

# The proapoptotic BH3-only proteins Bim and Puma are downstream of endoplasmic reticulum and mitochondrial oxidative stress in pancreatic islets in response to glucotoxicity

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Apoptosis of pancreatic beta cells is a feature of type 2 diabetes and its prevention may have therapeutic benefit. High glucose concentrations induce apoptosis of islet cells, and this requires the proapoptotic Bcl-2 homology domain 3 (BH3)-only proteins Bim and Puma. We studied the stress pathways induced by glucotoxicity in beta cells that result in apoptosis. High concentrations of glucose or ribose increased expression of the transcription factor CHOP (C/EBP homologous protein) but not endoplasmic reticulum (ER) chaperones, indicating activation of proapoptotic ER stress signaling. Inhibition of ER stress prevented ribose-induced upregulation of *Chop* and *Puma* mRNA, and partially protected islets from glucotoxicity. Loss of Bim or Puma partially protected islets from the canonical ER stressor thapsigargin. The antioxidant *N*-acetyl-cysteine also partially protected islets from glucotoxicity. Islets deficient in both Bim and Puma, but not Bim or Puma alone, were significantly protected from killing induced by the mitochondrial reactive oxygen species donor rotenone. Our data demonstrate that high concentrations of glucose induce ER and oxidative stress, which causes cell death mediated by Bim and Puma. We observed significantly higher *Bim* and *Puma* mRNA in islets of human donors with type 2 diabetes. This indicates that inhibition of Bim and Puma, or their inducers, may prevent beta-cell destruction in type 2 diabetes.

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Type 2 diabetes occurs as a result of progressive beta-cell dysfunction causing insufficient insulin secretion to compensate for insulin resistance, leading to hyperglycaemia. In addition to an insulin secretory defect, there is a contribution from a decrease in beta-cell mass as a result of apoptosis.<sup>1–3</sup> Because the loss of beta-cell mass is a central event in type 2 diabetes, its prevention is a key therapeutic target.

High concentrations of glucose (glucotoxicity) were reported to trigger apoptosis in cultured human and mouse islets<sup>4,5</sup> and in animal models of type 2 diabetes.<sup>6–8</sup> Using gene-targeted mice lacking critical regulators of cell death, we demonstrated that a high concentration of glucose, or the reducing sugar ribose, activates the intrinsic apoptosis pathway in beta cells. In particular, the apoptosis-initiating Bcl-2 homology domain 3 (BH3)-only proteins Bim and Puma, as well as the downstream apoptosis effector Bax, are required for glucotoxicity of beta cells in mouse islets.<sup>5</sup>

Endoplasmic reticulum (ER) stress occurs when there is accumulation of misfolded proteins in the lumen of the ER.

This activates different factors of the unfolded protein response (UPR) pathway.<sup>9</sup> Although there is some overlap in the regulation of downstream factors, UPR pathway molecules can be broadly divided into three main arms: PERK, IRE1 $\alpha$  and ATF6.<sup>10</sup> Markers of ER stress were increased after exposure of islets *in vitro* to saturated fatty acids (palmitate) and glucose, and also *in vivo* in islets from leptin-receptor-deficient *db/db* mice, hyperglycaemic rats,<sup>11</sup> human beta cell exposed to hyperglycaemic conditions<sup>12</sup> and pancreatic sections of type 2 diabetic patients.<sup>13,14</sup> ER stress has been reported to activate proapoptotic BH3-only proteins, particularly Bim and Puma, in diverse cell types. Bim is upregulated in response to ER stress through the transcription factor CHOP (C/EBP homologous protein) and post-translational phosphorylation that inhibits its proteasomal degradation.<sup>9,15</sup> Evidence from non-islet cells suggests that CHOP can also induce *Puma* gene expression.<sup>16–18</sup>

Increased production of reactive oxygen species (ROS) and subsequent oxidative stress represent another possible

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**Abbreviations:** ER, endoplasmic reticulum; ROS, reactive oxygen species; CHOP, C/EBP homologous protein; BH3, Bcl-2 homology domain 3; UPR, unfolded protein response; TUDCA, tauroursodeoxycholic acid; PBA, 4-phenyl butyric acid; NAC, *N*-acetylcysteine

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mechanism by which high concentrations of glucose could kill beta cells. Pertinently, beta cells have only low expression of antioxidant enzymes rendering them susceptible to damage by ROS.<sup>19,20</sup> Exposure of human and rat islets to high concentrations of glucose or ribose resulted in increased production of intracellular ROS,<sup>21,22</sup> and overexpression of the antioxidant enzyme glutathione peroxidase protected rat islets from ribose toxicity.<sup>22</sup> There is also some evidence that the intrinsic apoptosis pathway may be activated in beta cells by oxidative stress. Oxidative stress upregulated *Hrk/DP5* gene expression<sup>23</sup> and caused an almost 50% decrease in the expression of the caspase-9 inhibitor *CARD8*.<sup>24</sup>

Because of the proposed role of ER and oxidative stress in beta-cell loss in type 2 diabetes and our previous discovery that glucotoxicity activates the intrinsic apoptotic pathway in beta cells, we sought to determine whether ER and/or oxidative stress constitute the upstream activators of apoptosis in this setting. We found that activation of the proapoptotic BH3-only proteins Bim and Puma in response to glucotoxicity is dependent on the induction of both ER stress and mitochondrial oxidative stress. Furthermore, consistent with our findings in mouse islets, we observed significantly more proapoptotic BH3-only gene expression in islets of human subjects with type 2 diabetes.

## Results

**ER stress induces apoptosis in islets that is dependent on Bim and Puma.** To determine whether Bim and Puma act downstream of ER stress in beta cells, wild-type islets were incubated for 4–5 days with low-dose (5  $\mu$ M) thapsigargin and cell death was examined. Thapsigargin induced a marked increase in cells with fragmented DNA (a hallmark of apoptosis) in wild-type islets, and this was significantly reduced in Bim- or Puma-deficient islets (Figures 1a and b). Similar results were obtained when tunicamycin (10  $\mu$ g/ml) was used to induce ER stress (Figure 1c). This shows that the proapoptotic BH3-only proteins Bim and Puma are required for ER stress-induced islet cell death. These data are consistent with other studies using different inducers of ER stress in FACS-purified beta cells.<sup>25,26</sup>

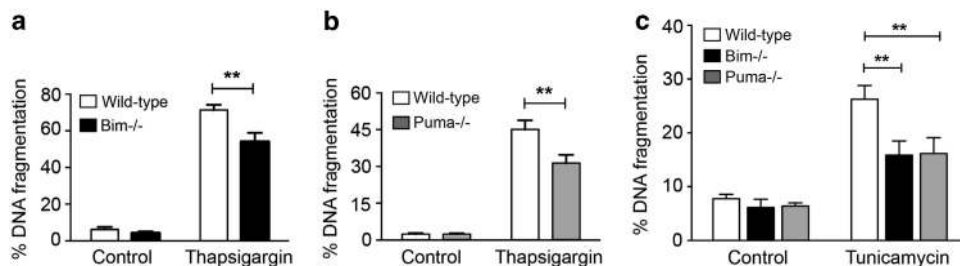
**Glucotoxicity induces ER stress that activates UPR signaling to initiate apoptosis.** We next determined whether ER stress-mediated UPR can be induced by

glucose. For *in vitro* treatment, we used the reducing sugar ribose (50 mM), which can enter the cell through glucose transporters and be metabolized through the pentose phosphate pathway and glycolysis. Like other reducing sugars, including glucose or fructose, ribose induces islet cell death through glycation and formation of ROS,<sup>22</sup> but over a shorter time frame. The levels of mRNAs for UPR pathway components were examined in wild-type islets by qPCR. Expression of *Chop* but not *ATF4* increased in islets cultured with ribose, and this increase was statistically significant after 2 days (Figure 2a). Moreover, IRE1 $\alpha$ -mediated splicing of XBP1 gradually increased with ribose treatment and was significantly increased after 3 days, whereas unspliced XBP1 mRNA expression did not change (Figure 2a). There was a marginal decrease in expression of the ATF6-regulated chaperones *BiP* and *Pdia4* after the first day of ribose incubation, but expression of the cochaperone *P58* was not affected by the exposure of islets to ribose (Figure 2a). Treatment of cultured islets with 33.3 mM glucose for 7 days produced the same results (data not shown).

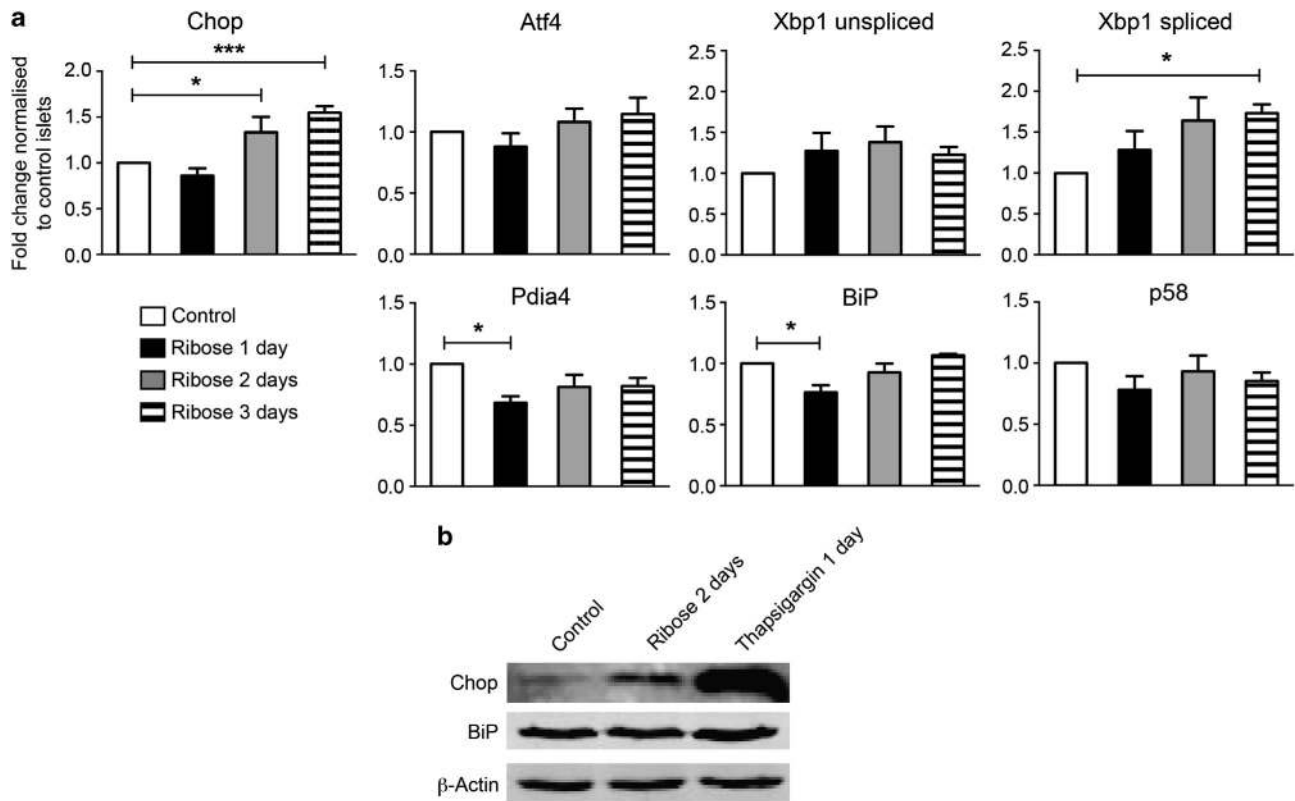
Consistent with the findings from the qPCR analysis, treatment of islets with ribose caused a marked increase in CHOP protein after 2 days, whereas BiP levels remained constant (Figure 2b).

**CHOP is required for glucotoxicity-mediated ER stress in islets.** Because CHOP was increased in islets in response to ribose and glucose exposure, the role of CHOP in mediating ER stress-induced islet cell apoptosis was further examined. Islets cultured with ribose plus the ER stress inhibitor tauroursodeoxycholic acid (TUDCA) had significantly lower expression of CHOP when compared with islets cultured with ribose alone (Figure 3a).

Interestingly, the glutathione precursor *N*-acetylcysteine (NAC) also reduced the impact of ribose on CHOP expression, suggesting that, in addition to ER stress, CHOP expression may also be enhanced by increased ROS levels as a consequence of protein misfolding in the ER lumen<sup>27,28</sup> (Figure 3a). Upon treatment with 50 mM ribose for 4 days or 33.3 mM glucose for 6 days, islets lacking CHOP had reduced numbers of apoptotic cells compared with wild-type islets (Figures 3b and c). These data indicate that CHOP is required for ER stress-induced apoptosis of islet cells in response to glucotoxicity.



**Figure 1** ER stress activates intrinsic apoptosis in islet cells. (a–c) Islets from wild-type C57BL/6, *Bim*<sup>-/-</sup> or *Puma*<sup>-/-</sup> mice were cultured in a control medium or in a medium containing 5  $\mu$ M thapsigargin for 5 days (a) or 4 days (b), or 10  $\mu$ g/ml tunicamycin for 4 days (c). The frequency of cells undergoing DNA fragmentation was measured by flow cytometry. Results represent mean  $\pm$  s.e.m. of  $n = 4$  independent experiments. \*\* $P < 0.01$  when comparing wild-type to *Bim*<sup>-/-</sup> or wild-type to *Puma*<sup>-/-</sup> islets cultured with thapsigargin or tunicamycin (two-way ANOVA)



**Figure 2** Glucose toxicity activates the PERK and IRE1 $\alpha$  arms of the UPR pathway. (a) Quantitative RT-PCR of wild-type C57BL/6 islets cultured for 1 to 3 days either in a control medium or in a medium containing 50 mM ribose. Relative mRNA expression levels for *Chop*, *Atf4*, *Bip*, *Pdia4*, *P58* and *XBP1*-unspliced/spliced were calculated by normalizing to the signal for  $\beta$ -Actin mRNA in each sample and comparison with islets cultured in a control medium. Results represent mean  $\pm$  s.e.m. of  $n = 3$ –6 independent experiments. \* $P < 0.05$ , \*\*\* $P < 0.001$  when comparing islets cultured in ribose with those cultured in a control medium (one-way ANOVA). (b) Eight hundred wild-type C57BL/6 islets were cultured in a control medium or in a medium containing 50 mM ribose. Positive control islets were cultured in 5  $\mu$ M thapsigargin. Western blotting was performed with antibodies to CHOP (31 kDa), BiP (78 kDa) and  $\beta$ -Actin (45 kDa) (loading control). Results are representative of three independent experiments

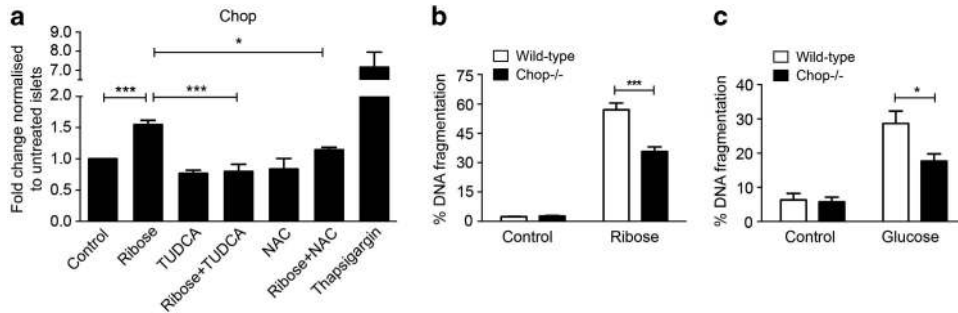
### CHOP regulates Puma expression in islets with ER stress

**Attenuation of ER stress reduces Puma expression:** To examine whether ribose-induced CHOP activation results in increased expression of downstream proapoptotic targets, gene expression of *Bim* and *Puma* was studied in ribose-treated islets. The expression of *Puma* increased by almost threefold in islets treated with ribose and this increase could be prevented by addition of the ER stress inhibitor TUDCA (Figure 4a). This demonstrates that CHOP and Puma are both regulated by TUDCA-sensitive processes. Thapsigargin also induced a marked increase in *Puma* mRNA expression. Although NAC efficiently diminished the ribose-induced increase in CHOP levels, it did not impair Puma induction (Figure 4a), suggesting that ribose can induce *Puma* expression independent of oxidative stress. The presence or absence of TUDCA or NAC had no impact on *Bim* mRNA in islets cultured for 3 days with ribose (Figure 4b). This reveals that *Bim* mRNA levels do not change with alterations in CHOP mRNA abundance.

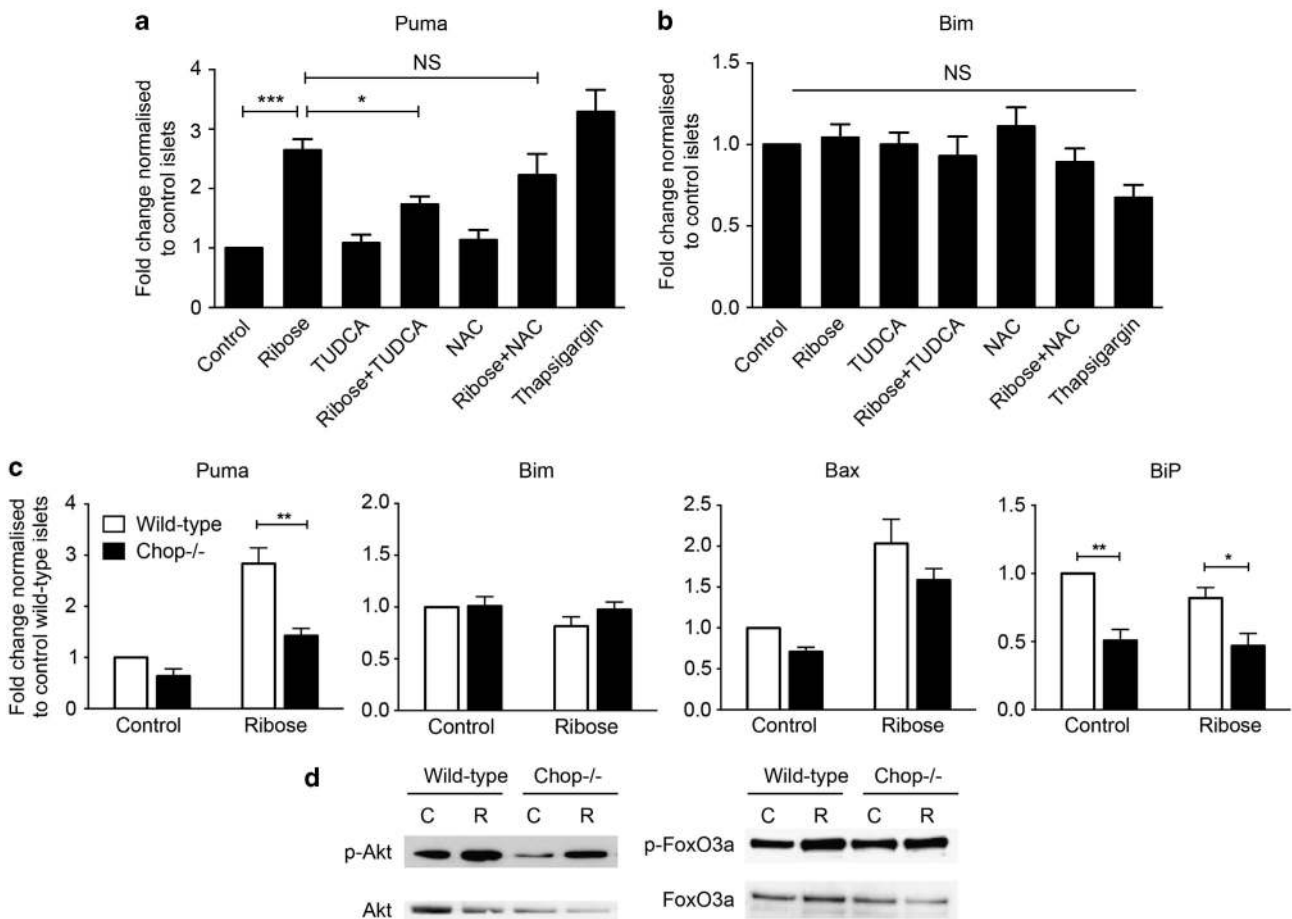
**Chop regulates Puma expression in islets:** To examine whether CHOP induces apoptosis in islets by increasing transcription of downstream proapoptotic targets,<sup>9</sup> we

studied the mRNA expression of *Bim*, *Puma* and *Bax* in wild-type and CHOP-deficient islets that had been treated with 50 mM ribose for 3 days (Figure 4c). The expression of *Puma* was significantly reduced in ribose treated *Chop*<sup>-/-</sup> islets when compared with similarly treated wild-type islets (Figure 4c). This indicates that CHOP regulates *Puma* expression in islets that are subject to ER stress. In contrast, loss of CHOP did not affect *Bim* or *Bax* mRNA expression in ribose-treated islets (Figure 4c). Compared with wild-type islets, islets from *Chop*<sup>-/-</sup> mice also had reduced *BiP* mRNA expression, perhaps indicating that CHOP deficiency changes the balance between the induction of proapoptotic and prosurvival factors in the UPR pathway, resulting in reduced baseline ER stress in islets (Figure 4c). Treatment with ribose did not increase *BiP* expression in wild-type and CHOP-deficient islets when they were compared with control (untreated) islets of similar genotype (Figure 4c).

**Puma regulation by Chop is not mediated by Akt-FoxO3a signaling:** It has been suggested that the transcription factor FOXO3a regulates the expression of *Bim* and *Puma* in neurons and haematopoietic cells.<sup>29–32</sup> Phosphorylated (activated) Akt was shown to cause inhibitory phosphorylation of FOXO3a, thereby preventing its nuclear translocation



**Figure 3** Downregulation or loss of Chop inhibits ER stress-induced apoptosis. (a) Quantitative RT-PCR of wild-type C57BL/6 islets cultured for 3 days in a control medium or in a medium containing 50 mM ribose, 0.5 mM TUDCA or 1.0 mM NAC for 3 days. Relative mRNA expression levels for *Chop* were calculated by normalizing to the signal for  $\beta$ -Actin mRNA in each sample and comparison with islets cultured in a control medium. Treatment with thapsigargin (5  $\mu$ M for 1 day) was used as a positive control. Results represent mean  $\pm$  s.e.m. of  $n = 5$  independent experiments. \* $P < 0.05$ , \*\*\* $P < 0.001$  (one-way ANOVA). (b and c) The frequency of cells undergoing DNA fragmentation was measured by flow cytometry in islets from wild-type and *Chop*<sup>-/-</sup> mice that had been cultured in a control medium containing 5.5 mM glucose or a medium containing 50 mM ribose for 4 days (b) or 33.3 mM glucose for 6 days (c). Results represent mean  $\pm$  s.e.m. of  $n = 3$  independent experiments. \* $P < 0.05$ , \*\*\* $P < 0.001$  when comparing islets from *Chop*<sup>-/-</sup> mice with those from wild-type mice (two-way ANOVA)



**Figure 4** CHOP controls Puma expression in islet cells undergoing ER stress. (a and b) Quantitative RT-PCR of wild-type C57BL/6 islets cultured for 3 days in a control medium or in a medium containing 50 mM ribose, 0.5 mM TUDCA or 1.0 mM NAC. Relative mRNA expression levels for *Puma* (a) and *Bim* (b) were calculated by normalizing to the signal for  $\beta$ -Actin mRNA in each sample and comparison with islets cultured in a control medium. Treatment with thapsigargin (5  $\mu$ M for 1 day) was used as a positive control. Results represent mean  $\pm$  s.e.m. of  $n = 3$ –5 independent experiments. NS = not significant. \* $P < 0.05$ , \*\*\* $P < 0.001$  (one-way ANOVA). (c) Quantitative RT-PCR of islets from wild-type C57BL/6 and *Chop*<sup>-/-</sup> mice that had been cultured for 3 days either in a control medium or in a medium containing 50 mM ribose. Relative mRNA expression levels for *Bim*, *Puma*, *Bax* and *BiP* were calculated by normalizing to the signal for  $\beta$ -Actin mRNA in each sample and comparison with wild-type islets cultured in a control medium. Results represent mean  $\pm$  s.e.m. of  $n = 5$  independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  when comparing islets cultured in ribose with those cultured in a control medium (two-way ANOVA). (d) Western blot analysis with antibodies to phosphorylated Akt (p-Akt, 60 kDa) and total Akt (loading control), and phosphorylated FoxO3a (p-FoxO3a, 95 kDa) and total FoxO3a (loading control). A total of 400–700 islets were cultured for 2 days in a control medium or in a medium containing 50 mM ribose. Results are representative of four independent experiments



and induction of target genes, such as *Bim* or *Puma*. ER stress was reported to inhibit phosphorylation of Akt in a CHOP-dependent manner.<sup>30</sup> The levels of phosphorylated (activated) FOXO3a and pAkt were similar in ribose-treated *Chop*<sup>-/-</sup> islets compared with similarly treated wild-type islets (Figure 4d). These data suggest that CHOP-dependent FOXO3a activation may not be required for regulating *Puma* expression in islets, indicating that ER stress must activate *Puma* through other processes in these cells.

**ER stress-induced *Bim* and *Puma* activation mediates glucotoxicity in islets.** To determine whether glucotoxicity-induced proapoptotic ER stress signaling results in *Bim*- and *Puma*-dependent cell death, we used the chemical inhibitors of ER stress TUDCA and 4-phenyl butyric acid (PBA). Addition of PBA or TUDCA partially protected islets from wild-type mice against ribose-induced cell death, as demonstrated by a reduction in cells exhibiting DNA fragmentation (Figures 5a and b). In islets lacking *Bim* or *Puma*, ribose-induced cell killing was significantly lower when compared with wild-type islets, as described previously<sup>5</sup>. The cell death in *Bim*- or *Puma*-deficient islets was further reduced when PBA or TUDCA was added to the culture medium, with the frequency of cells undergoing DNA fragmentation being not significantly different from islets cultured with PBA or TUDCA alone (Figures 5a and b). Similar results were obtained when islets were cultured with 33.3 mM glucose together with TUDCA (Figure 5c). These results suggest that inhibition of ER stress protects islets from glucotoxicity and deficiency of *Bim* or *Puma* reduces residual ER stress-mediated cell death in TUDCA-treated islets.

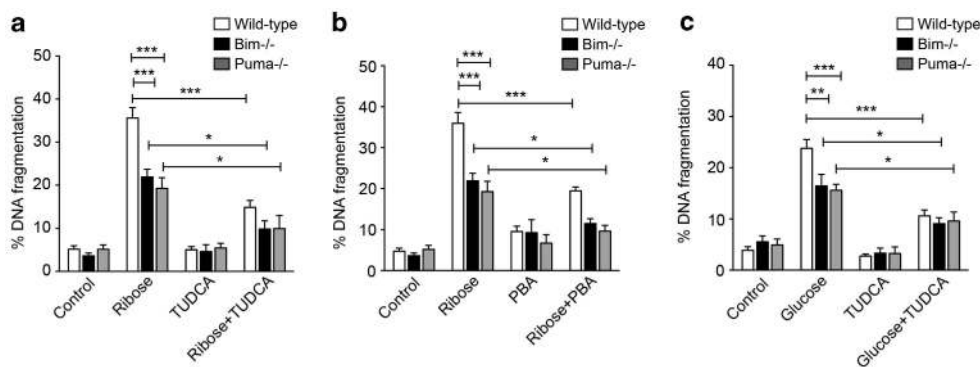
**Mitochondrial oxidative stress induces *Bim*- and *Puma*-dependent apoptosis in islets.** Previous reports showed that exposure to high concentrations of glucose or ribose lead to increased ROS production in rat and human islets.<sup>21,22</sup> We investigated the involvement of oxidative stress in glucose-induced islet cell killing. First, we cultured islets in hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) at low concentrations (30 μM) that have been shown to induce apoptosis but not necrosis in non-islet cells.<sup>33,34</sup> Absence of *Bim* or *Puma*, or both *Bim* and *Puma*, did not protect islets from H<sub>2</sub>O<sub>2</sub>-induced

DNA fragmentation (Figure 6a). Addition of the pancaspase inhibitor qVD.oph also failed to inhibit H<sub>2</sub>O<sub>2</sub> toxicity (Figure 6a). This suggests that in contrast to certain other cell types, inhibition of apoptosis does not protect islet cells from cell death caused by cytosolic oxidative stress.

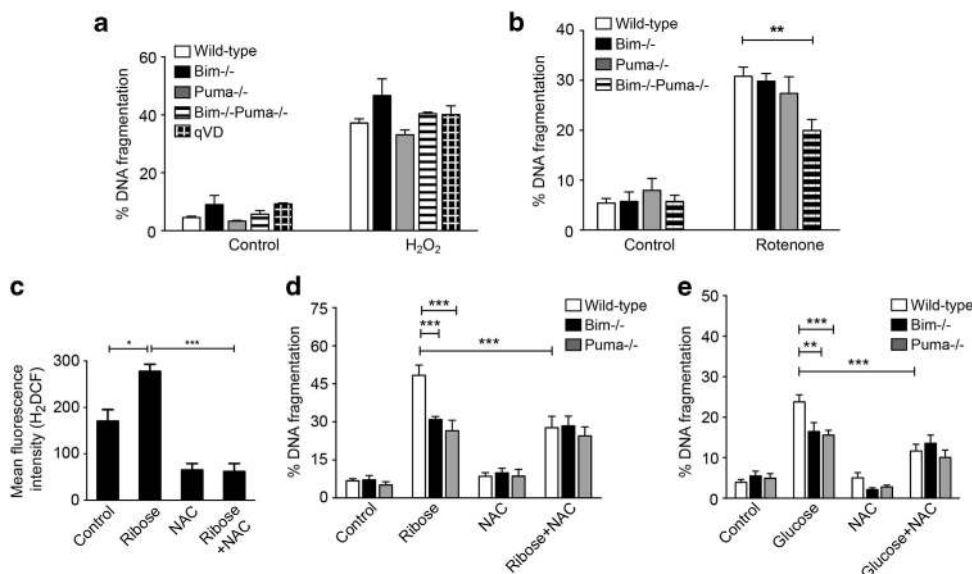
Next, islets were treated with the mitochondrial electron transport chain complex-I inhibitor rotenone (100 nM), which induces oxidative stress selectively in the mitochondria.<sup>35</sup> Combined loss of *Bim* and *Puma* significantly inhibited rotenone-induced islet cell death (Figure 6b). Protection from rotenone toxicity was only partial, possibly because rotenone is known to also induce some necrosis.<sup>35</sup> Overall, these data suggest that mitochondrial oxidative stress induces apoptosis in islet cells that utilizes both *Bim* and *Puma* proteins.

**Oxidative stress-induced *Bim* and *Puma* activation mediates glucotoxicity in islets.** We used dihydrodichlorofluorescein (H<sub>2</sub>DCF) to measure intracellular ROS. ROS production increased after ribose treatment for 3 days, and this was inhibited by the mitochondrial ROS scavenger NAC (1 mM)<sup>36</sup> (Figure 6c). When we cultured islets with ribose or glucose in the presence of NAC, DNA fragmentation of wild-type islets was reduced by almost 50% (Figures 6d and e). This suggests that oxidative stress is required for glucose-induced islet cell killing. In contrast to the findings with the ER stress inhibitors TUDCA and PBA (see above), loss of *Bim* or *Puma* did not cooperate with NAC to provide enhanced protection against ribose- or glucose-induced islet cell killing (Figures 6c and d). These results suggest that inhibition of oxidative stress protects islets from glucotoxicity, but deletion of either *Bim* or *Puma* on their own does not reduce the residual oxidative stress-mediated cell killing in NAC-treated islets. This is consistent with our observation that combined loss of *Bim* and *Puma* are required to block islet cell killing elicited by mitochondrial ROS toxicity.

**Expression of proapoptotic genes is higher in islets from human type 2 diabetic donors.** Hyperglycaemia is an important feature of type 2 diabetes. The presence of oxidative stress<sup>3,37</sup> and increased expression of UPR pathway factors such as CHOP<sup>14</sup> has been observed in islets from type 2 diabetic individuals. We investigated if



**Figure 5** Attenuation of ER stress protects islet cells from glucotoxicity. The frequency of cells undergoing DNA fragmentation was measured by flow cytometry after incubation of wild-type C57BL/6, *Puma*<sup>-/-</sup> or *Bim*<sup>-/-</sup> islets for 4 days with 50 mM ribose, 0.5 mM TUDCA (a) or 2.5 mM PBA (b) or 33.3 mM glucose and 0.5 mM TUDCA for 6–7 days (c). Control islets were incubated in a medium containing 5.5 mM glucose. Results represent mean ± s.e.m. of n ≥ 6 independent experiments. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 (two-way ANOVA)



**Figure 6** Inhibition of oxidative stress protects islet cells from glucotoxicity. **(a and b)** The frequency of cells undergoing DNA fragmentation was measured by flow cytometry after incubation of wild-type C57BL/6, *Bim*<sup>-/-</sup>, *Puma*<sup>-/-</sup>, *Bim*<sup>-/-</sup> or *Puma*<sup>-/-</sup> islets with 30  $\mu$ M H<sub>2</sub>O<sub>2</sub> and 50  $\mu$ M pancaspase inhibitor qVD.oph for 2 days **(a)**, or 100 nM Rotenone for 2 days **(b)**. Control islets were incubated for 2 days in a medium containing 5.5 mM glucose. Results represent mean  $\pm$  s.e.m. of  $n \geq 4$  independent experiments. \*\* $P < 0.01$  \*\*\* $P < 0.001$  (two-way ANOVA). **(c)** Mean fluorescence intensity of H<sub>2</sub>DCF staining measured by flow cytometry after incubation of MIN6 cells with 50 mM ribose with or without 1.0 mM NAC for 3 days. Results are mean  $\pm$  s.e.m. of  $n = 3-4$  independent experiments. \* $P < 0.05$ , \*\*\* $P < 0.001$  (one-way ANOVA). **(d and e)** The frequency of cells undergoing DNA fragmentation was measured by flow cytometry after incubation of wild-type C57BL/6, *Puma*<sup>-/-</sup> or *Bim*<sup>-/-</sup> islets with 50 mM ribose with or without 1.0 mM NAC for 4 days **(d)**, or 33.3 mM glucose with or without 1.0 mM NAC for 6–7 days **(e)**. Control islets were incubated in a medium containing 5.5 mM glucose. Results represent mean  $\pm$  s.e.m. of  $n \geq 5$  independent experiments. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (two-way ANOVA)

proapoptotic molecules are also upregulated in human type 2 diabetes. Islets were isolated from non-diabetic and age-matched type 2 diabetic donors (Supplementary Table S1) and gene expression of *Bim*, *Puma*, *Bax* and *Bid* were studied by qRT-PCR. *Bim* mRNA was almost threefold higher, whereas *Puma* and *Bax* were nearly twofold higher in islets of type 2 diabetic donors when compared with non-diabetic donors (Figure 7). Federici *et al.*<sup>38</sup> observed an increase in expression of *Bid*, a BH3-only molecule involved in the extrinsic apoptosis pathway in beta cells after culture in high glucose concentrations.<sup>38</sup> Although we did not observe a significant increase in *Bid* expression, it remains possible that its expression is increased in islets from a subset of type 2 diabetic individuals. Overall our data suggest that type 2 diabetes-associated oxidative and ER stress is accompanied by activation of intrinsic apoptotic signaling in islets.

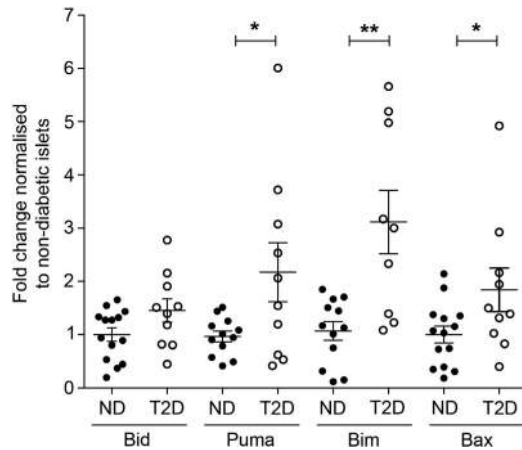
## Discussion

This study shows that exposure to high concentrations of glucose kills islet cells by inducing proapoptotic ER and oxidative stress signaling and downstream activation of the intrinsic apoptotic pathway. The ER stress pathway-associated transcription factor CHOP and the proapoptotic BH3-only proteins *Bim* and *Puma* are essential for this process. This is in contrast to physiological glucose concentration, which does not result in islet cell apoptosis.

Substantial inhibition of cell death in wild-type islets with TUDCA and PBA indicates that ER stress is a major contributor to beta-cell glucotoxicity. Deficiency of either *Bim*

or *Puma* alone partially reduced this cell death, indicating that ER stress can activate both of these proteins to initiate apoptosis, thereby suggesting overlapping, additive roles for these two molecules in glucotoxicity in islet cells. This is consistent with the previous finding that glucose- or ribose-induced islet cell death is reduced to background levels after combined deletion of *Bim* and *Puma*.<sup>5</sup> Overlapping actions of *Bim* and *Puma* have previously been detected with several apoptotic stimuli in a range of cell types (e.g. treatment of lymphoid cells with glucocorticoids<sup>39</sup>). The dominant role of *Bim* and *Puma* in the initiation of apoptosis can in part be explained by the fact that (unlike many other BH3-only proteins) they can bind and inhibit all prosurvival *Bcl-2* family members.<sup>40</sup>

Upregulation of the transcription factor CHOP represents an important mechanism through which ER stress initiates apoptosis. CHOP has been suggested to induce cell death through various mechanisms that include depleting cellular glutathione and sensitizing the cell to oxidative stress, upregulation of proapoptotic genes<sup>15</sup> and downregulation of the prosurvival molecule *Bcl-2*.<sup>9,41</sup> ER stress, through activation of CHOP, has been shown to activate the proapoptotic molecules *Bim* and *Puma* in several cell types and tissues.<sup>15,16,42</sup> Conversely, *Puma* was found to be a critical mediator of ER stress-induced apoptosis in mouse embryonic fibroblasts<sup>43</sup> and mouse motor neurons.<sup>44</sup> In mouse islets, *Puma* expression followed a similar pattern to the induction of CHOP. This suggests that CHOP is critical for the ER stress-induced increase in *Puma* expression in beta cells, similar to hepatocytes<sup>16</sup> and neuronal cells.<sup>42</sup> CHOP deficiency did not



**Figure 7** Proapoptotic intrinsic pathway genes are upregulated in human islets in type 2 diabetes. Quantitative RT-PCR of human islets isolated from non-diabetic (ND;  $n = 12-14$ ) and age-matched type 2 diabetic (T2D;  $n = 9-10$ ) donors. Relative mRNA expression levels for *Bim*, *Puma*, *Bax* and *Bid* were calculated by normalizing to the signal for *18S* and *Actin* mRNA in each sample. Results represent mean  $\pm$  s.e.m. \* $P < 0.05$ , \*\* $P < 0.01$  when comparing gene expression in islets of type 2 diabetic donors with those non-diabetic donors (unpaired *t*-test)

affect *Puma* expression in control (untreated) islets, suggesting that *Puma* mRNA levels may not be influenced by ER stress pathway factors under basal conditions.

*Bim* transcript abundance remained unchanged after exposure to ribose with or without addition of TUDCA, consistent with our previous report.<sup>5</sup> This raises the possibility that CHOP may be required for maintaining a high basal expression of *Bim* through a mechanism that does not affect *Bim* in conditions of increased ER stress. The finding that stronger ER stress inducers, such as thapsigargin, also failed to increase *Bim* expression supports this hypothesis. However, loss of CHOP did not cause a reduction in the basal levels of *Bim* expression, suggesting that ER stress may affect *Bim* expression post-translationally.<sup>15,45,46</sup>

The significant reduction in ribose- and glucose-induced cell death in wild-type islets afforded by treatment with NAC indicated that, in addition to ER stress, oxidative stress is also an important mediator of glucose and ribose toxicity. Mitochondrial oxidative stress, but not cytosolic oxidative stress, was more important in activating intrinsic apoptosis in islets. This is not surprising as increased metabolism caused by high glucose concentrations will result in upregulated electron transport chain activity and ROS generation in the mitochondria.<sup>47</sup> Consistent with our findings, a previous study showed that upregulation of *Puma* protein expression was seen in mitochondrial fractions of neuronal cells treated with drugs that induce oxidative stress.<sup>18</sup> The increase in *Bim* protein levels was also limited to the mitochondrial fraction and was much more sensitive to mitochondrial ROS than cytosolic ROS.<sup>18</sup> In contrast to ER stress, we observed that oxidative stress-induced apoptosis of islet cells required both *Bim* and *Puma*. It is possible that in contrast to the relatively mild toxicity induced by ER stress, ROS toxicity is more severe, possibly because of lower antioxidant expression in islet cells.<sup>19,20</sup> Therefore, combined loss of both *Bim* and

*Puma* is required to abrogate the intrinsic apoptosis triggered by this insult.

Increased expression of *Bim*, *Puma* and *Bax* in the islets of human type 2 diabetic subjects suggest that similar to mouse islets, oxidative and ER stress activate intrinsic apoptosis in human islets. While exposure of mouse islets to high glucose or ribose concentrations did not cause an increase in *Bim* expression, the increased expression observed in human islets indicates that prolonged and combined exposure to high glucose, lipids and amyloid (conditions that are normally seen in human type 2 diabetes) may be required to cause an increase in *Bim* gene expression. Although we did not directly demonstrate expression of these molecules in purified beta cells, there is sufficient evidence in the literature for expression and function of proapoptotic Bcl-2 family members in rodent beta cells and dispersed human islets to suggest that increased expression in beta cells at least in part contributes to the differences observed in islets from type 2 diabetic individuals.<sup>25,45,48-50</sup>

ER and oxidative stress are the major pathways by which beta cells are destroyed in type 2 diabetes. Activation of the intrinsic apoptosis pathway downstream of both of these stress pathways in islets, and their upregulation in islets from type 2 diabetic donors, suggests a central role for the BH3-only proteins *Bim* and *Puma* in the loss of beta-cell mass in type 2 diabetes. Thus, these stress and apoptosis pathways need to be inhibited to prevent beta-cell loss in type 2 diabetes. The challenge will now be to design inhibitors of these pathways that are suitable for *in vivo* use in patients.

#### Materials and Methods

**Mice.** Mice deficient for *Bim* (generated using 129SV-derived ES cells and backcrossed for > 10 generations onto the C57BL/6 background) and *Puma* (generated using C57BL/6-derived ES cells) have been described previously.<sup>5,51,52</sup> Mice deficient in *Chop* (generated using 129/Ola ES cells), backcrossed for 10 generations onto the C57BL/6 background, have been described previously.<sup>53</sup> All animal experiments were approved by the Institutional Animal Ethics Committees.

**Reagents.** D-Glucose (used at 33.3 mM) and D-ribose (50 mM) were purchased from Invitrogen (Invitrogen Corporation, Grand Island, NY, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Similar concentrations of L-glucose or L-ribose were used as hyperosmolarity controls (data not shown).<sup>5</sup> Thapsigargin (used at 5  $\mu$ M) was purchased from Calbiochem (Darmstadt, Germany). Anisomycin (Sigma-Aldrich) was used at 1  $\mu$ g/ml concentration. TUDCA (Sigma-Aldrich) was used at 0.5 mM, PBA (Sigma-Aldrich) at 2.5 mM, NAC (Sigma-Aldrich) at 1.0 mM, H<sub>2</sub>O<sub>2</sub> (Sigma-Aldrich) at 30  $\mu$ M and Rotenone (Sigma-Aldrich) at 100 nM. The concentrations of TUDCA, PBA, NAC, H<sub>2</sub>O<sub>2</sub> and Rotenone were titrated to determine optimal culture conditions. Control conditions contained the same concentration of diluent (DMSO for rotenone, tunicamycin and Q-VD-Oph, ethanol for thapsigargin or NaOH for PBA, other reagents were made up in complete CMRL medium) in the complete medium.

**Islet isolation, culture and DNA fragmentation assay.** Islets of Langerhans were isolated as described previously.<sup>54</sup> Islets were washed, hand-picked and cultured overnight at 37 °C in 5% CO<sub>2</sub> in CMRL medium-1066 (Invitrogen) supplemented with 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mmol/l glutamine and 10% FCS (JRH Biosciences, Lenexa, KS, USA) (referred to below as complete CMRL). Following incubation with different reagents, islets were trypsinized and DNA fragmentation was analysed by staining with PI and flow cytometric analysis as described previously.<sup>5</sup> MIN6 cells were a kind gift from Dr. Jun-ichi Miyazaki.<sup>55</sup> H<sub>2</sub>DCF staining was performed on MIN6 cells according to the manufacturer's instructions (Cell Biolabs, San Diego, CA, USA).



Human pancreata were obtained, with informed consent from next-of-kin, from heart-beating, brain-dead donors by the Australian Islet Transplant Consortium. The project was approved by the human ethics committees of the hospitals involved and the Australian Red Cross. Human islets were isolated as described previously.<sup>48</sup> Islets were immediately snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  before they were used to isolate RNA. Donor details are provided in Supplementary Table S1.

**Real-time quantitative RT-PCR analysis.** RNA was prepared using the NucleoSpin RNA XS (Macherey Nagel, Düren, Germany). First-strand cDNA was prepared from 200 to 300 ng RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). c-DNA was diluted (1:100) and real-time PCR was performed using the Rotor-Gene RG-3000 machine (Corbett Research; Qiagen, Hilden, Germany) and the TaqMan PCR Master Mix (AmpliTaQ Gold with GeneAmp kit; Applied Biosystems) in 20  $\mu\text{l}$  reaction volumes. Data analyses were performed with the  $\Delta\Delta\text{CT}$  method using  $\beta\text{-Actin}$  as an internal control. qRT-PCR analyses for *Atf4*, *Chop*, *Bip*, *Pdia4*, *P58*, *Bim*, *Puma* and *Bax* were performed using the commercially available TaqMan gene expression assay kits for mouse genes (Applied Biosystems). qRT-PCR for *Xbp1* (unspliced) and *Xbp1s* (spliced) was performed with Custom TaqMan gene expression assay primers and probes (Applied Biosystems) using the sequences published previously.<sup>56</sup> For human islets, qRT-PCRs were performed using the commercially available TaqMan gene expression assay kits (Applied Biosystems) using *Actin* and *18S* as housekeeping genes.

**Western blotting.** Islets (number of islets described in figures) were incubated for the indicated time points with the reagents described above, then washed with PBS and finally lysed in 15  $\mu\text{l}$  of lysis buffer (20 mmol/l Tris-HCl (pH 7.4), 135 mmol/l NaCl, 1.5 mmol/l  $\text{MgCl}_2$ , 1 mmol/l EGTA, 1% Triton X-100, 1  $\times$  protease inhibitor cocktail and 1  $\times$  phosphatase inhibitor cocktails 1 and 2). Western blotting was performed using standard procedures with antibodies to actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CHOP (Enzo Life Sciences, Farmingdale, NY, USA), BIP, phosphorylated and total Akt and FoxO3a (all from Cell Signaling Technology, Danvers, MA, USA). Detection of CHOP, FoxO3a and Akt was with horseradish peroxidase-conjugated secondary antibody (SouthernBiotech, Birmingham, AL, USA) and detection with ECL + Western blotting detection system (GE Healthcare, Little Chalfont, UK). For all other antibodies, Alexa Fluor 680 goat antibodies to mouse IgG (Invitrogen) or IR Dye 800CW donkey antibodies to rabbit IgG (LI-COR, Lincoln, NE, USA) were used as secondary antibodies, and blots were examined using the ODYSSEY infrared imager (LI-COR).

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism Software (San Diego, CA, USA). All data shown as bar graphs are mean  $\pm$  s.e.m. Data were analysed by one- or two-way ANOVA with Bonferroni's or Dunnett's post-test for comparison of multiple columns (as appropriate). Human qPCR data was analysed by unpaired *t*-test.

### Conflict of Interest

The authors declare no conflict of interest.

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