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Inhibition of Gsk3 β activity improves β -cell function in *c-Kit*^{Wv/+} male mice

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Previous studies have shown that the stem cell marker, c-Kit, is involved in glucose homeostasis. We recently reported that *c-Kit*^{Wv/+} male mice displayed the onset of diabetes at 8 weeks of age; however, the mechanisms by which c-Kit regulates β -cell proliferation and function are unknown. The purpose of this study is to examine if *c-Kit*^{Wv/+} mutation-induced β -cell dysfunction is associated with downregulation of the phospho-Akt/Gsk3 β pathway in *c-Kit*^{Wv/+} male mice. Histology and cell signaling were examined in C57BL/6J/*Kit*^{Wv/+} (*c-Kit*^{Wv/+}) and wild-type (*c-Kit*^{+/+}) mice using immunofluorescence and western blotting approaches. The Gsk3 β inhibitor, 1-azakenpauillone (1-AKP), was administered to *c-Kit*^{Wv/+} and *c-Kit*^{+/+} mice for 2 weeks, whereby alterations in glucose metabolism were examined and morphometric analyses were performed. A significant reduction in phosphorylated Akt was observed in the islets of *c-Kit*^{Wv/+} mice ($P < 0.05$) along with a decrease in phosphorylated Gsk3 β ($P < 0.05$), and cyclin D1 protein level ($P < 0.01$) when compared with *c-Kit*^{+/+} mice. However, *c-Kit*^{Wv/+} mice that received 1-AKP treatment demonstrated normal fasting blood glucose with significantly improved glucose tolerance. 1-AKP-treated *c-Kit*^{Wv/+} mice also showed increased β -catenin, cyclin D1 and Pdx-1 levels in islets, demonstrating that inhibition of Gsk3 β activity led to increased β -cell proliferation and insulin secretion. These data suggest that *c-Kit*^{Wv/+} male mice had alterations in the Akt/Gsk3 β signaling pathway, which lead to β -cell dysfunction by decreasing Pdx-1 and cyclin D1 levels. Inhibition of Gsk3 β could prevent the onset of diabetes by improving glucose tolerance and β -cell function.

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KEYWORDS: Akt/Gsk3 β /cyclin D1 signaling pathway; β -cell regeneration; *c-Kit*^{Wv/+} mice; Gsk3 β kinase inhibitor

Loss of pancreatic β -cell mass and varying degrees of insulin secretory defects are observed in both type 1 and 2 diabetes. Restoring insulin-producing cells show great potential for attaining normal blood glucose levels, and thus extensive research efforts are focused on developing methods to differentiate progenitor cells into β cells^{1–3} and maintain their viability and function.^{4,5}

Previous studies have emphasized that c-Kit (also known as CD117) together with its ligand, stem cell factor (SCF), are important in a number of cell types to control a variety of cellular processes, including hematopoiesis, gametogenesis and melanogenesis,^{6,7} as well as pancreatic β -cell survival and maturation.^{8–15} Stimulation of c-Kit by SCF leads to dimerization of the c-Kit receptor and subsequent autophosphorylation of intrinsic tyrosine kinases. Moreover, c-Kit receptor activation mediates multiple intracellular pathways, including the phosphatidylinositol-3-kinase (PI3K)

pathway¹⁶ for modulation of gene transcription, proliferation, differentiation, metabolic homeostasis and cell survival.^{17–19} Stimulation of PI3K is required for the regulation of glucose and preproinsulin gene expression as well as the nuclear translocation of Pdx-1.²⁰ Our previous *in vitro* studies of human fetal islets demonstrated that increased c-Kit activity leads to enhanced Pdx-1 and insulin expression, and cell proliferation via the activation of the PI3K/AKT pathway.^{21,22} Recently, our *in vivo* study showed that reduced c-Kit activity correlated with reduced β -cell function and proliferation, which is associated with significantly decreased Pdx-1 expression and glucose intolerance in *c-Kit*^{Wv/+} mice.¹³ Evidence from these studies suggests that in β cells, SCF/c-Kit is a modulator of the PI3K/Akt pathway, upstream of Pdx-1.^{13,21} Nevertheless, the signaling pathway under c-Kit including PI3K/Akt and downstream targets, involved in β -cell proliferation and function *in vivo*, remains unclear.

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Glycogen synthase kinase 3, a serine/threonine kinase, consists of two highly homologous α (Gsk3 α) and β (Gsk3 β) isoforms that share overlapping physiological functions in terms of regulating a diverse array of cellular processes. Gsk3 β is one of the substrates that are directly regulated by the PI3K/Akt pathway. Activation of Akt, which phosphorylates Gsk3 β at serine residue 9, leads to inactivation of Gsk3 β .^{23–25} Unphosphorylated Gsk3 β can directly phosphorylate downstream substrates, and modulate their activity.²⁶ Apart from the function from which it derives its name (ie, suppressing glycogen synthesis by phosphorylating glycogen synthase), studies have shown that Gsk3 β is involved in mediating cell cycle, motility and apoptosis,²⁶ and that Gsk3 β dysregulation is linked to several prevalent pathological diseases, including Alzheimer's disease, cancer and diabetes.²⁶ Indeed, mice with β -cell-specific overexpression of Gsk3 β displayed glucose intolerance owing to reduced β -cell mass and proliferation.²⁷ Furthermore, isolated adult human and rat islets incubated with Gsk3 β inhibitors demonstrated a significant increase in islet cell proliferation.²⁸ This evidence was further confirmed by mice with β -cell-specific ablation of Gsk3 β .²⁹ Although the beneficial effect of Gsk3 β inhibition has been demonstrated in islet biology, the physiological role of Gsk3 β under disrupted c-Kit signaling in β -cell has yet to be investigated.

Our recent *in vivo* studies using *c-Kit*^{Wv/+} male mice have shown that disrupted c-Kit receptor function is associated with severe loss of β -cell mass and function, resulting in early-onset of diabetes.¹³ However, the intracellular mechanisms that relate the c-Kit receptor to physiological changes in β -cells have yet to be elucidated. The aim of this study is to examine whether β -cell dysfunction in *c-Kit*^{Wv/+} male mice is associated with downregulation of the phospho-Akt/Gsk3 β pathway. Furthermore, 1-azakenpauillone (1-AKP), a specific organic inhibitor that prevents Gsk3 β phosphorylation of multiple downstream substrates by competitively binding at its ATP-binding site,³⁰ was used to investigate whether inhibition of Gsk3 β could improve β -cell proliferation and function, delaying the onset of diabetes in *c-Kit*^{Wv/+} mice. Here, we report that decreased β -cell proliferation and function in *c-Kit*^{Wv/+} mice was associated with dysregulated Akt and Gsk3 β phosphorylation as well as cyclin D1 protein level. Treating *c-Kit*^{Wv/+} mice with 1-AKP delayed onset of diabetes, maintained β -cell mass and enhanced β -cell function. This study demonstrates a functional role for c-Kit in mediating glucose homeostasis and β -cell function via the Akt/Gsk3 β axis in *c-Kit*^{Wv/+} mice.

MATERIALS AND METHODS

Heterozygotic C57BL/6J/*Kit*^{Wv/+} Male Mice

c-Kit^{+/+} and *c-Kit*^{Wv/+} male mice were obtained by breeding C57BL/6J/*Kit*^{Wv/+} parents heterozygous for the c-Kit point mutation (Jackson Laboratories, Bar Harbor, ME, USA).¹³ Mice were genotyped by differences in fur pigmen-

tation as described previously.¹³ Mice were maintained in an environment with constant temperature (20°C) and regular 12-h day and 12-h night cycles. Pancreata from both *c-Kit*^{+/+} and *c-Kit*^{Wv/+} male littermates at 8 weeks old were dissected for experimentation. The animal use protocol was approved by the Animal Use Subcommittee at the University of Western Ontario in accordance with the guidelines of the Canadian Council of Animal Care.

In Vivo Treatment with Gsk3 β Inhibitor and Metabolic Studies

To investigate whether inhibition of Gsk3 β activity could prevent early onset of diabetes at 8 weeks of age in *c-Kit*^{Wv/+} mice,¹³ both *c-Kit*^{+/+} and *c-Kit*^{Wv/+} 6-week-old mice were treated with either 1-AKP (VWR Canlab, Mississauga, ON, Canada) at 2 mg/kg or saline injection intraperitoneally every other day for 2 weeks to establish four experimental groups:^{31,32} *c-Kit*^{+/+}/saline, *c-Kit*^{+/+}/1-AKP, *c-Kit*^{Wv/+}/saline and *c-Kit*^{Wv/+}/1-AKP. Body weight was measured weekly, while fasting (4 h) blood glucose levels, glucose (IPGTT) and insulin (IPITT) tolerance tests were performed at 8 weeks of age (after 2 weeks of 1-AKP treatment). For the IPGTT and IPITT, an intraperitoneal injection of glucose (D-(+)-glucose (dextrose, 2 mg/kg; Sigma, St Louis, MO, USA) or insulin (Humalin 1 U/kg; Eli Lilly, Toronto, ON, Canada) was administered to each mouse, respectively.¹³ Blood glucose levels were examined at 0, 15, 30, 60, 90 and 120 min following injection.

In Vitro Islet Treatment with Gsk3 β Inhibitor

Islets were isolated from *c-Kit*^{+/+} and *c-Kit*^{Wv/+} mice at 8 weeks of age and cultured in RPMI 1640 media plus 10% FBS with or without 1-AKP (10 μ M) treatment for 24 h. Islets were harvested for protein preparation. All culture experiments were conducted with at least three repeats per group.

In Vivo and *Ex Vivo* GSIS Study and Insulin ELISA

To assess the effects of Gsk3 β inhibition on insulin secretion, glucose-stimulated insulin secretion (GSIS) both *in vivo* and on isolated islets was performed. For *in vivo* GSIS, at the end of either saline or Gsk3 β inhibitor treatment, mouse blood samples were collected from the tail vein following 4 h of fasting (0 min), and at 5 and 35 min after glucose loading.¹³ Assessment of GSIS on isolated islets involved islet isolation using the hand-pick method¹³ and incubation of the islets in an oxygenated Krebs–Ringer bicarbonate HEPES plus 0.5% BSA buffer³³ containing 2.2 mmol/l or 22 mmol/l glucose for 1 h. Glucose-stimulated insulin secretion was measured using an ultrasensitive (mouse) insulin ELISA (Alpco, Salem, NH, USA). A static glucose stimulation index was calculated by dividing insulin output from the high glucose (22 mmol/l) incubation by the insulin output during low glucose (2.2 mmol/l) incubation.¹³

Double Immunofluorescence and Morphometric Analysis

Pancreata collected from both *c-Kit*^{+/+} and *c-Kit*^{Wv/+} mice at 8 weeks of age with or without 1-AKP treatments were fixed in 4% paraformaldehyde, followed by a standard protocol of dehydration and paraffin embedding. Pancreatic tissue sections (4 μ m thick) were prepared and double-stained with primary antibodies of appropriate dilutions as listed (Supplementary Table S1). Secondary antibodies, fluorescein isothiocyanate (anti-mouse or anti-guinea-pig) and TexRed (anti-rabbit or anti-guinea-pig), were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Double-immunolabeled images were recorded by a Leica DMIRE2 fluorescence microscope with the Openlab image software (Improvision, Lexington, MA, USA).

Quantitative evaluation for islet number, islet size, and α - and β -cell mass was performed using computer-assisted image analysis, as described previously.¹³ To quantify cell proliferation (labeled by Ki67), cyclin D1 and Pdx-1-positive staining in β -cell nuclear, at least 10 random islet images per pancreatic section were captured and counted. The average percent colocalization of cyclin D1⁺/insulin⁺, Ki67⁺/insulin⁺ and Pdx-1⁺/insulin⁺ cells was calculated. A minimum of five pancreata per age per experimental group with at least 1000 cells was analyzed. Data are presented as a percentage of the total number of insulin⁺ cells counted.¹³

Western Blot Analysis

Protein level of Akt, Gsk3 β , β -catenin, cyclin D1 and Pdx-1 in both pancreata and islets from *c-Kit*^{+/+} and *c-Kit*^{Wv/+} mice at 8 weeks of age with or without 1-AKP treatment was analyzed. Equal amounts of lysate from each experimental group were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Mississauga, ON, Canada). Membranes were then incubated with primary antibodies as listed in Supplementary Table S1, followed by incubation with a goat anti-mouse or rabbit IgG secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA). As a control for sample loading, the blot was also probed with antibodies against total signaling or housekeeping proteins. Membranes were further incubated in chemiluminescence reagents (Perkin-Elmer, Wellesley, MA, USA) and exposed to BioMax MR Film (Kodak, Rochester, NY, USA) to visualize protein bands. Densitometric quantification of bands at subsaturating levels was performed using the Syngenetool gel analysis software (Syngene, Cambridge, UK) and normalized to the loading controls. Data are expressed as relative level of phosphorylated proteins to total protein levels or protein levels to the loading controls.¹³

RNA Extraction and Real-time RT-PCR

RNA was extracted from isolated islets of *c-Kit*^{+/+} and *c-Kit*^{Wv/+} mice with or without 1-AKP injection using the

miRNeasy kit (Qiagen, Germantown, MD, USA).¹³ Sequences of PCR primers are listed (Supplementary Table S2). Real-time PCR analyses were performed using the iQ SYBR Green Supermix kit in Chromo4 Real-time PCR (Bio-Rad Laboratories, Mississauga, ON, Canada). Relative levels of gene expression were calculated and normalized to the internal standard gene, 18S rRNA, with at least five repeats per experimental group.^{13,21,22}

Statistical Analysis

Data are expressed as means \pm s.e.m. Statistical significance was determined by unpaired Student's *t*-test, or ANOVA followed by least significant difference (LSD) *post-hoc* tests. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Reduction of Phospho-Akt, Phospho-Gsk3 β and Cyclin D1 in *c-Kit*^{Wv/+} Mice

Our recent study has demonstrated that male mice with mutated *c-Kit* develop early onset of diabetes with a loss of β -cell mass and proliferation, suggesting that *c-Kit* plays a critical role in β -cell proliferation and function.¹³ Moreover, experiments in isolated human fetal islet-epithelial cells^{21,22} revealed that the PI3K/Akt pathway is significantly upregulated upon exogenous SCF stimulation. Here, we showed that both whole pancreatic tissue and isolated islets from *c-Kit*^{Wv/+} male mice displayed a significant reduction in phosphorylated Akt at Ser473 ($P < 0.05$; Figure 1a), but not at Thr308 (Supplementary Figure S1a). In parallel, decreased protein level of phosphorylated Gsk3 β at Ser9 was observed in *c-Kit*^{Wv/+} mice when compared to *c-Kit*^{+/+} mice ($P < 0.05$; Figure 1b), but not at Tyr216 (Supplementary Figure S1b). No alteration in cyclin D1 protein level was observed in total pancreatic tissue extract. However, a significant reduction in cyclin D1 protein expression was noted in isolated islets of *c-Kit*^{Wv/+} mice ($P < 0.01$; Figure 2a) along with a 60% reduction in the proportion of cyclinD1⁺/insulin⁺ cells when compared with *c-Kit*^{+/+} mice ($P < 0.05$; Figures 2b and c).

Inhibition of Gsk3 β Activity Prevented Early Onset of Diabetes in *c-Kit*^{Wv/+} Mice

c-Kit^{+/+} and *c-Kit*^{Wv/+} male mice that received saline or 1-AKP treatment revealed no significant change in body weight at 8 weeks of age (Figure 3a). However, fasting blood glucose levels in *c-Kit*^{Wv/+}/1-AKP mice showed a significant improvement when compared with the *c-Kit*^{Wv/+}/saline group ($P < 0.01$; Figure 3b) and reached similar fasting blood glucose levels of the *c-Kit*^{+/+}/saline and *c-Kit*^{+/+}/1-AKP groups (Figure 3b). These improvements in glucose metabolism were further confirmed by glucose tolerance tests (Figure 3c). *c-Kit*^{Wv/+}/1-AKP mice showed similar glucose tolerance capacity to both *c-Kit*^{+/+}/saline and *c-Kit*^{+/+}/1-AKP groups, along with significant decreases in area under

the curve when the IPGTT was performed ($P < 0.001$; Figure 3c). Insulin tolerance tests revealed no significant differences between the four experimental groups (Figure 3d).

Furthermore, the effects of Gsk3 β inhibition on insulin secretion were examined by the GSIS assay *in vivo* and on isolated islets. No significant differences were found in basal plasma insulin levels between the four experimental groups. However, 5 min after glucose stimulation, an increase in plasma insulin release was noted in *c-Kit*^{Wv/+}/1-AKP mice when compared with the *c-Kit*^{Wv/+}/saline ($P < 0.001$) and *c-Kit*^{+/+}/saline ($P < 0.01$) groups (Figure 4a). There was a further increase in plasma insulin secretion at 35 min following stimulation in both 1-AKP-treated groups

($P < 0.05$ – 0.01 ; Figure 4a). To further confirm if these changes in insulin secretion upon glucose stimulation are observed in isolated islets, *in vitro* GSIS was performed using isolated islets from all experimental groups. Insulin secretion in response to 22 mM glucose was significantly increased in the islets of *c-Kit*^{Wv/+}/1-AKP mice, showing a twofold increase in insulin secretion compared with the *c-Kit*^{Wv/+}/saline group ($P < 0.05$; Figure 4b).

Inhibition of Gsk3 β Activity Preserved β -Cell Mass in *c-Kit*^{Wv/+} Male Mice

Our *in vivo* metabolic data suggest that inhibition of Gsk3 β activity in *c-Kit*^{Wv/+} mice could prevent early onset of

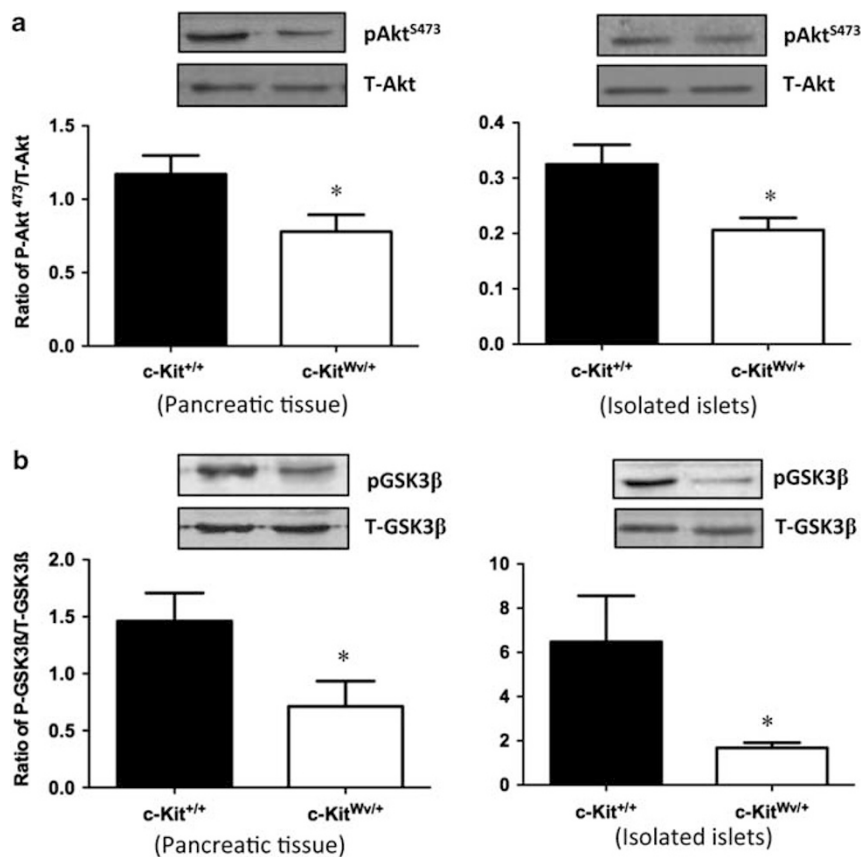
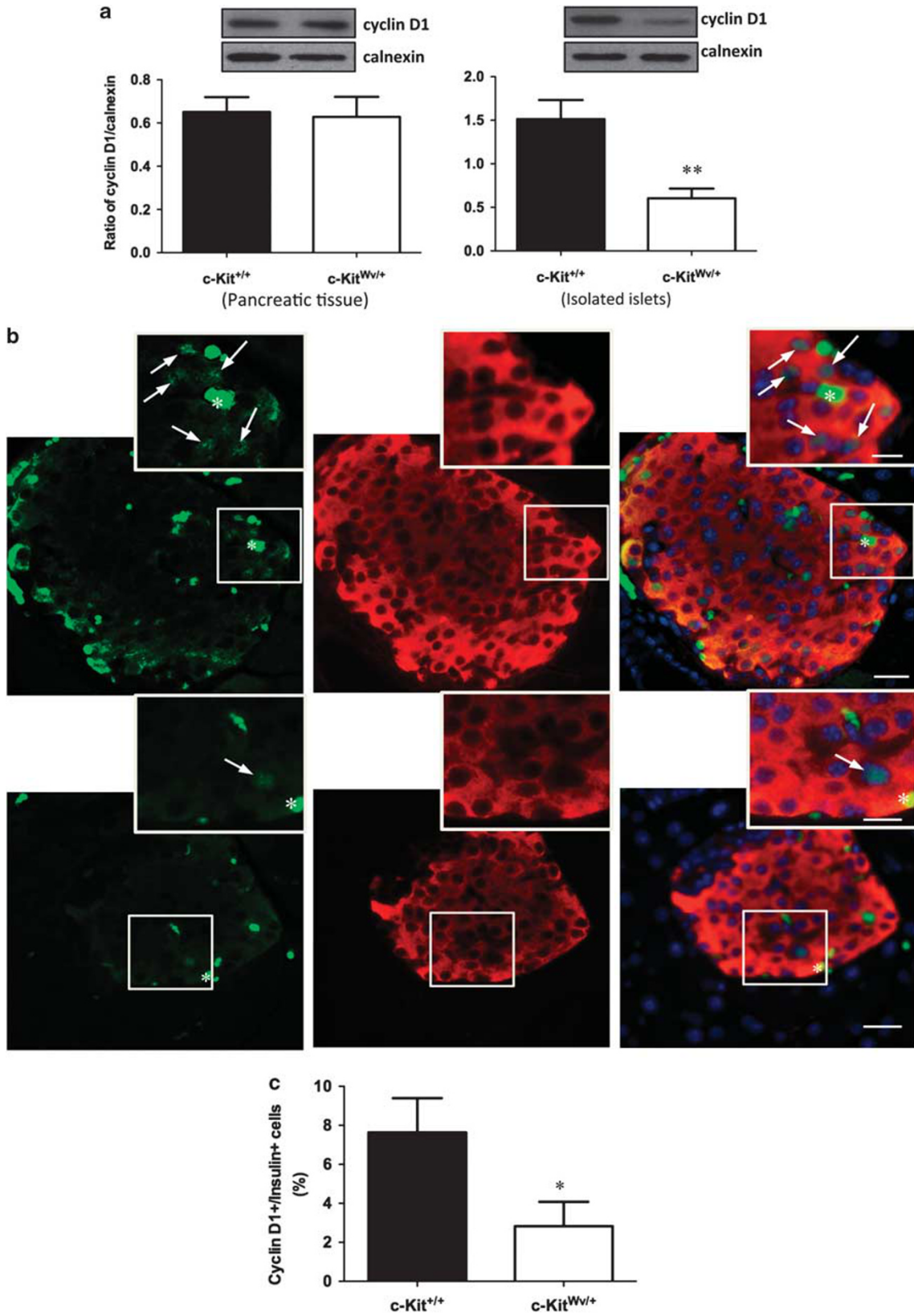


Figure 1 Downregulation of phosphorylated Akt/glycogen synthase kinase 3 β (Gsk3 β) signaling in the pancreas and isolated islet from *c-Kit*^{Wv/+} mice. Western blot analysis of S473-phosphorylated (P) and total (T) Akt (a), phosphorylated (P) and total (T) Gsk3 β (b) protein level in the pancreas and isolated islets of *c-Kit*^{+/+} and *c-Kit*^{Wv/+} male mice at 8 weeks of age. Representative blots are shown. Data are normalized to total protein and expressed as means \pm s.e.m. ($n = 11$ pancreas; $n = 3$ isolated islets per experimental group) * $P < 0.05$ vs *c-Kit*^{+/+} male mice.

Figure 2 Decreased cyclin D1 protein level in isolated islet of *c-Kit*^{Wv/+} mice. (a) Western blot analysis of cyclin D1 in the pancreas and isolated islets of *c-Kit*^{+/+} and *c-Kit*^{Wv/+} male mice at 8 week of age. Representative blots are shown. Data are normalized to loading control calnexin and expressed as means \pm s.e.m. ($n = 4$ pancreas; $n = 6$ isolated islets per experimental group) ** $P < 0.01$ vs *c-Kit*^{+/+} male mice. (b) Double immunofluorescence staining for cyclin D1 (green), insulin (red) of *c-Kit*^{+/+} and *c-Kit*^{Wv/+} male mice pancreatic section at 8 weeks of age. Nuclear counter stained by 4',6-diamidino-2-phenylindole (DAPI) (blue). Scale bar: 20 μ m (inset: 10 μ m). Arrows indicate cyclin D1/insulin double-positive cells. (c) The percent of cyclin D1⁺ cells in β -cells of *c-Kit*^{+/+} and *c-Kit*^{Wv/+} male mice at 8 weeks of age. Data are expressed as mean percent positive cells over insulin⁺ cells \pm s.e.m. ($n = 6$) * $P < 0.05$ vs *c-Kit*^{+/+} male mice.



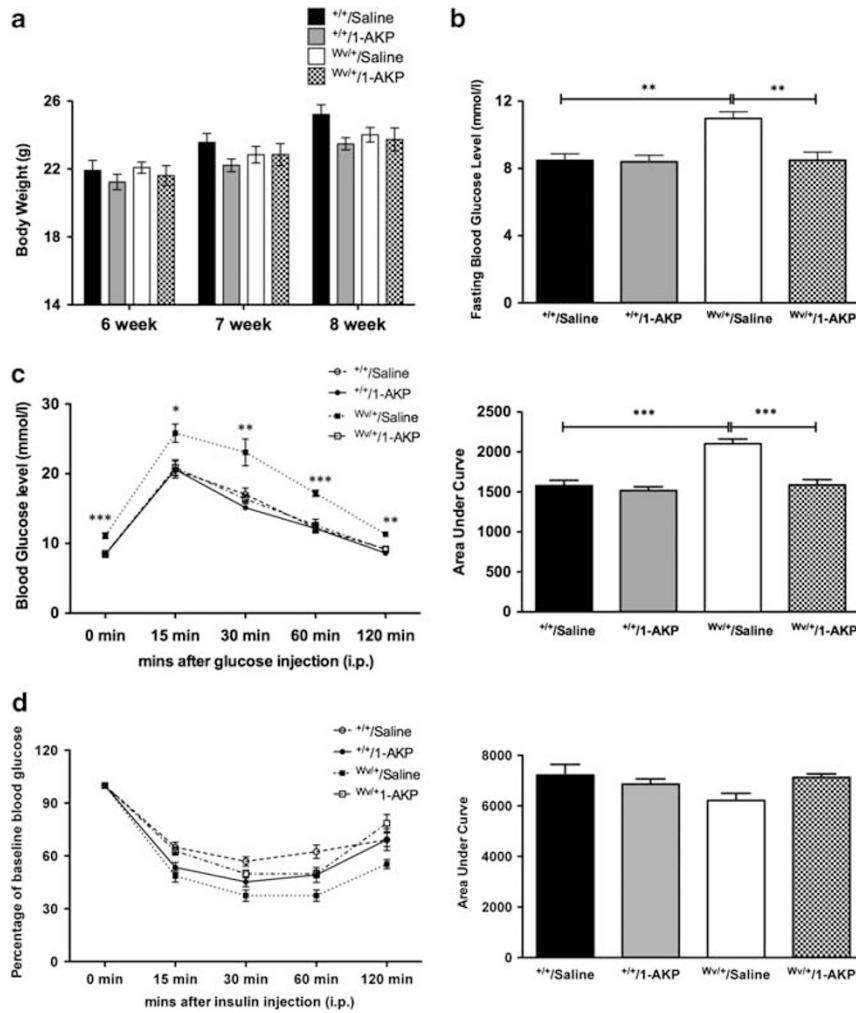


Figure 3 Effect of glycogen synthase kinase 3 β inhibitor on glucose homeostasis of *c-Kit*^{Wv/+}/1-AKP mice. Body weight (a) and fasting blood glucose level (b) of *c-Kit*^{+/+}/saline, *c-Kit*^{+/+}/1-AKP, *c-Kit*^{Wv/+}/saline and *c-Kit*^{Wv/+}/1-AKP groups. Intra-peritoneal glucose tolerance test (IPGTT) (c) and insulin tolerance test (IPITT) (d) in *c-Kit*^{+/+}/saline, *c-Kit*^{+/+}/1-AKP, *c-Kit*^{Wv/+}/saline and *c-Kit*^{Wv/+}/1-AKP mouse group. Glucose responsiveness of the corresponding experimental groups is shown as a measurement of area under the curve (AUC) of the IPGTT or IPITT graphs with units of ((mmol/l) min) shown on the Y axis. Data are expressed as means \pm s.e.m. (n = 6–8). *P < 0.05, **P < 0.01, ***P < 0.001 vs *c-Kit*^{Wv/+}/saline group. 1-AKP, 1-azakenpaullone.

diabetes. There was no observed difference in pancreatic weight between all experimental groups (data not shown). Morphometric analyses were performed to examine the effects of the Gsk3 β inhibitor on islet number and size as well as α - and β -cell mass in *c-Kit*^{+/+} and *c-Kit*^{Wv/+} male mice with or without 1-AKP treatments. A significant increase in the number of pancreatic islets was noted in *c-Kit*^{Wv/+}/1-AKP mice ($P < 0.01$; Figure 5a), with a significant increase in the number of small islets ($< 500 \mu\text{m}^2$) when compared with the *c-Kit*^{Wv/+}/saline group ($P < 0.05$; Figure 5b). Moreover, the number of large islets ($> 10\,000 \mu\text{m}^2$) was only slightly increased in *c-Kit*^{Wv/+}/1-AKP mice when compared with the *c-Kit*^{Wv/+}/saline group, while both *c-Kit*^{+/+}/saline and *c-Kit*^{+/+}/1-AKP groups had the most large islets ($P < 0.05$; Figure 5b). Furthermore, β -cell mass in *c-Kit*^{Wv/+}/1-AKP mice also increased by 24% when compared with the

c-Kit^{Wv/+}/saline group ($P < 0.01$; Figure 5d); however, it only reached 80–90% of the β -cell mass of the *c-Kit*^{+/+}/saline and *c-Kit*^{+/+}/1-AKP groups (Figure 5d). Increased β -cell mass was also correlated with increased expression of insulin I and II genes in the islets of 1-AKP-treated *c-Kit*^{+/+} and *c-Kit*^{Wv/+} mice (Figure 5e). No alterations were detected in α -cell mass among any of the four experimental groups (Figure 5c).

Inhibition of Gsk3 β Activity Preserved β -Catenin, Cyclin D1, Pdx-1 and Mafa Expression and Increased the Proliferative Capacity of β -Cells of *c-Kit*^{Wv/+} Male Mice

The effect of Gsk3 β inhibition on downstream signaling mediators of β -catenin protein level in the islets of *c-Kit*^{Wv/+}/1-AKP mice was significantly higher when compared with the *c-Kit*^{Wv/+}/saline group ($P < 0.05$; Figure 6a). However,

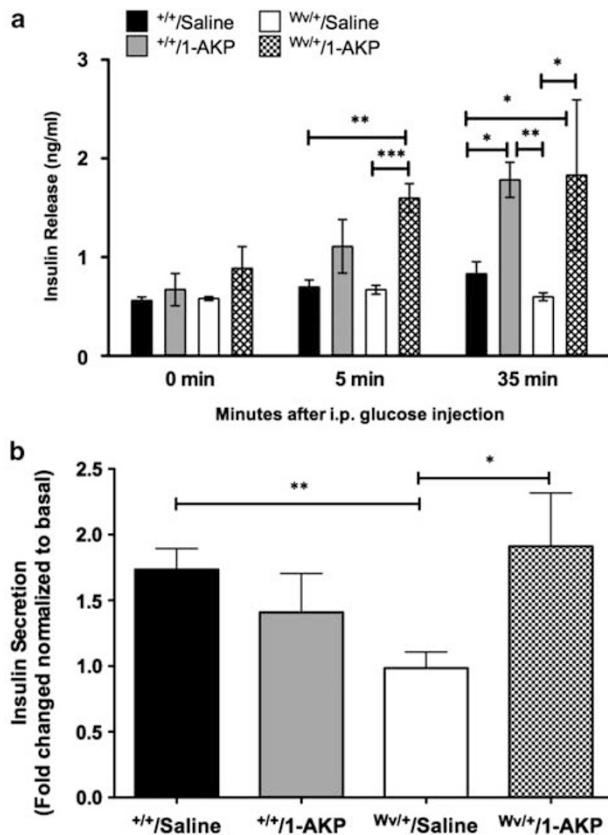


Figure 4 Effect of glycogen synthase kinase 3 β (Gsk3 β) inhibitor on β -cell function of $c\text{-Kit}^{Wv/+}/1\text{-AKP}$ mice. **(a)** *In vivo* glucose-stimulated insulin secretion of $c\text{-Kit}^{+/+}/\text{saline}$, $c\text{-Kit}^{+/+}/1\text{-AKP}$, $c\text{-Kit}^{Wv/+}/\text{saline}$ and $c\text{-Kit}^{Wv/+}/1\text{-AKP}$ groups. $c\text{-Kit}^{Wv/+}/1\text{-AKP}$ groups demonstrated an increase in plasma insulin release after glucose loading ($n = 3\text{--}8$). **(b)** Insulin secretion is improved in isolated islets from male $c\text{-Kit}^{Wv/+}/1\text{-AKP}$ mice in response to 22 mM glucose challenge; data are expressed as fold change normalized to basal (2.2 mM glucose) secretion ($n = 4\text{--}5$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs $c\text{-Kit}^{Wv/+}/\text{saline}$ group. 1-AKP, 1-azakenpaullone.

this protein level only reached 78% of both $c\text{-Kit}^{+/+}/\text{saline}$ and $c\text{-Kit}^{+/+}/1\text{-AKP}$ groups ($P < 0.05\text{--}0.01$; Figure 6a). The $c\text{-Kit}^{Wv/+}/\text{saline}$ group showed a significantly lower level of islet β -catenin protein than that of $c\text{-Kit}^{+/+}/1\text{-AKP}$ and $c\text{-Kit}^{+/+}/\text{saline}$ groups ($P < 0.001$; Figure 6a). In addition, $c\text{-Kit}^{+/+}/1\text{-AKP}$ mice showed slightly increased β -catenin protein level in islet protein extracts, but did not reach statistical significance when compared with the $c\text{-Kit}^{+/+}/\text{saline}$ group (Figure 6a). The protein level of β -catenin was also significantly higher in isolated islets from $c\text{-Kit}^{Wv/+}$ mice, when treated with 1-AKP for 24 h, compared with non-treated $c\text{-Kit}^{+/+}$ and $c\text{-Kit}^{Wv/+}$ islets ($P < 0.05$; Figure 6a). These increases in islet β -catenin corresponded with significantly elevated islet cyclin D1 protein level in $c\text{-Kit}^{Wv/+}/1\text{-AKP}$ mice and $c\text{-Kit}^{Wv/+}$ islets cultured with 1-AKP when compared with $c\text{-Kit}^{Wv/+}/\text{saline}$ mice and untreated isolated $c\text{-Kit}^{Wv/+}$ islets, respectively ($P < 0.05\text{--}0.01$; Figure 6b). These findings were further corroborated using q-RT-PCR

and double immunofluorescence, which revealed significantly increased *Ccnd1* mRNA (Figure 6c) and a higher number of cyclin D1⁺/insulin⁺ cells in $c\text{-Kit}^{Wv/+}/1\text{-AKP}$ mice ($P < 0.05$ vs $c\text{-Kit}^{Wv/+}/\text{saline}$ group; Figure 6d). Quantitative real-time RT-PCR analysis of *Pdx-1* and *Mafa* showed significantly increased mRNA levels in the islets of both 1-AKP-treated $c\text{-Kit}^{+/+}$ and $c\text{-Kit}^{Wv/+}$ mice (Figure 7a). Double immunofluorescence demonstrated a significant increase in the number of Pdx-1⁺ cells in β -cells of $c\text{-Kit}^{Wv/+}/1\text{-AKP}$ mice ($P < 0.01$ vs $c\text{-Kit}^{Wv/+}/\text{saline}$ group; Figure 7b). Increased *Mafa* staining intensity was also observed in $c\text{-Kit}^{Wv/+}/1\text{-AKP}$ mouse β -cells (Supplementary Figure S2). These results were further supported by western blot analysis and showed significantly higher Pdx-1 protein level in $c\text{-Kit}^{Wv/+}$ islets from either 1-AKP-injected mice or 1-AKP incubation when compared with $c\text{-Kit}^{Wv/+}/\text{saline}$ mice and $c\text{-Kit}^{Wv/+}$ islets without 1-AKP treatment, respectively ($P < 0.05$; Figure 7c). Furthermore, increased cyclin D1 expression (Figure 6b–d) was correlated with a significant increase in Ki67 immunoreactivity in β -cells of $c\text{-Kit}^{Wv/+}/1\text{-AKP}$ mice ($P < 0.05$ vs $c\text{-Kit}^{Wv/+}/\text{saline}$ group; Figure 7d).

DISCUSSION

This study demonstrates that diabetic $c\text{-Kit}^{Wv/+}$ male mice displayed reduced levels of phospho-Akt at Ser473 and phospho-Gsk3 β at Ser9 in both pancreatic and isolated islet protein extracts. These mice also possess decreased cyclin D1 expression in their β -cells. When treated with the Gsk3 β inhibitor, 1-AKP, $c\text{-Kit}^{Wv/+}$ mice demonstrated significant improvements in fasting glucose, glucose tolerance and insulin secretion along with increased β -cell mass and proliferation. These results indicate that the Akt/Gsk3 β pathway downstream of the c-Kit receptor is predominantly involved in regulating β -cell proliferation and function in $c\text{-Kit}^{Wv/+}$ male mice and that inhibition of Gsk3 β activation can significantly improve glucose tolerance and β -cell function (Figure 8).

The PI3K pathway is one of the major downstream pathways of c-Kit that triggers a variety of pro-survival signaling events, such as cell survival, cell growth and cell cycle entry via Akt phosphorylation.^{21,34} Akt, a serine/threonine protein kinase, exerts its effects after being phosphorylated by PI3K at two regulatory residues: Thr308 in the activation segment and Ser473 in the hydrophobic motif of the kinase domain.³⁵ In this study, we showed decreased protein levels of phospho-Akt at Ser473, but not at Thr308 in $c\text{-Kit}^{Wv/+}$ mice when compared with controls. $c\text{-Kit}^{Wv/+}$ mice are heterozygous for the *c-Kit* *Wv* mutation caused by substitution of a threonine to methionine at amino acid 660 in the kinase domains of the c-Kit receptor,³⁶ and this loss-of-function mutation leads to downregulation, but not abolition of c-Kit receptor activity. Previous research has demonstrated that Akt monophosphorylation on Thr308 showed only 10% kinase activity compared with 100% when Akt was doubly phosphorylated on Ser473 and Thr308,³⁵ suggesting that Ser473

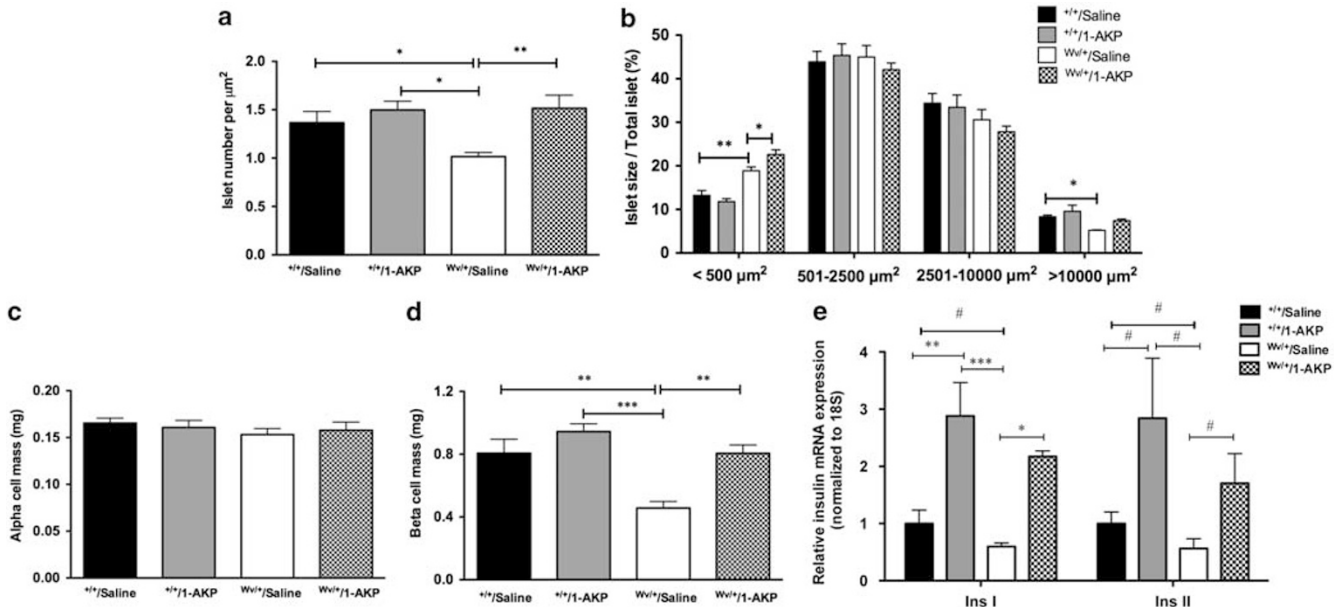


Figure 5 Effect of glycogen synthase kinase β (Gsk3 β) inhibitor on pancreatic morphology of $c\text{-Kit}^{Wv/+}$ /1-AKP mice. Morphometric analysis of islet number (a), islet size (b), α -cell mass (c) and β -cell mass (d) in $c\text{-Kit}^{+/+}$ /saline, $c\text{-Kit}^{+/+}$ /1-AKP, $c\text{-Kit}^{Wv/+}$ /saline and $c\text{-Kit}^{Wv/+}$ /1-AKP groups ($n=6-8$). (e) Quantitative RT-PCR analysis of insulin I and II mRNA in isolated islets of $c\text{-Kit}^{+/+}$ /saline, $c\text{-Kit}^{+/+}$ /1-AKP, $c\text{-Kit}^{Wv/+}$ /saline and $c\text{-Kit}^{Wv/+}$ /1-AKP mice. Data are expressed as means \pm s.e.m. ($n=5-8$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ was tested by one-way analysis of variance (ANOVA) analysis; # $P<0.05$ was analyzed by unpaired Student's t -test. 1-AKP, 1-azakenpaullone.

phosphorylation is required for maximal kinase activity of Akt.³⁵ Therefore, our results demonstrate that $c\text{-Kit}^{Wv/+}$ male mice showed significant downregulation of the primary Akt phosphorylation site required conferring maximal activity, Ser473.

It is well documented that Gsk3 β is the target of many important signaling molecules.³⁷ In particular, Akt blocks the catalytic activity of the kinase, and activates downstream signals repressed by active Gsk3 β .³⁴ In this study, we observed reduced Gsk3 β phosphorylation and cyclin D1 protein level in islets of $c\text{-Kit}^{Wv/+}$ male mice, which is consistent with the role of cyclin D1 as essential for postnatal β -cell growth.³⁸ Active Gsk3 β promotes destabilization of cyclin D1 and thus cell cycle arrest, which is associated with Akt inactivation.³⁹ Taken together, our results suggest that Akt/Gsk3 β axis dysregulation from the $c\text{-Kit}^{Wv}$ mutation contributed to loss of β -cell mass and function, which may have been responsible for the early onset of diabetes observed in $c\text{-Kit}^{Wv/+}$ male mice reported previously.¹³

It has been previously shown that Gsk3 β is involved in differentiation and proliferation of numerous tissue types including β -cells.^{28,29} Tanabe et al⁴⁰ showed that genetic ablation of Gsk3 β in $Irs^{-/-}$ mice preserved β -cell function and prevented the onset of diabetes. Moreover, mice with β -cell-specific ablation of Gsk3 β were resistant to high-fat diet-induced diabetes.²⁹ These studies suggest that endogenous Gsk3 β activity is involved in a highly specialized feedback inhibition mechanism with the insulin receptor, which primarily signals via the PI3K/Akt pathway. In this

study, $c\text{-Kit}^{Wv/+}$ mice received treatment of a Gsk3 β inhibitor for 2 weeks and showed significant improvements in glucose tolerance and β -cell function when compared with saline-treated $c\text{-Kit}^{Wv/+}$ controls, which showed diabetic symptoms. In addition, no significant differences in insulin tolerance were observed between the experimental groups, suggesting that this improvement in glucose tolerance and β -cell function was not due to enhanced peripheral insulin sensitivity, but primarily to improved β -cell proliferation and function.

Our morphological analyses revealed that improved glucose tolerance and insulin secretion was associated with preservation of β -cell mass and an increase in islet number in $c\text{-Kit}^{Wv/+}$ /1-AKP mice. The most notable change was a significant increase in the percentage of small islets (< 500 μm^2), with a relative increase in the number of large islets (> 10000 μm^2), suggesting that inhibition of Gsk3 β activity is associated with an increase in β -cell neogenesis and proliferation. β -Catenin and cyclin D1 are two potential targets of Gsk3 β , and have been implicated in β -cell growth.^{38,41} Preserved β -cell mass in the $c\text{-Kit}^{Wv/+}$ /1-AKP group was accompanied by increased β -cell proliferation (Ki67), and maintained β -catenin and cyclin D1 protein levels, which closely matched those of the $c\text{-Kit}^{+/+}$ groups. This is in agreement with the role of β -catenin in upregulating cyclin D1 expression to promote β -cell expansion.^{41,42} These results suggest that Akt/Gsk3 β axis-mediated β -catenin activation in islets may regulate β -cell growth and function in $c\text{-Kit}^{Wv/+}$ mice.⁴³

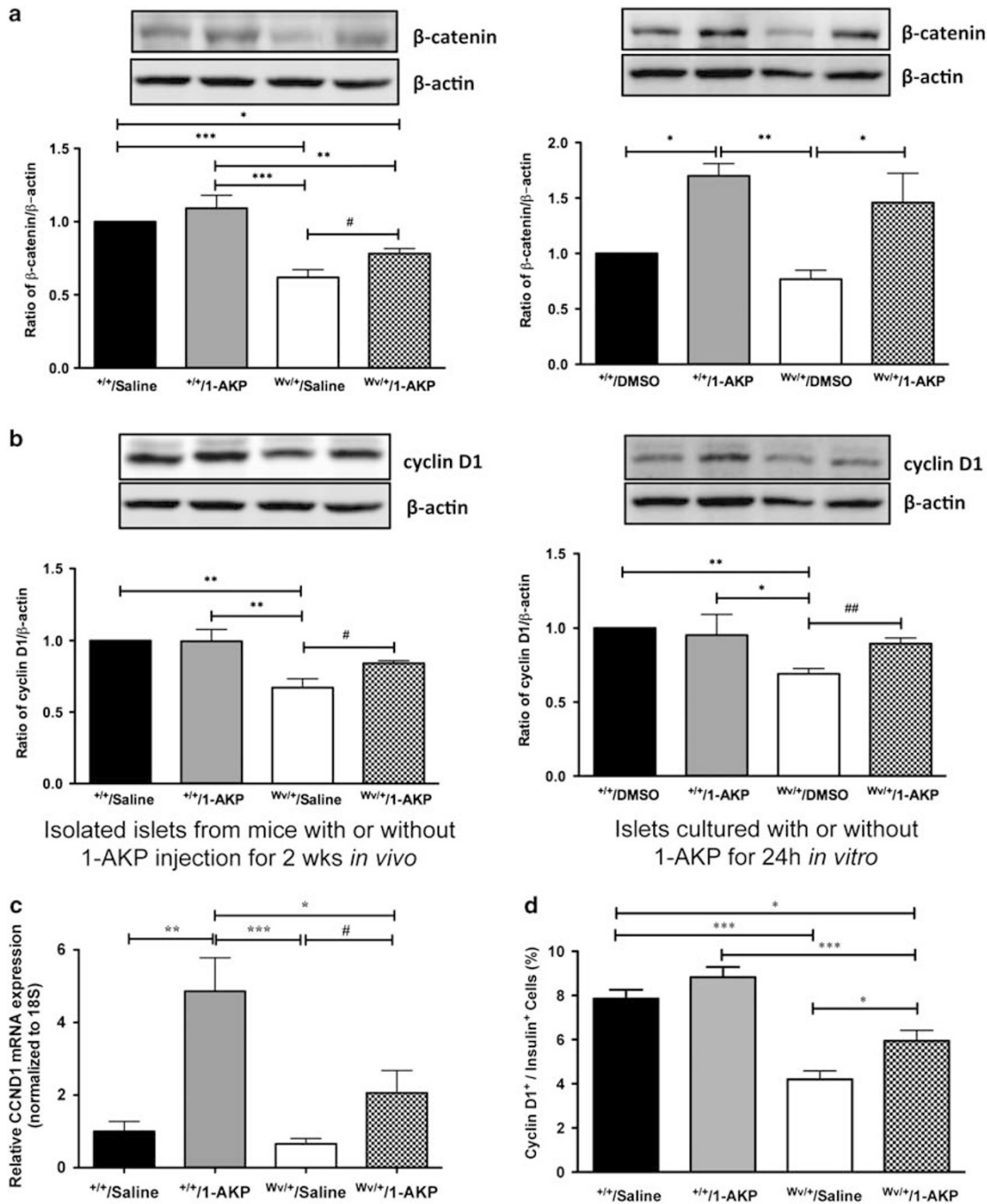


Figure 6 Inhibition of glycogen synthase kinase 3 β (Gsk3 β) maintained β -catenin and cyclin D1 (CCND1) protein level in $c\text{-Kit}^{Ww/+}/1\text{-AKP}$ mouse islets. Western blot analysis of β -catenin (a) and CCND1 (b) in isolated islets from $c\text{-Kit}^{+/+}/\text{saline}$, $c\text{-Kit}^{+/+}/1\text{-AKP}$, $c\text{-Kit}^{Ww/+}/\text{saline}$ and $c\text{-Kit}^{Ww/+}/1\text{-AKP}$ mice (left column) and isolated islets from 8 weeks of $c\text{-Kit}^{+/+}$ and $c\text{-Kit}^{Ww/+}$ mice cultured with or without 1-AKP treatment for 24 h (right column). Representative blots are shown. Data are normalized to β -actin and expressed as means \pm s.e.m. ($n = 4\text{--}5$) * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs $c\text{-Kit}^{Ww/+}/\text{saline}$ groups by one-way analysis of variance (ANOVA) analysis. # $P < 0.05$, ## $P < 0.01$ was analyzed by unpaired Student's t -test between $c\text{-Kit}^{Ww/+}$ islets with or without 1-AKP treatment. Quantitative analysis of CCND1 mRNA in isolated islets (c) and the number of nuclear CCND1 $^+$ cells in β -cells (d) of $c\text{-Kit}^{+/+}/\text{saline}$, $c\text{-Kit}^{+/+}/1\text{-AKP}$, $c\text{-Kit}^{Ww/+}/\text{saline}$ and $c\text{-Kit}^{Ww/+}/1\text{-AKP}$ mice. Data are expressed as means \pm s.e.m. ($n = 5\text{--}8$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs $c\text{-Kit}^{Ww/+}/\text{saline}$ group by one-way ANOVA analysis. # $P < 0.05$ was analyzed by unpaired Student's t -test. 1-AKP, 1-azakenpaullone.

The critically important pancreatic transcription factor, Pdx-1, has been shown to regulate β -cell survival, function and pancreatic development.^{44,45} Previous studies have also

demonstrated that reduction of Pdx-1 impairs islet insulin secretion,^{46,47} while a partial reduction in Pdx-1 has a major effect on β -cell survival, with reductions in Bcl_{XL} and Bcl2

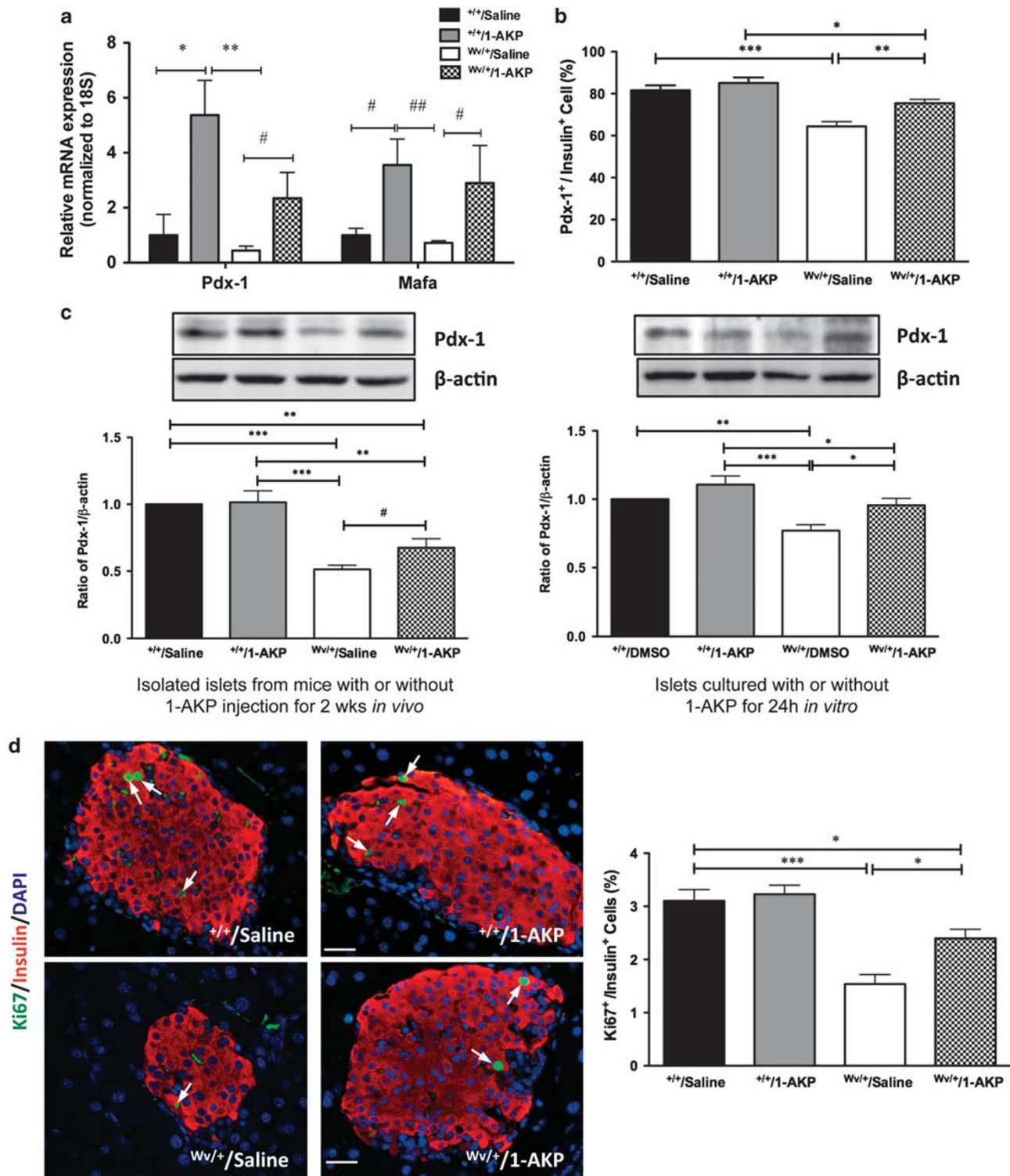


Figure 7 Inhibition of glycogen synthase kinase 3 β (Gsk3 β) resulted in increased Pdx-1 and Mafa expression, β -cell proliferation in *c-Kit*^{Ww/+}/*1-AKP* mice. Quantitative analysis of Pdx-1 and Mafa mRNA in isolated islets (a) and the number of Pdx-1⁺ (b) and Ki67⁺ (d, right) cells in β -cells of *c-Kit*^{+/+}/*saline*, *c-Kit*^{+/+}/*1-AKP*, *c-Kit*^{Ww/+}/*saline* and *c-Kit*^{Ww/+}/*1-AKP* mice. (c) Western blot analysis of Pdx-1 protein level in isolated islets from *c-Kit*^{+/+}/*saline*, *c-Kit*^{+/+}/*1-AKP*, *c-Kit*^{Ww/+}/*saline* and *c-Kit*^{Ww/+}/*1-AKP* mice (left column) and isolated islets from 8-week-old *c-Kit*^{+/+} and *c-Kit*^{Ww/+} mice cultured with or without 1-AKP treatment for 24 h (right column). (d, left) Double immunofluorescence staining of Ki67 (green) with insulin (red) and nuclear stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). Arrows indicate Ki67/insulin double-positive cells. Scale bar: 20 μ m. Data are expressed mean \pm s.e.m. (*n* = 5–8) **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs *c-Kit*^{Ww/+}/*saline* group by one-way analysis of variance (ANOVA) analysis. #*P* < 0.05, ##*P* < 0.01 was analyzed by unpaired Student's *t*-test. 1-AKP, 1-azakenpaullone.

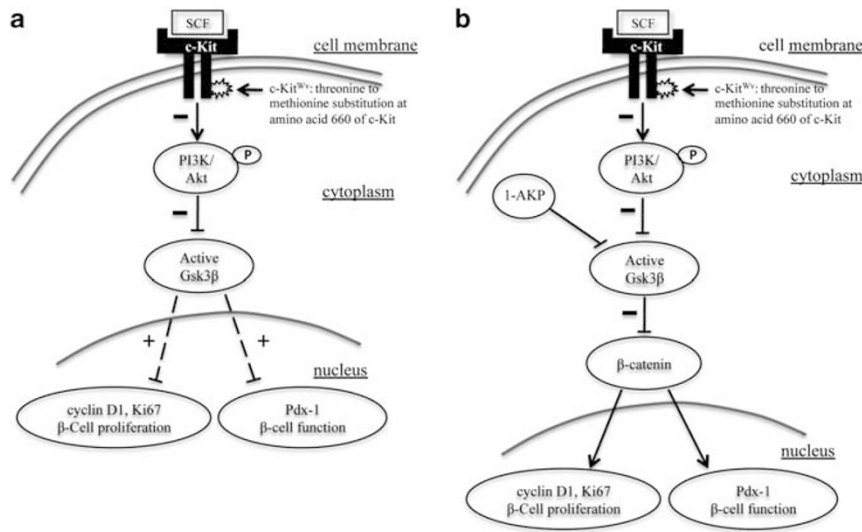


Figure 8 Schematic of proposed model involving c-Kit/Akt/glycogen synthase kinase 3 β (Gsk3 β) signaling pathways in β -cell survival and function in *c-Kit*^{Wv/+} mice. (a) Mutating the c-Kit receptor at the Wv locus lead to a downregulation of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway via reduced phosphorylation of Akt at Ser473, which resulted in enhanced active Gsk3 β and increased inhibition of Pdx-1 and cyclin D1 expression. (b) Direct inhibition of active Gsk3 β with 1-AKP preserved β -catenin protein level, along with maintained cyclin D1 and Pdx-1 expression that allowed improvement of β -cell proliferation and function and prevented the onset of diabetes in *c-Kit*^{Wv/+} mice. '+': enhanced; '-': reduced; '↓': stimulation; '⊥' inhibition. 1-AKP, 1-azakenpaullone.

expression.⁴⁵ Using *c-Kit*^{Wv/+} mice, we observed a significant reduction in Pdx-1 expression (~50% of wild-type controls) along with impaired β -cell function¹³ and an upregulation in the Fas-associated cell death pathway (unpublished data). In this study, Pdx-1 expression was maintained upon Gsk3 β inhibition, suggesting that preservation of Pdx-1 expression is associated with increased insulin secretion in *c-Kit*^{Wv/+} / 1-AKP mice. Previous studies in isolated mouse islets have shown that Pdx-1 is negatively regulated in a Gsk3 β -dependent manner.⁴⁸ In addition, inhibition of Gsk3 β by activated Akt could ameliorate Pdx-1 phosphorylation, leading to a consequential delay of its degradation, which suggests that Pdx-1 stability in β -cells may be mediated by the Akt/Gsk3 β axis.⁴⁹ Further *in vivo* studies have demonstrated that Gsk3 β ablation in mouse β -cells lead to increased Pdx-1 protein level.^{29,40} In addition, a significant upregulation of *Mafa* mRNA expression in *c-Kit*^{Wv/+} / 1-AKP mouse islets was observed, which may have contributed to the observed improvements in β -cell insulin secretion. Overexpression of *Mafa* in neonatal rat β -cells led to enhanced glucose-responsive insulin secretion and β -cell maturation.⁵⁰ Taken together, the findings of this study showed that Akt/Gsk3 β signaling, downstream of c-Kit, is not only required for β -cell proliferation, but also for mediating Pdx-1 and *Mafa* expression, which is essential for β -cell function and differentiation.

This study demonstrates that the Gsk3 β inhibitor, 1-AKP, predominantly improved β -cell proliferation and function, and had no significant effects in peripheral tissues, like muscle or liver insulin sensitivity. However, previous studies

have shown that treatment of Gsk3 β inhibitors improved glucose homeostasis by ameliorating muscle and liver insulin resistance in non-insulin-dependent obese animal models.⁵¹⁻⁵³ The *c-Kit* Wv point mutation primarily causes defect in melanocytic, hematopoietic and endocrine cell lineages, with no apparent muscle and liver dysfunction.^{13,36,54} This is in contrast to non-insulin-dependent diabetic animal models, such as ZDF rat and ob mice that have primary defects in muscle and liver insulin-stimulated peripheral glucose transport activity.⁵¹⁻⁵³ Thus, we speculate that the effects of Gsk3 β inhibition depend on the injury of the animal. Indeed, wild-type mice in our analysis showed less responsiveness in lowering blood glucose when treated with Gsk3 β inhibitors.

In summary, this study elucidates a novel link between the c-Kit receptor and Akt/Gsk3 β /cyclin D1 signaling pathways and their effects on β -cell proliferation and function *in vivo* (Figure 8). Our results suggest that dysregulated Akt/Gsk3 β /cyclin D1 downstream from the *c-Kit* Wv mutation is the possible signaling mechanism that contributes to the loss of β -cell mass and function in *c-Kit*^{Wv/+} male mice reported in our previous publication.¹³ Furthermore, chronic inhibition of Gsk3 β in *c-Kit*^{Wv/+} male mice resulted in increased β -catenin, cyclin D1 and Pdx-1 protein expression, suggesting that regulation of downstream signaling increased β -cell proliferative capacity, mass, as well as later improved glucose tolerance, and helped prevent early onset of diabetes. Taken together, the findings of this study emphasizes that understanding the complex intracellular pathways that affect β -cell proliferation and function *in vivo*, which the c-Kit receptor

regulates, will enable the development of more successful treatment protocols for diabetes.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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