Rgs16 and Rgs8 in embryonic endocrine pancreas and mouse models of diabetes

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SUMMARY
Diabetes is characterized by the loss, or gradual dysfunction, of insulin-producing pancreatic β-cells. Although β-cells can replicate in younger adults, the available diabetes therapies do not specifically target β-cell regeneration. Novel approaches are needed to discover new therapeutics and to understand the contributions of endocrine progenitors and β-cell regeneration during islet expansion. Here, we show that the regulators of G protein signaling Rgs16 and Rgs8 are expressed in pancreatic progenitor and endocrine cells during development, then extinguished in adults, but reactivated in models of both type 1 and type 2 diabetes. Exendin-4, a glucagon-like peptide 1 (Glp-1)/incretin mimetic that stimulates β-cell expansion, insulin secretion and normalization of blood glucose levels in diabetics, also promoted re-expression of Rgs16::GFP within a few days in pancreatic ductal-associated cells and islet β-cells. These findings show that Rgs16::GFP and Rgs8::GFP are novel and early reporters of G protein-coupled receptor (GPCR)-stimulated β-cell expansion after therapeutic treatment and in diabetes models. Rgs16 and Rgs8 are likely to control aspects of islet progenitor cell activation, differentiation and β-cell expansion in embryos and metabolically stressed adults.

INTRODUCTION
Diabetes affects over 246 million people worldwide and accounts for about 6% of annual global mortality (www.idf.org). This disease is characterized by defective glucose metabolism and hyperglycemia resulting from the destruction of insulin-producing β-cells within the pancreas (type 1), or defects in insulin signaling (type 2). Diabetes has no cure, although there are palliative treatments to control its symptoms. There is a great need to understand the cellular and molecular basis for islet cell proliferation and differentiation in an effort to generate β-cell regenerative therapies for diabetic patients. Although groundbreaking work has advanced our ability to drive stem cells towards the pancreatic endocrine cell fate in culture (D’Amour et al., 2005; D’Amour et al., 2006; Kroon et al., 2008), much remains unknown about the molecular pathways regulating the differentiation of islet cell lineages (Lammert et al., 2001; Cleaver and Melton, 2003; Lammert et al., 2003; Collombat et al., 2006; Oliver-Krasiński and Stoffers, 2008) and the mechanisms underlying islet regeneration (Dor et al., 2004; Bonner-Weir et al., 2008; Xu et al., 2008).

New tools required for the development of diabetes therapies can be fashioned using embryonic genes that are expressed during pancreas development and later reactivated during pancreatic β-cell regeneration in models of diabetes (Inada et al., 2008; Xu et al., 2008). The earliest candidate genes are expressed in pancreatic ‘progenitor cells’ within the pre-pancreatic endoderm at around embryonic day (E) 8.75–9.0 (Golosow and Grobstein, 1962; Gittes and Rutter, 1992; Kim and MacDonald, 2002; Yoshitomi and Zaret, 2004). By E12.5–14.5, endocrine progenitor cells proliferate, delaminate and begin coalescing into small islet-like clusters. During postnatal development, these clusters acquire recognizable islet anatomy; in mice, this consists of a core of β-cells (that produce insulin) surrounded by a mantle of mostly α-cells (that produce glucagon), but also δ-cells (somatostatin), ε-cells (ghrelin) and PP (pancreatic polypeptide) cells (Kim and MacDonald, 2002; Cleaver and Melton, 2003; Collombat et al., 2006). In adulthood, there is little endocrine cell proliferation unless animals experience metabolic stresses that challenge their glucose homeostasis.

The cellular origin of the new endocrine cells remains controversial. Studies from Melton and others demonstrate that new β-cells derive from replication of pre-existing β-cells rather than through proliferation of endogenous specialized progenitors (Dor et al., 2004; Teta et al., 2007). Work from Bonner-Weir, by contrast, supports the existence of ‘foci of regeneration’ or pools of endocrine progenitors within the pancreatic ducts (Bonner-Weir et al., 2004). Recent work by Heinberg and colleagues has shown that the adult pancreatic ducts have the ability to generate new β-cell formation in response to extreme pancreatic injury (Gradwohl et al., 2000; Xu et al., 2008). It is therefore plausible that both mechanisms occur, but depend on unspecified signals within the microenvironment. New biomarkers are therefore needed to further identify and examine expanding islets in different injury or disease models. These biomarkers will provide direct and rapid in vivo validation of conditions that stimulate β-cell replication and expansion.

G protein-coupled receptor (GPCR) signaling pathways have been associated with β-cell neogenesis. Glucagon-like peptide 1 (Glp-1) and exendin-4 (Byetta) are GPCR agonists that stimulate β-cell replication and neogenesis and improve glucose tolerance in mouse models of type 1 diabetes (Xu et al., 1999; Tourrel et al.,...
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islet progenitor cell differentiation and sensitive reporters of conditions that stimulate the early stages of treatment with the GPCR agonist exendin-4. Rgs16 and Rgs8 are model of type 2 diabetes (Chua et al., 2002; Prentki and Nolan, 2006). Identifying the Rgs genes is likely to further the development of novel therapies for metabolic diseases.

The Rgs genes were first implicated in glucose homeostasis when we discovered that hepatic glucose production elevates the expression of Rgs16 in hepatocytes during fasting (Huang et al., 2006). To identify Rgs8/16 in other tissues that regulate glucose homeostasis, we used two lines of bacterial artificial chromosome (BAC) transgenic mice that express either Rgs16::GFP or Rgs8::GFP. These reporter genes faithfully reproduce endogenous Rgs16 and Rgs8 mRNA expression in mice (Gong et al., 2003; Su et al., 2004; Huang et al., 2006; Morales and Hatten, 2006). Identifying the ligands, conditions and cell types that induce the expression of these Rgs genes is likely to further the development of novel therapies for metabolic diseases.

Here, we show that Rgs16::GFP and Rgs8::GFP are expressed during pancreatic endocrine cell development and proliferation. Pancreatic progenitor cells and differentiating β-cells express Rgs16 and Rgs8, beginning in the dorsal pancreatic anlage and continuing throughout embryogenesis. In the perinatal pancreas, Rgs8-expressing cells aggregate into islets in tight association with pancreatic blood vessels. In adults, pancreatic expression of Rgs16 and Rgs8 becomes quiescent. However, under conditions of chronic glucose stress, Rgs16 and Rgs8 are re-expressed in adult pancreatic islets. Here, we demonstrate Rgs16::GFP expression in four different models of adult β-cell expansion, including in (1) PANIC-ATTAC mice, a model of type 1 diabetes (Wang et al., 2008); and, together with Rgs8 expression, in (2) obese, hyperglycemic ob/ob mice, a model of type 2 diabetes (Chua et al., 2002; Prentki and Nolan, 2006); (3) midgestation pregnant females; and (4) following treatment with the GPCR agonist exendin-4. Rgs16 and Rgs8 are sensitive reporters of conditions that stimulate the early stages of islet progenitor cell differentiation and β-cell expansion in development and disease.

RESULTS

Rgs8/16 gene expression in endocrine progenitors during development

Two separate lines of GFP-expressing BAC transgenic mice (Fig. 1A,B) (Gong et al., 2003) were used to determine the expression of the tandemly duplicated Rgs16 and Rgs8 genes during pancreatic development. Both Rgs16::GFP and Rgs8::GFP were expressed throughout embryonic and neonatal pancreas development (Fig. 1C,D; Fig. 2 and Table 1).

Rgs16::GFP was expressed throughout the early gut tube endoderm (E8.5) from the foregut to the tip of the open hindgut (Fig. 2A and data not shown) and became restricted to the early liver and dorsal pancreatic bud epithelium after embryonic turning (E9.5-10.5) (Fig. 2B,C). During dorsal bud outgrowth, Rgs16 was expressed in a punctate pattern within the pancreatic epithelium in a subpopulation of cells known to contain mostly epithelial and endocrine cell types (Fig. 2C-E). In postnatal stages, Rgs16 expression became restricted to aggregates of endocrine cells forming the islets of Langerhans (Fig. 2F).

In contrast to the broad distribution of early Rgs16::GFP expression in the endoderm, Rgs8::GFP expression was initially localized to a distinct dorsal patch in a region fated to give rise to the pancreatic endoderm (E8.5) (Fig. 2G) (Wells and Melton, 1999). This striking expression was initiated prior to any cellular or molecular evidence of pancreas specification, such as expression of Pdx1/lpf1 or Ngn3 (Villasenor et al., 2008). By E9.5, Rgs8 expression, like Rgs16, became largely restricted to the forming pancreatic bud (Fig. 2H,I). Later, Rgs8 was expressed in a pattern that was almost identical to that of Rgs16, in scattered clusters of cells in the central region of the developing bud (Fig. 2J), but was also weakly present in the exocrine pancreas (Fig. 2K,L). Overall, both patterns of expression were consistent with endocrine cell distribution until postnatal stages (Fig. 2K,L).

Because the spatiotemporal distribution of Rgs16- and Rgs8-expressing cells was reminiscent of endocrine precursors, their expression was compared with that of Ngn3, a well-characterized endocrine progenitor marker (Gradwohl et al., 2000). Rgs16 and
Rgs8 were expressed in a very similar pattern to Ngn3 throughout development and until approximately 2 weeks postnatal, when all three genes were extinguished in islets. However, the Rgs::GFP genes were expressed earlier than Ngn3::GFP (Fig. 2A,G,M) (see also Villasenor et al., 2008). Outside the pancreatic bud, endodermal Rgs8/16 and Ngn3 expression diverged, with Rgs16::GFP in the budding liver (Fig. 2B), Rgs8::GFP in the adjacent dorsal endoderm (Fig. 2H) and Ngn3::GFP faint in the posterior duodenal endoderm (Fig. 2O).

In contrast to Rgs8::GFP and Ngn3::GFP, both of which were extinguished shortly after birth, Rgs16::GFP remained strong in scattered cells along veins, ducts and arteries for the first 3-4 weeks of postnatal development (see below). Rgs16::GFP expression was on average about twofold longer than that of Rgs8::GFP. Endogenous Rgs16 mRNA was also about twofold more abundant than Rgs8 mRNA in E14.5 pancreas [quantitative PCR (qPCR); data not shown]. Therefore, further investigation was focused on Rgs16::GFP expression during development and in adult models of islet regeneration/expansion.

Rgs16::GFP in embryonic pancreatic endocrine cells
To determine whether endocrine cells within the pancreas expressed Rgs16, the co-expression of Rgs16::GFP and endocrine cell markers was analyzed (Fig. 3). We examined Rgs16 expression at E8.75 through to E10.5, as the bud first evaginates and the first transition endocrine cells emerge, and at E15.5 during the rapid wave of endocrine expansion and differentiation called the 'secondary transition'. Rgs16::GFP was expressed initially in most...
Following the secondary transition at E15.5, Rgs16::GFP expression was largely excluded from the tubular epithelium. Most Rgs16::GFP-positive (Rgs16::GFP+) cells co-expressed synaptophysin and islet hormones in delaminating endocrine cells (Fig. 3K-M). Only a small number of Rgs16::GFP+ cells co-expressed either E-cadherin (3.5%) or Sox9 (5%) in the epithelium (Fig. 3F,G; supplementary material Fig. S2A,B and Table S1 and data not shown). Interestingly, Rgs16::GFP was never co-expressed with Ngn3 (Fig. 3H; supplementary material Fig. S2C and Table S1).

In contrast to the lack of overlap with Ngn3, we found that 53% of Rgs16::GFP co-localized with Pdx1 (Fig. 3I; supplementary material Fig. S2H and Table S1), a pancreatic progenitor marker that also partially overlaps with insulin expression at E15.5 (Offield et al., 1996). Rgs16::GFP co-localized with other markers of endocrine cell fate, such as 52% with Nkx6.1 and 97% with synaptophysin (Fig. 3J,K; supplementary material Fig. S2D and Table S1). Rgs16::GFP was co-expressed with terminal endocrine differentiation markers such as glucagon and insulin (Fig. 3L,M and supplementary material Fig. S2E-G), indicating that Rgs16 is expressed in islet lineages. Consistent with these expression patterns, greater than 98% of Rgs16::GFP+ cells were found to be Ki67 negative at E15.5, indicating that, at this stage, the vast majority of Rgs16::GFP-expressing cells were non-replicating (only 1.45% were Rgs16+/Ki67+) (supplementary material Fig. S3 and Table S1, and data not shown). As expected, Rgs16 expression did not co-localize with amylase-positive cells in acinar tissue (Fig. 3N).

Rgs16::GFP co-localized with markers of pancreatic ducts (Fig. 5A) and lymphatics (LYVE-1) (Fig. 5E). In addition, VDACs were often in close proximity to developing islets expressing synaptophysin (Fig. 5D) and, later, differentiation markers such as insulin (Fig. 5E). Indeed, Rgs16::GFP+ cells were often observed around the periphery of islets or along duct/vessels triads in proximity to islets (supplementary material Fig. S10).

Table 1. Expression of Rgs16::GFP in embryonic and postnatal pancreas

<table>
<thead>
<tr>
<th>Stage</th>
<th>Islet (Rgs16::GFP expression)</th>
<th>VDACS (Rgs16::GFP expression)</th>
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<tbody>
<tr>
<td>E8.5</td>
<td>+/-</td>
<td>NA</td>
</tr>
<tr>
<td>E8.75-9.0</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>E9.5-16.5</td>
<td>+++</td>
<td>NA</td>
</tr>
<tr>
<td>P0</td>
<td>+++</td>
<td>NA</td>
</tr>
<tr>
<td>P1</td>
<td>+++</td>
<td>NA</td>
</tr>
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<td>P3</td>
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<td>NA</td>
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<td>P7</td>
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<td>P10</td>
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<td>P11</td>
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<td>P14</td>
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<td>++</td>
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<td>P15</td>
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<td>++</td>
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<tr>
<td>P16-30</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Adult (2 months)</td>
<td>–</td>
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<td>Adult (3 months)</td>
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Arbitrary expression levels were assigned by independent visual analysis of relative intensity of fluorescence (two observers). Live dissected pancreatic tissue was examined in PBS, under fluorescence microscopy. P, pancreas-stage postnatal day. Expression could be detected either in forming islets (middle column) or within scattered cells along ducts and blood vessels (VDACs, right column); NA, not applicable because strong expression in islets at these stages masks potential VDAC expression; (−) absent; (+) present; (++)) medium; (+++) strong.

To elucidate the identity of Rgs16-expressing VDACs, a range of endocrine markers of early versus late endocrine differentiation were examined. A small proportion of VDACs (~5%) expressed the earliest endocrine progenitor marker Sox9 (Fig. 5F), but none expressed later markers of endocrine differentiation such as synaptophysin, insulin, glucagon, Glut2, somatostatin, PP or ghrelin (supplementary material Fig. S4 and data not shown). Furthermore, VDACs did not express markers of vascular endothelial, smooth muscle or macrophage lineages (supplementary material Fig. S5). Some Rgs16+ VDACs are therefore similar to early progenitor cells in the epithelium in their expression of Sox9 and all are distinct from delaminating embryonic Rgs16+ endocrine cells (Fig. 3C-F).
To determine whether Rgs16-expressing cells re-emerge in the pancreas of metabolically stressed mice, the pancreata of pregnant females were examined at different times during gestation. Islet mass in females expands during pregnancy (Van Assche, 1978). Although Rgs16::GFP expression was never observed in normal adult pancreas, either within islets or as VDACs, Rgs16 was re-expressed along vessel and duct tracts in mid- to late-gestation female pancreas (supplementary material Fig. S5). Indeed, starting at approximately 8 days of gestation, rare GFP+ cells could be readily observed along the central axes of lateral pancreatic branches (supplementary material Fig. S5A), and increasing numbers of VDACs were present between E10.5-15.5 along many different branches (supplementary material Fig. S5B-E). Rgs16::GFP expression was coincident with the initiation of a known phase of β-cell expansion in pregnant females (Gupta et al., 2007), but then faded between E16.5 and E18.5 (supplementary material Fig. S5F; data not shown). Between these stages, all pregnant females expressed Rgs16::GFP in VDACs but only one expressed Rgs16::GFP in the islets. By contrast, lactating females did not express Rgs16::GFP in either VDACs or islets (data not shown). Rgs16::GFP expression along the ducts in pregnant females suggests that increased maternal metabolic demands might be conveyed by GPCR signaling and regulated by RGS proteins.

Rgs16::GFP re-activated in islets of regenerating β-cells of type 1 diabetic mice

Given the correlation of Rgs16 expression with β-cell proliferation, both in the embryo and pregnant adults, we asked whether Rgs16::GFP might also be re-expressed in islets during β-cell regeneration. Recently, islet regeneration was shown to occur following ablation in PANIC-ATTAC (pancreatic islet β-cell
apoptosis through targeted activation of caspase 8) mice (Wang et al., 2008). In this model of type 1 diabetes, pancreatic β-cells were targeted for cell death by the regulated expression of an FKBP-caspase 8 fusion protein, resulting in hyperglycemia. To assess the expression of Rgs16 during islet regeneration, the Rgs16::GFP transgene was crossed into the background of the PANIC-ATTAC mice. Following β-cell apoptosis, Rgs16::GFP was co-expressed with insulin in a subset of pancreatic β-cells during the first two weeks of islet regeneration (Fig. 6). The percentage of Rgs16::GFP+ cells was higher in hyperglycemic mice with more severe hyperglycemia and islet destruction than in mice with moderate glucose levels (Fig. 6A,B; supplementary material Fig. S6). Rgs16 was specifically expressed in β-cells, as assessed by either insulin (Fig. 6A,B) or Glut2 co-staining (Fig. 6F), and was not observed in other endocrine cell types, such as α-cells (Fig. 6C). Rgs16::GFP was also not expressed in the pancreas of either parental PANIC-ATTAC (Fig. 6D) or normoglycemic Rgs16::GFP;PANIC-ATTAC mice (Fig. 6E; supplementary material Fig. S6A,B,E).

A time course study showed that hyperglycemia (>300 mg/dl) appeared in about half the males during the first three to five days of FKBP-ligand injection (supplementary material Fig. S6A,B). Rgs16::GFP was induced by chronic, but not acute, hyperglycemia. For example, Rgs16::GFP was not visible in day 5 hyperglycemic mice (n=2) and only first appeared by day 7 in VDACs and a few cells within one or a small cluster of neighboring islets (supplementary material Fig. S6E, panel a). Rgs16::GFP expression expanded to more cells in more islets throughout the pancreas over a 50-day interval of β-cell regeneration (supplementary material Fig. S6D,E). Of note, if the blood insulin was at least 0.5 ng/ml, the day 5 blood glucose levels were good predictors of later Rgs16::GFP expression (supplementary material Fig. S6B-D).

Interestingly, the Rgs16::GFP+ cells in this model of β-cell regeneration displayed a higher proliferative capacity than the...
Rgs16::GFP+ cells at E15.5 during embryogenesis (supplementary material Table S2). Over 5% of Rgs16::GFP+ cells in the PANIC-ATTAC mice incorporated bromodeoxyuridine (BrdU), compared with the surrounding Rgs16-negative endocrine cells, which showed a rate of 0.98%. These data indicate that Rgs16 expression is found in adult regenerating islets within the proliferative β-cell compartment.

Rgs16::GFP re-activated in pancreas of ob/ob hyperglycemic mice

β-Cell proliferation also occurs in obese, diabetic ob/ob mice, a model of type 2 diabetes (Chua et al., 2002; Prentki and Nolan, 2006). Therefore, Rgs16::GFP expression was assessed in normal and hyperglycemic ob/ob mice (Fig. 7). Rgs16::GFP expression in ob/ob mice correlated with chronic increases in both blood glucose and insulin (Fig. 7A–C; supplementary material Fig. S7). Consistent with this observation, Rgs16::GFP+ cells were never found in the pancreas of 4–5-week-old Rgs16::GFP;ob/ob mice, which had near-normal or recently elevated levels of glucose and insulin (supplementary material Fig. S7D, panel a; and data not shown). In the early phase of induction, especially in mice with high levels of either glucose or insulin and a modest elevation of the other, Rgs16::GFP was expressed in VDACs (supplementary material Fig. S10) and in a few cells in a small cluster of neighboring islets (supplementary material Fig. S7D, panels b,c,g). The expression expanded in islets throughout the pancreas as mice became both hyperglycemic and hyperinsulinemic (supplementary material Fig. S7D, panels b,c,g).
was similar to that observed in the islets of Rgs16::GFP;PANIC-ATTAC mice. As anticipated, Rgs16::GFP+ islet β-cells were found in close association with islet capillaries (Fig. 7G) and ducts (Fig. 7H). Additionally, Rgs8::GFP was similarly induced specifically in the islets of hyperglycemic and hyperinsulinemic ob/ob mice (supplementary material Fig. S8).

In older ob/ob mice with high glucose and insulin levels (as in Fig. 7C and supplementary material Fig. S7C,D, panel k), Rgs16::GFP expression was observed in up to half of the pancreatic islets (supplementary material Fig. S9), each containing 1-5% Rgs16::GFP+ β-cells (in the three animals counted, 78% of ob/ob islets expressed Rgs16 in 0.5-5% of total endocrine cells, 17% expressed Rgs16 in 6-20% of total cells and 5% expressed Rgs16 in 21-50% of total cells). The majority of Rgs16::GFP+ islets were found along the central region of the main axis of the pancreas (Fig. 7C) but Rgs16::GFP expression appeared in islets of all sizes throughout the pancreas (supplementary material Fig. S7D).

Although β-cells in islets of ob/ob mice are known to proliferate (Bock et al., 2003), relatively modest numbers of Ki67+ cells were observed in hyperglycemic animals (36% of the ob/ob islets had 0.5-1% Ki67+ cells, 59% had 1-3% Ki67+ cells and about 5% had 3-5% Ki67+ cells). In addition, Rgs16::GFP+ β-cells of the islets of older ob/ob mice did not display a more highly proliferative state than Rgs16::GFP+ β-cells (supplementary material Table S3). Indeed, Rgs16-expressing cells co-stained for Ki67 at the same rate as the surrounding β-cells, at slightly over 1% (supplementary material Table S3). The total number of β-cells expressing Rgs16::GFP declined in the pancreas of ob/ob mice as glycemia was lowered following initial β-cell expansion, then increased with hyperinsulinemia in older ob/ob mice (supplementary material Fig. S7).

Exendin-4 induces Rgs16::GFP in adult pancreas

Finally, to assess Rgs16::GFP expression using another model of β-cell proliferation, we stimulated β-cell neogenesis and islet expansion by injection of Rgs16::GFP transgenic mice with exendin-4, a known GPCR agonist (Tourrel et al., 2001). Within 3 days of twice-daily injections of exendin-4 plus glucose, Rgs16 expression was induced in some VDACs but rarely, if at all, in islets (Fig. 8A,B). Later, by the sixth day of injection, Rgs16::GFP expression was observed in more VDACs but rarely, if at all, in islets (Fig. 8A,B). As seen previously in PANIC and ob/ob mice, Rgs16 expression first appeared in a small cluster of neighboring islets (Fig. 8G,H). Rgs16::GFP+ islets were also observed in mice injected daily with exendin-4 alone (three of five mice). As seen previously in PANIC and ob/ob mice, Rgs16 expression was induced in some VDACs but rarely, if at all, in islets (Fig. 8A,B). By contrast, expression was not observed in islets in mice injected with glucose alone or PBS (Fig. 8G and supplementary material Fig. S11). These results confirmed that Rgs16::GFP can be used as a reporter of GPCR-stimulated β-cell neogenesis in the pancreas.

DISCUSSION

In this study, we introduce regulators of G protein signaling as genetic beacons of β-cell expansion during development and disease. Rgs16 and Rgs8 are expressed in pancreatic progenitor and endocrine cells during development, then extinguished in adults, but reactivated in models of both type 1 and type 2 diabetes. Furthermore, Rgs16 expression is activated in response to exendin-4, a GPCR agonist that is used to stimulate β-cell expansion in type
2 diabetes (Xu et al., 1999; Tourrel et al., 2001; Fineman et al., 2003; Kendall et al., 2005; Kodama et al., 2005; Xu et al., 2006; Chu et al., 2007; Sherry et al., 2007; Wang et al., 2008). Based on their endocrine expression and known biochemical activities, Rgs16 and Rgs8 are likely to have redundant functions in the endocrine pancreas and may promote β-cell differentiation and maturation, although definitive functional characterization awaits simultaneous deletion of both genes in β-cells. Rgs16::GFP and Rgs8::GFP transgenic mice are unique tools for comparative analysis of the endocrine pancreas during β-cell expansion/regeneration in development, disease and response to therapy.

Rgs8/16::GFP expression in embryonic pancreas

Using Rgs8/16 BAC transgenic reporter lines, we assessed expression during key time points of pancreas development. Our results showed that, similar to Pdx1 and Ngn3 (Kim and MacDonald, 2002; Villasenor et al., 2008; Cleaver and MacDonald, 2009), Rgs16::GFP and Rgs8::GFP are first observed throughout the pancreatic epithelium, but later become restricted to β-cells. Both Rgs16::GFP and Rgs8::GFP are first expressed in the ‘proto-differentiated’ early pancreatic epithelium at E8.75, when the essential progenitor cells are set aside for later pancreas development (Offield et al., 1996; Zhou et al., 2007; Cleaver and MacDonald, 2009). At this early developmental stage, Rgs16::GFP is co-expressed with the pancreatic progenitor marker Sox9 in most multipotent progenitors. Interestingly, Rgs16::GFP and Rgs8::GFP are induced in the pancreatic bud in this early epithelium prior to Ngn3::GFP, a well-characterized marker of pancreatic endocrine progenitors (Villasenor et al., 2008).

With the onset of endocrine differentiation, Rgs16::GFP expression becomes restricted to endocrine cells. By E15.5, Rgs16::GFP expression is predominantly restricted to delaminated and/or differentiated endocrine cells that co-express synaptophysin, but not Ngn3. These Rgs16::GFP+ endocrine cells are primarily non-replicating, as less than 1.5% of Rgs16::GFP+ cells are Ki67+. Given these data, Rgs16 may promote β-cell differentiation and/or inhibit cell cycle re-entry in delaminating endocrine cells.

During development, Rgs16 expression identifies transient cell states, an earlier progenitor state, and a later state that anticipates and initiates hormone expression. The two ‘waves’ of Rgs16 expression, in the early pancreatic epithelium and later endocrine lineages, are similar to the ‘biphasic’ expression of pancreatic genes, such as Pdx1, Hhex9 and Ngn3, before and after the secondary transition (Pictet and Rutter, 1972; Offield et al., 1996; Li and Edlund, 2001; Villasenor et al., 2008; Cleaver and MacDonald, 2009).

For example, Pdx1 expression is first observed throughout the pancreatic epithelium and its derivatives, but after birth it becomes restricted to differentiating islets (Offield et al., 1996). The qualitatively different expression domains for Rgs16 may signify temporal regulation within a single cell lineage or expression in two separate lineages, the first appearing in the early epithelium of the pancreatic bud and the other following transient expression of Ngn3.

Rgs8/16::GFP expression in models of diabetes

Despite the strong expression of Rgs16 and Rgs8 throughout embryonic and neonatal isletogenesis, their expression ceases after 4 weeks of age in endocrine pancreas under normal conditions. However, both genes were re-expressed in models of β-cell expansion or regeneration, suggesting that these genes are likely to play a role in either endocrine proliferation, differentiation, or in response to chronic changes in glucose metabolism. The absence of RGS::GFP expression in normal adult pancreatic endocrine cells suggests that, if Rgs16 and Rgs8 have a physiological role in adult islets, low basal levels are sufficient to regulate daily postprandial insulin release and glucose homeostasis. Dietary approaches to alter blood glucose and metabolism, such as high-fat or high-disaccharide diets, or twice-daily intraperitoneal injections of glucose, failed to induce Rgs8/16 expression in otherwise-normal pancreatic islets (data not shown). However, the pancreatic expression of Rgs16 in pregnant females during midgestation, in exendin-4-treated mice, and in the islets of hyperglycemic type 1 and type 2 diabetic mice suggests that Rgs16 is a biomarker for cells that have reactivated an aspect of the embryonic β-cell program during adult β-cell proliferation/regeneration in response to glucose stress.

Rgs16::GFP becomes re-expressed in hyperglycemic PANIC-ATTAC and ob/ob mice during the early phase of β-cell regeneration, indicating that Rgs16 may regulate β-cell replication during compensatory islet expansion. In these two models, the pattern of expression is strikingly similar during disease progression and recovery. Hyperglycemia precedes Rgs16 induction in both PANIC and ob/ob mice (supplementary material Figs S6 and S7, respectively). This lag in expression suggests that Rgs16 is not directly induced in the pancreas by elevated blood glucose, but by physiologic responses to chronic glucose stress. Elevated insulin may contribute to increased Rgs16::GFP expression but is not sufficient, as some young hyperinsulinemic ob/ob mice express little or no Rgs16::GFP (supplementary material Fig. S7). However, combined hyperglycemia and hyperinsulinemia (and severe obesity) in older ob/ob mice are strongly correlated with Rgs16::GFP expression (supplementary material Fig. S7).

Another striking similarity during disease progression is Rgs16 distribution. In both mouse models of diabetes, Rgs16 expression is first observed in the β-cells of one or a small cluster of neighboring islets. As the disease progresses, Rgs16::GFP expression expands to more cells, within more islets, throughout the pancreas (supplementary material Tables S2 and S3), reflecting expression during embryogenesis and neonatal isletogenesis (supplementary material Table S1). This progressive and heterogeneous expression of Rgs in islets during β-cell expansion in PANIC and ob/ob mice
reflects the known heterogeneous response to hyperglycemia and early diabetes (Atkinson and Gianani, 2009).

Rgs16 has been suggested to inhibit β-cell expansion during postnatal development and in ob/ob mice (Poy et al., 2009). Indeed, Rgs16 and Rgs8 feedback may inhibit the GPCR signaling that initiates β-cell expansion. Endogenous Rgs16 and Rgs8 genes are similarly induced in the islets of 10-week-old ob/ob mice in both C57BL/6 and BTBR genetic backgrounds (Keller et al., 2008), where β-cell expansion occurs in the C57BL/6 mice but not in BTBR mice. Blood insulin levels are elevated in both backgrounds of mice, but modestly in ob/ob BTBR mice (to about 10 ng/ml) in a constrained effort to manage insulin resistance and hyperglycemia. This indicates that BTBR islets perceive chronic glucose stress and induce Rgs16 and Rgs8, even though they fail to undergo β-cell expansion. These signals may be transmitted within the local environment of responding islets and augment cellular responses to elevated glucose and insulin (Llona, 1995). Feedback inhibition by Rgs16 (and Rgs8) could promote the cell cycle exit or prevent the cell cycle re-entry of maturing β-cells that are still exposed to mitogens during diabetic islet expansion.

Rgs16::GFP a biomarker of GPCR stimulation

Exendin-4 is an incretin mimetic prescribed for type 2 diabetes to improve glucose homeostasis by suppressing glucagon secretion, stimulating insulin release and promoting β-cell expansion (Xu et al., 1999; Tourrel et al., 2001; Kodama et al., 2005; Bond, 2006; Lee et al., 2006; Sherry et al., 2007). Exendin-4 is a protease-resistant homolog of mammalian Glp-1, and both are agonists of the Glp-1 receptor (Glp-1R). Agonist binding evokes Gs-mediated increases in intracellular cAMP levels, promotes mitogenic and anti-apoptotic signals, and activates c-fos and other early response genes (Lee and Nielsen, 2009). However, RGS proteins are not Gs-GAPs (Berman 1996). Therefore, Glp-1R either activates Gi or Gq proteins as well, or stimulates the synthesis or release of other agonists of Gi/q-coupled GPCRs in the pancreas, which would be regulated by Rgs16 and Rgs8 (Wang et al., 2006).

Daily injection of exendin-4 induced Rgs16::GFP expression in the pancreas. Rgs16::GFP expression was initiated in VDACs, whereas β-cell expression was observed in islets by day 6. Interestingly, Glp-1R is specifically expressed in pancreatic ducts and islet β-cells, and exendin-4 was reported to promote the emergence of insulin-positive cells in cultured pancreatic ducts (Xu et al., 2006; Torrehave et al., 2008). The pattern of Rgs16::GFP induction by exendin-4 is strikingly similar to the early phase in PANIC and ob/ob mice, in which small numbers of VDACs appear transiently, and islet expression is first seen in one or a few closely clustered islets (supplementary material Fig. S10). Although speculative, the location of VDACs along pancreatic ducts, and their appearance early in the time course of the hyperglycemic response in mouse models of diabetes or in exendin-4 treatment, raises the alluring possibility that these represent an endocrine progenitor cell type. The contribution of endocrine progenitors within adult ducts to β-cell regeneration is controversial. Future lineage-tracing experiments will be required to conclusively determine the endocrine potential of Rgs16+ VDACs.

The induction of Rgs genes in VDACs and β-cells implies regulation of endogenous GPCRs that are important in the process of β-cell differentiation during development and β-cell regeneration in models of metabolic stress and disease. Many GPCRs are expressed in mouse islets but their functions are unknown (Regard et al., 2007). Furthermore, GPCRs that are expressed in embryonic endocrine progenitors, such as the Gq-coupled receptor Gpr56 (Gu et al., 2004) (www.genepaint.org), may be induced during β-cell expansion in diabetes. A subset of these GPCRs may be regulated by Rgs16 and/or Rgs8 during pancreas development and islet regeneration. A challenge is to identify additional mitogens that stimulate β-cell expansion. A practical approach would be to identify molecules that induce Rgs16 expression in surrogates of pancreatic ducts and/or endocrine progenitors, such as primary cultured cells from pancreatic ductal adenocarcinoma (Aguirre et al., 2003), ‘endocrine-differentiated’ embryonic stem (ES) cells (Kroon et al., 2008; Serafimidis et al., 2008), or induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006; Nakamura et al., 2009). Superlative candidate molecules could be identified by in vivo validation in Rgs16::GFP transgenic mice, as demonstrated here for exendin-4.

We propose that Rgs16 and Rgs8 are fetal pancreatic endocrine genes that have redundant functions during embryonic pancreas and β-cell development and, later in adults, during islet regeneration in type 1 diabetes (PANIC-ATTAC) and islet expansion in type 2 diabetes (ob/ob) (Herrera et al., 1994; Nir et al., 2007; Cano et al., 2008; Wang et al., 2008). Future work will identify the ligands and receptor-signaling pathways that are regulated by Rgs16 and Rgs8 in hyperglycemic type 1 and type 2 diabetic mice. These ligands, GPCRs and regulatory RGS proteins are likely to be important signaling molecules in the progression towards diabetes, and key targets for diabetes therapy.

METHODS

Green fluorescent protein (GFP) visualization in the pancreas from embryos, pups and adults

Rgs16::GFP and Rgs8::GFP BAC transgenic mice were generated by the Gene Expression Nervous System Atlas (GENSAT) project (Gong et al., 2003; Morales and Hatten, 2006). Pancreata were collected from Rgs16::GFP and Rgs8::GFP embryos (E8.5 through to E16.5) and from postnatal stages P0 (birth) to P28. Pups were weaned at P16 for time course studies of Rgs16::GFP expression post-weaning. Tissues were dissected and transferred into ice-cold 1× PBS buffer. GFP visualization was accomplished by removing the embryonic gut tube and isolating the midgut, including the pancreas, stomach and spleen. Tissue fragments were equilibrated in 40% glycerol for viewing. The pancreas was visualized using a Zeiss NeoLumar fluorescent microscope and photographed using an Olympus DP70 camera. Ngn3::eGFP embryos were generated by mating Ngn3::eGFP heterozygous males (generously provided by Klaus Kaestner) with CD1 females. Embryos were dissected at different developmental stages, and pancreata were dissected and visualized as described above. Pancreata from adult pregnant females and ob/ob;Rgs16::eGFP transgenic mice were treated similarly to those from embryos, as described above.

Immunofluorescence staining of frozen sections

E15.5 dorsal pancreata were dissected from Rgs16::eGFP pregnant females and were fixed overnight in 4% PFA in 1× PBS at 4°C. The next day the tissues were washed several times with 1× PBS,
equilibrated in 30% sucrose overnight and embedded in optimal cutting temperature (OCT) medium (Tissue-Tek). Cryosections (10 μm) of complete pancreata were mounted on SuperfrostPlus slides (Fisher) and immunofluorescence was carried out using: chicken anti-GFP (1:500; Aves Labs), guinea pig anti-insulin (1:300; DakoCy), rabbit anti-glucagon (1:1000; LINCO), rat anti-CD31 (1:300; BD Pharmingen), rabbit anti-synaptophysin (1:200; DakoCy), 5 μg/μl DBA (1:200; Vector Labs), rabbit anti-Pdx1 (1:600) and mouse anti-αngn3 (1:4000; Beta Cell Biology Consortium, kindly provided by Dr Raymond MacDonald). TRITC secondary antibodies were from Jackson ImmunoResearch Laboratories and anti-chicken Alexa 488 was from Invitrogen. Slides were counterstained with DAPI and mounted with ProLong Gold antifade reagent (Invitrogen). Images were acquired on a LSM 510 META Zeiss confocal microscope. For PANIC-ATTAC samples, histology and immunofluorescence were performed as described previously (Wang et al., 2008). Briefly, the pancreas was fixed in 10% buffered formalin overnight. Paraffin sections (5 μm) were incubated with guinea pig anti-insulin (1:500; DakoCy) and rabbit anti-GFP (1:100; Invitrogen). The secondary antibodies used were donkey anti-guinea pig FITC (1:250; Jackson ImmunoResearch) and donkey anti-rabbit Cy3 (1:500; Jackson ImmunoResearch). Images were taken on a Leica TCS SP5 confocal microscope (Leica).

**Immunofluorescence on paraffin sections**
E15.5 dorsal pancreata (Rgs16::eGFP) or adult pancreata from ob/ob;Rgs16::eGFP or PANIC-ATTAC;Rgs16::eGFP mice were dissected and fixed overnight in 4% PFA in 1× PBS at 4°C. The next day, the tissues were washed several times with 1× PBS, dehydrated and paraffin embedded in Paraplast Plus tissue embedding medium (McCormick). Sections (10 μm) of complete pancreata were mounted on SuperfrostPlus slides. Sections were de-waxed in xylene; rehydrated through an ethanol series; washed several times in 1× PBS; treated with R-Buffer A or R-Buffer B (Electron Microscopy Sciences) in the 2100 Retriever; blocked for 1-2 hours with CAS-Block (Invitrogen); and incubated with chicken anti-GFP (1:200), rabbit anti-sox9 (1:400; Chemicon), mouse anti-e-cadherin (1:200; Invitrogen), rabbit anti-glut2 (1:100; Abcam) and the four-hormones cocktail: guinea pig anti-glucagon (1:1000; kindly provided by Raymond MacDonald), guinea pig anti-insulin (1:600; DakoCy), goat anti-ghrelin (1:300; Beta Cell Consortium) and goat anti-somatostatin (1:300; Santa Cruz). TRITC secondary antibodies were from Jackson ImmunoResearch and anti-chicken Alexa 488 was from Invitrogen. Tissues were dehydrated, cleared with BABB (1:2 mix of benzyl alcohol:benzyl benzoate), and visualized using a Zeiss NeoLumar fluorescent microscope and photographed using an Olympus DP70 camera or LSM 510 META Zeiss confocal microscope.

**Quantification of rates of replication**
Immunofluorescence in paraffin sections of E15.5 dorsal pancreata (Rgs16::eGFP) or adult pancreata from ob/ob;Rgs16::eGFP and PANIC-ATTAC;Rgs16::eGFP transgenic mice were performed utilizing chicken anti-GFP (1:200) and either rabbit anti-Ki67 (1:200; Invitrogen) or rat anti-Brdu (1:50; Serotec). A total of eight fields of three different E15.5 embryonic pancreata, 70 islets of three different ob/ob;Rgs16::eGFP pancreata, and 13 islets for PANIC-ATTAC;Rgs16::GFP pancreata were studied. The total number of endocrine cells per islet/field, the total number of Ki67+ cells, the total number of Rgs16+ cells, and the total overlap between Ki67 and Rgs16 were all determined, and rates of replication were calculated.

**Immunofluorescence staining of frozen sections for VDACs**
P15 pancreata from Rgs16::eGFP mice were harvested and immediately embedded in OCT medium and frozen. Sections (10 μm) were cut and allowed to dry overnight at room temperature. Sections were then fixed for 5 minutes in ice-cold acetone and rinsed several times in 1× TBST (1× TBS supplemented with 0.2% Tween). Sections were then blocked in 20% Aquablock in 1× TBST, and stained using chicken anti-GFP (1:100) and one of the following primary antibodies: rabbit anti-sox9 (1:1000; Chemicon), guinea pig anti-insulin (1:200; DakoCy), rabbit anti-glucagon (1:300; LINCO), goat anti-somatostatin (1:300; Santa Cruz), rabbit anti-synaptophysin (1:300; DakoCy), rabbit anti-glut2 (1:200; Millipore), 25 μg/ml rat anti-MECA32 (Rolf Breken), 20 μg/ml rabbit anti-NG2 (Millipore), 20 μg/ml rat anti-Mac1 (AbD Serotec). Sections were stained overnight at 4°C then washed three times in 1× TBST. Bound primary antibody was visualized using appropriate Cy3- or FITC-conjugated secondary antibodies. Nuclei were visualized with DAPI. Images were taken using a Nikon Eclipse E600 microscope.

**Whole-mount immunofluorescence**
Pancreata were fixed for 1 hour in 4% PFA in PBS, washed and dehydrated to 70% ethanol. Embryos were then washed in 50% methanol for 1 hour and rinsed twice in 1× PBS. The tissue was permeabilized for 1 hour in 1% TritonX 100 in 1× PBS, blocked in Cas-Block (Zymed), and immunofluorescence was then carried out using: chicken anti-GFP (1:250; Aves Labs), guinea-pig anti-insulin (1:200; DakoCy), rabbit anti-glucagon (LINCO), rat anti-CD31 (1:200; BD Pharmingen), rabbit anti-synaptophysin (1:200; DakoCy), 5 μg/μl DBA (1:200; Vector Labs), and rabbit anti-LYVE (1:1000; Ambion). TRITC secondary antibodies were from Jackson ImmunoResearch and anti-chicken Alexa 488 was from Invitrogen. Tissues were dehydrated, cleared with BABB (1:2 mix of benzyl alcohol:benzyl benzoate), and visualized using a Zeiss NeoLumar fluorescent microscope and photographed using an Olympus DP70 camera or LSM 510 META Zeiss confocal microscope.

**ob/ob;Rgs16::eGFP and ob/ob;Rgs8::eGFP mice**
ob/+ breeder mice were obtained from Jackson Labs. Double heterozygous Rgs16::eGFP;ob/+ mice were intercrossed to obtain ob/ob, ob/Rgs16::eGFP and ob/+;Rgs16::eGFP mice. Animals were fed a standard rodent chow diet (Teklad) ad libitum. Blood was acquired through tail clipping. Glucose levels were measured by using a glucometer (AscensiaElite), and insulin levels were measured by using a rat/mouse insulin ELISA kit (Millipore) and read with a Sunrise microplater reader (Tecan).

**PANIC-ATTAC;Rgs16::eGFP transgenic mice**
PANIC-ATTAC transgenic mice were generated as described previously (Wang et al., 2008). Briefly, the rat insulin promoter was used to drive the expression of an FKBP-caspase 8 fusion protein. Homozygous PANIC-ATTAC animals were crossed to Rgs16::eGFP transgenic mice. All progeny were hemizygous for the PANIC-ATTAC transgene. At about 12 weeks of age, animals were grouped into hemizygous PANIC-ATTAC;Rgs16::eGFP transgenic mice (n=2), hemizygous PANIC-ATTAC;Rgs16::eGFP-negative mice (n=2), and heterozygous PANIC-ATTAC;Rgs16::eGFP-positive mice (n=2).
(n=4) and FVB control mice (n=3). The dimerizer AP20187 was administered to animals according to the manufacturer’s recommendations (Ariad Pharmaceuticals). For hemizygous PANIC-ATTAC mice, dimerizer (0.2 mg/g body weight) was injected either twice per day, every other day for eight days, or once per day at 12.00 h for five consecutive days. Fed glucose levels were monitored using a glucometer and strips (Abbott Diabetes Care). After five days treatment, hemizygous PANIC-ATTAC males showed either moderate (glucose <300 mg/dl) or severe (glucose >300 mg/dl) hyperglycemia. Animals were sacrificed two weeks later, with the pancreas processed for immunofluorescent staining (Fig. 6), or at the times indicated for analysis of GFP expression (supplementary material Fig. S6). Insulin levels were assessed as per ab/ob mice.

Exendin-4+/−, glucose or PBS treatment
Male mice aged 8-12 weeks (30-40 g) were injected, intraperitoneally, twice daily at zeitgeber time ZT6 (6 hours after lights on) and ZT11. Exendin-4 was injected at a concentration of 200 ng/mouse time point in a volume of 100 μl glucose at 4.5 g/kg, and PBS at a volume of 100 μl.

GFP quantification of exendin-4/glucose images
Original JPEG RGB images, at a resolution of 1360×1024 pixels, of mouse pancreas taken with a 48X objective magnification were converted into 8-bit gray-scale format without rescaling in ImageJ (NIH). Background levels with a rolling ball radius of 50 pixels were subtracted from images. Varying lower threshold adjustments were selected based on the contours of GFP+ regions. Integrated densities as the sum of 8-bit gray values, on a 0-255 scale per pixel, were obtained from each particle with a size of three pixels or greater. The sum of analyzed integrated densities as a total GFP value was calculated using MS Excel. Three-dimensional bar graphs correlating the levels of blood glucose, insulin, Rgs16::GFP expression in islets, and the age of ob/ob mice or the time after treatment in PANIC mice, were created by Matlab.

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COMPETING INTERESTS
The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS
O.C and T.M.W. conceived the project and, with A.V., Z.V.W., R.A.B. and P.E.S., designed the experiments. A.V., Z.V.W., L.B.R., O.O., I.W.A., O.C. and T.M.W. performed the experiments. All authors analyzed data and edited the manuscript. A.V., O.C. and T.M.W. wrote the manuscript.

SUPPLEMENTAL MATERIAL
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Rgs8/16 reactivated in expanding islets

TRANSLATIONAL IMPACT

Clinical issue
Diabetes exacts one of the highest annual costs for treatment of any disease in the world and is the seventh leading cause of death in the USA. Most cases of both type 1 and type 2 diabetes involve the actual or functional loss of insulin-producing pancreatic β-cells. Although β-cells can replicate, little is known about how the process is regulated and available therapies for diabetes cannot specifically regulator β-cell regeneration. There is hope that understanding the signals that control islet cell proliferation and differentiation will help generate targeted β-cell regenerative therapies for diabetic patients.

Results
This study shows that two members of the gene family regulators of G protein signaling (RGS), Rgs8 and Rgs16, coordinate β-cell expansion with metabolic need. Rgs8 and Rgs16 are expressed during islet development and in mouse models of type 1 and type 2 diabetes. Exendin-4, a Glp-1/incretin mimetic that stimulates β-cell expansion in diabetics, also promotes the expression of Rgs8/16::GFP in pancreatic ductal-associated cells and islet β-cells in mice. As the name implies, RGS members regulate the frequency and duration of G protein-coupled receptor (GPCR) signaling. Since GPCR pathways are also associated with β-cell expansion, this suggests that RGS proteins serve as sensitive and early beacons of G protein signaling in β-cell progenitor expansion and regeneration during development and in metabolically stressed adults.

Implications and future directions
This study indicates that the RGS proteins Rgs8 and Rgs16 coordinate GPCR signaling with islet development and, later in life, with metabolic stress. The early response of these proteins to metabolic changes suggests that they may be useful screening indicators. Furthermore, understanding the mechanisms that regulate GPCR induction of pancreatic β-cell proliferation may provide a crucial inroad to diabetes therapy. Future work should determine the potential of RGS proteins to promote β-cell development and islet regeneration.

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