CREB-binding protein (CBP) regulates β -adrenoceptor (β -AR) – mediated apoptosis

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Catecholamines regulate the β -adrenoceptor/cyclic AMP-regulated protein kinase A (cAMP/PKA) pathway. Deregulation of this pathway can cause apoptotic cell death and is implicated in a range of human diseases, such as neuronal loss during aging, cardiomyopathy and septic shock. The molecular mechanism of this process is, however, only poorly understood. Here we demonstrate that the β -adrenoceptor/cAMP/PKA pathway triggers apoptosis through the transcriptional induction of the pro-apoptotic BH3-only Bcl-2 family member *Bim* in tissues such as the thymus and the heart. In these cell types, the catecholamine-mediated apoptosis is abrogated by loss of *Bim*. Induction of *Bim* is driven by the transcriptional co-activator CBP (CREB-binding protein) together with the proto-oncogene c-Myc. Association of CBP with c-Myc leads to altered histone acetylation and methylation pattern at the *Bim* promoter site. Our findings have implications for understanding pathophysiology associated with a deregulated neuroendocrine system and for developing novel therapeutic strategies for these diseases.

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Cyclic adenosine monophosphate (cAMP) is a second messenger that is highly conserved throughout evolution. In metazoans, its primary role is to act as an intracellular carrier of metabolic information, regulating hormonal responses,¹ in triggering apoptotic cell death and in regulating ontogeny.^{2,3} Studies with transgenic mouse models have revealed that deregulation of the cyclic AMP-regulated protein kinase A (cAMP/PKA) pathway can cause apoptosis.⁴ Specifically, β -adrenergic receptor (β AR) activation by catecholamines or autoantibodies, as seen in Grave's disease,⁵ can cause PKA-mediated cardiomyocyte apoptosis, resulting in heart failure (HF).⁶ Furthermore, administration of catecholamines, such as epinephrine, is often the last resort for treatment of patients suffering from septic shock,⁷ and this is associated with profound alterations in immune function, similar to those observed in haemorrhagic shock where all lymphocyte subsets are decreased owing to excessive apoptosis.⁸ Similarly, loss of dopamine receptor containing neurons during aging has been attributed to the apoptosis-inducing effects of the catecholamine, dopamine⁹ and chronic stress-induced immune modulation has been attributed to increased levels of circulating epinephrine.¹⁰

The molecular mechanisms of cAMP/PKA pathway-mediated apoptosis are only poorly defined. The BH3-only Bcl-2 family protein *Bim* is considered to be an essential

initiator of apoptosis in a wide variety of physiological settings, including deregulated calcium flux, growth factor withdrawal,¹¹ endoplasmic reticulum stress¹² and T cell receptor¹³ as well as B cell receptor activation¹⁴ in autoreactive lymphocytes. Downregulation of *Bim* appears to be a common denominator in many cell survival signalling pathways in cancers, and many anticancer therapeutics kill cancer cells by inducing *Bim* expression.^{15,16} We have previously reported that the cAMP/PKA pathway can regulate *Bim* protein levels by phosphorylation and stabilization.¹⁷ However, the present work demonstrates that in many cell types and tissues, transcriptional induction of *Bim* by the cAMP/PKA pathway has a major role in cell death regulation.

Here we elucidate the molecular mechanism of the cAMP/PKA-triggered apoptotic pathway downstream of β AR activation. We demonstrate that PKA activation results in the transcriptional induction of the pro-apoptotic BH3-only Bcl-2 family gene *Bim* and subsequent *Bim*-dependent apoptosis in diverse cell types, including thymocytes and cardiomyocytes. We also provide proof for the engagement of the *Bim* promoter by the proto-oncogene c-Myc, together with the transcriptional co-activator CREB (cAMP response element-binding protein)-binding protein (CBP). Our results shed light on the role of increased sympathetic nerve activity and apoptosis in the myocardium that can lead to HF¹⁸, in the

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Abbreviations: cAMP/PKA, cyclic AMP-regulated protein kinase A; β AR, β -adrenergic receptor; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; 4-OHT, 4-hydroxytamoxifen; FTOC, foetal thymic organ culture; MHC, myosin heavy chain; Prkar1a, PKA regulatory subunit 1a; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay

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development of the thymus during embryogenesis¹⁹ and in stress-induced thymic atrophy and immune modulation.²⁰

Results

cAMP/PKA activation induces BIM in a variety of tissues. A critical role for the pro-apoptotic BH3-only Bcl-2 family member Bim (*Bcl2L11*) in cAMP/PKA-triggered apoptosis has been reported in the S-49 T-cell lymphoma line.²¹ To test the generality of this phenomenon, we used a variety of systems to induce PKA activity. PKA exists as a tetrameric holoenzyme composed of two catalytic (C) and two regulatory (R) subunits. Binding of cAMP causes the dissociation of the holoenzyme into its constituent subunits, that is, PKA regulatory subunit 1a (Prkar1a) and PKA α .

However, cAMP regulation of PKA can be bypassed by ectopic overexpression of PKA α .²² 4-hydroxytamoxifen (4-OHT)-induced expression of PKA α HA-tagged in mouse embryonic fibroblasts (MEFs) resulted in the induction of Bim, both at the protein and mRNA levels (Figure 1a). Similarly, β AR stimulation by agonists, such as isoproterenol, leads to cAMP flux and PKA activation.²¹ Consistent with this, treatment of MEFs with isoproterenol led to a robust induction of Bim both at the protein and mRNA levels (Figure 1b and Supplementary Figure S1d).

The primary and secondary lymphoid organs, such as the developing thymi as well as the spleen and lymph nodes, are innervated by the autonomic, mainly sympathetic, nervous system, allowing neuroimmune modulation.²³ Postnatal infection and stress can cause profound involution of the thymus

Figure 1 cAMP/PKA activation induces Bim in a variety of tissues. (a) MEFs expressing PKA α under 4-OHT regulation. First two lanes are samples from a clone, which expresses very little PKA α used as control. pCREB is the surrogate marker for PKA activation. (b) MEFs treated with isoproterenol for various time points and analyzed by western blot or by qPCR. *Nur77* transcripts are used as a marker for PKA activation in the qPCR analysis. (c and d) Thymocytes in FTOC or cardiomyocytes in culture were treated with isoproterenol and rolipram (10 μ M each) and analyzed for various transcripts by qPCR. (e) β^2 AR transgenic mouse (β^2 AR expressed under MHC promoter or MHC α) and littermate control on C57BL/6 background (at 6 months) were analyzed by echocardiogram, TUNEL staining for apoptosis or by Masson's trichrome staining for collagen accumulation. (f) LV sections were stained with Bim-specific antibodies or the antibodies pre-incubated with the peptide epitope against which the antibodies were raised to show the specificity of the staining. (g) RT-qPCR analysis of the heart tissues for *Bim* transcripts and (h) western blot analysis of the transgenic heart tissues with myocardial infarct (MI) as a positive control. Error bars: $\pm$ S.E.M., $n = 3$, one-tailed *T*-tests. #*P* = 0.004; **P* = 0.023 versus untreated control (b); #*P* = 0.002–0.0012 versus untreated control (c); #*P* = 0.0014 versus untreated control (d); #*P* = 0.013 versus wild-type (WT) control

gland, and this is attributed to increased secretion of catecholamines by the sympathetic nervous system.²⁴ As *Bim* is critical for the killing of thymocytes by diverse apoptotic stimuli,¹¹ we tested whether thymocytes could upregulate *Bim* in response to catecholamines. Treatment of thymocytes in foetal thymic organ culture (FTOC) with isoproterenol resulted in a robust induction of *Nur77*, a marker for PKA activation²⁵ and *Bim*, whereas the expression of other BH3-only genes, such as *Puma* (*Bbc3*) and *Noxa* remained unchanged (Figure 1c).

β -adrenergic stimulation leads to PKA-mediated apoptosis in adult cardiomyocytes.²⁶ Accordingly, isoproterenol treatment induced *Nur77* and *Bim* expression in cardiomyocytes isolated from adult mice by Langendorff perfusion (Figure 1d). Similar results were obtained in *in vivo* mouse models. Similar to what was seen in β^1 AR transgenic animals,²⁷ cardiomyocyte-specific transgenic overexpression of β^2 AR led to cardiomyocyte apoptosis, hypertrophy and ultimately HF.²⁸ These transgenic mice had enlarged hearts, dilated left ventricles (LV) and increased fibrosis, as revealed by echocardiogram, TUNEL and collagen staining (Figure 1e and Supplementary Figure S1a). Immunohistochemical staining of heart sections demonstrated that hearts in these transgenic mice expressed higher levels of *Bim* compared with hearts from wild-type (WT) mice (Figure 1f). Quantitative PCR (qPCR) and western blot analyses of these heart tissues indicated that *Bim* was induced in these mice both at the mRNA and protein levels (Figures 1g and h). The heart is richly innervated by sympathetic nerves, which regulate cardiac function by the release of catecholamines that act on β ARs. Excessive catecholamine levels are correlated with failing myocardium.²⁹ Consistent with this notion, induction of myocardial infarction by left coronary artery ligation resulted in increased levels of both adrenaline and nor-adrenaline in the serum of C57BL/6 mice, and this was accompanied by increased *Bim* protein expression (Supplementary Figures S1b and c). Similar to what we observed in thymocytes, this induction was specific to *Bim*, as we did not detect any induction of other BH3-only proteins, such as *Puma* (Supplementary Figure S1c).

Loss of *Bim* inhibits cAMP/PKA-induced apoptosis. Consistent with the reports from T hybridoma cells,²¹ our results show that *Bim* is a downstream target of the cAMP/PKA pathway in diverse tissues. We therefore examined whether *Bim* was required for PKA-induced apoptosis. Apart from the sympathetic nervous system, immune cells themselves can synthesize and metabolize catecholamines and stimulate β ARs. Immune cells are therefore considered to be 'a new, diffusely distributed adrenergic organ'.⁴ Catecholamines regulate lymphocyte proliferation, differentiation and apoptosis via an autocrine loop.³⁰ Therefore, we tested whether loss of *Bim* could protect thymocytes from β AR-mediated apoptosis in FTOC. Treatment of FTOC with isoproterenol resulted in *Bim* protein induction (Supplementary Figure S2a) accompanied by apoptosis of the WT thymocytes. Loss of *Bim*, not loss of *Puma* rendered thymocytes completely resistant to this treatment (Figures 2a and b). This was not because of attenuated PKA activation in *Bim*^{-/-} cells, as treatment with isoproterenol activated PKA

to similar extent in both *Bim*^{-/-} and WT cells, as indicated by the increase in phospho-CREB (pCREB) and cAMP levels (Supplementary Figure S2a, lanes 2 and 4 and Figure 2c). (Constitutive levels of both pCREB and cAMP levels were higher in *Bim*^{-/-} cells; this might be because of the survival of thymocytes with high cAMP/PKA activity in the absence of *Bim*.)

In an acute mouse model of HF, injection of isoproterenol (15 mg/kg per day for 7 days) causes ventricular remodelling, leading to cardiac hypertrophy.³¹ Although isoproterenol injection resulted in impaired contractile function of the LV (significantly lower fractional shortening and lower fractional area change) in the WT mice, the *Bim*^{-/-} mice did not develop this pathology (Figures 2d–f). Accordingly, many more TUNEL+ (apoptotic) cells were seen in WT mice compared with the *Bim*^{-/-} animals (Supplementary Figure S2b). Similarly, whereas cardiomyocytes isolated from adult WT mice by Langendorff perfusion underwent substantial apoptosis after treatment with isoproterenol, those from *Bim*^{-/-} mice were resistant (Supplementary Figure S2c).

Apart from rescuing the catecholamine-induced pathology, *Bim* ablation also resulted in constitutive physiological hypertrophy of the heart. This is characterized by increased weight of LV and whole heart, and this could be attributed to increased size of cardiomyocytes (Figures 3a and b and Supplementary Figure S2d). Furthermore, gene expression analysis of WT and *Bim*^{-/-} heart tissues indicated that the cardiac hypertrophy is not associated with any pathology, as we did not observe any of the pathological hypertrophy-associated gene induction in the *Bim*^{-/-} hearts (Figure 3c).

***Prkar1a* deletion induces apoptosis and this can be inhibited by loss of *Bim*.** The discovery that the exchange protein activated by cAMP (Epac) is a direct target of cAMP with an affinity comparable to the regulatory subunit of PKA³² questions the long-held notion that PKA is the only direct downstream target of cAMP. To rule out that Epac is critical for the induction of *Bim*, we used a genetic model in which PKA can be activated without inducing cAMP flux or adenylyl cyclase activation. Deletion of the ubiquitously expressed regulatory subunit of PKA, *R1 α* (*Prkar1a*), leads to constitutive activation of PKA.³³ Accordingly, MEFs from *Prkar1a*^{fl/fl} mice were infected with lentiviruses expressing 4-OHT-inducible CRE.³⁴ This resulted in acute loss of *R1 α* with consequent PKA activation (as seen by increased levels of phospho-PKA substrates) and in a robust induction of *Bim* both at the mRNA and protein levels (Figure 4a). Induction of *Bim* triggered by *R1 α* deletion resulted in a substantial reduction in the clonogenic survival of these MEFs. Remarkably, this could be prevented by the loss of *Bim* (Figure 4b). This was also seen in short-term survival assay as well as when *Prkar1a*-deficient MEFs were treated with isoproterenol (Figure 4c).

c-Myc regulates *BIM* transcription during β AR stimulation. To identify the transcription factor(s) involved in β AR stimulation-mediated induction of *Bim*, we used the MEFs with 4-OHT-inducible PKA α expression (Figures 1a and 5a, inset). Transient transfection of these cells with a *Bim* promoter-luciferase reporter resulted in a robust induction of luciferase upon addition of 4-OHT. Using this assay,

Figure 2 cAMP/PKA-mediated apoptosis is Bim dependent. **(a and b)** Analysis of thymocytes apoptosis in FTIC in response to β AR activation. **(c)** cAMP measurement in thymocytes from WT and *Bim*^{-/-} mice in response to β AR activation. **(d)** Representative echocardiographic 2D short-axis view of the LV of mice injected with isoproterenol (15 mg/kg per day i.p. for 8 days) compared with untreated controls (UT). **(e and f)** Measurement of fractional shortening and fractional area change in the isoproterenol-treated mice compared with control. Error bars: $\pm$ S.E.M., $n = 3-5$, $\#P = 0.0053-0.0078$ (**b and c**); $n = 5-7$, $\#P = 0.0067-0.0094$ (**e and f**); $*P = 0.432-0.562$; one-tailed *T*-tests

we mapped the *Bim* promoter and identified a conserved hexa-nucleotide region 140 bp upstream of the transcription start site, which was essential for PKA-mediated *Bim* upregulation (Figure 5a). This site potentially represents a non-canonical E-box capable of binding the proto-oncogene c-Myc,³⁵ and binding of Myc to this site could indeed be confirmed in electrophoretic mobility shift assays (EMSA) (Supplementary Figure S3e). A critical role for c-Myc in *Bim* mRNA upregulation was first reported in the *E μ -Myc* mouse pre-B/B lymphoma model. *Bim* mRNA and Bim protein levels were elevated in the apoptosis-prone B lymphoid cells of *E μ -Myc* transgenic mice, and Bim-deficient *E μ -Myc* mice had increased numbers of slgM⁺ B cells.³⁶ However, a direct relationship between c-Myc and *Bim* transcription is yet

to be established. Expression of c-Myc under 4-OHT-regulation in MEFs resulted in a robust induction of Bim at both the mRNA and protein levels as, for example, seen in the luciferase assay (Figure 5b). This transcriptional induction of the *Bim* reporter construct could be abrogated either by mutating the conserved E-Box or by using the PKA inhibitor H-89 (Figure 5b). Furthermore, β AR stimulation-triggered induction of the *Bim* reporter in MEFs could also be abrogated by 10058-F4,³⁷ a specific inhibitor of c-Myc–Max interaction. This indicates that Myc–Max heterodimerization is crucial for this transcriptional upregulation of *Bim* (Figure 5c). Consistent with these results, acute loss of c-Myc by CRE-mediated deletion in *c-Myc*^{fl/fl} MEFs resulted in failure to induce Bim in response to β AR activation,

Figure 3 Bim ablation leads to increased cardiomyocyte size. (a) Wheat germ agglutinin staining of heart section from mice treated with isoproterenol (ISO). (b and d) Cardiomyocyte size determination from the WT and Bim^{-/-} heart sections. (c) qPCR analysis of pathology-associated gene expression in the WT and Bim^{-/-} hearts. Error bars: $\pm$ S.E.M., $n=5-7$, # $P=0.0009$; * $P=0.2309-0.8809$; paired one-tailed T -tests

although both *c-Myc*^{fl/fl} and *c-Myc*^{-/-} MEFs were equally capable of activating the β AR pathway, as evidenced by the increase in p-CREB levels (Figure 5d).

Whole genome chromatin immunoprecipitation (ChIP) and Next Generation Sequence analysis data generated as part of the ENCODE consortium by the Iyer lab (University of Texas, Austin) demonstrated that the *Bim* promoter was occupied by c-Myc in a variety of human cell lines (Figure 4e; http://www.genome.ucsc.edu/cgi-bin/hgTracks?hgS_doOtherUser=submit&hgS_otherUserName=Vishy&hgS_otherUserSessionName=bim_Myc). The c-Myc-binding region identified by ChIP spanned the sequence identified in the luciferase reporter assays corroborating our results (Figure 5e). To validate and expand these findings, we generated a knock-in mouse strain in which the conserved c-Myc-binding sites within the *Bim* promoter were mutated (Supplementary Figure S3a). In MEFs derived from these mutant mice, *Bim* transcription was not upregulated upon enforced c-Myc expression or β AR activation (Figure 5f and Supplementary Figure S3b). This demonstrates that c-Myc is essential for β AR-mediated *Bim* induction.

CBP is a required cofactor for c-Myc in *Bim* transcriptional induction. The data so far suggest that the transcription factor c-Myc is the critical driver of *Bim* transcriptional induction in response to cAMP/PKA stimulation. However, the link between PKA and c-Myc remains to be established. The only published report linking c-Myc directly with PKA is the transcriptional induction of the PKA catalytic subunit beta.³⁸ We first examined whether c-Myc was a direct phosphorylation substrate of PKA, as c-Myc protein has canonical PKA phosphorylation sites (¹⁵⁸RKDS¹⁶¹ and ²⁷⁶KRSES²⁸⁰ as predicted by the IMP Bioinformatics Group algorithm, Austria). Indeed, both WT and S²⁷⁹A mutant c-Myc could be readily phosphorylated by PKA *in vitro*, whereas S¹⁵⁹A mutant c-Myc could not be phosphorylated (Supplementary Figure S3c). This mutation did, however, not have any discernable effect on the DNA-binding activity of c-Myc, as shown by EMSA and transcriptional activation of the *Bim* reporter construct or on the induction of Bim in MEFs when stably expressed, using 4-OHT-inducible lentiviral system (Supplementary Figures S3d–f). The transcriptional activity of c-Myc can be augmented by the recruitment of the

Figure 4 Activation of cAMP/PKA by targeted deletion of *Prkar1a* in MEFs induces Bim-mediated apoptosis. (a) CRE-mediated deletion of *Prkar1a* in MEFs using 4-OHT-inducible lentiviral system induces Bim both at protein and RNA levels. (b) 100 cells of various genotypes (*Prkar1a^{fl/fl}*; *Prkar1a^{-/-}*; *Prkar1a^{fl/fl}, Bim^{-/-}* and *Prkar1a^{-/-}, Bim^{-/-}*) were plated for clonogenic survival on six-well plates and stained with crystal violet and (c) MEFs of various genotypes as shown were treated with 4-OHT to induce CRE expression, and then treated with isoproterenol (10 μ M) in serum-free medium and apoptosis was measured. Error bars: $\pm$ S.E.M., $n = 3$, $\#P = 0.006$ – 0.042 (b); $\#P = 0.072$ (c); one-tailed *T*-tests

cofactor CBP.³⁹ We therefore tested whether such a process is critical for PKA-induced c-Myc transcriptional activity by RNAi-mediated knockdown of *Cbp* in MEFs. Interestingly, this resulted in the blockage of Bim induction, although both the WT and *Cbp*-knockdown cells were equally responsive to the β AR agonist, as reflected by CREB phosphorylation (Figure 6a). Furthermore, induction of the *Bim* luciferase reporter was markedly impaired in *Cbp*-knockdown cells (Figure 6b). Consequently, these cells were resistant to β AR agonist-induced apoptosis (Supplementary Figure S4a). To prove that CBP regulates *Bim* expression through c-Myc, we used the *Bim*-luciferase reporter assay. Transfection of MEFs with CBP and the WT *Bim* promoter-luciferase construct resulted in a robust induction of this reporter in response to treatment with isoproterenol, whereas such induction was not seen with the reporter in which the c-Myc-binding site had been mutated (Figure 6c).

Inactivating mutations in CBP are frequently found in acute lymphoblastic leukemia and in diffuse large cell B cell lymphomas. A large proportion of these mutations reside in the histone acetyltransferase (HAT) domain of CBP.⁴⁰ Moreover, epigenetic silencing of *Bim* is a feature in some types

of B cell lymphoma.⁴¹ We therefore examined whether HAT-deficient mutants of CBP were impaired in their ability to transcriptionally induce *Bim*. WT CBP could readily acetylate c-Myc in 293T cells but the HAT-deficient mutants H1451C or Y1234C⁴⁰ had lost this ability (Figure 6d). Consistent with this, WT CBP could induce the *Bim* reporter, whereas the mutants failed to do so (Figure 6d). These results were further corroborated in the human lung cancer-derived cell line LK-2, which does not have a functional CBP protein owing to a deletion of exon 3 of the *Cbp* gene.⁴² β AR activation could readily induce Bim expression in the osteosarcoma cell line (143B) or B cell lymphoma cell line (Ramos), both of which have functional CBP, but not in CBP-deficient LK-2 cells (Supplementary Figure S4b). However, introduction of WT *Cbp* could restore β AR stimulation-triggered Bim induction in these cells (Figure 6e and Supplementary Figures S4c and d). These results demonstrate an essential role for CBP in c-Myc-mediated *Bim* induction during β AR activation.

Epigenetic regulation of *Bim* during β AR signalling. The *Bim* gene is subject to epigenetic changes both by histone modification and by DNA methylation. In many cancers, such

Figure 5 c-Myc regulates *Bim* transcription during cAMP/PKA activation. **(a)** Mapping of the *Bim* promoter by luciferase assay using MEFs expressing PKAC α under 4-OHT-regulation. Western blot showing *Bim* induction is shown in the inset. The sequence identified is shown in blue (WT) or red (mutant). **(b)** Confirmation of c-Myc regulation in reporter assays using MEFs expressing c-Myc under 4-OHT-regulation as in **(a)**. H-89 is used as the PKA inhibitor. **(c)** Myc-Max inhibitor, 10058-F4 blocks *Bim* induction in luciferase assays and in western blots (inset). **(d)** Deletion of *c-MYC* in MEFs abrogates β AR-mediated *Bim* induction. pCREB acts as the marker for cAMP/PKA activation. Deletion of *c-Myc*^{fl/fl} allele is shown by PCR. **(e)** Whole genome ChIP analysis of c-Myc binding to the *Bim* promoter in different human cell lines. Base overlap is shown on the X axis. The conserved c-Myc-binding sequence on the *Bim* promoter is shown at the bottom. **(f)** MEFs from WT and the *Bim* promoter knock-in mouse where c-Myc-binding sites were mutated, were infected with lentiviruses expressing c-Myc under 4-OHT-regulation (top panel) or were treated with isoproterenol (bottom panel). The knock-in cells show lack of *Bim* induction. Non-specific band (indicated by asterisk) acts as the loading control. Error bars: $\pm$ S.E.M., $n = 3$, $\#P = 0.007$ – 0.016 versus untreated controls **(a–c)**; one-tailed *T*-tests

as multiple myeloma, treatment with histone deacetylase inhibitors increases *Bim* expression and triggers apoptosis in a manner that is dependent (at least in part) on *Bim*.⁴³ In chronic myeloid leukemia, changes in *Bim* promoter methylation status are associated with differences in *Bim* gene expression.⁴⁴ As CBP is a cofactor in c-Myc-regulated *Bim* expression, and CBP mutations in the HAT domain are associated with tumor development,⁴⁰ we investigated whether epigenetic changes in the *Bim* promoter occur during β AR signalling. Bisulphite sequence analysis of the *Bim* promoter from β AR responsive Ramos cells and the non-responsive LK-2 (CBP negative) cells showed a significant increase in CpG island methylation in the non-responsive cells. This could be partially reversed by the ectopic expression of CBP from a lentiviral vector (Figure 7a). Finally, ChIP analysis using MEFs expressing the catalytic subunit of PKA under 4-OHT regulation showed

that both c-Myc and CBP bind constitutively to the *Bim* promoter, irrespective of the PKA activation status (Figure 7b). This is consistent with the whole genome ChIP analysis (Figure 5e), which showed that c-Myc could constitutively bind to the *Bim* promoter in different cell lines. However, histone 4 acetylation at the *Bim* promoter occurred specifically during PKA activation, corroborating the role of CBP in histone modification (Figure 7b last lane). These results establish a role for CBP in the cAMP/PKA pathway in inducing epigenetic changes in the *Bim* gene.

Discussion

Increased sympathetic nervous activity in the myocardium is a classical feature of patients with HF.¹⁸ Similarly, catecholamines, the primary transmitter of the sympathetic nervous system, regulate lymphocyte homeostasis by both autocrine

Figure 6 CBP is the co-factor for c-Myc for *Bim* induction. (a and b) RNAi knockdown of CBP in MEFs results in lack of *Bim* induction up on β AR activation in western blot and in luciferase assays. (c) CBP-mediated *Bim* induction is dependent on intact c-Myc-binding sites on the *Bim* promoter. (d) HAT-deficient mutants of CBP fail to induce *Bim*. Acetylation of c-Myc by CBP or by the mutant variants in 293T cells is shown in the inset. (e) LK-2 cells that lack functional CBP respond to β AR signalling by inducing *Bim* protein in CBP-dependent manner. Error bars: $\pm$ S.E.M., $n=3$, $\#P=0.0022$ versus untreated control (b); $\#P=0.008$ versus isoproterenol-treated CBP control (c); $\#P=0.064$ versus CBP control (e); paired one-tailed *T*-tests

and paracrine signalling.⁴ In cardiac β AR signalling, it is well established that chronically increased stimulation and subsequent PKA activation could result in a robust apoptotic response.⁶ Moreover, it was shown that the pro-apoptotic BH3-only protein Bim has a crucial role in cAMP-induced apoptosis in the T cell hybridoma line S49.²¹ Therefore, our initial task was to explore the generality of this phenomenon in different tissues. We could demonstrate PKA activation and subsequent *Bim* induction both at the protein and mRNA levels by a variety of stimuli and in a variety of tissues (Figures 1 and 4). We could also demonstrate that in

thymic and heart tissues, Bim has a critical role in β AR-mediated apoptosis (Figure 2). Our results also demonstrate that Bim has an important role in the heart development. In MEFs and thymocytes, there was a lag time between mRNA induction and protein accumulation. This could be partly due to the Bim protein stabilization by PKA, as we previously reported.¹⁷ Lag time between transcript induction and protein accumulation has been reported in many previous studies,⁴⁵ particularly for the β AR pathway where feedback inhibition and receptor decoupling leads to receptor desensitization.⁴⁶

Figure 7 *Bim* promoter region is subject to epigenetic changes during β AR signalling. (a) Bisulphite sequencing analysis of RAMOS, LK-2 and LK-2 cells expressing full-length CBP using primers flanking the region between -1513 and -948 (from the transcription initiation site). The numbers shown are the potential CpG methylation sites. (b) ChIP analysis of the *Bim* promoter in MEFs expressing PKAC α using antibodies as indicated. (c) A model for *Bim* induction by cAMP/PKA pathway. PKA activation of CBP bound to the *Bim* promoter through its interaction with c-Myc results in histone 4 acetylation, leading to *Bim* transcriptional induction

Apart from protecting from β AR-induced cardiomyopathy, *Bim*^{-/-} mice had a significantly larger heart (Supplementary Figure S2d). This is not associated with hyperplasia rather because of increased size of cardiomyocytes, which does not change with isoproterenol treatment (Figures 3a and b). As cardiac sympathetic nervous system is a strong regulator of cardiomyocyte size via β AR-dependent repression of proteolysis,⁴⁷ we believe that this size increase is a function of elevated β AR activity in the absence of Bim, similar to the observation in thymocytes. Furthermore, this hypertrophy is not associated with any pathology, as we could not detect any upregulation of pathology-associated genes such as *Myh6* and 7; *Serca2* or *Col1a1* and *Col3a1*.⁴⁸ However, cardiac skeletal α -actin (*Aska*) was significantly increased in the KO tissue. On the basis of gene knock-out studies, it is believed that *Aska* contributes to muscular strength and contractility,⁴⁹ and thus our results suggest that blocking Bim expression in the heart will maintain the β AR-mediated inotropic function and only prevent the apoptotic arm of the signal transduction pathway.

The observation that deletion of *Prkar1a* results in Bim-dependent apoptosis is significant in yet another context (Figure 4). Inactivation of *Prkar1a* has been attributed to the

development of cancer, such as multiple endocrinal neoplasia, myxoma and thyroid tumors.⁵⁰ Therefore, *Prkar1a* is believed to be a tumor suppressor.⁵⁰ However, our results suggest that cancer associated with *Prkar1a* mutations can develop only if there are cooperating oncogenic mutations that inhibit Bim-induced apoptosis. This could involve either genetic or epigenetic changes in the *Bim* gene or mutations that counter *Bim* induction, such as overexpression of antiapoptotic Bcl-2 family members. Given that *Bim* is a tumor suppressor in many cancers,^{41,51} it is possible that in those cancers where *Prkar1a* loss of heterozygosity causes elevated PKA activity, *Bim* is a critical tumor suppressor.

Our data show for the first time that c-Myc regulates the transcriptional induction of *Bim* during β AR activation. CREB is one of the main downstream targets of PKA and based on expression analysis, it was reported that *Bim* was a downstream target of CREB.⁵² However, our results, obtained through a variety of methods, demonstrate that it is c-Myc that is mediating *Bim* transcriptional induction (Figure 5). This finding is also relevant to *E μ -myc* transgenic mice, a model of Burkitt's lymphoma, where deregulated c-Myc expression leads to increased Bim protein levels in B cells,³⁶ and thereby suppresses lymphoma development. However, c-Myc was

considered to upregulate Bim indirectly. The results presented here represent the first demonstration of a direct role for c-Myc in *Bim* transcriptional upregulation.

Yet another significant finding in this study is the role of CBP in *Bim* induction. Our results demonstrated an absolute requirement of this transcriptional co-factor for β AR stimulation-triggered *Bim* induction (Figure 6). Mutations in CBP are found in B cell lymphomas.⁴⁰ It is therefore possible that Bim downregulation in these cells may contribute to tumorigenesis, given that Bim functions as a tumour suppressor in B cell lymphoma.³⁶ Our results also show that although LK-2 cells have normal expression of p300, Bim can be upregulated only when CBP is forcibly expressed (Figure 6e and Supplementary Figures S4c and d). This indicates that there is no functional redundancy between Cbp and p300 in inducing *Bim* transcription in spite of the extensive sequence similarity between these two proteins, in agreement with previous report.⁵³ Finally, our findings of epigenetic changes at the *Bim* locus (Figure 7), both in terms of histone acetylation and DNA methylation during PKA activation/CBP expression can be reconciled as CBP is a HAT and the interplay between these two processes in gene regulation has long been established.⁵⁴ On the basis of these findings, the following model (Figure 7c) is proposed where β AR-mediated PKA activation leads to activation of CBP. The activated CBP leads to histone acetylation (and demethylation) at the *Bim* promoter locus, leading to increased gene expression.

Taken together, these results provide a clear link between cAMP/PKA pathway activation and *Bim* induction and consequent Bim-mediated apoptosis. Catecholamines are the prime mediators of cardiomyocyte apoptosis.⁶ In the immune system, apart from the sympathetic nervous innervation of lymphoid organs, lymphocytes and macrophages themselves can be a source of catecholamines.^{4,24} This is known to contribute to thymocyte apoptosis and thymic involution can exert immune-modulatory effect as a prototypic stress response.²⁴ The present work is the first demonstration of the molecular pathway of β AR-mediated apoptosis. Understanding the molecular mechanism of this apoptotic pathway may provide critical clues for developing new drugs for the treatment of diseases associated with increased β -adrenergic activation.

Materials and Methods

Cell culture and luciferase assay. Cells were grown in DMEM supplemented with 10% foetal calf serum at 10% CO₂ and at 37 °C. Transient transfections were performed using Eugene (Roche, Indianapolis, IN, USA). For PKAC α or c-Myc-expressing cells, proteins expression was induced 24 h after transfection with 5 nM 4-OHT overnight, followed by luciferase assay using the Dual Luciferase (Promega, Alexandria, NSW, Australia) kit. For catecholamine induction, cells were treated 24 h after transfection, with isoproterenol (10 μ M) and rolipram (10 μ M) in serum-free medium overnight before the luciferase assay.

Animal experimentation. All animal experiments were conducted according to the La Trobe University Animal Ethics committee and the Alfred Medical Research and Education Precinct Animal Ethics Committee guidelines.

Lentiviral infection. To generate stable, transformed MEFs, primary MEFs were infected with lentiviral particles expressing SV40 T antigen. To generate conditional knock-out MEFs, transformed MEFs (*Prkar1a*^{fl/fl} and *c-Myc*^{fl/fl}) were infected with 4-hydroxytamoxifen (4-OHT)-inducible CRE-expressing lentiviral particles and clones were selected. CRE deletion was induced by the addition of

4-OHT (5 nM). Production of lentiviral particles and infections were carried out as described.¹⁷

Foetal thymic organ culture. Thymic organ culture was performed as described.⁵⁵ Thymus lobes obtained from E15 embryos were cultured in DMEM medium supplemented with 50 μ M β -mercaptoethanol (Sigma, Castle Hill, NSW, Australia) plus 10% FCS. After 12 days, thymocytes were treated with 10 μ M isoproterenol and 0.5 mM IBMX. Cells were isolated from these thymi for FACS, qPCR, western blot and cAMP analyses.

RT-quantitative PCR. Total RNA from thymocytes was isolated using TRIzol. Complementary DNA (cDNA) was synthesized from 2 μ g of total RNA using the Superscript III RT-PCR system (Catalogue number 18080-051 Invitrogen, Carlsbad, CA, USA), using oligo-dT primer, according to manufacturer's instructions. qPCR was carried out using Brilliant II SYBER Green QPCR master Mix (Catalogue number 600828 Stratagene, La Jolla, CA, USA). All cDNA samples were tested in triplicate using a Light cycler 480 Real-time PCR instrument (Roche). Following primers were used for qPCR reactions: *Bim* (F): 5'-GAGTTGTGACAAAGTCAACACAAACC-3'; *Bim* (R): 5'-GAAGATAAAGCGTAACAGTTGTAAGATA-3'; *Nur77* (F): 5'-CCTGTTGCTAGAGTCTGCCTTC-3'; *Nur77* (R): 5'-CAATCCAATCACCAGGACGACG-3'; *Puma* (F): 5'-ATGCCCTGCCTCACCTTCATCT-3'; *Puma* (R): 5'-AGCACAGGATTCACAGTCTGGA-3'; *Noxa* (F): 5'-ACTGTGGTTCTGGCGCAGAT-3'; *Noxa* (R): 5'-TTGAGCACACTCGTCTCTCAA-3'; *c-Myc* (F): 5'-CAAATCCTGTACCTCGTCCGATT-3'; *c-Myc* (R): 5'-CTTCTTCTTCTTCTTCTCAGAGTCGC-3'; *Serca2a* (F): 5'-TCGACCAAGTCAATCTTACAG-3'; *Serca2a* (R): 5'-GGGACAGGGTCAGTATGCTT-3'; *Myh6* (F): 5'-GTCACCAACAACCCATACGACTAC-3'; *Myh6* (R): 5'-CAGCACATCAAAGGC ACTATCAGT-3'; *Myh7* (F): 5'-AGAACCTACTGCGGCTCCA-3'; *Myh7* (R): 5'-CTA CTCCTCATTCAGGCC-3'; *Col1a1* (F): 5'-GGAGATGATGGGGAAGCTG-3'; *Col1a1* (R): 5'-AATCCACGACGACCCCTGA-3'; *Col3a1* (F): 5'-GGAATGGAGCAAGACAGTCTTTG-3'; *Col3a1* (R): 5'-TGCATATCTATGATGGGTAGTCTCA-3'; *Aska* (F): 5'-CCTGCCATGTATGTGGCTATC-3'; *Aska* (R): 5'-CCCCAGAATCC AACACGAT-3'; *18s rRNA* (F): 5'-CCGCTCCCAAGATCCAACTA-3'; *18s rRNA* (R): 5'-TTGAGGGCAAGTCTGGTG-3'.

Chromatin immunoprecipitations. DNA for ChIP analysis was prepared from cells expressing PKAC α under 4-OHT regulation using EZ-ChIP (Millipore, Billerica, MA, USA) kit, following manufacturer's instructions. The following antibodies were used: anti-Myc (N-262, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CBP (C-20, Santa Cruz Biotechnology), anti-acetyl-Histone H3 (17-658, Millipore), anti-acetyl-Histone H4 (17-630, Millipore). Immunoprecipitated DNAs were subjected to PCR using oligos specific to *Bim* promoter F: 5'-GTGAAGTGCTAACTAGATTGCAC-3' and R: 5'-GAAACCCAGCAGTGGTGGAC-3'. Primers specific to the 3'UTR were used as negative controls, that is, F: 5'-TGCTTCTCTGCTGATTGAC-3' and R: 5'-AAACGTAAAGGAAGCCAGGG-3'.

Western and northern blot analyses. Western blot and northern blot analyses were performed as described before.¹⁷ The following antibodies were used for western blot analyses: anti-Bim (3C5), anti-HA (6E2, Cell Signaling, Boston, MA, USA), anti-CBP, anti-p300 (N-15, Santa Cruz Biotechnology), anti pCREB Ser¹³³, Affinity Bioreagents, Golden, CO, USA), anti HSP70, anti-acetyl lysine (9441, Cell Signaling), anti PRKAR1A (610609, BD Biosciences, CA, USA), anti-PKA substrate (9621, Cell Signaling), anti-PUMA (3043, ProSci, Poway, CA, USA).

cAMP measurement. cAMP in cellular extracts was measured using cAMP Direct Immunoassay Kit (Cat# ab65355, Abcam, Cambridge, MA, USA).

Cardiomyocyte isolation. Cardiomyocytes were isolated by Langendorff perfusion technique, as described.⁵⁶

In vitro PKA kinase assay. *In vitro* PKA kinase assay was performed as described.¹⁷

Catecholamine measurements. Blood samples were collected from anesthetized animals by cardiac puncture. Plasma were prepared by centrifugation and stored at -80 °C, until assay with high-performance liquid chromatography (HPLC).⁵⁷ This method allowed simultaneous determination of norepinephrine and epinephrine. Catecholamines were adsorbed with activated alumina, separated by HPLC, and quantified by electrochemical detection.

Histology. Hearts were fixed in 10% formalin in phosphate-buffered saline, paraffin-embedded, serially sectioned (5 μ M) and stained with Picrosirius red or Masson's trichrome.⁵⁸

Echocardiography. Animals were anesthetized by isoflurane. Using an IE33 ultrasound system and a linear 15 MHz probe, two-dimensional (2D) short-axis loop of the LV was acquired for measurement of LV cross-sectional areas at the diastole and systole. LV fractional shortening was calculated from LV diameters at the diastole and systole from the M-mode traces derived from 2D image, as described before.⁵⁸

Promoter methylation analysis. Promoter methylation was analysed by bisulphite sequencing of the *BIM* promoter using MethylDetector kit (Active Motif, Carlsbad, CA, USA). Sulphonated DNA samples (from Ramos, LK-2 and LK-2 cells expressing functional CBP) were amplified by PCR using primers specific for CpG island corresponding to bases -1513 to -948 (relative to the transcription start site on human *BIM* gene). PCR-amplified fragments were cloned into PCRIL-TOPO vector using TOPO TA Cloning kit (Life Technologies, Grand Island, NY, USA). Ten clones from each cloning were sequenced and analysed using BISMA software (Jacobs University Bremen, Germany).

Conflict of Interest

The authors declare no conflict of interest.

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Author Contributions

HP, X-JD, PB and Id A designed the experiments. YYL, MD, LG, RW, AR, DM, MH, X-JD and HP performed the experiments and YYL, X-JD and HP analysed the data, and HP prepared the manuscript.

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