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Foxo-mediated *Bim* transcription is dispensable for the apoptosis of hematopoietic cells that is mediated by this BH3-only protein

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The BH3-only protein Bim is a critical initiator of apoptosis in hematopoietic cells. Bim is upregulated in response to growth factor withdrawal and *in vitro* studies have implicated the transcription factor Foxo3a as a critical inducer. To test the importance of this regulation *in vivo*, we generated mice with mutated Foxo-binding sites within the *Bim* promoters (*Bim*^{ΔFoxo/ΔFoxo}). Contrary to Bim-deficient mice, *Bim*^{ΔFoxo/ΔFoxo} mice had a normal hematopoietic system. Moreover, cytokine-dependent haematopoietic cells from *Bim*^{ΔFoxo/ΔFoxo} and wt mice died at similar rates. These results indicate that regulation of *Bim* by Foxo transcription factors is not critical for the killing of hematopoietic cells.

Keywords: apoptosis; Bcl-2; BH3-only proteins; Bim; hematopoiesis

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INTRODUCTION

The 'Bcl-2-regulated' apoptotic pathway has a critical role in the hematopoietic system [1]. It is controlled by the complex interactions of three sub-groups of the Bcl-2 protein family: the pro-survival Bcl-2-like proteins, the Bax/Bak multi-BH domain pro-apoptotic proteins and the pro-apoptotic BH3-only proteins, which are induced on growth factor deprivation [2,3]. The BH3-only proteins activate Bax/Bak (directly or indirectly by binding to the Bcl-2 pro-survival relatives), and this unleashes cell demolition [3].

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The BH3-only protein Bim is a critical regulator of the hematopoietic system [4–6]. Bim is essential for the deletion of auto-reactive T and B cells and the killing of effector lymphocytes during shutdown of immune responses [7–10].

Experiments using dominant-negative or constitutively active mutants of Foxo3a implicated this transcription factor as a critical inducer of Bim in apoptosis triggered by cytokine deprivation [11–14]. To explore the physiological relevance of this process, we generated mice in which all Foxo-binding sites within the *Bim* promoters [15] had been mutated (called *Bim*^{ΔFoxo/ΔFoxo} mice). Contrary to Bim-deficient mice, *Bim*^{ΔFoxo/ΔFoxo} and *Foxo3a*^{−/−} mice had a normal hematopoietic system. Moreover, in contrast to Bim-deficient cells, diverse lymphoid cell subsets and mast cells from *Bim*^{ΔFoxo/ΔFoxo} mice were normally sensitive to cytokine deprivation. These results indicate that direct transcriptional induction of *Bim* by Foxo is not critical for homeostasis and cytokine deprivation-induced apoptosis of hematopoietic cells.

RESULTS

Identification of Foxo-binding sites in the *Bim* promoters

Previous overexpression studies showed that Foxo3a enhances Bim expression by binding to two specific sequence motives (5'-GTAAACAA-3') within its promoters [11–14]. As Bim is critical for cytokine deprivation-induced apoptosis in diverse hematopoietic cell subsets [5,16], it was concluded that growth factor withdrawal kills cells through transcriptional upregulation of Bim by Foxo3a [11–14].

Our bioinformatics analysis of the *Bim* gene revealed the two published [11–14] and two additional Foxo3a-binding sites (−625 and +2,509 from Exon1) (Fig 1A; supplementary Fig 2A online). To examine whether Foxo3a can bind to these sites, we performed electrophoretic mobility shift assay (EMSA) with nuclear extracts from 293T cells that transiently expressed a tamoxifen (4-OHT)-inducible HA-tagged Foxo3a (HA-Foxo3a(A3)ER) (Fig 1B). We only observed Foxo3a-specific binding to site 4; this band

Fig 1 | Identification of candidate Foxo-binding sites in the *Bim* promoters. (A) Map showing four Foxo-binding sites within the *Bim* promoters (numbered according to the predicted start site reported by Bouillet *et al.*[15]). (B) EMSA to test for binding of Foxo3a to the Foxo-binding sites in the *Bim* promoters. ³²P-labelled oligos corresponding to the four sites were incubated with nuclear extracts from 293T cells transiently transfected with a control plasmid or tamoxifen-inducible active HA-tagged Foxo3a (HA-Foxo3A(A3)ER) that had been treated for 24 h with tamoxifen. (C) Control and HA-Foxo3a(A3)ER nuclear extracts from (B) were left untreated or incubated with either HA- or Foxo3A-specific antibodies to verify the specificity of Foxo3A binding to site 4 in the *Bim* promoters. Arrows indicate specific bands in (B) and (C). EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; UTR, untranslated region; wt, wild type.

disappeared when nuclear extracts of HA-Foxo3A(A3)ER were incubated with HA or Foxo3A-specific antibodies (Fig 1B,C). Additionally, we could efficiently compete Foxo3A binding to site 4 (supplementary Fig 1 online). These results show that site 4 in the mouse *Bim* promoters is the dominant site bound by Foxo3a.

***Bim*^{ΔFoxo/ΔFoxo} mice have a normal hematopoietic system**

Foxo3a belongs to a transcription factor family with partially overlapping expression patterns [17]. To examine the physiological role of Foxo-driven *Bim* activation, we generated mice in which the invariable core sequence of the Foxo-binding sequences in the *Bim* promoters were exchanged from 5'-AACAA-3' to 5'-TCGA-3' for site 1 or to 5'-GTGG-3' for sites 2, 3 and 4 (supplementary Fig 2A,B online). To verify the functional impact of these mutations, we generated T-cell blasts from wt and *Bim*^{ΔFoxo/ΔFoxo} mice and ectopically expressed HA-Foxo3a(A3)ER; this elicited *Bim* messenger RNA (mRNA) upregulation only in wt but not *Bim*^{ΔFoxo/ΔFoxo} T cells (supplementary Fig 2C online). Additionally we observed no compensatory Foxo protein upregulation in thymocytes from *Bim*^{ΔFoxo/ΔFoxo} mice (supplementary Fig 2D online).

We examined the hematopoietic system of the *Bim*^{ΔFoxo/ΔFoxo} mice using wt, *Foxo3a*^{-/-} and *Bim*-deficient animals as controls. As previously reported [5], *Bim*^{-/-} mice had a marked increase of mature CD4⁺8⁻ and CD4⁺8⁺ thymocytes compared with wt controls (Fig 2A). In contrast, *Bim*^{ΔFoxo/ΔFoxo} and *Foxo3a*^{-/-} mice had normal thymic cell subset distribution (Fig 2A). *Bim* mRNA and *Bim* protein levels in thymocytes from *Bim*^{ΔFoxo/ΔFoxo} and *Foxo3a*^{-/-} mice were comparable with those seen in wt thymocytes (Fig 2B,C).

Similarly, only *Bim*^{-/-} mice but not the *Bim*^{ΔFoxo/ΔFoxo} or *Foxo3a*^{-/-} animals had abnormally increased numbers of T as well as B cells and granulocytes in the spleen (Fig 3A). Accordingly, spleen cells from *Bim*^{ΔFoxo/ΔFoxo} and *Foxo3a*^{-/-} mice expressed normal levels of *Bim* mRNA and *Bim* protein (Fig 3B,C).

Bim activation by Foxo dispensable for thymocyte apoptosis

Bim is critical for thymocyte apoptosis triggered by diverse cytotoxic insults [5]. To examine the contribution of Foxo-mediated transcriptional induction of *Bim* in this scenario, we isolated thymocytes from *Bim*^{ΔFoxo/ΔFoxo}, *Foxo3a*^{-/-}, *Bim*^{-/-}

Fig 2 | *Bim*^{ΔFoxo/ΔFoxo} mice have normal T lymphoid cell subset composition in the thymus. (A) Cell numbers of CD4⁺CD8⁻ progenitors, CD4⁺CD8⁺ immature, CD4⁺CD8⁻ and CD4⁺CD8⁺ mature thymocytes were determined by flow cytometry in wt, *Bim*^{ΔFoxo/ΔFoxo}, *Foxo3a*^{-/-} and *Bim*^{-/-} mice (*n* indicates the numbers of mice analysed for each genotype). The data represent means $\pm$ s.d.; **P* < 0.05. (B) Western blot analysis to determine the levels of Bim protein expression in extracts from thymocytes of wt, *Bim*^{ΔFoxo/ΔFoxo}, *Foxo3a*^{-/-} and *Bim*^{-/-} mice. Probing for β-actin served as a loading control. (C) qRT-PCR analysis to determine the levels of *Bim* mRNA expression in extracts from thymocytes of wt, *Bim*^{ΔFoxo/ΔFoxo} and *Foxo3a*^{-/-} mice. The expression of *Bim* mRNA was normalised to HMBS mRNA. The data represent means $\pm$ s.d.; ***P* < 0.01. HMBS, hydroxymethylbilane synthase; mRNA, messenger RNA; qRT-PCR, quantitative real time polymerase chain reaction; wt, wild type.

Fig 3 | *Bim*^{ΔFoxo/ΔFoxo} mice have normal hematopoietic cell subset composition in the spleen. (A) The numbers of B cells, CD4⁺CD8⁻ and CD4⁺CD8⁺ mature T cells, macrophages (Mac-1⁺Gr-1⁻) and granulocytes (Mac-1⁺Gr-1⁺) in the spleens of wt, *Bim*^{ΔFoxo/ΔFoxo}, *Foxo3a*^{-/-} and *Bim*^{-/-} mice were determined by flow cytometry (*n* indicates the numbers of mice for each genotype analysed). The data represent means $\pm$ s.d.; **P* < 0.05, ****P* < 0.0005, *****P* < 0.0001. (B) Western blot analysis to determine the levels of Bim protein expression in extracts from spleen cells of wt, *Bim*^{ΔFoxo/ΔFoxo}, *Foxo3a*^{-/-} and *Bim*^{-/-} mice. Probing for HSP70 served as a loading control. (C) qRT-PCR analysis to determine the levels of *Bim* mRNA expression in extracts from spleen cells of wt, *Bim*^{ΔFoxo/ΔFoxo} and *Foxo3a*^{-/-} mice. The expression of *Bim* mRNA was normalised to HMBS mRNA. HMBS, hydroxymethylbilane synthase; mRNA, messenger RNA; qRT-PCR, quantitative real time polymerase chain reaction; wt, wild type.

and wt animals, and cultured them in simple medium or treated them with ionomycin and dexamethasone. Phorbol ester an apoptotic stimulus that does not depend on Bim [5] served as a control. The *Bim*^{-/-} and to a lesser extent the *Foxo3a*^{-/-}

thymocytes showed abnormally increased survival in simple medium, but the *Bim*^{ΔFoxo/ΔFoxo} thymocytes died at the same rate as their wt counterparts (Fig 4). As previously reported [5], loss of Bim substantially reduced ionomycin-induced killing of

Fig 4 | Thymocytes from *Bim*^{ΔFoxo/ΔFoxo} mice are normally sensitive to a broad range of apoptotic stimuli that kill these cells in a Bim-dependent manner. Survival of thymocytes from wt, *Bim*^{ΔFoxo/ΔFoxo}, *Foxo3a*^{-/-} and *Bim*^{-/-} mice in culture, either in simple medium (no added growth factors, to simulate growth factor withdrawal) or after treatment with dexamethasone, ionomycin or phorbol ester, was determined at the indicated time points by flow cytometry (*n* indicates numbers of mice from each genotype). The data represent means $\pm$ s.d.; **P* < 0.05, ***P* < 0.01, ****P* < 0.0005, *****P* < 0.0001. wt, wild type.

thymocytes, but cells from the *Bim*^{ΔFoxo/ΔFoxo} and *Foxo3a*^{-/-} mice behaved like wt thymocytes (Fig 4). Finally, thymocytes from *Bim*^{-/-} and to a lesser extent those from *Foxo3a*^{-/-} mice survived treatment with dexamethasone better than their wt counterparts or cells from the *Bim*^{ΔFoxo/ΔFoxo} mice (Fig 4).

***Bim*^{ΔFoxo/ΔFoxo} cells die normally by cytokine withdrawal**

Previous studies showed that cytokine deprivation activates Foxo3a and that the resulting apoptosis can be inhibited by expression of a dominant-negative Foxo3a [11–13]. It was therefore concluded that Foxo3a-mediated transcriptional induction of *Bim* is essential for cytokine deprivation-induced apoptosis.

Mature T cells require IL-7 for efficient survival [18] and the cytokine deprivation-induced death of these cells requires Bim [5]. To examine the role of Foxo-mediated transcriptional upregulation of *Bim* in this setting, we isolated CD4⁺ and CD8⁺ T cells from the spleens of *Bim*^{ΔFoxo/ΔFoxo}, *Foxo3a*^{-/-}, *Bim*^{-/-} and wt mice and cultured them in the presence or absence of IL-7. CD4⁺ as well as CD8⁺ T cells of all genotypes survived well in the presence of IL-7, but only the *Bim*^{-/-} T cells showed extended survival in the absence of this cytokine, whereas the *Foxo3a*^{-/-} and *Bim*^{ΔFoxo/ΔFoxo} T cells died at similar rates as the wt cells (Fig 5A, upper panel).

Bim is also critical for cytokine deprivation-induced apoptosis in mature B cells [5]. When FACS-sorted transitional (IgM⁺IgD⁺) and follicular B cells (IgM^{low}IgD⁺) from the spleens were cultured in simple medium, only the *Bim*^{-/-} cells showed extended survival, whereas *Foxo3a*^{-/-} and *Bim*^{ΔFoxo/ΔFoxo} B cells died at similar rates as wt B cells (Fig 5A, lower panel).

During shutdown of immune responses, a substantial fraction of activated T cells are killed to minimise the risk of immunopathology and to make space for subsequent immune responses against different pathogens [19]. As Bim, triggered by a reduction in

cytokine levels, is essential for this death of activated T cells [9], Foxo transcription factors have been implicated as the critical inducers of *Bim* in this process [13]. We examined the importance of Foxo-mediated transcriptional induction of *Bim* in cytokine deprivation-induced killing of activated T cells. In the presence of IL-2, mitogen-stimulated T-cell blasts of all genotypes survived comparably. *Bim*^{-/-} T-cell blasts were almost completely protected from cytokine deprivation, and those from the *Foxo3a*^{-/-} mice showed modest (albeit significant) protection (Fig 5B). In contrast, the *Bim*^{ΔFoxo/ΔFoxo} T-cell blasts died at the same rate as wt cells (Fig 5B). IL-2 withdrawal caused similar increases of *Bim* mRNA and Bim protein levels in *Bim*^{ΔFoxo/ΔFoxo}, *Foxo3a*^{-/-} and wt T-cell blasts (Fig 5C).

In order to examine whether direct transcriptional induction of Bim by Foxo3a or related Foxo family members is critical for growth factor deprivation-induced apoptosis of myeloid cells, we generated IL-3/SCF-dependent mast cells from the bone marrow of *Bim*^{ΔFoxo/ΔFoxo}, *Bim*^{-/-}, *Foxo3a*^{-/-} and wt mice. As reported [20], mast cells from *Bim*^{-/-} and *Foxo3a*^{-/-} mice survived cytokine deprivation significantly better than wt cells, whereas the *Bim*^{ΔFoxo/ΔFoxo} mast cells died at a normal rate (Fig 5D). Cytokine deprivation caused comparable levels of Bim protein upregulation in wt, *Foxo3a*^{-/-} and *Bim*^{ΔFoxo/ΔFoxo} mast cells (Fig 5E).

No impact of *Bim*^{ΔFoxo} mutation on *Em-myc* lymphomagenesis

Both, the pro-apoptotic BH3-only protein Bim and Foxo transcription factors have been shown to act as tumour suppressors [17,21]. Loss of both or even one allele of *Bim* substantially accelerated pre-B/B lymphoma development in *Em-myc* transgenic mice [22]. To test whether Foxo-mediated transcriptional induction of *Bim* is critical for its tumour suppressive function, we crossed the *Bim*^{ΔFoxo/ΔFoxo} mice with

Fig 5 | Impact of the *Bim*^{ΔFoxo} mutation or loss of Foxo3a on cytokine deprivation-induced killing of lymphoid and myeloid cells. (A) Survival (%living = %AnnexinV/PI-negative cells) of FACS-sorted CD4⁺8⁺ or CD4⁺8⁺ mature T cells and transitional or follicular B cells from the spleens of wt, *Bim*^{ΔFoxo/ΔFoxo}, *Foxo3a*^{-/-} and *Bim*^{-/-} mice in culture in simple medium (no added growth factors) was determined at the indicated time points by flow cytometry (*n* indicates number of mice from each genotype). The data represent means ± s.d.; **P* < 0.05, ***P* < 0.01, ****P* < 0.0005, *****P* < 0.0001. (B) Survival of mitogen-stimulated T-cell blasts from wt, *Bim*^{ΔFoxo/ΔFoxo}, *Foxo3a*^{-/-} and *Bim*^{-/-} mice in culture in simple medium (after removal of IL-2) was determined at the indicated time points by flow cytometry (*n* indicates number of mice from each genotype). The data represent means ± s.d.; **P* < 0.05, ***P* < 0.01, ****P* < 0.0005, *****P* < 0.0001. (C) qRT-PCR and western blot analyses to determine the levels of *Bim* mRNA and Bim protein expression, respectively, in the T-cell blasts described in (B), in the presence of IL-2 or 8 h after removal of IL-2 (in the presence of the pan-caspase inhibitor QVD-OPH (20 μM)). For qRT-PCR analysis *Bim* mRNA levels were normalised to HMBS. Probing for HSP70 served as a loading control. (D) Survival of IL-3/SCF-dependent mast cells from wt, *Bim*^{ΔFoxo/ΔFoxo}, *Foxo3a*^{-/-} and *Bim*^{-/-} mice in simple medium (after removal of IL-3 and SCF) was determined at the indicated time points by flow cytometry (*n* indicates number of mice from each genotype). The data represent means ± s.d.; **P* < 0.01, ***P* < 0.0005, *****P* < 0.0001. (E) Western blot analysis to determine the levels of Bim protein expression in the mast cells described in (D), in the presence of IL-3/SCF or 8 h after removal of these growth factors (in the presence of 20 μM QVD-OPH). Probing for β-actin served as a loading control. FACS, fluorescence-activated cell sorting; HMBS, hydroxymethylbilane synthase; IL, interleukin; mRNA, messenger RNA; PI, propidium iodide; qRT-PCR, quantitative real time polymerase chain reaction; SCF, stem cell factor; wt, wild type.

the *Em-myc* transgenic mice [23] and monitored tumour development. There was no significant difference in the rate of lymphoma development or immunophenotype (~60% B220⁺slg⁻ pre-B and ~40% B220⁺slg⁺ B) between *Em-myc* (control), *Em-myc*;*Bim*^{ΔFoxo/wt} and *Em-myc*;*Bim*^{ΔFoxo/ΔFoxo} mice (supplementary Fig 3A,B online). We therefore conclude that

direct transcriptional induction of *Bim* by Foxo transcription factors is not critical for Bim's tumour suppressive function.

DISCUSSION

The pro-apoptotic BH3 only Bcl-2 family member Bim is critical for several developmentally programmed pathways to cell death

in the hematopoietic system [5,7,8]. Studies *in vitro* have shown that Bim is essential for the death of diverse cell types triggered by a broad range of apoptotic stimuli [5].

Despite the importance of Bim in so many processes, no transcriptional and/or post-transcriptional regulator has been certified *in vivo*. This is particularly the case for cytokine deprivation-induced apoptosis, a process that is critical for at least some of the functions of Bim within the whole animal [24]. Experiments using immortalized cytokine-dependent cell lines and enforced expression of dominant-negative or constitutively active Foxo3a mutants have indicated that this transcription factor binds within the *Bim* promoters and is essential for the activation of Bim in cytokine deprivation-induced apoptosis [11–13].

To determine the impact of loss of transcriptional induction of *Bim* by all Foxo family members, we generated mice in which all potential Foxo-binding sites in the *Bim* promoters had been mutated. In contrast to Bim-deficient cells [5], T as well as B lymphoid cells and mast cells from these animals were normally sensitive to cytokine deprivation. How can these results be reconciled with previous studies demonstrating a role for Foxo transcription factors in Bim activation in response to cytokine deprivation [11–13]? It remains theoretically possible that Foxo transcription factors can directly activate *Bim* expression by binding to sites other than the four that we have mutated. However, our sequence analysis and overexpression of Foxo3a in T-cell blasts from *Bim^{ΔFoxo/ΔFoxo}* mice (supplementary Fig 2C online) did not reveal any additional candidate Foxo-binding sites in the *Bim* promoter region. It also remains possible that Foxo proteins regulate Bim expression indirectly, for example by inducing another transcription factor that does bind to the *Bim* promoter or by controlling the expression of microRNAs that regulate Bim expression levels. In this regard, it is noteworthy that the mir17–92 cluster has been shown to regulate apoptosis by repressing Bim expression [25]. Alternatively, cytokine deprivation might activate Bim by a mechanism that is entirely independent of the Foxo transcription factors. This question might be resolved by generating cells that lack all Foxo family members, although such cells might be non-viable because of the loss of essential functions of Foxo transcription factors that are not related to transcriptional induction of *Bim*. It must also be noted that our studies were conducted with hematopoietic cells only. *Bim* expression was reported to be regulated by Foxo transcription factors in neurons [14], hence it will be interesting to use our *Bim^{ΔFoxo/ΔFoxo}* mice to examine the physiological relevance of this finding.

It is interesting that loss of Foxo3a (but not the *Bim^{ΔFoxo}* mutation) could significantly delay spontaneous and dexamethasone-induced thymocyte apoptosis and cytokine deprivation-induced apoptosis of T-cell blasts and mast cells, but did not reduce Bim expression. This indicates that Foxo3a must regulate a critical pro-apoptotic gene other than *Bim* under these stress conditions. Notably, Foxo3a was shown to upregulate *Puma* expression in response to cytokine deprivation [26] and to cooperate with Bim in spontaneous and dexamethasone-induced thymocyte killing, and IL-2 or IL-3/SCF withdrawal-induced killing of T-cell blasts or mast cells, respectively [6,27]. This indicates that Foxo3a might have a critical role in these pathways to apoptosis by transcriptional induction of *Puma*.

Finally, we showed that although Bim suppresses Myc-induced lymphomagenesis [22], mutation of all Foxo-binding sites in the

Bim promoters did not alter the incidence or rate of pre-B/B lymphoma development in *Eμ-myc* transgenic mice. It remains possible that Foxo proteins could regulate the expression and thereby the tumour suppressive action of Bim indirectly. Alternatively, the activation of Bim during tumour suppression is mediated by a yet to be identified process that is independent of Foxo transcription factors.

METHODS

Cell culture, cell survival assays and retroviral transduction of T-cell blasts. Thymocytes (5×10^4) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 50 μM beta-mercaptoethanol and 100 μM asparagin (simple medium) alone or in combination with 30 nM dexamethasone (Mayne Pharma Pty Ltd.), 2 ng/ml PMA (Sigma) or 1 μg/ml Ionomycin (Sigma). To obtain T-cell blasts, 2×10^6 spleen cells were stimulated with recombinant mIL-2 and 2 μg/ml concanavalin A (ConA)(Sigma) in simple medium for 3 days. Subsequently, cells were washed five times to remove IL-2 and ConA and then cultured in the presence or absence of recombinant mIL-2. Mast cells were generated by culturing 5×10^5 bone marrow cells with weekly medium change for 4 weeks in simple medium supplemented with 12.5 ng/ml recombinant SCF and recombinant mIL-3. Sorted quiescent T and B cell populations from the spleen were cultured in simple medium alone or in the case of T cells, alone or with recombinant mIL-7. For retroviral transduction, T-cell blasts were generated as described above. After removal of ConA, cells were maintained in IL-2 for the duration of the whole experiment. Viral particles were produced by transiently transfecting HEK 293T cells with the gene of interest (pBABE or pBABE/HA-Foxo3a(A3)ER) alongside the packaging plasmids using standard calcium phosphate transfection protocols. After 2 days, retroviral supernatants were centrifuged for 45 min at 3,500 r.p.m. at 32 °C onto plates coated with RetroNectin (Takara). T-cell blasts were cultured overnight in the presence of the virus and 2 days later transduced cells were selected in 5 μg/ml puromycin. Afterwards, cells were either left untreated or incubated for 24 h with 1 μM tamoxifen to induce nuclear translocation and hence activation of HA-Foxo3a(A3)ER. RNA was isolated from these cells to measure *Bim* mRNA levels.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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M.J.L. performed some experiments. L.T. performed gene-targeting in ES cells. T.K., R.G. and S.G. performed the EMSA experiments. P.B., T.K. and A.S. conceived study, planned the experiments, interpreted data and wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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