Intraislet SLIT–ROBO signaling is required for beta-cell survival and potentiates insulin secretion

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Edited by Mu-ming Poo, University of California, Berkeley, CA, and approved August 28, 2013 (received for review August 20, 2012)

We previously cataloged putative autocrine/paracrine signaling loops in pancreatic islets, including factors best known for their roles in axon guidance. Emerging evidence points to nonneuronal roles for these factors, including the Slit–Roundabout receptor (Robo) family, in cell growth, migration, and survival. We found SLIT1 and SLIT3 in both beta cells and alpha cells, whereas SLIT2 was predominantly expressed in beta cells. ROBO1 and ROBO2 receptors were detected in beta and alpha cells. Remarkably, even modest knockdown of Slit production resulted in significant beta-cell death, demonstrating a critical autocrine/paracrine survival role for this pathway. Indeed, recombinant SLIT1, SLIT2, and SLIT3 decreased serum deprivation, cytokine, and thapsigargin-induced cell death under hyperglycemic conditions. SLIT treatment also induced a gradual release of endoplasmic reticulum luminal Ca2+, suggesting a unique molecular mechanism capable of protecting beta cells from endoplasmic reticulum stress-induced apoptosis. SLIT treatment was also associated with rapid actin remodeling. SLITs potentiated glucose-stimulated insulin secretion and increased the frequency of glucose-induced Ca2+ oscillations. These observations point to unexpected roles for local Slit secretion in the survival and function of pancreatic beta cells. Because diabetes results from a deficiency in functional beta-cell mass, these studies may contribute to therapeutic approaches for improving beta-cell survival and function.

Emerging evidence highlights the important role of locally released pancreatic islet peptide factors on beta-cell mass growth, maintenance, and survival (1–12). We have published a list of 233 ligands and 234 receptors expressed in islets and/or beta cells (12). Although our list is undoubtedly not comprehensive, it provides a starting point for the investigation of factors in adult islets that had previously only been reported in other cell types or in fetal pancreas (12). We identified a group of secreted molecules known to provide axonal guidance cues during neuronal development, comprising members of the netrin, slit, semaphorin, and ephrin families (13). The parallels between cell fate decisions in neurons and the endocrine pancreas prompted us to examine some factors in detail and discover that netrin treatment modulates beta-cell survival signaling (12).

The Slit ligands and their Roundabout receptors (Robo) were discovered in Drosophila as regulators of axon guidance during development (14–17). Mammalian homologs of Slit and Robo with functions outside of axon guidance have since been identified (18, 19). Slit ligands have been implicated in liver, kidney, lung, and mammary development by modulating cell adhesion, migration, differentiation, and death (18, 20, 21). It was not known whether Slit–Robo signaling functions in beta cells. Here, we report that Slit expression can be regulated by stress and that local Slit production is required for beta-cell survival and optimal function via a mechanism involving endoplasmic reticulum (ER) Ca2+ homeostasis and actin remodeling. Our work provides examples of local guidance factors that are required for beta-cell survival and suggests avenues for protecting functional beta-cell mass.

**Results**

**Slits Are Expressed in Adult Mouse and Human Islets.** The mammalian genome contains three Slit ligands and four Robo receptors. Our bioinformatic studies identified the expression of several Slit and Robo family members in adult human and rodent pancreatic islet cells (12), and Robo1 was identified by others as a transcript enriched in pancreatic endocrine cells during development (22). Nevertheless, no in-depth studies of these proteins have been reported. We detected Slit1, Slit2, and Slit3 transcripts in 6- and 30 wk-old mouse islets, with higher expression of Slit2 and Slit3 (Fig. 1 A). In human islets, SLIT1, SLIT2, and SLIT3 expression was similar. Robo1 and Robo2 were expressed in MIN6 cells, mouse islets, and human islets (Fig. 1 F). SLIT1, SLIT2, SLIT3, ROBO1, and ROBO2 were confirmed at the protein level (Fig. 1 B–F). Secretion of SLIT2 and SLIT3 from mouse islets was detected following incubation under 3 mM and 15 mM glucose conditions (Fig. 1 B). Although SLIT1 secretion from islets and SLIT1 protein content within islets fell below the detection threshold of the ELISA kit, the protein could be detected by immunoblotting and immunostaining (Fig. 1 B–F). SLIT2 immunoreactivity was more predominant in beta cells, whereas SLIT1 and SLIT3 were detected in both beta and alpha cells with the same intensity (Fig. 1 E and F). Deconvolution microscopy illustrated that SLIT2 colocalized with insulin-positive granules, whereas SLIT1 and SLIT3 were present in distinct granules (Fig. 1 F). These data suggest that SLIT2 may act in an autocrine manner on beta cells, whereas SLIT1 and SLIT3 may play both autocrine and paracrine roles.

Next, we assessed the mechanism of local Slit signaling by investigating the expression and localization of Slit receptors. ROBO1 and ROBO2 staining was detected in both beta and alpha

Significance

There is an unmet need for factors that can protect pancreatic islet beta cells from apoptosis and improve insulin secretion in the context of diabetes. There are many candidate factors produced locally in islets. We investigated the role of axon guidance factors and found that the Slit–Roundabout receptors system is present, where it responds to stress. Full expression of SLIT ligands is essential for optimal beta-cell survival. Recombinant SLIT promotes survival and increases insulin secretion via mechanisms involving Ca2+ and actin.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freedly available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1214312110/-/DCSupplemental.

PNAS | October 8, 2013 | vol. 110 | no. 41

16480–16485

www.pnas.org/cgi/doi/10.1073/pnas.1214312110
However, although ROBO1 could be found in the plasma membrane and cytosolic compartments typical for receptors of soluble ligands, ROBO2 displayed nuclear localization (Fig. 1F). Further biochemical assays, such as nuclear fractionation, will be required to support this observation. Although ROBO1 has been reported to be nuclear in some cell types (23), we are unaware of reports of nuclear ROBO2. Our data suggest that SLIT ligands act via ROBO1 receptors on islet cell plasma membranes.

It is not well understood in any cell type whether the Slit–Robo system can be dynamically regulated, for example by stresses (24, 25). Quantitative RT-PCR (qRT-PCR) revealed that cytokines, thapsigargin, and palmitate down-regulated Slit3, and serum deprivation down-regulated Slit2 (Fig. 1G). Slit1 expression could not be consistently detected under all of the treatment conditions or up-regulated under stress. In contrast to the situation in primary islets, thapsigargin and palmitate up-regulated Slit1 and Slit2 in MIN6 cells (Fig. S1). Slit3, which is typically absent in MIN6 cells, was robustly induced by ER stress (Fig. S1). These data suggest that the production of Slit ligands can be regulated in response to specific cellular stresses, with differential effects observed between primary islet cells and MIN6 insulinoma cells. We also examined the effects of stress on the Robo genes, and observed significant regulation (Fig. 1G).

Knockdown of Endogenous Slits Decreases Beta-Cell Survival. The regulation of Slit expression by stress suggests that Slit–Robo signaling play a role in beta-cell survival. A loss-of-function approach was used to determine the role of endogenous Slit ligands in beta-cell survival. In mouse islet cells, simultaneous siRNA targeting of all Slit ligands (to circumvent isoform compensation) resulted in a 48% knockdown of Slit1, 46% of Slit2, and 49% of Slit3 mRNA (Fig. 2A). Remarkably, even this incomplete reduction in endogenous Slit production significantly reduced beta-cell survival in serum-free conditions (Fig. 2B). Similarly, knockdown of Slit1/2/3 in MIN6 cells had significant negative effects on beta-cell survival (Fig. S2). These studies demonstrate that the local production of Slit ligands is required for optimal beta-cell survival.
SLIT3 also reduced islet cell death in response to serum deprivation in low-glucose conditions, suggesting a context-dependent switch. Cell effects were only seen in the context of high-glucose and never in low-glucose serum-free (SF) conditions (Fig. S4). Mouse islet cells were transfected with siRNA for SLIT1, SLIT2, SLIT3, or scramble siRNA as control, and examined by qRT-PCR after 72 h (2^ΔΔCt; n = 6, *P < 0.05 compared with control). (B) Mouse islet cells transfected with Slit siRNAs were stained with 0.05 μg/mL Hoechst and 0.5 μg/mL propidium iodide (PI) 48 h following transfection and imaged. Cells were in SF conditions with 20 mM and 5 mM glucose. Percent PI-positive cells and area under the curve (AUC) were calculated for indicated time intervals (n = 12, *P < 0.05 compared with control). (C) Mouse islet cells transfected with Slit siRNAs were incubated in 5 mM glucose SF conditions supplemented with 10 nM SLIT1-3 (n = 10, *P < 0.05 compared with scramble control, *P < 0.05 compared with Slit knockdown without SLIT supplement). (D) Mouse islet cells transfected with Slit siRNAs were stained and treated with cytokine mixture (25 ng/mL TNF-α, 10 ng/mL IL-1β, 10 ng/mL IFN-γ), 1 μM thapsigargin, or 1.5 mM palmitate in 20 mM glucose. (Inset) AUC from 0 to 60 h (n = 8–10).

We asked whether supplementing islet cell cultures with recombinant SLIT would be sufficient to rescue the effects of Slit1/2/3 knockdown. Indeed, although Slit1 and Slit2 alone could not rescue the elevated level of cell death observed under 5 mM glucose serum-free condition (Fig. 2C), Slit3 and a combination of all SLITs reversed the effects of Slit knockdown on islet cell death (Fig. 2C). We did not observe significant differences in cell survival between control and Slit knockdown in primary islet cells and MIN6 treated with cytokines, thapsigargin, or palmitate (Fig. 2D and Fig. S3). Collectively, our data indicate that SLIT ligands have acute protective effects on islet cells.

Exogenous SLITs Increase Beta-Cell Survival During Stress and Hyperglycemia. Next, we tested whether exogenous Slit1, Slit2, and Slit3 could protect beta cells from multiple forms of death. We first sought to determine whether the glucose milieu altered the protective effects of Slit treatment, as we have observed with notrin and Notch signaling (12, 26). Indeed, treatment with Slit1 and Slit2 recombinant proteins significantly reduced thapsigargin-induced death in MIN6 cells under high-, but not low-glucose conditions (Fig. S4 A and B). In mouse islet cells, Slit1, Slit2, and Slit3 also reduced islet cell death in response to serum deprivation alone and in combination with cytokines (Fig. 3 A and B). These effects were only seen in the context of high-glucose and never in low-glucose conditions, suggesting a context-dependent switch. Cell death induced by exposure to thapsigargin under serum deprivation was rescued with combination treatment of SLIT1-3 (Fig. 3C). Slit1 and Slit2 also reduced the level of cell death induced by thapsigargin treatment alone (Fig. S4C). Palmitate-induced cell death was not significantly reduced with SLITs (Fig. 3D). These data show that exogenous SLIT ligands, especially in combination, exert significant protection against several harsh stresses.

Slits Protect Beta Cells by Suppressing Apoptosis and ER Stress. To assess the molecular mechanisms associated with Slit action in beta cells, we examined markers of ER stress and apoptosis. Significant increases in annexin V-positive cells were observed in Slit1/2/3 knockdown cell lines compared with control (Fig. 4 A–C), pointing to an effect on apoptotic cell death. Treatment of mouse islets with exogenous SLIT decreased expression of Chop, Gadd34, and s3bp1 mRNA, but only in high glucose (Fig. 4D). In low glucose, SLIT increased the expression of Bip, a protective chaperone (Fig. 4D and Fig. S5). Consistent with the down-regulation of Chop observed in mouse islet cells, we also found a decrease in thapsigargin-induced CHOP protein upon treatment with SLIT2 in high glucose (Fig. S6A). Treating mouse islets with SLITs reduced cleaved caspase-3 and cleaved PARP (Fig. 4E).

Upon induction of ER stress, IRE1α activation can lead to the downstream activation of NF-κB and ASK1-p38 MAPK/JNK signaling cascades. Treatment with SLITs significantly reduced phospho-JNK and phospho-p38 MAPK, indicative of the down-regulation of these signaling cascades (Fig. 4E). Downstream mediators of p38 MAPK pathway, p53 and HSP27, were also down-regulated. Treatment of MIN6 cells with SLIT2 following ER-stress induction reduced ASK1 activation (Fig. S6B). Thapsigargin-induced cleaved caspase-3 and cleaved caspase-12 were also down-regulated by SLIT2 (Fig. S6 C–E). Serum deprivation-induced cleaved caspase-3 levels were also down-regulated upon SLIT1 and SLIT2 treatments under high-glucose, but not low-glucose, conditions (Fig. S6D). These data show the protective effects of SLITs on beta cells, especially in the context of high-glucose conditions.

Fig. 2. Knockdown of endogenous Slits increases cell death. (A) Dispersed mouse islet cells were transfected with siRNA for Slit1, Slit2, and Slit3 or scramble siRNA as control, and examined by qRT-PCR after 72 h (2^ΔΔCt; n = 6, *P < 0.05 compared with control). (B) Mouse islet cells transfected with Slit siRNAs were stained with 0.05 μg/mL Hoechst and 0.5 μg/mL propidium iodide (PI) 48 h following transfection and imaged. Cells were in SF conditions with 20 mM and 5 mM glucose. Percent PI-positive cells and area under the curve (AUC) were calculated for indicated time intervals (n = 12, *P < 0.05 compared with control). (C) Mouse islet cells transfected with Slit siRNAs were incubated in 5 mM glucose SF conditions supplemented with 10 nM SLIT1-3 (n = 10, *P < 0.05 compared with scramble control, *P < 0.05 compared with Slit knockdown without SLIT supplement). (D) Mouse islet cells transfected with Slit siRNAs were stained and treated with cytokine mixture (25 ng/mL TNF-α, 10 ng/mL IL-1β, 10 ng/mL IFN-γ), 1 μM thapsigargin, or 1.5 mM palmitate in 20 mM glucose. (Inset) AUC from 0 to 60 h (n = 8–10).

Fig. 3. Slits reduce stress-induced islet cell death under high glucose conditions. Dispersed mouse islet cells were stained with Hoechst and PI and imaged. The percentage of PI-positive cells was determined following treatments with 10 nM SLIT1, SLIT2, and/or SLIT3 in 20 mM glucose SF (A), 20 mM glucose SF with cytokine mixture (B), 20 mM glucose SF with 1 μM thapsigargin (C), and 20 mM glucose SF with 1.5 mM palmitate (D). (Inset) Area under the curve from 0 to 50 h (n = 8, *P < 0.05 compared with untreated control).
Interestingly, SLIT treatment increased the frequency of glucose-glucose, SLIT treatment had little or no effect on cytosolic Ca\(^{2+}\) associated with increased insulin secretion (27, 29, 30, 34). At basal phospho-HSP27 (p-38, cleaved PARP, phospho-JNK, phospho-p38, phospho-p53, and phospho-ROBO signaling on cell death signaling were significantly decreased). Together, these experiments indicated that CHOP-mediated apoptosis signaling in beta cells can be controlled by luminal Ca\(^{2+}\) (24, 25), we predicted that the ROBO signaling on cell death signaling were downstream of the modulation of ER Ca\(^{2+}\).

**Fig. 4.** Slits down-regulate proapoptotic and ER-stress signaling. MIN6 cells transfected with Slit1, Slit2, and Slit3 siRNAs were stained with Hoechst, PI, and Alexa Fluor 647-conjugated Annexin V 48 h following transfection. Cells were cultured in 12 mM (A and B) and 5 mM (C) glucose in the presence or absence of FBS and imaged. (Inset) Area under the curve from 20 to 40 h (n = 10, *P < 0.05 compared with scramble siRNA control). (D) Mouse islet cells were treated with SLIT1 or SLIT2 for 4 h before RNA isolation and qRT-PCR analysis (n = 3, *P < 0.05). (E) Mouse islet cells were treated with SLIT1-3 under 20 mM glucose SF condition. Immunoassay for cleaved caspase-3, cleaved PARP, phospho-JNK, phospho-p38, phospho-p53, and phospho-HSP27 (n = 6–8, *P < 0.05).

**Fig. 5.** Slits modulate cytosolic Ca\(^{2+}\) and ER Ca\(^{2+}\) signaling and actin remodeling. Dispersed mouse islet cells loaded with Fura-2-AM. Representative single-cell traces following 10 nM SLIT1, SLIT2, or SLIT3 treatment in 15 mM glucose (A). Single-cell oscillation frequency of cytosolic Ca\(^{2+}\) (n = 91–104, *P < 0.05) (B). Representative single-cell traces following exposure to 10 nM SLIT2 in 3 mM glucose (C). (D) Dispersed mouse islet cells were transfected with D1ER cameleon to image ER Ca\(^{2+}\). Cells were exposed to 1 μM Tg for 15 min following treatment with 10 nM SLIT1, SLIT2, or SLIT3 (colored lines) or untreated (black line) in 15 mM glucose (n = 13–14). (Inset) D1ER FRET/CFP ratios normalized to the time point following Tg addition. (Right) AUC for the 15 min preincubation with 15 mM glucose and 15 min treatment with SLIT1-3 (n = 13–14, *P < 0.05 compared with untreated). (E) Mouse islet cells treated with 10 nM SLIT1, SLIT2, or SLIT3 in 11 mM glucose-containing RPMI. Beta cells stained for insulin and phalloidin and peak intensity of cortical actin staining was normalized to untreated cells at the same time point (n = 39–60 cells).

Slits deplete Ca\(^{2+}\) and the level of depletion (25). SLITs partially depleted ER Ca\(^{2+}\) because thapsigargin treatment led to further depletion of ER Ca\(^{2+}\) (Fig. 5D and Fig. S7). The partial depletion of ER Ca\(^{2+}\) was maintained throughout a 6-h treatment with SLITs (Fig. S7, Bottom). These experiments demonstrate that Slit signaling has direct effects on ER luminal Ca\(^{2+}\), a parameter known to modulate ER stress and insulin secretion (24, 25, 28).

**SLITs Potentiate Glucose-Stimulated Insulin Secretion.** Given the roles of Ca\(^{2+}\) and actin on insulin secretion, we investigated whether SLITs affect insulin release. Static incubation showed that mouse islets cultured in 15 mM glucose secreted more insulin in the presence of SLIT (control: 9.6 ± 2.8 ng/mL, SLIT1: 21.6 ± 8.2 ng/mL, SLIT2: 19.1 ± 8.4 ng/mL). This observation led us to conduct islet perifusion experiments. Indeed, glucose-stimulated insulin secretion was significantly potentiated in the presence of SLIT1, SLIT2, or SLIT3 (Fig. 6A). SLIT treatment did not potentiate insulin secretion when beta cells were depolarized with 30 mM KCl (Fig. 6A), ruling out effects distal to the opening of voltage-gated Ca\(^{2+}\) channels and pointing to effects on glucose sensing/signaling. SLITs did not affect Ins1 and Ins2 transcription (Fig. S8). Thus, Slit proteins can both protect beta cells and increase insulin secretion, which itself is antiapoptotic (8, 9, 39).

**SLITs Accelerate Ca\(^{2+}\) Oscillations and Modulate ER Luminal Ca\(^{2+}\).** Ca\(^{2+}\) homeostasis, especially within the ER, plays a key role in both beta-cell survival (24, 25, 27, 28) and glucose responsiveness (27, 29, 30). Given that SLIT2 signaling in neurons involves Ca\(^{2+}\) release from the ER (31–33), we examined this mechanism in beta cells. Interestingly, SLIT treatment increased the frequency of glucose-stimulated cytosolic Ca\(^{2+}\) oscillations (Fig. 5A and B), a phenomenon that is known to be modulated by the ER Ca\(^{2+}\) filling state and associated with increased insulin secretion (27, 29, 30, 34). At basal glucose, SLIT treatment had little or no effect on cytosolic Ca\(^{2+}\) (Fig. 5C). However, in cells transfected with luminal ER Ca\(^{2+}\) sensor, D1ER (25, 35), we observed that SLIT induced a gradual release of Ca\(^{2+}\) from the ER filled after exposure to high glucose (Fig. 5D and Fig. S7). These effects correlate with the conditions under which SLIT proteins protect beta cells from ER stress induced by cytokines and by thapsigargin, a drug that blocks ER Ca\(^{2+}\) refilling. This result fits with a model whereby ER stress-induced cell death is dependent on the rate at which Ca\(^{2+}\) is

low-glucose conditions (Fig. S6 F and G). Cleaved caspase-7 and cleaved caspase-12 levels were also significantly decreased in cells treated with SLIT1 under hyperglycemic serum-free conditions (Fig. S6 H and I). Together, these results indicate that SLIT protects beta cells by broad suppression of the ER stress-induced apoptosis pathway. Because we have previously shown that the CHOP-caspase axis in beta cells can be controlled by luminal Ca\(^{2+}\) (24, 25), we predicted that the effects of SLIT-ROBO signaling on cell death signaling were downstream of the modulation of ER Ca\(^{2+}\).
Discussion
The present study was conducted to determine the expression pattern, regulation, and roles of the Slit family of secreted factors and their Robo receptors in pancreatic islet cells. Slit and Robo transcripts and protein were detected in adult mouse and human islets and regulated in stress conditions. We identified an important role for this local autocrine/paracrine network in beta-cell survival and function. We identified a unique anti-ER stress and antiapoptotic mechanism of action involving the controlled release of Ca\(^{2+}\) from the ER lumen (Fig. 6B).

The roles of Slit–Robo signaling in cell survival remain poorly understood. Knockout mouse studies, along with the observed loss of expression of ROBO1 in some human cancers, provide evidence that the loss of ROBO is tumorigenic. SLIT1 and SLIT3 are candidate tumor suppressor genes (40), although SLIT2 is up-regulated in prostate tumors (41). In islet cells, a modest knockdown of Slits significantly increased cell death, suggesting that endogenous SLIT secretion plays an important role in cell survival. Conversely, SLIT1, SLIT2, and/or SLIT3 supplementation reduced stress-induced cell death. We observed significant decreases in both ER stress- and serum starvation-induced cell death, but only under hyperglycemic conditions. Our data suggest that Ca\(^{2+}\)-dependent mechanisms are important for the protective effects of Slit, which is in line with a role for Ca\(^{2+}\) in Slit–Robo signaling in other cell types (24, 25, 27, 28, 31–33). In particular, our results implicate a controlled depletion in ER Ca\(^{2+}\) and an increase in the frequency of Ca\(^{2+}\) oscillations.

It is possible that Slit may protect beta cells via interaction with receptors for other guidance factors. DCC, UNC5, and Neogenin are dependence receptors that can increase cell survival in the presence of netrins and induce cell death in the absence of netrins (42, 43). Interaction between ROBO1 and DCC in a Slit-dependent manner decreases netrin-induced chemotraction in neurons (44, 45). Perhaps Slit–Robo signaling regulates beta-cell survival through down-regulation of netrin receptor-induced apoptosis. Additionally, because Slit–Robo signaling induces tumor angiogenesis by attracting endothelial cells, perhaps local production of SLITs could improve islet engraftment following transplantation (46). The stress-induced up-regulation of Slit expression, the survival effects of exogenous SLITs under high-glucose conditions, and the protective effects of endogenous SLITs suggest an autocrine/paracrine compensatory survival network.

In addition to the protective effects on beta cells, we uncovered effects of SLIT ligands on proximal glucose sensing/signaling, which were associated with changes in Ca\(^{2+}\) and actin. It was not surprising to find that SLITs potentiate glucose-stimulated insulin secretion given that Cdc42, Rac1, and RhoA play important roles in Slit-induced cellular migration (33, 47, 48). The down-regulation of cortical F-actin staining observed upon SLIT treatment is in agreement with the requirement for Cdc42 and RhoA inactivation via increasing Rho GTPase-activating proteins for the repulsive effects of SLITs in neurons and olfactory ensheathing cells (33, 47). Our current data do not allow us to distinguish whether the biphasic F-actin depletion following SLIT1 and SLIT3 treatments directly or indirectly mediates the potentiation of insulin secretion, but this could be a fruitful area for future investigation. In addition to the release of antiapoptotic insulin (8, 9, 39, 49–51), modulation of actin polymerization may directly promote survival (52–55). We have not determined whether the survival effects of SLITs are due to direct down-regulation of apoptotic pathways or indirect effects through up-regulation of insulin secretion (39, 56).

In conclusion, we provide detailed evidence that Slit ligands and their Robo receptors are present in pancreatic islet cells and define multiple roles for the Slit-secreted factors in beta-cell physiology. Our results reveal that Slit signaling depletes ER Ca\(^{2+}\) and protects beta cells from ER stress. Together, our results point to the Slit–Robo pathway and a novel area for investigations around beta-cell survival and function.

Materials and Methods
Detailed methods can be found in SI Materials and Methods.

Primary Islet Isolation, Cell Culture, and Perifusion. Islets were isolated from C57BL/6J mice using collagenase and filtration (12, 56). Human islets (>80% pure) were provided by Garth Warnock (University of British Columbia, Vancouver), collected via protocols approved by the University of British Columbia Research Ethics Board. Islets and MIN6 cells were cultured as described (8, 12, 57). Insulin secretion was measured by perifusion and RIA (26).

siRNA, RT-PCR, and Immunoblotting. MIN6 and dispersed islet cells were cultured as described (8, 12, 57). Insulin secretion was measured by perifusion and RIA (26).

diagram

Fig. 6. Slits modulate insulin secretion. (A) Mouse islets perfused with Krebs-Ringer buffer containing 3 or 15 mM glucose in combination with 10 nM SLIT1, SLIT2, or SLIT3 (n = 5–6). (Right) Baseline subtracted AUC for the 15 mM glucose- and KCl-stimulated insulin secretion, along with first- and second-phase insulin secretion (n = 5–6, *P < 0.05 compared with control). (B) Model of Slit–Robo signaling in beta cells. TCA, tricarboxylic acid.

Imageing. MIN6 and dispersed islet cells stained as described (12). For cell death assays, cells were seeded into 96-well plates and exposed to 0.05 μg/mL Hoechst 33342 (Invitrogen), 0.5 μg/mL propidium iodide (Sigma), and Alexa-Fluor647-conjugated Annexin V (1:250; Invitrogen) (25). Cells were imaged...
with ImageXpress MICRO (Molecular Devices) every 1 or 2 h at 37 °C and 5% (vol/vol) CO₂ (12). Cytosolic Ca²⁺ was imaged using Fura-2-AM (Invitrogen) (57). To measure changes in 
Ca²⁺, mouse islet and MIN6 cells were transfected with the D1ER sensor (24, 25). To detect F-actin, mouse islet cells were fixed with Z-FIX (Anatech Ltd.) and stained with Alexa Fluor 488-conjugated phalloidin (Invitrogen). Beta cells were identified by insulin staining (Santa Cruz Biotechnology). LINE scans were performed using ImageJ (version 1.41o; National Institutes of Health) and mean peak-intensity calculated.

ACKNOWLEDGMENTS. We thank Keshika Nanda, Xiaoake Hu, and Dr. Kwan Yi Chu for technical assistance. These studies were supported by grants from the JDRF–Johnson & Johnson (to J.D.J.) and Canadian Diabetes Association (to P.E.M.).