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Role of BH3-Only Molecules Bim and Puma in β-Cell Death in Pdx1 Deficiency

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ISLET STUDIES

Mutations in pancreatic duodenal homeobox-1 (*PDX1*) are associated with diabetes in humans. *Pdx1*-haploinsufficient mice develop diabetes due to an increase in β-cell death leading to reduced β-cell mass. For definition of the molecular link between *Pdx1* deficiency and β-cell death, Pdx1-haploinsufficient mice in which the genes for the BH3-only molecules Bim and Puma had been ablated were studied on a high-fat diet. Compared with Pdx1$^{+/-}$ mice, animals haploinsufficient for both Pdx1 and Bim or Puma genes showed improved glucose tolerance, enhanced β-cell mass, and reduction in the number of TUNEL-positive cells in islets. These results suggest that Bim and Puma ablation improves β-cell survival in Pdx1$^{+/-}$ mice. For exploration of the mechanisms responsible for these findings, Pdx1 gene expression was knocked down in mouse MIN6 insulinoma cells resulting in apoptotic cell death that was found to be associated with increased expression of BH3-only molecules Bim and Puma. If the upregulation of Bim and Puma that occurs during Pdx1 suppression was prevented, apoptotic β-cell death was reduced in vitro. These results suggest that Bim and Puma play an important role in β-cell apoptosis in *Pdx1*-deficient diabetes.

A progressive reduction in β-cell mass occurs in the evolution of diabetes and an increase in β-cell death due to an increase in apoptosis has been documented as an essential element in many studies (1). In human pancreatic tissue from patients with type 2 diabetes, β-cell mass is reduced and the frequency of β-cell apoptosis is increased (2). Thus, understanding the mechanisms responsible for β-cell apoptosis is important not only for understanding the pathogenesis of diabetes but also for developing novel approaches to prevention and treatment.

Pancreas and duodenal homeobox-1 (Pdx1) plays important roles in pancreas development and maintaining β-cell function and survival. Islet-specific disruption of *Pdx1* causes impaired insulin release, glucose intolerance, and diabetes (3–6). Previous studies have shown that islets from heterozygous *Pdx1*$^{+/-}$ mice are reduced in number, are smaller in size, and show increased susceptibility to apoptosis (7–9).

Transcriptional profiling of MIN6 insulinoma cells in which Pdx1 had been suppressed revealed increased expression of Bim and Puma (10). The current study was undertaken to determine whether these two proapoptotic molecules play a role in mediating pancreatic β-cell death associated with Pdx1 suppression.

RESEARCH DESIGN AND METHODS

MIN6 Cell Culture, Quantification of mRNA Levels, and Lentivirus-Mediated Short Hairpin RNA Expression

MIN6 cell culture, RNA isolation, and first-strand cDNA synthesis, mouse pancreatic islet isolation, and preparation of pLKO.1-Pdx1 short hairpin RNA (shRNA) lentivirus all were performed as previously described (10). Applied Biosystems (Foster City, CA) TaqMan assay numbers were as follows: Hmbs, Mm00660262_g1; Pdx1, Mm00435565_m1; Bim, Mm00437796_m1; and Puma, Mm00519268_m1. The pLKO-Bim shRNA (TRCN0000009692 and 9693), Puma shRNA (TRCN0000009712 and 9713), and Pdx1 (8) and lentiviral vectors (TRCN0000086031) were purchased from Thermo Scientific. Bim shRNA targets all Bim isoforms.

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Lentivirus was added to the medium on day 1. The blots were probed with antibodies against Pdx1 (07-696; Millipore), Puma (7467; Cell Signaling), Bim (202000; Calbiochem), cleaved caspase3 (9661; Cell Signaling), and actin (A-2066; Sigma). Antibody detection was accomplished using an enhanced chemiluminescence method and LAS-3000 imaging system (FUJIFILM).

Quantitation of Cell Death

Cell death was quantified by propidium iodide (PI) staining (11), followed by flow cytometric analyses using a FACS Caliber (BD Bioscience) and FlowJo software. Pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD) (20 μmol/L) was added to the medium 2 h prior to treating MIN6 cells by Pdx1 shRNA lentivirus. Z-VAD was added to the cells on days 1 and 3.

In Vivo Characterization of Mice

The *Pdx1*$^{+/-}$ mice have previously been described (6). *Bim*$^{+/-}$ and *Puma*$^{+/-}$ mice (12,13) were provided by E.H.C. Male mice were fed a high-fat diet (HFD) containing 42% fat (Harlan Laboratories Inc.) from 5 weeks of age and provided with water ad libitum. Intraperitoneal glucose tolerance tests were performed after a 16-h fast (1 g/kg dextrose) at age 17–18 weeks. Insulin levels were measured after a 16-h fast and 10 min after glucose challenge. Insulin tolerance tests were performed after a 4-h fast by administering human recombinant insulin (0.75 units/kg). The relative β-cell area was measured from anti-insulin–stained pancreas sections counterstained with hematoxylin using ImageJ software. At least five pancreatic serial sections (6 μm) spaced 70 μm apart per block were stained for each animal. The TUNEL and Ki-67 staining was performed as previously described (10). More than 20,000 β-cells and 300 islets were counted after TUNEL and Ki-67 staining, and at least three mice were counted per group. All of the experiments in this study using animal protocols were approved by the University of Chicago Animal Studies Committee.

Confocal Imaging Studies of Pancreatic Islets

Formalin-fixed pancreas sections underwent antigen retrieval in boiling citrate buffer (pH 6.0) for 10 min before labeling with antibodies against insulin (catalog no. A0564; DAKO), glucagon (G2654; Sigma), and DAPI (P-36931; Invitrogen).

Statistical Analysis

Multiple experimental groups were compared using one-way ANOVA. The two-tailed unpaired Student *t* test was used to assess the statistical significance of differences between two sets of data. Differences were considered significant when $P < 0.05$. In all experiments, the number of asterisks is used to designate the following levels of statistical significance: ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$ compared with the control group or wild-type (*WT*) group and ####$P < 0.001$, ##$P < 0.01$, and #$P < 0.05$ compared with the the Pdx1 knockdown (KD) or *Pdx1*$^{+/-}$ group. Results are presented as mean ± SEM.

RESULTS

Bim and Puma Are Upregulated in MIN6 Cells After Pdx1 Suppression

Pdx1 KD induced a 2.2-fold increase in *BimEL* mRNA and a 7.5-fold increase in *Puma* mRNA in MIN6 cells (Fig. 1*A*). Western blotting showed a corresponding increase in BIM, PUMA, and cleaved caspase-3 protein levels (Fig. 1*B*). The three protein isoforms of Bim (Bim_{EL}, Bim_L, and Bims) were increased sixfold, sevenfold, and sixfold, respectively (Fig. 1*B* and *C*). Pdx1 KD induced a threefold increase in PUMA protein and a >10-fold increase in cleaved caspase-3 protein (Fig. 1*B* and *C*). Immunohistochemical staining also showed increased Bim and Puma in Pdx1 KD cells (Fig. 1*D*). BimEL and Puma mRNA levels were examined in pancreatic islets isolated from 5- to 6-week-old *Pdx1*$^{+/-}$ mice. BimEL and Puma mRNA levels were increased by ~2.5-fold (Fig. 1*E*), recapitulating the results in MIN6 cells after Pdx1 KD. Immunofluorescence staining further confirms that Pdx1 suppression induces Bim and Puma upregulation in β-cells (Supplementary Fig. 1).

Effect of Puma and Bim Ablation in Adult Pdx1$^{+/-}$ Mice

The effects of Bim and Puma on β-cell death in vivo were assessed by crossing *Pdx1*$^{+/-}$ mice onto homozygous null Bim or Puma backgrounds. All mice were fed an HFD to promote insulin resistance and accelerate the glucose intolerant/diabetic phenotype (Supplementary Fig. 1). β-Cell mass was reduced in *Pdx1*$^{+/-}$ mice and the islets contained reduced numbers of β-cells as noted previously (10) (Fig. 2*A*). The architecture of the pancreatic islets from *Bim*$^{-/-}$ and *Puma*$^{-/-}$ mice was not significantly different from wild-type mice (Fig. 2*A*). The *Pdx1*$^{+/-}$*Bim*$^{-/-}$ and *Pdx1*$^{+/-}$*Puma*$^{-/-}$ mice showed an increase in β-cell mass compared with *Pdx1*$^{+/-}$ mice by 107 and 42%, respectively (all $P < 0.001$) (Fig. 2*B*). However, the abnormal distribution of α-cells in the islet core seen in the *Pdx1*$^{+/-}$ islets was not normalized (Fig. 2*A*). The increase in TUNEL labeling present in β-cells from *Pdx1*$^{+/-}$ islets was significantly reduced in *Pdx1*$^{+/-}$*Bim*$^{-/-}$ and *Pdx1*$^{+/-}$*Puma*$^{-/-}$ mice (Fig. 2*C*). Interestingly, proliferation of β-cells (Ki-67$^+$) was significantly increased in *Pdx1*$^{+/-}$*Bim*$^{-/-}$ and *Pdx1*$^{+/-}$*Puma*$^{-/-}$ islets compared *Pdx1*$^{+/-}$ islets (all $P < 0.001$) (Fig. 2*D*).

Pdx1$^{+/-}$ Mice With Bim or Puma Gene Ablation Have Improved Glucose Tolerance

For determination of whether Bim and Puma ablation prevents hyperglycemia in *Pdx1*$^{+/-}$ mice, glucose tolerance tests were performed on *Pdx1*$^{+/-}$, *Pdx1*$^{+/-}$*Bim*$^{-/-}$, and *Pdx1*$^{+/-}$*Puma*$^{-/-}$ mice maintained on an HFD. *Pdx1*$^{+/-}$ mice fed an HFD develop increased fasting blood glucose and impaired glucose tolerance (Fig. 3*A* and *D*). *Pdx1*$^{+/-}$*Bim*$^{-/-}$ and *Pdx1*$^{+/-}$*Puma*$^{-/-}$ mice exhibited significantly lower fasting blood glucose and improved glucose tolerance (Fig. 3*A* and *D*). The area under the blood glucose curve decreased 22% and 27% in *Pdx1*$^{+/-}$*Puma*$^{-/-}$ mice and *Pdx1*$^{+/-}$*Bim*$^{-/-}$ mice compared with *Pdx1*$^{+/-}$ mice, respectively ($P < 0.01$) (Fig. 3*B* and *E*). Interestingly, *Bim*$^{-/-}$ and *Puma*$^{-/-}$ mice had a better response

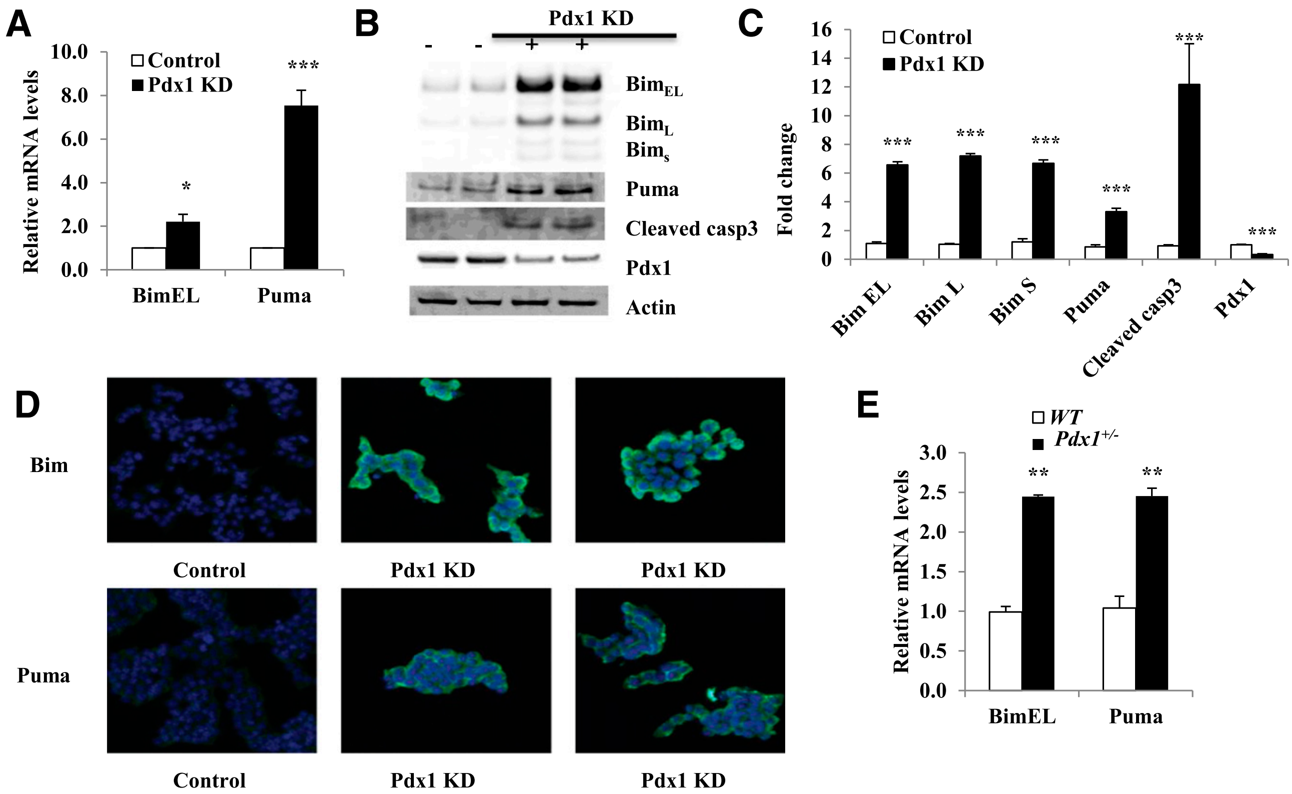

Figure 1—Bim and Puma are upregulated in MIN6 cells after Pdx1 suppression. *A*: Bim and Puma mRNA levels in control and Pdx1 KD cells. Five days after Pdx1 KD in MIN6 cells, BimEL and Puma mRNA levels were measured by real-time RT-PCR in MIN6 cells (n = 3). **P* < 0.05, ****P* < 0.001 compared with control group. *B*: Western blot of Pdx1 KD cells. Three days after Pdx1 KD in MIN6 cells, immunoblot analysis was performed to determine Pdx1, Bim, Puma, and cleaved caspase-3 levels in Pdx1 KD MIN6 cells. *C*: The bar graph depicts the relative changes in the levels of the indicated proteins using densitometry analysis of the Western blots in *B* (n = 3). Values are mean ± SEM. *D*: Three days after Pdx1 KD in MIN6 cells, endogenous Bim (green) and Puma (green) were detected by immunofluorescence using anti-Bim and anti-Puma antibodies, respectively, followed by Alexa Fluor 488–conjugated secondary antibody. The nuclei (blue) are stained by DAPI. Original magnification ×400. *E*: BimEL and Puma mRNA levels were measured by RT-PCR in islets from 5- to 6-week-old male $Pdx1^{+/-}$ mice on normal chow (n = 4–6). ***P* < 0.01 compared to the *WT* group.

to glucose challenge than wild-type mice (Fig. 3*A*, *B*, *D*, and *E*). The reduction in blood glucose after insulin administration is similar in $Pdx^{+/-}$ and $Pdx1^{+/-}Bim^{-/-}$ or $Pdx1^{+/-}Puma^{-/-}$ mice indicating that differences in insulin sensitivity are not responsible for the observed differences in glucose concentration (Fig. 3*C* and *F*). Insulin levels were decreased in the $Pdx^{+/-}$ mice under basal conditions and after glucose challenge (Fig. 3*G*). In comparison, insulin concentrations were increased in $Pdx1^{+/-}Bim^{-/-}$ and $Pdx1^{+/-}Puma^{-/-}$ mice (Fig. 3*G*). Together with the above results, we conclude that $Bim^{-/-}$ or $Puma^{-/-}$ ablation improves the diabetic phenotype in the $Pdx1^{+/-}$ mouse by preserving glucose tolerance and augmenting insulin secretion. These effects are due to an increase in β-cell mass and a decrease in β-cell apoptosis.

Suppression of Bim and Puma Upregulation Reduced β-Cell Apoptosis Induced by Pdx1 Suppression in MIN6 Cells

To define the role of Bim and Puma upregulation in the pancreatic β-cell death seen after Pdx1 suppression, we used shRNA to knock down Bim and Puma in MIN6 cells. Both Bim and Puma shRNA lentiviruses suppress Bim and Puma expression by >50% and prevented the *Bim* or *Puma* mRNA upregulation induced by Pdx1 suppression (Fig. 4*A* and *B*). Pdx1 suppression increased Bim_{EL} protein and Puma protein by more than twofold and fivefold, respectively (Fig. 4*A* and *B*). Bim and Puma KD significantly inhibited BIM and PUMA protein increase induced by Pdx1 KD (Fig. 4*A* and *B*). Caspase-3 activation was also inhibited by Bim and Puma KD (Fig. 4*A* and *B*). Double KD (DKD) of Pdx1 and Bim or Puma decreased cell death (PI staining) and apoptosis (TUNEL staining) compared with Pdx1 KD alone (Fig. 4*C* and *D*). After Pdx1 KD, 49.5 ± 4.9% of the MIN6 cells took up the PI stain. In the Bim/Pdx1 DKD group, only 29.3 ± 2.9% (***P* < 0.01 compared with Pdx1 alone) took up the PI stain, indicative of a 20% increase in cell viability (Fig. 4*C*). Puma KD also significantly reduced the proportion of MIN6 cells that took up the PI stain from 54.7 ± 1.7% when Pdx1 alone was knocked down to 40.1 ± 1.9% with Pdx1/Puma DKD, i.e., an improvement of 14% in cell viability

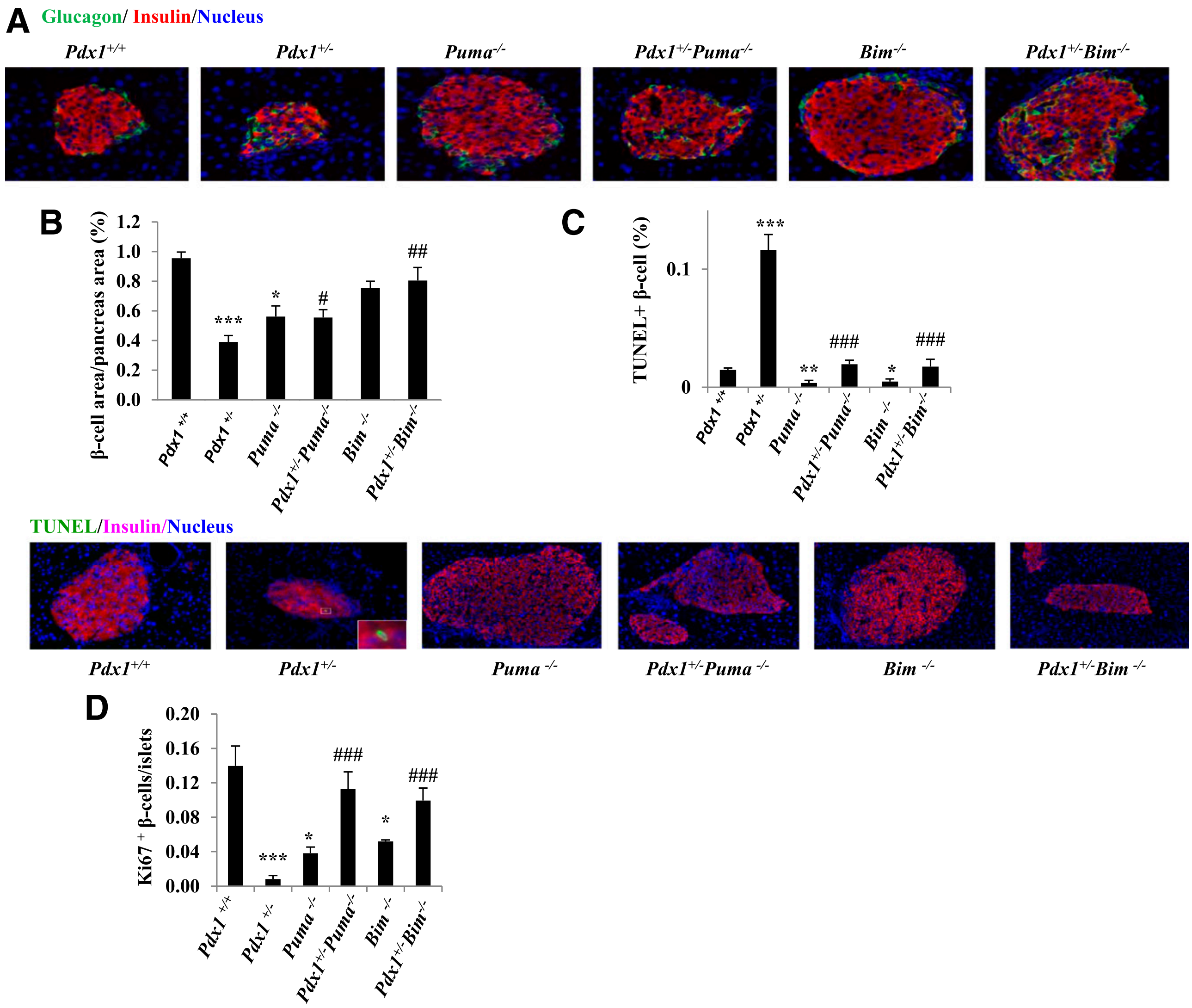

Figure 2—Effect of Puma and Bim ablation in adult $Pdx1^{+/-}$ mice. *A*: Islet morphology in adult mouse after 12 weeks on an HFD; anti-insulin and anti-glucagon antibodies were used to stain β-cells (red) and α-cells (green), respectively. *B*: Quantitation of β-cell mass is shown (n = 3–4 per group). *P < 0.05, ***P < 0.001 compared with the $Pdx1^{+/+}$ mice. #P < 0.05, ##P < 0.01 compared with $Pdx1^{+/-}$ mice. Values are mean ± SEM. *C*: TUNEL labeling of adult pancreatic β-cells. Quantitative TUNEL data are shown. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the $Pdx1^{+/+}$ mice. ###P < 0.001 compared with $Pdx1^{+/-}$ mice. Original magnification ×200. Values are mean ± SEM. *D*: Ki-67 staining of adult pancreatic β-cells. Quantitative data are shown. *P < 0.05, ***P < 0.001 compared with the $Pdx1^{+/+}$ mice. ###P < 0.001 compared with $Pdx1^{+/-}$ mice.

(Fig. 4*C*). TUNEL staining also showed that both Bim and Puma suppression in the Pdx1-insufficient state significantly decreased TUNEL staining (Fig. 4*D*). We confirmed the results by using a second shRNA (Supplementary Fig. 2). Collectively, these findings show that both Bim and Puma contribute to apoptotic death induced by Pdx1 suppression in MIN6 cells.

DISCUSSION

BH3-only molecules Bim and Puma play essential roles in mitochondrial-dependent apoptosis. Bim and Puma can be activated by different death stimuli in a cell type–specific manner (14–16). Our understanding of the relative roles of Bim and Puma in β-cells is limited. Puma is regulated by cytokines and endoplasmic reticulum (ER) stress in β-cells (17), and Bim is upregulated by ER stress (18). Recent studies have indicated that glucose and ribose toxicity–induced β-cell apoptosis required Bim, Puma, and Bax rather than Bid, Noxa, and Bak (19). Interestingly, dexamethasone induced Pdx1 downregulation and Bim activation in β-cells through glucocorticoid receptor activation (20). The present experiments demonstrated that in *Pdx1* deficiency, KD of Bim or Puma significantly reduced β-cell apoptosis. The current studies also demonstrate that a reduction in the expression of Bim or Puma protects β-cell mass in the pancreas of adult $Pdx1^{+/-}$ mice with an HFD. Apoptosis is clearly increased with increased TUNEL staining and proliferation of β-cells is decreased in islet β-cells from

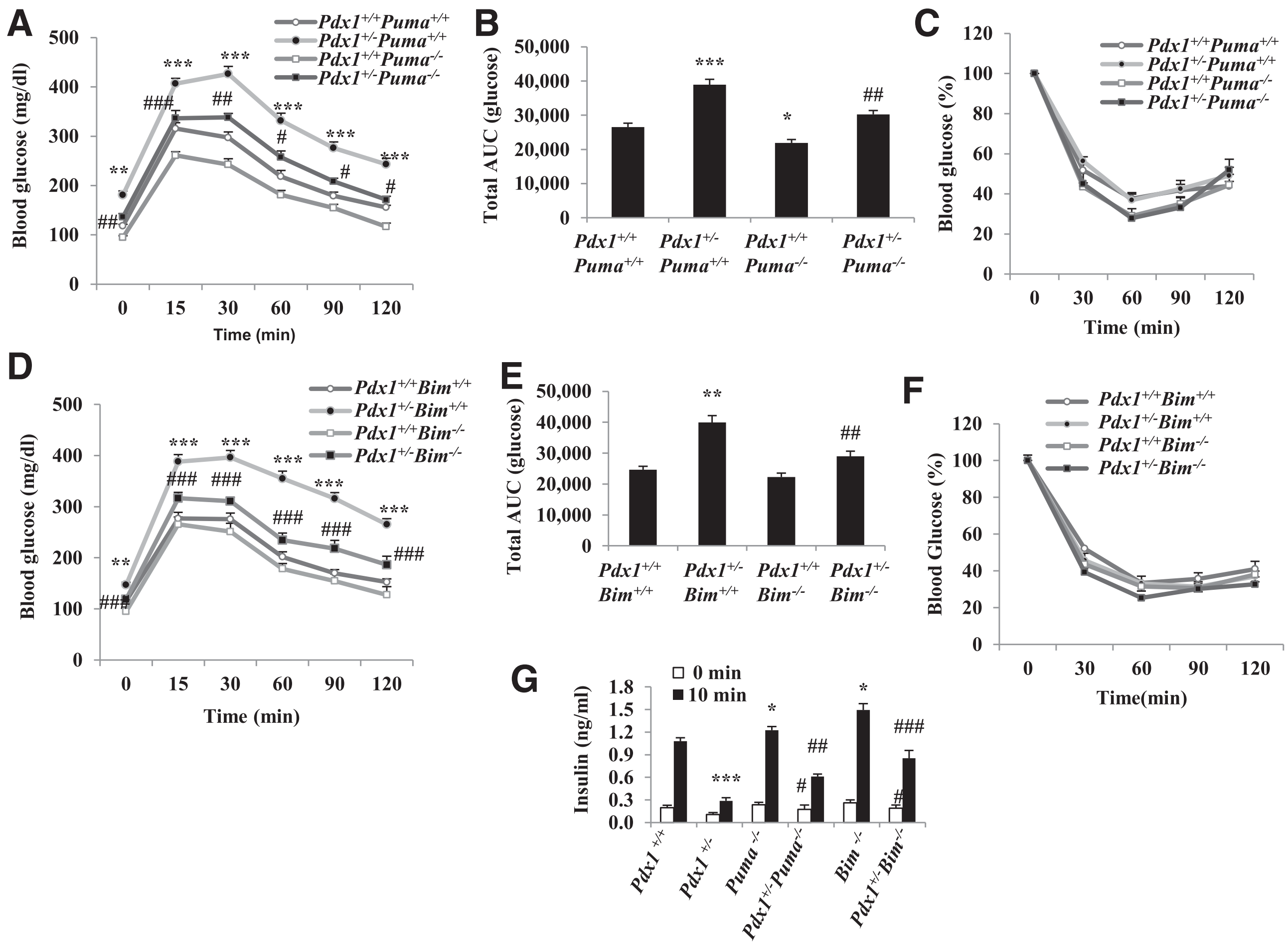

Figure 3—*Pdx1*$^{+/-}$ mice with Bim or Puma gene ablation have improved glucose tolerance. *A* and *D*: Glucose levels after injection of dextrose (1g/kg i.p.) in the mouse groups indicated. **P < 0.01, ***P < 0.001 compared with the *Pdx1*$^{+/+}$ mice. #P < 0.05, ##P < 0.01, ###P < 0.001 compared with *Pdx1*$^{+/-}$ mice. Values are mean ± SEM. *B* and *E*: Area under the blood glucose curves (AUC) using the data from *A* (panel *B*) or *D* (panel *E*) (n = 8–15) in the mouse groups designated. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the *WT* mice. ##P < 0.01 compared with *Pdx1*$^{+/-}$ mice. Values are mean ± SEM. *C* and *F*: Glucose levels in response to 0.75 units/kg body wt insulin in the mouse groups designated (n = 8–10). *G*: Insulin levels measured fasting and 10 min after intraperitoneal dextrose (n = 8–10). *P < 0.05, ***P < 0.001 compared with the *Pdx1*$^{+/+}$ mice. #P < 0.05, ##P < 0.01, ###P < 0.001 compared with *Pdx1*$^{+/-}$ mice. Values are mean ± SEM.

Pdx1$^{+/-}$ mice. Both *Pdx1*$^{+/-}$*Bim*$^{-/-}$ and *Pdx1*$^{+/-}$*Puma*$^{-/-}$ mice demonstrated increased proliferation of β-cells, preserved insulin secretion and β-cell mass compared with *Pdx1*$^{+/-}$ mice.

The caspase inhibitor, Z-VAD, significantly reduced but did not completely inhibit β-cell death induced by Pdx1 suppression. One interpretation of this result is that Pdx1 suppression induces death of β-cells by other mechanisms in addition to apoptosis that are not influenced by caspase inhibition. Our recent studies have shown that autophagy and necrosis are also implicated in β-cell death induced by Pdx1 deficiency (8,9). Another study recently showed Pdx1 occupancy of Puma and Noxa by chromatin immunoprecipitation in human and mouse islets (21). Pdx1 was also reported to increase β-cell insulin secretion; thus, we cannot rule out that Bim and Puma may be involved in the regulation of β-cell function besides inhibiting β-cell death (22). It should be noted that the mice in this study were fed an HFD. Since the HFD may cause lipotoxicity, the roles of Bim and Puma in Pdx1 haploinsufficiency need to be investigated on a normal chow diet to determine whether similar results would be obtained under those experimental conditions.

In conclusion, we have shown that BH3-only molecules Bim and Puma play a role in mediating β-cell apoptosis caused by Pdx1 deficiency. Genetic ablation of Bim and Puma protects β-cells from apoptosis and preserves insulin secretion and β-cell mass in *Pdx1*$^{+/-}$ mice. These results suggest novel targets for therapeutic interventions in diabetes associated with reduced Pdx1.

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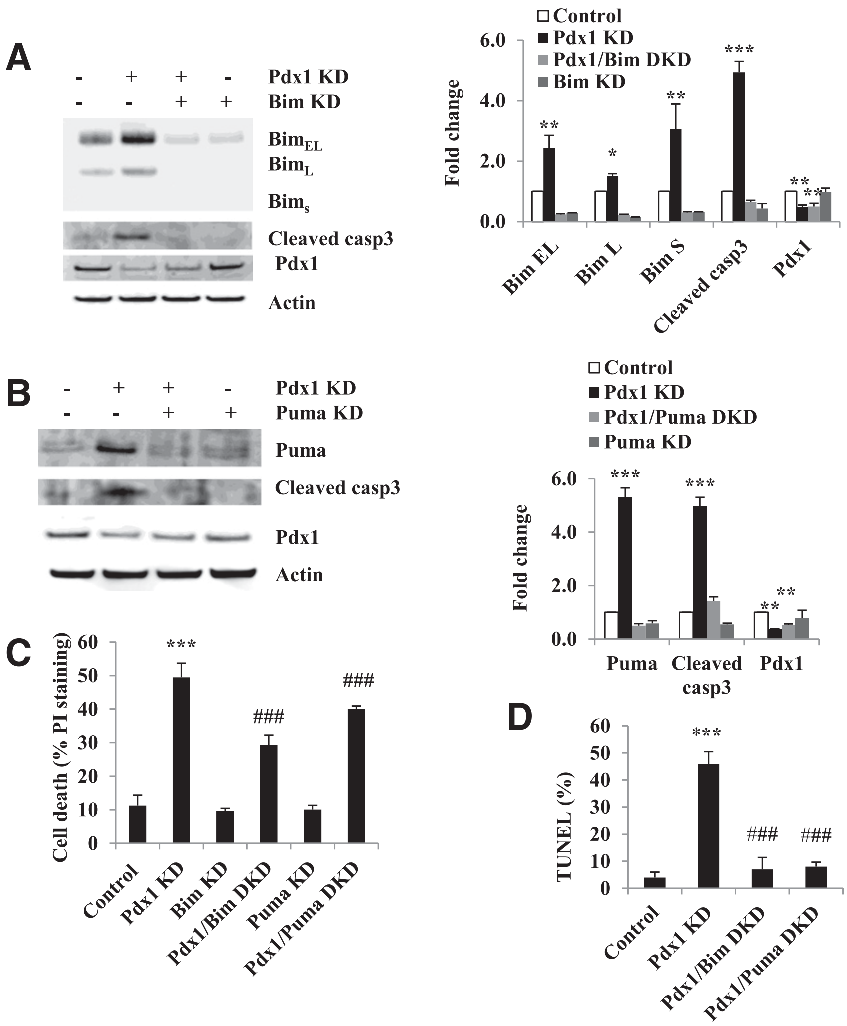

Figure 4—Bim and Puma upregulation is necessary for β-cell apoptosis induced by Pdx1 suppression. *A* and *B*: Immunoblot of Pdx1, Bim, and Puma and cleaved caspase-3 (casp3) in Bim/Pdx1 or Puma/Pdx1 DKD MIN6 cells. Bar graphs represent quantification using densitometry of the relative amounts of the indicated proteins determined by Western blots. *C*: Measurement of cell death. Four days after Bim/Pdx1 DKD or Puma/Pdx1 DKD in MIN6 cells, cell death was determined by PI staining ($n = 3$). Values are mean ± SEM. *D*: Measurement of apoptosis. Five days after Bim/Pdx1 DKD or Puma/Pdx1 DKD in MIN6 cells, apoptosis was determined by TUNEL staining ($n = 3$). Values are mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to the control group. ###$P < 0.001$ compared to the Pdx1 KD group.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. D.R. designed research, performed research, analyzed data, and wrote the manuscript. J.S., C.W., H.Y., and L.M. performed research. E.H.C. and G.I.B. contributed new reagents/analytic tools. K.S.P. designed research, analyzed data, and wrote the manuscript. D.R. and K.S.P. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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