of FOXO3a induces cell death. Treatment of PC12 cells with a p38 inhibitor caused a reduced electrophoretic mobility of FOXO3a after sodium arsenite treatment (supplemental Fig. 1), indicative of FOXO3a phosphorylation. Indeed, inhibition of p38 increases FOXO3a phosphorylation at T32; an inhibitory phosphorylation site that is phosphorylated by the pro-survival kinase Akt [25]. Thus p38 and ERK1/2 activate or inhibit the pro-apoptotic activity of FOXO3a, respectively, most likely through different mechanisms. While the inhibitory effect of ERK1/2 is mediated through direct phosphorylation, p38 most likely activates FOXO3a indirectly, perhaps by inducing the expression of a phosphatase which dephosphorylates the inhibitory phosphorylation site of FOXO3a.

In summary, our data demonstrate that sodium arsenite-induced apoptosis requires FOXO3a induction of Bim_{EL} transcription and that this process is regulated by p38. Together with our previous report that arsenite-induced apoptosis requires p38 phosphorylation and activation of Bim_{EL} [9], we hypothesize that p38 induces apoptosis by regulating Bim_{EL} both transcriptionally and post-translationally (Fig. 9). This mechanism may also apply to other forms of p38-induced apoptosis, including those triggered by anti-cancer drugs.

Acknowledgements

This work was supported by grants National Institutes of Health (NIH) R01 ES 012215 and NS44069 (ZX).

Electronic supplementary material The online version of this article (doi:10.1007/s10495-008-0218-5) contains supplementary material, which is available to authorized users.

References

1. Ghavamzadeh A, Alimoghaddam K, Ghaffari SH, Rostami S, Jahani M, Hosseini R, Mossavi A, Baybordi E, Khodabadeh A, Iravani M, et al. Treatment of acute promyelocytic leukemia with arsenic trioxide without ATRA and/or chemotherapy. *Ann Oncol* 2006;17:131–134. [PubMed: 16227315]
2. Mathews V, George B, Lakshmi KM, Viswabandya A, Bajel A, Balasubramanian P, Shaji RV, Srivastava VM, Srivastava A, Chandy M. Single-agent arsenic trioxide in the treatment of newly diagnosed acute promyelocytic leukemia: durable remissions with minimal toxicity. *Blood* 2006;107:2627–2632. [PubMed: 16352810]
3. Soignet SL, Frankel SR, Douer D, Tallman MS, Kantarjian H, Calleja E, Stone RM, Kalaycio M, Scheinberg DA, Steinherz P, et al. United States multicenter study of arsenic trioxide in relapsed acute promyelocytic leukemia. *J Clin Oncol* 2001;19:3852–3860. [PubMed: 11559723]
4. Deacon KMP, Chernoff J, Blank JL, Patel R. p38 Mitogenactivated protein kinase mediates cell death and p21-activated kinase mediates cell survival during chemotherapeutic drug-induced mitotic arrest. *Mol Biol Cell* 2003;14:2071–2087. [PubMed: 12802076]
5. Losa JH, Parada Cobo C, Viniegra JG, Sánchez-Arevalo Lobo VJ, Ramón y Cajal S, Sánchez-Prieto R. Role of the p38 MAPK pathway in cisplatin-based therapy. *Oncogene* 2003;22:3998–4006. [PubMed: 12821934]
6. Brancho D, Tanaka N, Jaeschke A, Ventura JJ, Kelkar N, Tanaka Y, Kyuuma M, Takeshita T, Flavell RA, Davis RJ. Mechanism of p38 MAP kinase activation in vivo. *Genes Dev* 2003;17:1969–1978. [PubMed: 12893778]
7. Namgung U, Xia Z. Arsenite-induced apoptosis in cortical neurons is mediated by c-Jun N-terminal protein kinase 3 and p38 mitogen-activated protein kinase. *J Neurosci* 2000;20:6442–6451. [PubMed: 10964950]
8. Namgung U, Xia Z. Arsenic induces apoptosis in rat cerebellar neurons via activation of JNK3 and p38 MAP kinases. *Toxicol Appl Pharmacol* 2001;174:130–138. [PubMed: 11446828]
9. Cai B, Chang SH, Becker EB, Bonni A, Xia Z. p38 MAP kinase mediates apoptosis through phosphorylation of BimEL at Ser-65. *J Biol Chem* 2006;281:25215–25222. [PubMed: 16818494]
10. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. *Science* 2004;305:626–629. [PubMed: 15286356]
11. Willis SN, Adams JM. Life in the balance: how BH3-only proteins induce apoptosis. *Curr Opin Cell Biol* 2005;17(6):617–625. [PubMed: 16243507]
12. Molton SA, Todd DE, Cook SJ. Selective activation of the c-Jun N-terminal kinase (JNK) pathway fails to elicit Bax activation or apoptosis unless the phosphoinositide 3'-kinase (PI3K) pathway is inhibited. *Oncogene* 2003;22:4690–4701. [PubMed: 12879014]
13. Okuno SSA, Hayashi T, Chan PH. The c-Jun N-terminal protein kinase signaling pathway mediates Bax activation and subsequent neuronal apoptosis through interaction with Bim after transient focal cerebral ischemia. *J Neurosci* 2004;24:7879–7887. [PubMed: 15356200]
14. Becker EB, Howell J, Kodama Y, Barker PA, Bonni A. Characterization of the c-Jun N-terminal kinase-BimEL signaling pathway in neuronal apoptosis. *J Neurosci* 2004;24:8762–8770. [PubMed: 15470142]
15. Biswas SC, Greene LA. Nerve growth factor (NGF) downregulates the Bcl-2 homology 3 (BH3) domain-only protein Bim and suppresses its proapoptotic activity by phosphorylation. *J Biol Chem* 2002;277:49511–49516. [PubMed: 12388545]
16. Putcha GV, Le S, Frank S, Besirli CG, Clark K, Chu B, Alix S, Youle RJ, LaMarche A, Maroney AC, Johnson EM Jr. JNK-mediated BIM phosphorylation potentiates BAX-dependent apoptosis. *Neuron* 2003;38:899–914. [PubMed: 12818176]
17. O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S, Huang DC. Bim: a novel member of the Bcl-2 family that promotes apoptosis. *EMBO J* 1998;17:384–395. [PubMed: 9430630]
18. Bouillet P, Zhang LC, Huang DC, Webb GC, Bottema CD, Shore P, Eyre HJ, Sutherland GR, Adams JM. Gene structure alternative splicing, and chromosomal localization of pro-apoptotic Bcl-2 relative Bim. *Mamm Genome* 2001;12:163–168. [PubMed: 11210187]

19. Putcha GV, Moulder KL, Golden JP, Bouillet P, Adams JA, Strasser A, Johnson EM. Induction of BIM, a proapoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis. *Neuron* 2001;29:615–628. [PubMed: 11301022]
20. Whitfield J, Neame SJ, Paquet L, Bernard O, Ham J. Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. *Neuron* 2001;29:629–643. [PubMed: 11301023]
21. Essafi A, Fernández de Mattos S, Hassen YA, Soeiro I, Mufti GJ, Thomas NS, Medema RH, Lam EW. Direct transcriptional regulation of Bim by FoxO3a mediates STI571-induced apoptosis in Bcr-Abl-expressing cells. *Oncogene* 2005;24:2317–2329. [PubMed: 15688014]
22. Gilley J, Coffey PJ, Ham J. FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons. *J Cell Biol* 2003;162:613–622. [PubMed: 12913110]
23. Urbich C, Knäuper A, Fichtlscherer S, Walter DH, Bruhl T, Potente M, Hofmann WK, de Vos S, Zeiher AM, Dimmeler S. FOXO-dependent expression of the proapoptotic protein Bim: pivotal role for apoptosis signaling in endothelial progenitor cells. *FASEB J* 2005;19:974–976. [PubMed: 15824087]
24. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 1995;270:1326–1331. [PubMed: 7481820]
25. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* 1999;96:857–868. [PubMed: 10102273]
26. Dérjard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M, Davis RJ. JNK1: a protein kinase stimulated by uv light and HA-ras binds to and activates the c-jun activation domain. *Cell* 1994;76:1025–1037. [PubMed: 8137421]
27. Hetman M, Kanning K, Cavanaugh JE, Xia Z. Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular-signal-regulated kinase and phosphatidylinositol-3 kinase. *J Biol Chem* 1999;274:22569–22580. [PubMed: 10428835]
28. Wang X, Finegan KG, Robinson AC, Knowles L, Khosravi-Far R, Hinchliffe KA, Boot-Handford RP, Tournier C. Activation of extracellular signal-regulated protein kinase 5 downregulates FasL upon osmotic stress. *Cell Death Differ* 2006;13:2099–2108. [PubMed: 16710360]
29. Susters A, Madureira PA, Pomeranz KM, Aubert M, Brosens JJ, Cook SJ, Burgering BM, Coombes RC, Lam EW. Paclitaxel-induced nuclear translocation of FOXO3a in breast cancer cells is mediated by c-Jun NH2-terminal kinase and Akt. *Cancer Res* 2006;66:212–220. [PubMed: 16397234]
30. Tan TT, Degenhardt K, Nelson DA, Beaudoin B, Nieves-Neira W, Bouillet P, Villunger A, Adams JM, White E. Key roles of BIM-driven apoptosis in epithelial tumors and rational chemotherapy. *Cancer Cell* 2005;7:227–238. [PubMed: 15766661]
31. Egle A, Harris AW, Bouillet P, Cory S. Bim is a suppressor of Myc-induced mouse B cell leukemia. *Proc Natl Acad Sci USA* 2004;101:6164–6169. [PubMed: 15079075]
32. Lei K, Davis RJ. JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. *Proc Natl Acad Sci USA* 2003;100:2432–2437. [PubMed: 12591950]
33. Yuan J, Lipinski M, Degterev A, Putcha GV, Le S, Frank S, Besirli CG, Clark K, Chu B, Alix S, et al. Diversity in the mechanisms of neuronal cell death JNK-mediated BIM phosphorylation potentiates BAX-dependent apoptosis. Regulation of neuronal survival and death by extracellular signals during development. *Neuron* 2003;40:401–413. [PubMed: 14556717]
34. Ley R, Balmanno K, Hadfield K, Weston C, Cook SJ. Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein, Bim. *J Biol Chem* 2003;278:18811–18816. [PubMed: 12646560]
35. Ley R, Ewings KE, Hadfield K, Howes E, Balmanno K, Cook SJ. Extracellular signal-regulated kinases 1/2 are serum-stimulated “Bim(EL) kinases” that bind to the BH3-only protein Bim(EL) causing its phosphorylation and turnover. *J Biol Chem* 2004;279:8837–8847. [PubMed: 14681225]
36. Harada H, Quearry B, Ruiz-Vela A, Korsmeyer SJ. Survival factor-induced extracellular signal-regulated kinase phosphorylates BIM, inhibiting its association with BAX and proapoptotic activity. *Proc Natl Acad Sci USA* 2004;101:15313–15317. [PubMed: 15486085]
37. Lu J, Quearry B, Harada H. p38-MAP kinase activation followed by BIM induction is essential for glucocorticoid-induced apoptosis in lymphoblastic leukemia cells. *FEBS Lett* 2006;580:3539–3544. [PubMed: 16730715]

38. Yang JY, Zong CS, Xia W, Yamaguchi H, Ding Q, Xie X, Lang JY, Lai CC, Chang CJ, Huang WC, et al. ERK promotes tumorigenesis by inhibiting FOXO3a via MDM2-mediated degradation. *Nat Cell Biol* 2008;10:138–148. [PubMed: 18204439]
39. Liu L, Cavanaugh JE, Wang Y, Sakagami H, Mao Z, Xia Z. ERK5 activation of MEF2-mediated gene expression plays a critical role in BDNF-promoted survival of developing but not mature cortical neurons. *Proc Natl Acad Sci USA* 2003;100:8532–8537. [PubMed: 12826611]
40. Pi X, Yan C, Berk BC. Big mitogen-activated protein kinase (BMK1)/ERK5 protects endothelial cells from apoptosis. *Circ Res* 2004;94:362–369. [PubMed: 14670836]

Fig. 1.

Sodium arsenite-induced apoptosis requires p38 and Bim_{EL}. (a) Kinetics and dose response of sodium arsenite-induced apoptosis in PC12 cells. (b) Transfection of PC12 cells with p38 siRNA protects against arsenite-induced apoptosis. PC12 cells were transfected with 50 nM siRNA to p38 (si-p38) or a non-specific control (si-NS). Twenty-four hours later, cells were treated with 0 or 15 μ M sodium arsenite for 24 h. Apoptosis in the total population was scored. (c) Expression of shRNA to Bim blocks sodium arsenite-induced apoptosis. PC12 cells were transfected with plasmid DNA encoding an empty vector control, shRNA of scrambled phosphodiesterase 2 (PDE2) as a non-specific control (shControl), or shRNA specifically targeting Bim_{EL} (shBim_{EL}). Transfected cells were identified by autofluorescence of a co-

transfected eGFP. One day later, cells were treated with vehicle control or 10 μ M sodium arsenite, and apoptosis in transfected cells (eGFP⁺) was scored 1 day later

Fig. 2. Sodium arsenite treatment upregulates Bim_{EL} protein in a p38-dependent manner. **(a)** Kinetics of Bim_{EL} protein induction after sodium arsenite treatment (15 μM). **(b)** Transient transfection of p38 siRNA blocks arsenite induction of Bim_{EL} protein. PC12 cells were transfected and treated as in Fig. 1b. Cell lysates were collected for Western analysis 16 h after arsenite treatment. β-Actin serves as a loading control

Fig. 3.

Sodium arsenite-induced apoptosis requires de novo transcription and is accompanied by an induction of Bim promoter activity. **(a)** Pretreatment of PC12 cells with actinomycin D (ActD, 100 ng/ml, 0.5 h), a general transcription inhibitor, attenuates apoptosis induced by arsenite (15 μM , 24 h). V, vehicle control; NT, not treated. **(b)** Dose dependent activation of Bim promoter-driven luciferase reporter by arsenite (16 h). **(c)** Pretreatment of PC 12 cells with ActD (100 or 200 ng/ml, 0.5 h) inhibits arsenite induction of Bim promoter-driven luciferase activity. **(d)** Pretreatment of PC 12 cells with ActD (100 ng/ml, 0.5 h) blocks arsenite induction of Bim protein

Fig. 4. Sodium arsenite activation of Bim promoter requires p38. (a) Pre-treatment with p38 inhibitors SB 203580 or SB 202190 (10 μ M, 1 h) attenuates Bim promoter activity after arsenite treatment. (b) Transfection of a dnMKK3 inhibits arsenite induction of Bim promoter activity

Fig. 5. p38 activation is sufficient to stimulate Bim promoter activity. PC12 cells were transfected with plasmid DNA encoding constitutive active (ca) MKK3 and wild type p38 α to activate p38 signaling in transfected cells. p38 inhibitor SB 203580 (10 μ M) (+) or vehicle control (-) was added 16 h after transfection. Cell lysates were harvested 16 h later for luciferase activity assay

Fig. 6.

Sodium arsenite treatment induces FOXO3a nuclear translocation, a process that requires p38 activity. (a) Subcellular localization of FOXO3a in control and arsenite-treated (15 μ M, 16 h) PC12 cells analyzed by anti-FOXO3a immunostaining. Scale bar: 20 μ m. Images were taken using a deconvolution microscope. (b) Quantification of cells with nuclear FOXO3a staining. PC12 cells were pre-treated with 10 μ M SB 203580 (SB) or vehicle control (V) for 1 h before arsenite treatment

Fig. 7. FOXO3a mediates sodium arsenite-induced Bim_{EL} expression. **(a)** FOXO3a activates Bim promoter. PC12 cells were cotransfected with plasmids encoding Bim promoter-luciferase and FOXO3a. One day after transfection, cells were treated with vehicle control or sodium arsenite (10 μ M) for 16 h. **(b)** RNAi silencing of FOXO3a blocks arsenite induction of Bim protein expression. siRNA against FOXO3a (si-FOXO3a) or a non-specific control (si-NS) was transfected 1 day before sodium arsenite treatment (15 μ M, 16 h). Protein level of Bim and FOXO3a was measured by western blotting; β -actin serves as a loading control. **(c)** RNAi silencing of FOXO3a inhibits arsenite activation of Bim promoter. PC12 cells were transfected with 100 nM si-FOXO3a or si-NS. One day later, cells were transfected again with plasmid

DNA encoding Bim promoter-luciferase. Cells were treated 1 day after plasmid transfection with vehicle control or sodium arsenite (15 μ M, 16 h)

Fig. 8. FOXO3a is required for sodium arsenite-induced apoptosis PC12 cells were transfected with si-FOXO3a or si-NS. One day later, cells were treated with vehicle control or sodium arsenite (15 μ M) for 16 h. Apoptosis in the total population was scored

Fig. 9.

Hypothesis model. Based on data reported here and published earlier [9], we propose that p38 mediates arsenite-induced apoptosis by both transcriptional and post-translational mechanisms. p38 induces nuclear translocation of FOXO3a and de novo transcription and expression of Bim_{EL}. It also phosphorylates Bim_{EL} on S65, activating Bim_{EL}'s pro-apoptotic activity