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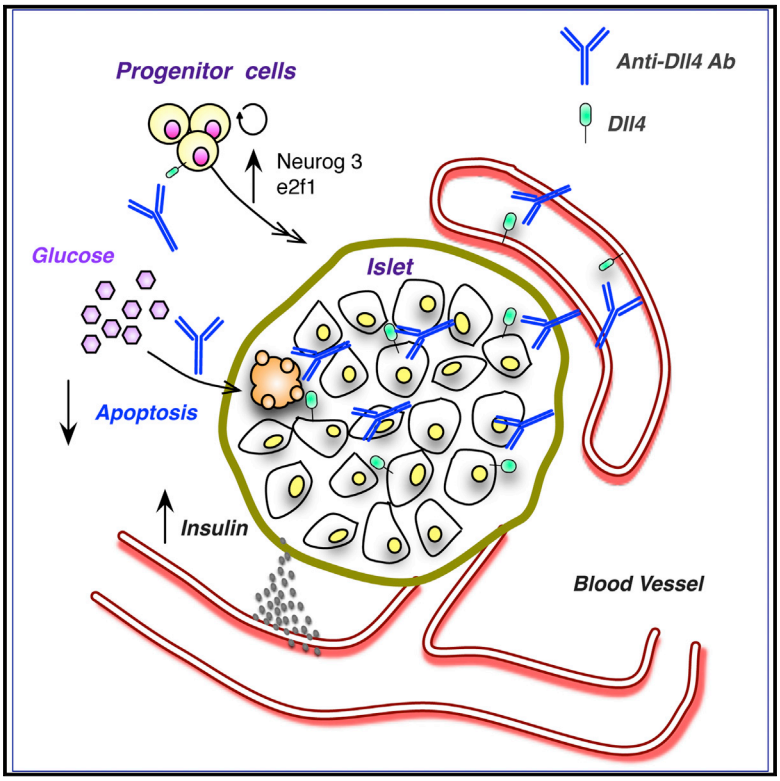
# Delta-like Ligand-4-Notch Signaling Inhibition Regulates Pancreatic Islet Function and Insulin Secretion

## Graphical Abstract

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## In Brief

The conserved Notch signaling pathway is required for adult tissue homeostasis. Billiard et al. show that Dll4-Notch signaling inhibition supports the health of β-islet cells and affects insulin production by driving differentiation of insulin-producing cell progenitors and blockade of islet apoptosis.

## Highlights

- Dll4-Notch signaling blockade preserves islets and reverses diabetes in NOD mice
- Inhibition of the Dll4 pathway restores β-islet cell function in STZ-treated mice
- Anti-Dll4 antibody enhances β-islet cell proliferation and differentiation
- Islet Dll4-blockade may provide a target in compromised insulin-producing states

## Data and Software Availability

PXD005333
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# Delta-like Ligand-4-Notch Signaling Inhibition Regulates Pancreatic Islet Function and Insulin Secretion

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## SUMMARY

**Although Notch signaling has been proposed as a therapeutic target for type-2 diabetes, liver steatosis, and atherosclerosis, its direct effect on pancreatic islets remains unknown. Here, we demonstrated a function of Dll4-Notch signaling inhibition on the biology of insulin-producing cells. We confirmed enhanced expression of key Notch signaling genes in purified pancreatic islets from diabetic NOD mice and showed that treatment with anti-Dll4 antibody specifically abolished Notch signaling pathway activation. Furthermore, we showed that Notch inhibition could drive proliferation of β-islet cells and confer protection from the development of STZ-induced diabetes. Importantly, inhibition of the Dll4 pathway in WT mice increased insulin secretion by inducing the differentiation of pancreatic β-islet cell progenitors, as well as the proliferation of insulin-secreting cells. These findings reveal a direct effect of Dll4-blockade on pancreatic islets that, in conjunction with its immunomodulatory effects, could be used for unmet medical needs hallmarked by inefficient insulin action.**

## INTRODUCTION

Notch, an evolutionary conserved signaling pathway, is important in regulating cell fate and lineage decisions in multiple tissues (Afelik and Jensen, 2013; Maillard et al., 2005; Radtke et al., 2010), and it has also been implicated in several neoplasias (Noguera-Troise et al., 2006; Thurston et al., 2007). Although Notch antagonists are in clinical testing against malignant tumors (Wolfe, 2009), emerging evidence indicates a critical role for Notch signaling in the maintenance of adult tissue homeostasis via its immune (Billiard et al., 2011) and metabolic effects (Pajvani et al., 2013; Pajvani et al., 2011). Along these lines, recent data showed that Notch inhibition modulates Foxo1-dependent gluconeogenesis, as shown by improvement of glucose tolerance in *L-Rbpj* mice (Pajvani et al., 2011). Furthermore, Notch signaling is considered as a therapeutic target for type-2 diabetes, liver steatosis, and atherosclerosis (Fowler et al., 2011; Valenti et al., 2013), whereas the direct effect of Notch signaling on pancreatic islets remains unknown. Here, we demonstrate a function of Dll4-Notch signaling inhibition on the biology of insulin-producing cells. We confirmed enhanced expression of key Notch signaling genes in purified pancreatic islets from non-obese diabetic (NOD) mice and demonstrated that treatment with anti-Dll4 antibody (anti-Dll4 Ab) (Billiard et al., 2011) specifically abolished Notch downstream signaling pathway activation, as shown by the inhibition of Notch-1 activation (Jarriault et al., 1995). In addition, we showed that administration of anti-Dll4 Ab could drive the proliferation of β-islet cells and confer protection from the development of streptozotocin (STZ)-induced diabetes. Importantly, inhibition of the Dll4 pathway in wild-type control mice drove differentiation of pancreatic β-islet cell progenitors and increased insulin secretion by inducing the proliferation of insulin-secreting cells. These findings reveal the direct effect of Dll4 blockade on pancreatic islets that, in conjunction to its immunomodulatory effects (Billiard et al., 2012), may provide a target in states of compromised insulin production.

Recent findings suggest a critical role for Notch signaling in homeostasis, whereas in disease models, inhibition of Notch signaling was found to induce a spectrum of protective responses to overcome pancreatic insult (Billiard et al., 2012; Fukuda et al., 2012; Valenti et al., 2013). Along these lines, our group demonstrated that *in vivo* administration of anti-Dll4 Ab inhibits the Notch signaling pathway and reverses established hyperglycemia in the NOD model of diabetes (Billiard et al., 2012). We attributed this effect to alternative differentiation of T cell

progenitors in the thymus leading to natural regulatory T (Treg) cell expansion two weeks after anti-Dll4 Ab treatment, thus promoting a tolerogenic environment essential for the maintenance of glucose homeostasis in NOD mice (Billiard et al., 2012). However, this observation could not explain the reversal of established hyperglycemia three days after anti-Dll4 Ab injection. Based on previous findings, we sought to characterize the specific contribution of the Dll4-Notch signaling pathway in the regulation of pancreatic islets functions under steady-state conditions and in mouse models of induced insulin insufficiency and islet toxicity.

## RESULTS

### Dll4-Notch Signaling Blockade Preserves Islets and Reverses Diabetes in NOD Mice

In this study, we hypothesized that the acute reduction of hyperglycemia by anti-Dll4 Ab in the NOD mouse model could be due to increased insulin availability and/or secretion. In NOD mice, a single injection of anti-Dll4 Ab rescued established hyperglycemia when the glucose level range was between 250 and 400 mg/dL (Figure 1A). Similarly, and in agreement with previously published findings (Billiard et al., 2012), this treatment resulted in reduced immune cell infiltration of the islets as compared with the control-treated group (Figure 1B). In contrast, the same anti-Dll4 Ab regimen had no effect on severe hyperglycemia (blood glucose levels >400 mg/dL) (Figure 1A). Most importantly, correction of hyperglycemia (blood glucose levels of 250–400 mg/dL) was associated with preserved insulin content in the remaining islets as revealed by positive c-peptide staining (Figure 1C).

Here, we sought to examine the role of Dll4 signaling in islets purified from the pancreas of diabetic NOD mice. We detected increased levels of mRNA expression of key components (*HEY1, NOTCH 1, HES1,* and *NRARP*) of the Notch signaling pathway in pancreatic islets, isolated from diabetic NOD mice, in comparison with islets from non-diabetic control mice (Figure 1D). In agreement with this result, we demonstrated that pancreatic islets purified from NOD mice previously treated with the isotype control antibody (Ab), showed enhanced Notch signaling activation as indicated by western blot using an Ab against V1744, a reagent identifying an epitope that is revealed on gamma-secretase cleavage of Notch-1 (NICD) (Hori et al., 2013) (Figure 1E). Importantly, we found that anti-Dll4 Ab treatment of NOD diabetic mice inhibited Notch-1 activation on purified pancreatic islets (Figure 1E). The latter provides a direct link between diabetes-induced Notch signaling activation and the specificity of anti-Dll4 Ab in reversing this effect. Further, we cannot exclude a potential implication of infiltrating immune cells in diabetes remission. The above findings raised the hypothesis for a physiological role of the Dll4-Notch signaling pathway in pancreatic islet function.

### Anti-Dll4 Ab Administration Prevents the Development of Diabetes in STZ-Treated Mice and Restores β-Islet Cell Function

To assess the ability of anti-Dll4 Ab to control hyperglycemia in a non-immune model of diabetes due to induced islet destruction, we administered anti-Dll4 Ab to wild-type (WT) C57BL/6 mice before administration of STZ. This treatment controlled the development of hyperglycemia over time (Figure 2A) and increased glucose clearance, as demonstrated by the intraperitoneal glucose tolerance test (IP-GTT) (Figure 2A). In anti-Dll4-Ab-treated mice (Figure S1A), insulin levels during IP-GTT were only slightly increased at 30 min (no statistical significance), possibly due to the low basal insulin levels and the limits in assay sensitivity. This prophylactic anti-Dll4 Ab administration protected the islets from STZ-induced immune cell infiltration (Figure 2B) and inhibited the associated apoptosis as assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Figure 2C). Importantly, prophylactic anti-Dll4 Ab treatment improved the circulating insulin levels following exposure to STZ (Figure 2D). Interestingly, the administration of dibenzazepine (DBZ), a γ secretase inhibitor (γGSI) that blocks the activation of the Notch pathway, had no effect on glucose tolerance (Figure S1 and Petersen et al., 2015). This finding suggests that simultaneous inhibition of all the components of Notch signaling (as by DBZ) is not effective in glucose regulation under these specific conditions. The similar blood glucose levels in control-Ab-treated and anti-Dll4-Ab-treated mice three weeks post-STZ administration (Figure S1C) suggest that prophylactic anti-Dll4 Ab administration is not sufficient to preserve pancreatic islet function after STZ exposure.

To assess the ability of chronic treatment with anti-Dll4 Ab to protect pancreatic islet function, such as over the course of STZ-induced diabetes, we extended the prophylactic anti-Dll4 Ab treatment for a period of six weeks following STZ administration (Therapeutic Protocol). Indeed, prolonged anti-Dll4 Ab treatment rescued the pancreatic islets from the deleterious effects of STZ, as reflected in the corresponding glucose and insulin levels six weeks following STZ administration (Figures 2E and 2F). In agreement with the finding described above (Figure S1B), DBZ administration did not have any effect, as compared with the anti-Dll4-Ab-treated group (Figures 2E and 2F). Consistently, anti-Dll4-Ab-treated mice presented with increased number of islets (Figure 2G), as well as bromodeoxyuridine (BrdU)-positive cells within the islets (Figure 2H) as compared with the STZ control-Ab-treated mice. Further, anti-Dll4 Ab treatment prevented STZ-induced apoptotic islet cell death as shown by caspase-3 staining (Figure 2I). DBZ treatment had no effect on the proliferation or survival of pancreatic islets of STZ-exposed mice (Figures 2H and 2I). These findings provide the first *in vivo* evidence for a protective role of anti-Dll4 Ab in STZ-induced injury of mature insulin-producing β-islet cells.

### Anti-Dll4 Ab Is Involved in Homeostatic Regulation of Pancreatic Insulin Processing

To test the hypothesis that Dll4-Notch signaling may also be involved in the steady-state regulation of pancreatic function, we treated WT C57BL/6 mice on a normal diet (ND) with anti-Dll4 Ab for 10 weeks. This treatment had no effect on body weight or adiposity as assessed from the different fat depots of the mice (Figures S2A and S2B). Similarly, anti-Dll4 Ab did not alter the energy expenditure or the respiratory ratio of the treated mice (Figures S2C and S2D). Further, treated mice had

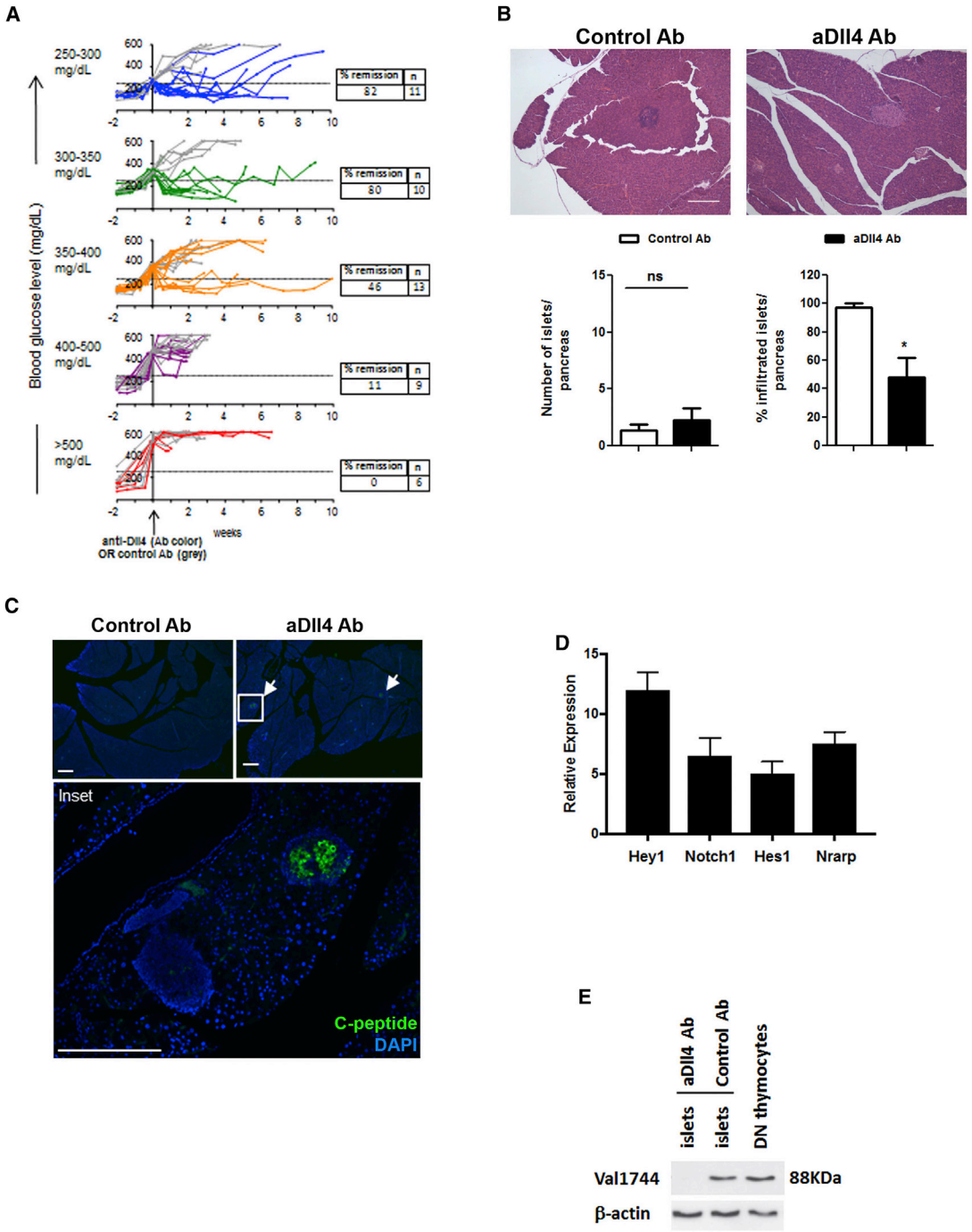


**Figure 1. Dll4-Notch Signaling Blockade Preserves Islets and Reverses Diabetes in NOD Mice**

(A) Blood glucose levels of NOD mice treated with one injection of anti-Dll4 Ab or control Ab at the onset of diabetes (week = 0).

(B) Upper: H&E staining of the pancreata of NOD mice treated with anti-Dll4 Ab (scale bar, 200 μm). Lower: Islet abundance in the pancreas (left) and percentage of infiltration per individual islet (right) (n = 10 mice per group).

(C) C-peptide staining of pancreas sections from anti-Dll4-treated (right) or control-Ab-treated (left) NOD mice. Four to six samples per group were analyzed. A representative staining from mice injected when their blood glucose level reached 250 and 350 mg/dL. The control mice's blood glucose level exceeded 500 mg/dL, and anti-Dll4-Ab-treated mice showed remission of diabetes at time of sacrifice (scale bar, 200 μm).

(D) Relative expression of key Notch signaling pathway genes in diabetic versus non-diabetic NOD mice. Anti-Dll4 Ab treatment inhibits Notch-1 activation on purified islets from NOD diabetic mice.

(E) Purified double-negative (DN) thymocytes from WT C57BL/6 mice are used as a positive control.

Data are expressed as mean ± SEM. *$p < 0.05$ indicates significant differences between groups as determined by Student's t test.

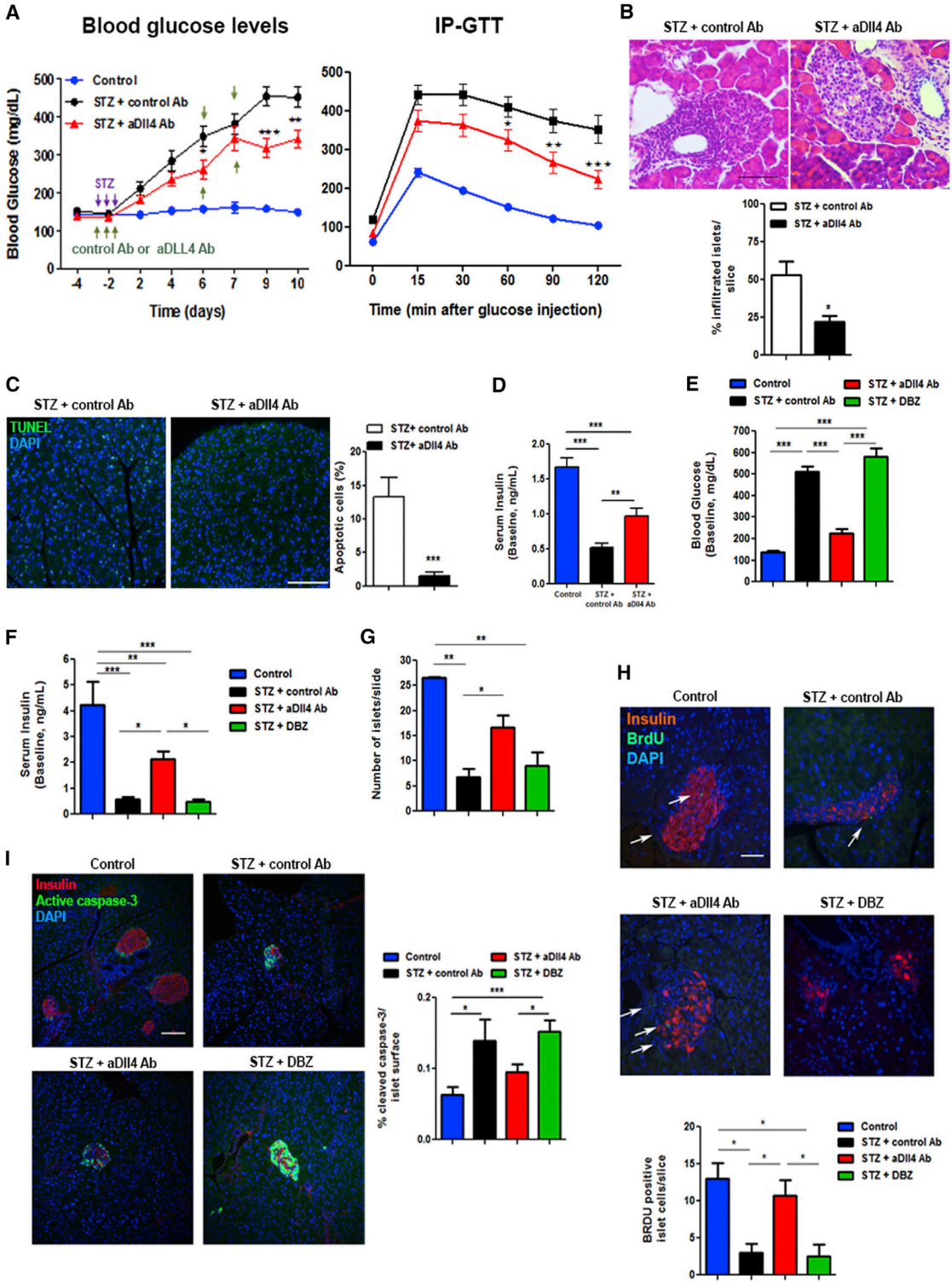


**Figure 2. Anti-Dll4 Ab Administration Prevents the Development of Diabetes in STZ-Treated Mice and Restores β-Islet Cell Function**

(A) Blood glucose level monitoring (left) and IP-GTT performed on day 12 post-STZ treatment (right) of prophylactic anti-Dll4 Ab (red) or control Ab (black) treatment (arrows: days of treatment with STZ, anti-Dll4 Ab, or control Ab at 10 mg/kg). Untreated mice (blue) are shown as controls (n = 13–14 mice per group). Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01; ***p < 0.001, compared with the group treated with STZ + control Ab.

(B) H&E staining of the pancreas (scale bar, 50 μm) (upper) and percentage of infiltration per individual islets (lower) on day 7 post-STZ treatment of prophylactic anti-Dll4 Ab or control Ab treatment (anti-Dll4 Ab or control Ab was given as described in Experimental Procedures) (n = 3 mice per group).

*(legend continued on next page)*

higher insulin and lower circulating glucose levels, without any change in their serum glucagon (Figure 3A). In addition, the anti-Dll4-Ab-treated mice showed better regulation of blood glucose after the intraperitoneal insulin tolerance test (IP-ITT) (Figure 3B) performed six hr after food deprivation. These responses were most likely due to increased insulin sensitivity and/ or increased baseline insulin levels (Figure 3A), likely associated with the increased pancreatic insulin content in response to anti-Dll4 treatment (Figure 3C). Notably, the average islet size was increased on anti-Dll4 treatment (Figure 3D), although there was no change in the total number of islets (Figure 3E) or the rate of islet apoptosis based on caspase-3 staining (Figure 3F). These findings point to induction of islet hyperplasia as a potential mechanism for the increase in insulin availability on anti-Dll4 Ab treatment.

Next, we assessed the time-dependent effects of anti-Dll4 Ab on pancreatic islets functionality. We found no changes in fasted insulin levels over the course of Ab treatment (Figure S2E). Further, IP-GTT performed at three distinct time points over the course of treatment identified gradual enhancement in glucose clearance (Figure S2F). In line, treated mice showed improved time-dependent pyruvate tolerance as per the intraperitoneal pyruvate tolerance test (IP-PTT), suggesting a reduced hepatic glucose output (Figure S2G). As expected, expression of *g6pase* was significantly inhibited in mice treated with anti-Dll4 Ab as compared with the control group (Figure S2H). The above findings are in agreement with the reported effects of γGSIs (Pajvani et al., 2011). Next, we sought to determine the mechanism for the induction of glucose-stimulated insulin secretion (GSIS) by anti-Dll4 Ab using an *ex vivo* system. GSIS experiments in islets purified from the pancreata of ND-fed C57BL/6 mice confirmed statistically significant induction of insulin secretion on treatment with anti-Dll4 Ab (Figure 3G); the basal and stimulated insulin levels during the GSIS are depicted in Table S1. In support the effects of the anti-Dll4 Ab on insulin secretion described above, RNA sequencing of pancreatic islet cells from a single WT C57BL/6 mouse (Xin et al., 2016) showed that the Notch-1 gene was predominantly expressed in β-islet cells as compared to alpha, pancreatic polypeptide (PP), and delta islet cell types (Figures S3A and S3B). Overall, our results demonstrate the cumulative effect of prolonged Dll4-Notch signaling inhibition on the pancreas via the improvement of β-islet cell function and more efficient insulin processing. These effects of anti-Dll4 Ab in the pancreas complement published data on the role of the Notch pathway in insulin-responsive tissues, such as liver, muscle, and adipose tissue (Pajvani et al., 2011; Valenti et al., 2013), that may also be targeted by the anti-Dll4 Ab treatment.

## Anti-Dll4 Ab Affects Insulin Production by Driving Differentiation and Proliferation of Insulin-Producing Cells

To further investigate the impact of Notch signaling pathway inhibition in the pancreatic protein profile, we performed ultra-high precision quantitative proteomic analysis in ND-fed WT C57BL/6 mice after 10 weeks of anti-Dll4 Ab treatment. A total of 4,749 proteins (peptide level false discovery rate [FDR] p value $\leq$ 0.05) (Table S2) were analyzed with at least two unique peptides, of which 1,695 were differentially expressed in the pancreata of anti-Dll4-Ab-treated mice, as compared with control samples (Table S3). Pathway map analysis using MetaCore of the differentially expressed proteome after anti-Dll4 Ab treatment versus control Ab treatment showed significant enrichment for the "Protein folding and maturation_Insulin processing" pathway (FDR-corrected p value = 5.0E−6) (Figure 4A). As shown, insulin abundance was increased after anti-Dll4 Ab treatment (Insulin-1: mean isobaric tags for relative and absolute quantification (iTRAQ) $\log_2$ ratio = 0.68 ± 0.09; p < 0.001; Insulin-2: mean iTRAQ $\log_2$ ratio = 0.46 ± 0.09; p < 0.001 after anti-Dll4 Ab versus control). Furthermore, carboxypeptidase H (CPH) (also known as carboxypeptidase E) (Figures 4A [box with dashed line] and 4B), an enzyme involved in the biosynthesis of insulin (Normant and Loh, 1998; O'Rahilly et al., 1995), and neuroendocrine convertase 2 (also known as proprotein convertase subtilisin/kexin type 2 [PC2]) (Figures 4A [box with solid line] and 4B), which participates in the proteolytic activation of insulin (Davidson et al., 1988), were both increased after treatment with anti-Dll4 Ab compared with control Ab (CPH: mean iTRAQ $\log_2$ ratio = 0.97 ± 0.30; p = 0.001; PC2: mean iTRAQ $\log_2$ ratio = 0.81 ± 0.33; p = 0.002). In contrast, the levels of insulin-degrading enzyme (IDE), a large zinc-binding protease that plays a central role in insulin degradation (Valera Mora et al., 2003), were decreased by anti-Dll4 Ab treatment (IDE: mean iTRAQ $\log_2$ ratio = −0.7 ± 0.45; p = 0.0001) (Figure 4B). The non-targeted and in-depth quantitative proteomic analysis of the pancreatic tissue supports our findings on insulin secretion in mice treated with anti-Dll4 Ab. Additional support was

(C) Left: Representative images from pancreatic slices of STZ-control Ab or anti-Dll4 Ab, prophylactic-treated mice after performance of TUNEL assay on day 12 after STZ-treatment (scale bar, 100 μm). Right: TUNEL-positive cells (green) were counted and reported as a percentage of 4',6-diamidino-2-phenylindole (DAPI)-positive cells (blue) (n = 5 mice per group).

(D) Serum insulin levels in mice from the prophylactic treatment in (A) at the time of sacrifice (day 14 after STZ treatment) (n = 9–12 mice per group).

(E and F) Baseline blood glucose (E) and insulin levels (F) of WT C57BL/6 mice after 6 weeks of anti-Dll4 Ab or DBZ treatment following STZ injections (n = 13 mice per group for STZ + control Ab and STZ + anti-Dll4 Ab groups and n = 6 mice per group for control and STZ + DBZ groups).

(G) Measurement of the number of mouse islets per slide in anti-Dll4 Ab, control Ab, and DBZ treated groups of (E). All islets from one slide per mouse were counted (n = 6 mice per group for STZ + control Ab and STZ + anti-Dll4 Ab groups and n = 4 mice per group for control and STZ + DBZ groups).

(H) Representative images of immunofluorescence (IF) co-staining for insulin and BrdU (scale bar, 50 μm) (upper) and quantification of BrdU-positive islet cells (lower) in mice from (E). At least 60 islets per group were counted (n = 6 mice per group for STZ + control Ab and STZ + anti-Dll4 Ab groups and n = 4 mice per group for vehicle and STZ + DBZ groups).

(I) Left: Cleaved caspase-3 and insulin IF co-staining was performed to measure apoptosis (scale bar, 100 μm). Right: At least 50 islets per group were counted to calculate the percentage of cleaved caspase-3-positive surface (n = 5 mice per group).

Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 indicate significant differences between groups as determined by two-way ANOVA.

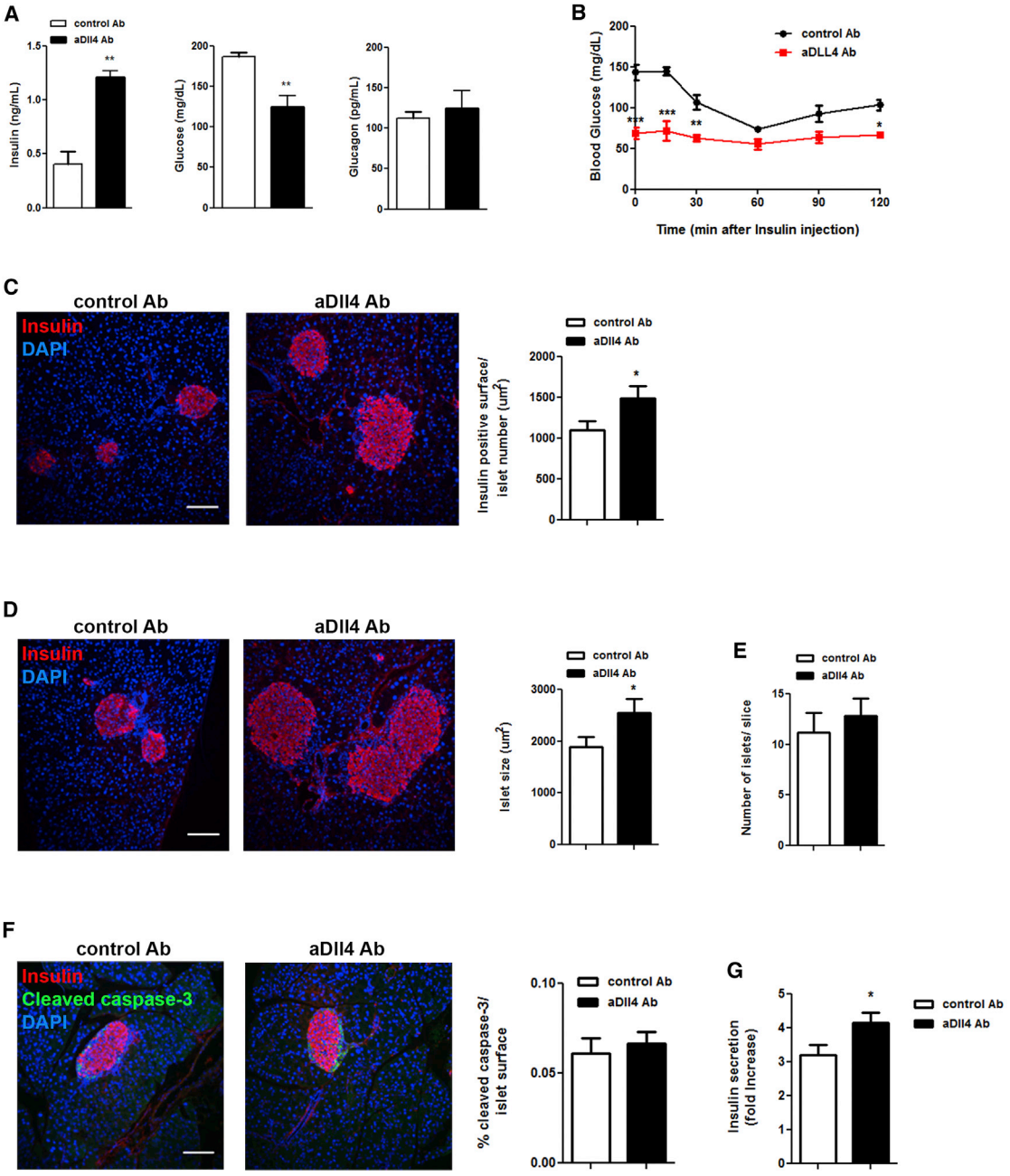


**Figure 3. Anti-Dll4 Ab Is Involved in Homeostatic Regulation of Pancreatic Insulin Processing**

(A and B) Mice were maintained on ND conditions and treated with anti-Dll4 Ab or control Ab at 10 mg/kg for 10 weeks. Blood insulin, glucose, and glucagon levels were measured (A) and IP- ITT was performed (B) after treatment (n = 4–5 mice per group). ***p < 0.0001 (B).

(C) IF for insulin (scale bar, 100 μm) (left) and total surface (right). At least 125 islets per group were counted to calculate the total insulin content (n = 5 mice per group).

(D) Representative IF insulin staining (scale bar, 100 μm) (left) and islet size (right) after anti-Dll4 Ab or control Ab (10 mg/kg per week) treatment for 10 weeks. At least 125 islets per group were counted to calculate islet size (n = 5 mice per group). Anti-Dll4 treatment did not change islet number.

(E) All islets from one slide per mouse were counted (n = 5 mice/ group).

(F) Left: Cleaved caspase-3 and insulin IF co-staining was performed to measure apoptosis (scale bar, 100 μm). Right: At least 125 islets per group were counted to calculate the percentage of cleaved caspase-3-positive surface (right) (n = 5 mice per group).

(G) Glucose-stimulated insulin secretion (GSIS) assay with primary isolated islets from pancreata of ND-fed C57BL/6 mice treated with anti-Dll4 Ab or control Ab (10 mg/kg) for 10 weeks.

Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01 indicate significant differences between groups as determined by Student's t test.

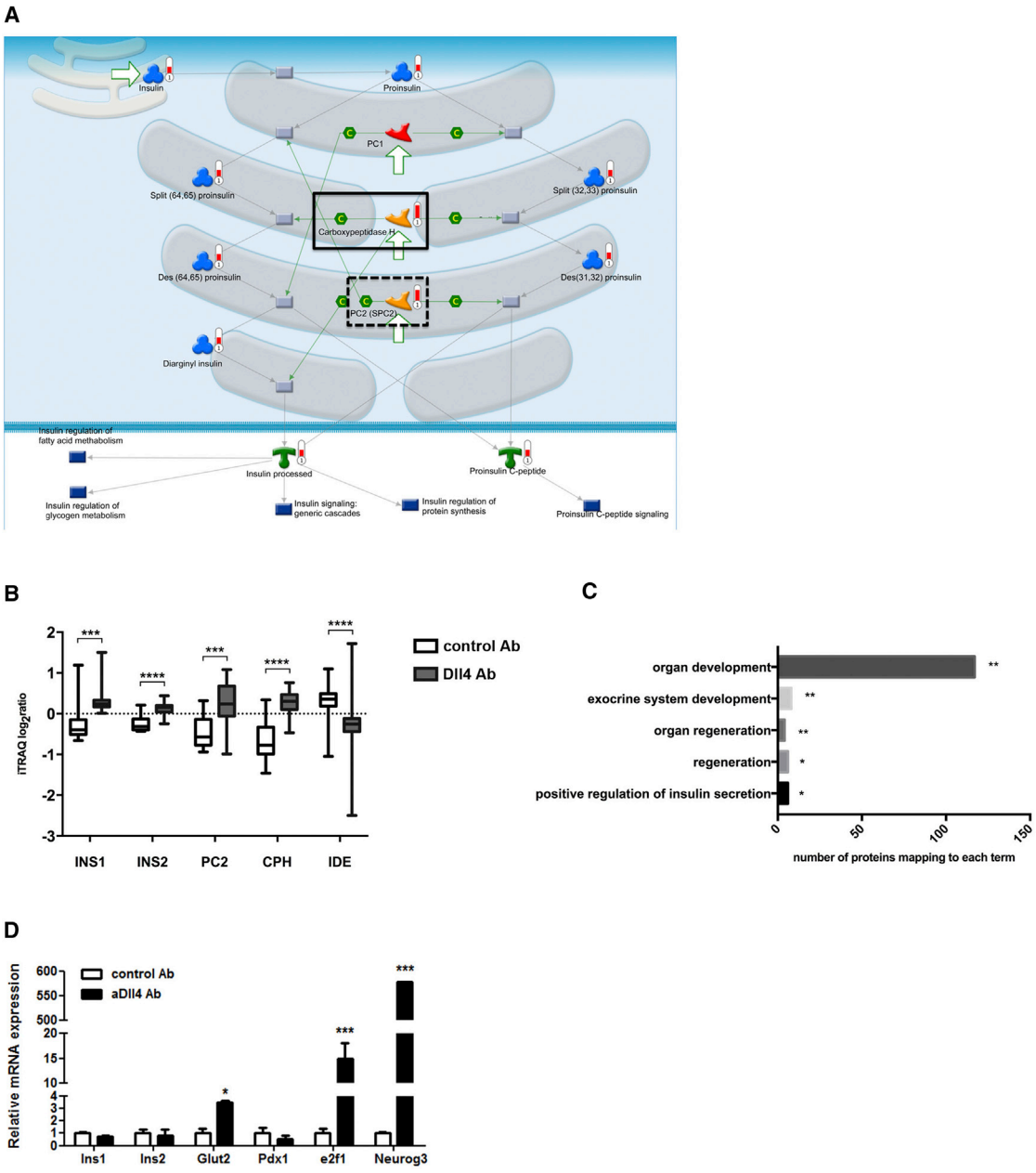


**Figure 4. Anti-Dll4 Ab Affects Insulin Production by Driving Differentiation and Proliferation of Insulin-Producing Cells**

(A) Quantitative proteomic analysis after treatment with anti-Dll4 Ab versus control Ab for 10 weeks. Pathway map analysis using MetaCore of the differentially expressed proteome after anti-Dll4 Ab treatment versus control Ab treatment for 10 weeks showed a significant enrichment for the "Protein folding and maturation_Insulin processing" pathway. Analyzed proteins are denoted with a thermometer, and a red thermometer signifies upregulation. In this pathway, insulin, CPH (box with solid line), and PC2 (box with dashed line) were upregulated after treatment with anti-Dll4 Ab versus control Ab.

(B) Relative expression of pancreatic proteins analyzed with proteomics: insulin 1(INS1), insulin 2 (INS2), CPH, and PC2 were found to increase, whereas insulin-degrading enzyme (IDE) was found to decrease after anti-Dll4 Ab treatment versus control Ab treatment.

(C) Gene ontology BiNGO analysis showed that positive regulation of insulin secretion, organ regeneration, exocrine system development, and organ development were significantly enriched terms in the differentially expressed proteins after anti-Dll4 Ab treatment.

(D) Relative expression of pancreatic genes involved in differentiation of pancreatic progenitors (n = 4–5 mice per group).

Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 indicate significant differences between groups as determined by Student's t test.

provided by gene ontology analysis. Indeed, using biological networks gene ontology (BiNGO), we confirmed "positive regulation of insulin secretion" and "organ regeneration" as significantly enriched terms in the differentially expressed pancreatic proteins after anti-Dll4 Ab treatment (FDR corrected p value = 2.6E−3 for both terms) (Figure 4C) (Table S4). Similarly,

upregulation of *GLUT2* mRNA (Figure 4D) was consistent with the corresponding changes in insulin levels following anti-Dll4 Ab treatment (Figures 3A and 3G), whereas there was no profound effect in *INSULIN* (*INS*) *1* and *INS2* transcripts levels (Figure 4D).

Interestingly, gene ontology analysis revealed enrichment of components of organ regeneration (Figure 4C), raising the hypothesis that anti-Dll4 Ab, in addition to the insulin-producing cells, may target β-islet cell progenitors, resulting in the expansion of the mature, functional islets. Indeed, we identified dramatically increased expression levels of key transcription factors driving differentiation of pancreatic progenitors in the pancreas of anti-Dll4-Ab-treated mice (Figure 4D). More specifically, anti-Dll4 Ab treatment resulted in significant activation of *E2F1* transcriptional factor and neurogenin-3 (*NUEORG3*) genes, the main transcription factors involved in islet development (Figure 4D). As previously demonstrated, *E2F1* activation drives the development of insulin-producing cells (Annicotte et al., 2009; Grouwels et al., 2010; Kim and Rane, 2011), while co-expression of *NEUROG3* is required for endocrine fate determination in the developing mouse pancreas (Gradwohl et al., 2000). This finding may provide an explanation for the increase of BrdU-positive cells within the islets of control mice following anti-Dll4 Ab treatment (Figure S4). Together, our results demonstrate that Dll4-Notch inhibition induces expansion of pancreatic progenitors *in vivo*, suggesting that the Notch signaling pathway could regulate pancreatic homeostasis, which is in line with reported findings in other organ systems (Pajvani et al., 2011; Valenti et al., 2013)

## DISCUSSION

Over the past several years, evidence has emerged illustrating the critical role of Notch signaling in the maintenance of adult tissue homeostasis under steady-state conditions via its immune (Billiard et al., 2011) and metabolic effects (Pajvani et al., 2013; Pajvani et al., 2011). Our findings expand on previous findings and provide a mechanistic foundation to explore the therapeutic potential of targeting the Notch pathway in pancreatic tissue. In this study, we found an important role of Dll4-Notch signaling inhibition in supporting the health of islet cells and affecting insulin production and secretion at multiple levels.

Our results suggest specific effects of Dll4-Notch signaling inhibition in the insulin-producing cells of the islets, as it significantly improved the function of β-islet cells. The latter has been suggested by the experiments described above, and it was further supported by single-pancreatic-cell analysis that revealed expression of the NOTCH-1 gene predominantly in the β-islet cells. However, these results do not exclude a potential contribution from other cell types.

Further, we show that anti-Dll4 Ab has complementary but distinct effects on pancreatic islets, as it induces differentiation and proliferation of insulin producing cells, inhibits islet apoptosis, and increases circulating insulin levels. Therefore, we suggest a mechanism of direct action of Dll4 inhibition in pancreatic islets that complements the reported effects of Notch inhibition in the regulation of carbohydrate metabolism and the restoration of insulin sensitivity (Fowler et al., 2011; Pajvani et al., 2011). Emerging evidence suggests that naturally occurring β-islet cell regeneration is not enough to achieve remission of diabetes due to the low number of β-islet cells and, in type I diabetes specifically, the continuing activity of the β-islet-cell-specific autoreactive T cells (Accili and Arden, 2004). An emerging suggested approach for the cure of diabetes could be via tissue regeneration, such as *in vivo* expansion of β-islet cell mass via the induced proliferation of existing β-islet cells (Thurston et al., 2007) and the differentiation of β-islet cell progenitors (Wolfe, 2012), similar to the effects of the anti-Dll4 Ab we report here. Our findings suggest that pancreatic Notch-Dll4 may provide a potentially targetable pathway for metabolic dysregulation associated with insulin resistance (Pajvani et al., 2011).

To date, components of the Notch signaling pathway have been validated as drug targets in various disease settings, such as malignancies and Alzheimer's disease (Lobry et al., 2011; Pajvani et al., 2013; Wolfe, 2012), although significant limitations for the use of these compounds have also been reported (Milano et al., 2004; van Es et al., 2005). Thus, a careful evaluation of the adverse effects in relation to therapeutic doses is essential for the advancement of Notch antagonists in clinical practice. We envision that selective blockade of the Notch signaling pathway could be applied in unmet medical needs hallmarked by inefficient insulin action, such as diabetes and sepsis, and possibly in regenerative medicine approaches for established pancreatic dysfunction.

## EXPERIMENTAL PROCEDURES

### Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dimitris Skokos (dimitris.skokos@regeneron.com).

### Antibody Treatment

Anti-Dll4 Ab was produced in house as described previously (Billiard et al., 2012). It recognizes both human and mouse Dll4. Human Fc (immunoglobulin G1 [IgG1], control Ab) was used as a control. C57BL/6 or NOD mice were injected subcutaneously (5 or 10 mg/kg) with anti-Dll4 or control Ab at various intervals, depending on the experiment. Therapeutic protocol for NOD mice involves a single administration of the anti-Dll4 Ab (10 mg/kg) at the time of diabetes onset. Remission percentage was calculated by dividing the number of mice showing an immediate blood glucose level drop on injection by the total number of mice injected. STZ-induced diabetic WT mice were injected with anti-Dll4 or control Ab according to the experimental design. Thus, the prevention protocol involves the administration of anti-Dll4 or control Ab at 10 mg/kg on day −3, −2, and −1 before and days 6 and 9 after STZ injection, whereas the therapeutic protocol after STZ treatment involves the administration of anti-Dll4 Ab (10 mg/kg) or DBZ (5 μm/kg) or control Ab (10 mg/kg) before STZ (on days −3, −2, and −1) and after STZ once per week for 6 weeks. The experimental protocols used in this study were approved by the Veterinary Authorities of the Prefecture of Athens, Greece (reference No. 2055/05-04-2016).

### Statistical Analysis

Statistical significances were calculated using the two-tailed unpaired Student's t test or two-way ANOVA test for multiple groups with 95% confidence intervals (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Please refer to Supplemental Experimental Procedures for a detailed description of other experiments.

## DATA AND SOFTWARE AVAILABILITY

The accession number for the mass spectrometry proteomics data reported in this paper is ProteomeXchange: PXD005333. The accession number for the RNA sequencing data (Xin et al., 2016) is GEO: GSE77980.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at 10.1016/j.celrep.2017.12.076.

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## AUTHOR CONTRIBUTIONS

S.K., F.B., B.W., D.S., A.D., Y.X., Y.K., W.Z., J.G., A.J.M., and G.D.Y. designed and performed the experiments and analyzed and interpreted the data. S.K., I.S., and E.N. isolated mouse islets and performed the GSIS assays. A.M. performed the proteomics experiments and analyzed and interpreted the data. S.D.G. designed and supervised the proteomics experiments and interpretation of results. P.T. performed the image processing and analysis. S.K., F.B., K.K., and D.S. designed the study. K.K. and D.S. supervised all experiments and interpretation of the results with S.K., and F.B., S.K., K.K., D.S., S.D.G., M.S., and A.M. drafted and revised the manuscript.

## DECLARATION OF INTERESTS

F.B., A.D., B.W., Y.X., W.Z., M.S., A.J.M., G.D.Y., and D.S. are all employees of Regeneron Pharmaceuticals, Inc.

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