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ORIGINAL PAPER

GGPPS-mediated Rab27A geranylgeranylation regulates β cell dysfunction during type 2 diabetes development by affecting insulin granule docked pool formation

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Abstract

Loss of first-phase insulin secretion associated with β cell dysfunction is an independent predictor of type 2 diabetes mellitus (T2DM) onset. Here we found that a critical enzyme involved in protein prenylation, geranylgeranyl pyrophosphate synthase (GGPPS), is required to maintain first-phase insulin secretion. GGPPS shows a biphasic expression pattern in islets of *db/db* mice during the progression of T2DM: GGPPS is increased during the insulin compensatory period, followed by a decrease during β cell dysfunction. *Ggpps* deletion in β cells results in typical T2DM β cell dysfunction, with blunted glucose-stimulated insulin secretion and consequent insulin secretion insufficiency. However, the number and size of islets and insulin biosynthesis are unaltered. Transmission electron microscopy shows a reduced number of insulin granules adjacent to the cellular membrane, suggesting a defect in docked granule pool formation, while the reserve pool is unaffected. *Ggpps* ablation depletes GGPP and impairs Rab27A geranylgeranylation, which is responsible for the docked pool deficiency in *Ggpps*-null mice. Moreover, *GGPPS* re-expression or GGPP administration restore glucose-stimulated insulin secretion in *Ggpps*-null islets. These results suggest that *GGPPS*-controlled protein geranylgeranylation, which regulates formation of the insulin granule docked pool, is critical for β cell function and insulin release during the development of T2DM.

Keywords: GGPPS; Rab27A; insulin secretion; docked pool; protein geranylgeranylation

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No conflicts of interest were declared.

Introduction

Type 2 diabetes mellitus (T2DM) is characterized by peripheral insulin resistance and pancreatic β cell dysfunction and the latter is associated with defects in insulin release [1]. A reduction in first-phase insulin release is the earliest detectable defect of β cell dysfunction in individuals destined to develop T2DM [2,3], even though β cells may contain sufficient amounts of insulin [4,5]. Clinical restoration of early-phase insulin secretion improves glucose homeostasis in T2DM patients [6,7]. Therefore, accurate regulation of insulin secretion is a promising therapeutic approach for preventing T2DM development [6].

Glucose stimulates insulin release from pancreatic β cells via the highly organized secretory vesicle/granule system [8]. Within a β cell, insulin is sorted into secretory vesicles and presented in two separate cytosolic pools, according to releasability: a releasable docked pool, consisting of an immediately releasable pool (IRP) and a readily releasable pool (RRP), which are responsible for glucose-stimulated biphasic insulin secretion; and a larger reserve pool, containing > 95% of the insulin granules, which serves to maintain adequate insulin storage and sustainably provide insulin to the releasable pool [8,9]. Upon a glucose challenge, a transient release of insulin granules is observed within 2–5 min, referred to as 'first-phase secretion', which rapidly reduces blood glucose and facilitates glucose uptake and glycogen synthesis. A lasting release follows, referred to as 'second-phase secretion', which reinforces the hypoglycaemic effects by promoting glucose utilization, lasting from 15 min to 3 h if necessary. This biphasic pattern is crucial to maintain glucose homeostasis [8,10]. Physiologically, insulin granules contained in the docked pool are necessary and sufficient for this biphasic secretion to handle a single meal. However, the islets of T2DM patients normally

show a deficiency in this pool [5]. Thus, any defects in the formation of the docked pool would result in an ablated glucose-responsive insulin release and insulin deficiency, as observed in β cell dysfunction in T2DM [11,12].

Emerging evidence implicates critical roles for Rab GTPases in insulin exocytosis in β cells. Notably, Rab27A specifically localizes in the dense-core secretory granules and is involved in the regulation of exocytosis [13,14]. In β cells, Rab27A is required for building the docked granule pool by controlling the functional IRP and determining the size of the RRP [13]. Thus, inactivation of Rab27A reduces the number of insulin granules docked at the plasma membrane and decreases exocytotic events [15]. Most previous studies have focused on GDP–GTP exchange in the activation of Rab proteins during insulin exocytosis [13,16]. Additionally, Rab proteins undergo geranylgeranylation through the attachment of one or two 20-carbon geranylgeranyl pyrophosphate (GGPP) molecules to the C-terminus, which is required for membrane anchoring and activation [17,18]. GGPP has been reported to induce glucose-stimulated insulin secretion (GSIS) in β cells [19]. Additional studies, in which geranylgeranylation was blocked using mevalonate pathway inhibitor statins [20,21] or specific silencing of Rab–geranylgeranyl transferase (RGGT) [22], have also demonstrated that Rab geranylgeranylation is essential in physiological insulin secretion (reviewed in [23,24]).

As a substrate of protein geranylgeranylation, GGPP synthesis is catalysed by geranylgeranyl pyrophosphate synthase (GGPPS), a key enzyme in the mevalonate pathway [17], which has been reported to decrease in the islets of T2DM patients [25]. Here, we found that GGPPS in islets is correlated to β cell function, which was elevated in the islets of *db/db* mice during the compensatory hyperinsulinaemia period and reduced when the β cells underwent dysfunction during progression of T2DM. Through studying β cell specific *Ggpps* knockout mice, we revealed that GGPPS plays a crucial role in maintaining β cell function during T2DM development by controlling the geranylgeranylation and activation of Rab27A that is required for insulin docked pool formation and insulin release.

Materials and methods

Animal studies

Db/db C57BL/KsJ (*db/db*), C57BL/KsJ (BKs), C57BL/6 J (B6) mice and *RIP–Cre* transgenic mice were purchased from the Model Animal Research Center of Nanjing University. *Ggpps*-floxed mice were generated through homologous recombination, as previously described [26,27]. β cell-specific *Ggpps* knockout mice (β*Ggpps* KO) were generated by crossing *Ggpps*-floxed mice with *RIP–Cre* mice. All the mice used in this research were male and housed in a normal 12:12 h light:dark cycle, with a regular chow diet (4% fat; Xietong, Jiangsu, China) or a high-fat diet (60% fat; Research Diets, NJ, USA); the animals had access to water *ad libitum*. All animal procedures were carried out in accordance with the Animal Care and Use Committee at the Model Animal Research Centre of Nanjing University, China.

Intraperitoneal glucose and insulin tolerance tests (ipGTT and ipITT) and histology

Glucose and insulin tolerance tests were performed in 8 week-old mice, as previously described [28]. Blood glucose was determined at the indicated time from tail blood using Accu-Chek glucometers (Roche Diagnostics). For histological analyses, pancreata were isolated, processed to paraffin embedding [27] and 5 μm serial sections were cut. Islet morphology was determined by haematoxylin and eosin (H&E) staining. To measure the α:β cell ratio, sections were stained with anti-insulin antibody (Sigma-Aldrich), anti-glucagon antibody (Dako) and DAPI. Cell proliferation was assessed by Ki67immunofluorescence, while cell apoptosis was evaluated using the DeadEnd™ Colorimetric TUNEL System (Promega), according to the manufacturer's instructions. The number and area of islets and the number of positive-staining β cells were traced and analysed manually using Image-Pro Plus v. 6.0 (Media Cybernetics, Silver Spring, USA); > 100 islets/mouse were analysed in each group.

Islet isolation, cell culture, adenovirus infection and siRNA transfection

Intact islets were isolated as previously described [29]. Islets were cultured in RPMI-1640 containing 11.1 mM glucose supplemented with 10% fetal bovine saline (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37 °C. Adherent islets were then infected with adenovirus carrying full-length mouse *Ggpps* or control adenovirus, or were transfected with Lipofectamine® 2000 Transfection Reagent (Invitrogen) and the indicated siRNAs (see supplementary material, Table S1), according to the manufacturer's instructions.

Glucose-stimulated insulin secretion (GSIS) and insulin measurement

Isolated mouse islets (eight islets/well) were seeded in 48-well plates. For the GSIS assay, islets were pre-incubated with HEPES-balanced Krebs–Ringer buffer (KRBH) containing 2 mM glucose. The islets were then treated with KRBH containing basal glucose (2.8 mM), stimulatory glucose (22.2 mM) or KCl (50 mM) for 1 h, after which supernatants were collected for insulin measurement and islets were lysed for measuring insulin and total protein contents. For *in vivo* GSIS, plasma insulin was collected at 0, 1, 3, 5, 15 and 30 min after an i.p. injection of 1.2 g/kg glucose, following 16 h of fasting. Insulin was detected using the

Rat/Mouse Insulin ELISA Kit (Millipore), according to the manufacturer's instructions.

Transmission electron microscopy (TEM)

Isolated islets were prepared for TEM and images acquired as previously described [30]. The dense core granules and empty granules, granule diameter and distance from the cellular membrane were manually counted and quantified using Image-Pro Plus v. 6.0 (Media Cybernetics).

Subcellular fractionation, immunoprecipitation and western blot

To assess membrane-anchoring of Rab27A and H-Ras, subcellular fractionation of islets was performed using the Triton X-114 partition method and/or ultracentrifugation [26]. These subcellular fractions were immunoprecipitated with Rab27A antibody (BD Biosciences) or H-Ras antibody (Santa Cruz), according to a standard protocol [31], and then subjected to western blot analysis.

Real-time PCR and HPLC–MS/MS measurement of GGPP

RNA was prepared using TRIzol (Takara Bio, Japan), according to the manufacturer's protocol. Real-time PCR was carried out to detect mRNA levels. A complete list of PCR primers is shown in Table S2 (see supplementary material). GGPP was extracted from isolated WT and *GGPPS*-null islets and detected using HPLC–MS/MS, as previously described [26].

Statistical analysis

All data are presented as mean ± standard error of the mean (SE). Statistical calculations were analysed using GraphPad Prism 5 (San Diego, CA, USA), using two-tailed Student's *t*-test; $p < 0.05$ was considered statistically significant and $p < 0.01$ highly significant.

Results

GGPPS shows biphasic expression in the islets of *db/db* mice during the development of T2DM

Pancreatic β cells respond to a chronic phase, excess surfeit of fuel to increase insulin release. In the prediabetic, insulin compensates for the reduced insulin sensitivity and maintains β cell normoglycaemia; this process involves expansion of β cell mass and enhanced function (insulin biosynthesis and secretion). As time goes by, a progressive β cell function proceeds to T2DM decline [11,12]. GEO data suggested that *GGPPS* is decreased in the islets of T2DM patients compared to non-diabetic islets (http://www.ncbi.nlm.nih.gov/geoprofiles/71202350) (see supplementary material, Figure S1) [25]. To determine the function of GGPPS in β cells, we conducted a time course observation of T2DM mouse model *db/db* mice aged 4, 6, 10 and 16 weeks. The body weight of *db/db* mice was significantly increased compared to control mice (Figure 1A). Moreover, *db/db* mice had higher glucose levels at all time-points (Figure 1B), although the insulin level was first increased at 4 and 6 weeks and then progressively declined in 10 and 16 week-old *db/db* mice (Figure 1C). The decrease in plasma insulin indicated that β cells had undergone dysfunction, as they could not compensate for insulin requirements and finally progressed to T2DM. Interestingly, GGPPS in islets showed a similar pattern – highly induced in prediabetic *db/db* mice (aged 4 weeks) and then gradually declining in β cell dysfunction (at age 16 weeks) at both the protein (Figure 1D) and mRNA levels (Figure 1E). These results suggest that decreased GGPPS in islets is associated with β cell dysfunction during the development of T2DM.

GGPPS expression is related to β cell function in isolated islets

We next investigated the correlation of GGPPS with insulin release in β cells. When we stimulated cultured islets of normal mice with high glucose (22.2 mM) to release insulin (Figure 2A), GGPPS was highly induced at both the protein and mRNA levels (Figure 2B, C). Likewise, mice fed high-fat diet (HFD; containing 60% fat) that exhibited elevated insulin release (Figure 2D) also showed significantly enhanced GGPPS expression in islets compared to mice fed with regular chow (RC; Figure 2E, F). In contrast, prolonged incubation of β cells with fatty acids has been shown to reduce the release of insulin in response to high glucose stimulation (Figure 2G) [32,33], an effect that was correlated with GGPPS down-regulation (Figure 2H, I). These data suggest that GGPPS is related to pancreatic β cell function in terms of glucose-responsive insulin release.

GGPPS mediates insulin secretion through protein geranylgeranylation

To investigate whether GGPPS contributes directly to insulin release, primary islets isolated from C57BL/6 J (B6) mice were infected with adenovirus carrying full-length mouse *Ggpps* (see supplementary material, Figure S2A, B). GSIS was measured 48 h after infection. As shown in Figure 2 J, over-expression of GGPPS increased insulin release in high glucose-stimulated islets. When GGPPS was reduced by siRNA targeting *Ggpps* (see supplementary material, Figure S2C, D), insulin secretion was significantly inhibited (Figure 2 K). Meanwhile, digeranyl bisphosphate (DGBP), a potent inhibitor of GGPPS [34], also effectively blocked insulin release in isolated islets (Figure 2 L). As reported previously, GGPP, a product synthesized by GGPPS and used for protein geranylgeranylation, had a similar effect to GGPPS over-expression (Figure 2 M), implying that protein

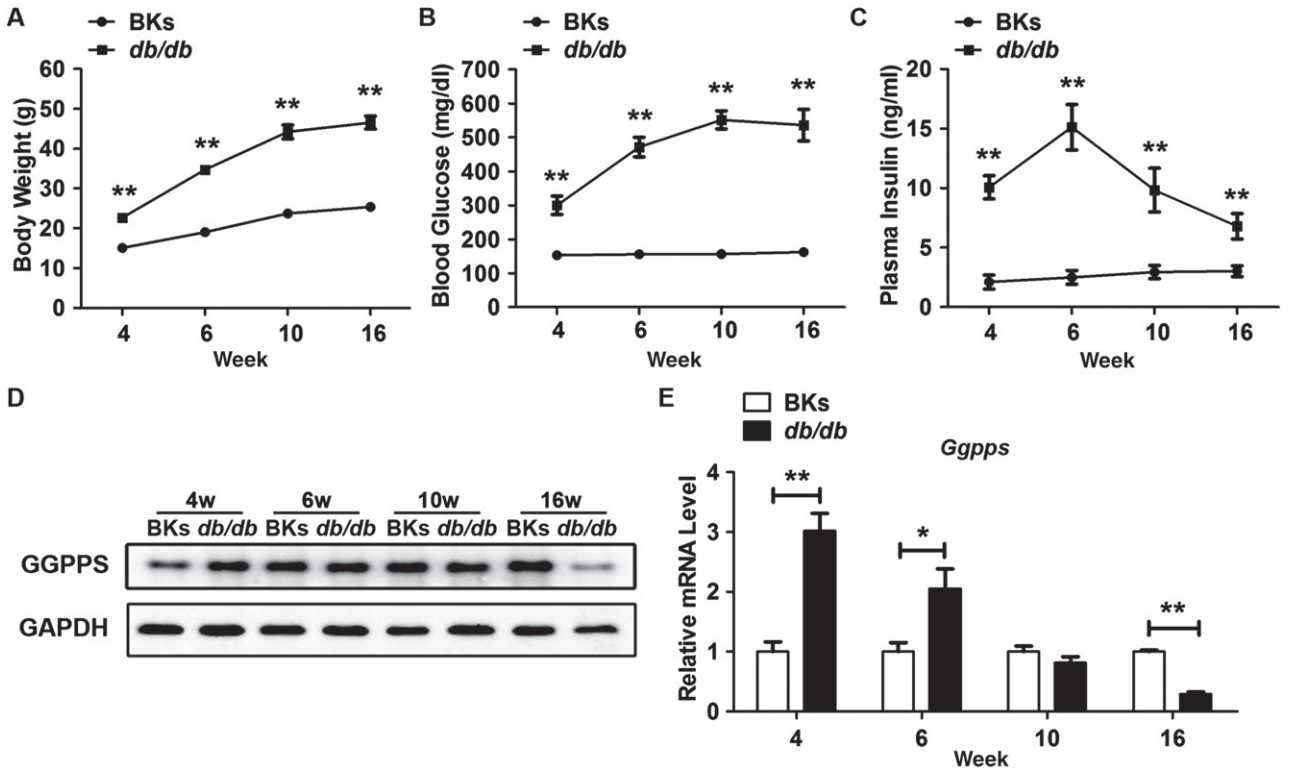

Figure 1. GGPPS shows a two-phasic expression pattern in the islets of *db/db* mice during the development of T2DM. Time course observation of the type 2 diabetes mouse model *db/db* C57BL/KsJ (*db/db*) mice ($n = 6$) and C57BL/KsJ (BKs) mice ($n = 6$) at age 4, 6, 10 and 16 weeks. Profiles of (A) body weight, (B) re-feeding blood glucose and (C) re-feeding plasma insulin in BKs and *db/db* mice. (D) GGPPS protein and (E) mRNA levels in the islets of BKs ($n = 6–8$) and *db/db* mice ($n = 5–8$). $^{*}p < 0.05$, $^{**}p < 0.01$

geranylgeranylation might be involved in GSIS [19]. To evaluate the function of protein geranylgeranylation in GSIS, isolated islets were pretreated with GGTI-2133 (inhibitor of GGTase-I) or siRNA targeting *Rggt*β (see supplementary material, Figure S2E). Blockage of protein geranylgeranylation through inhibition of GGTase-I or of RGGT effectively suppressed GSIS (Figure 2 N, O), as reported elsewhere [19,22]. These *ex vitro* experiments suggest that GGPPS is required for glucose-stimulated insulin secretion in pancreatic β cells through the regulation of protein geranylgeranylation.

GGPPS deficiency in β cells is related to β cell dysfunction during T2DM development

To further confirm the role of GGPPS in pancreatic β cells, we generated β cell-specific *Ggpps* knockout mice (β*Ggpps* KO) by breeding germline *Ggpps*-floxed mice with transgenic mice expressing Cre recombinase under the control of the rat insulin II promoter (*RIP–Cre* mice) (see supplementary material, Figure S3A, B). GGPPS was reduced in β*Ggpps* KO mouse islets by approximately 84% and 74% at the protein and mRNA levels, respectively (see supplementary material, Figure S3C, D). Due to leaky expression of *RIP–Cre* in the arcuate nucleus of the hypothalamus [35], we also detected GGPPS expression that was only partially decreased in the hypothalamus of β*Ggpps* KO mice (see supplementary material, Figure S3E, F). Because *RIP–Cre* mice have also been reported to develop glucose intolerance caused by the toxicity of *Cre* expression [36], we compared the metabolic parameters of *RIP–Cre* mice with wild-type (*Ggpps*-floxed, WT) mice, which showed no obvious differences (see supplementary material, Figure S3G–K). Hence, *RIP–Cre* and WT mice were used as controls for β*Ggpps* KO mice.

Ggpps deletion in pancreatic β cells did not affect body weight (Figure 3A) or food intake (Figure 3B). However, β*Ggpps* KO mice developed hyperglycaemia under both fasting and re-feeding conditions (Figure 3C) and plasma insulin levels were significantly lower (Figure 3D). When given a glucose challenge, β*Ggpps* KO mice exhibited glucose intolerance compared to WT mice (Figure 3E), while peripheral insulin sensitivity was unaffected, evidenced by insulin tolerance tests (ITTs; Figure 3 F). These results indicate that the hyperglycaemia caused by *Ggpps* deletion originates from a decline of insulin release from β cells, rather than systemic insulin resistance. Moreover, reduced insulin secretion due to GGPPS deficiency resembles β cell dysfunction during the progression of T2DM.

GGPPS deficiency does not affect the development of islets

The impaired glucose tolerance and reduced plasma insulin in β*Ggpps* KO mice suggested that β cell function was damaged. Thus, we further examined islets in WT and β*Ggpps* KO mice. The weight and size of the pancreases from β*Ggpps* KO mice were

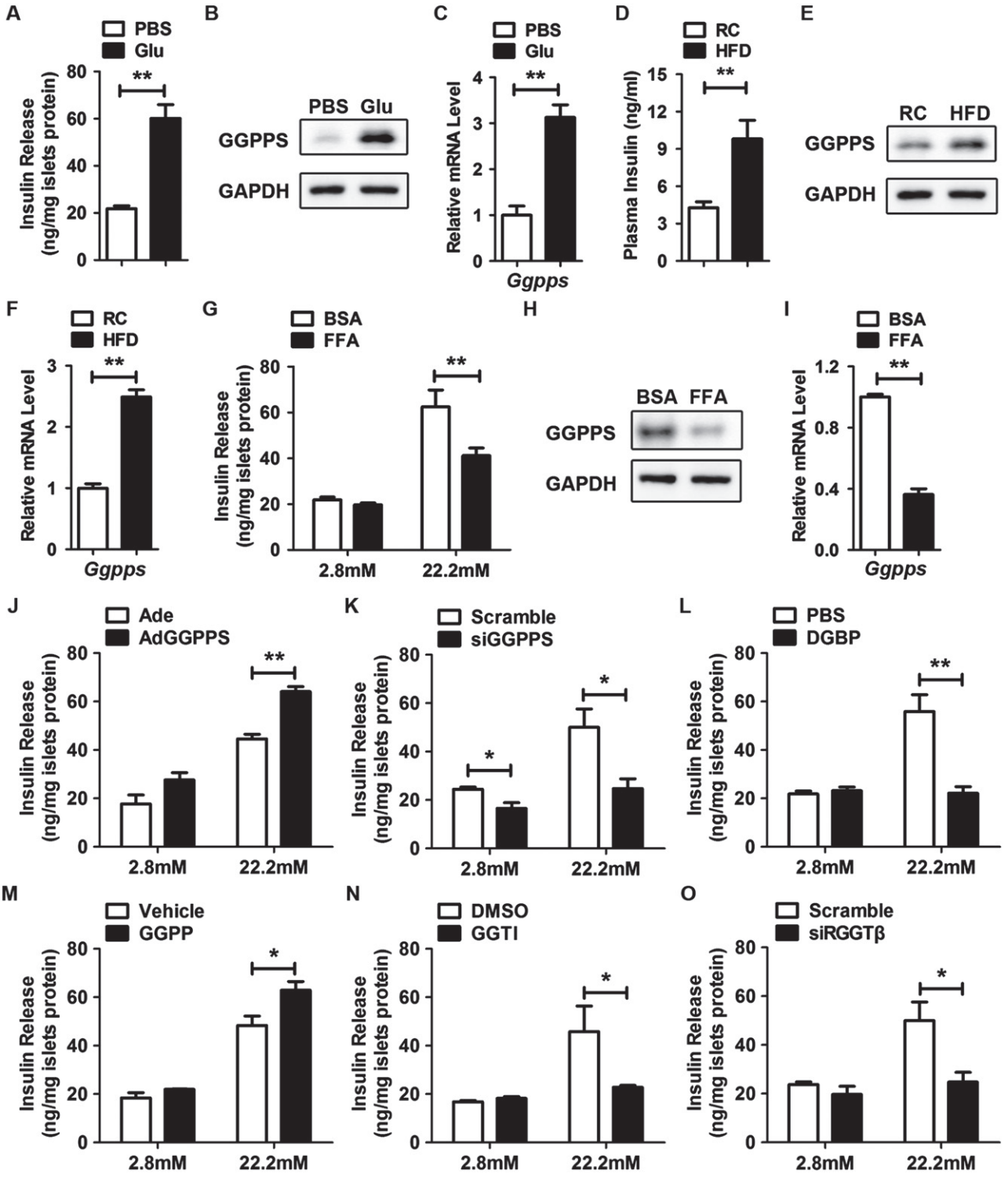

Figure 2. GGPPS regulates β cell insulin secretion through protein geranylgeranylation. (A–C) Islets were isolated from 8 week-old male C57BL/6J mice ($n = 5$) and incubated with medium containing 22.2 mM glucose for the indicated time periods. (A) The supernatant insulin concentration was detected 4 h later; insulin release was normalized to the total islet protein. (B, C) GGPPS protein (B) and mRNA (C) levels in isolated islets after treatment with glucose for 4 h. (D–F) C57BL/6J mice were fed regular chow (RC, containing 4% fat; $n = 5$) or a high-fat diet (HFD, containing 60% fat; $n = 5$) for 6 weeks: (D) re-feeding plasma insulin; (E) GGPPS protein; and (F) mRNA levels in isolated islets. (G–I) Islets isolated from 8 week-old C57BL/6J mice incubated with 1 mM FFA mixture (oleic:palmitic acids = 2:1) for 48 h; (G) the medium was replaced with fresh medium containing 2.8 and 22.2 mM glucose for 1 h and the insulin concentration of the supernatant was measured, normalized to total islets; (H) GGPPS protein and (I) mRNA levels. (J–O) Islets from 8 week-old C57BL/6J mice ($n = 5$ each sample preparation; three repeats) were treated with the indicated reagents: the medium was exchanged for fresh medium containing 2.8 and 22.2 mM glucose, 48 h after infection or transfection or 24 h after other treatments, and the insulin concentration of the supernatant was measured after 1 h: (J) islets were infected with adenovirus carrying mouse *Ggpps* and control (Ade) or (K) transfected with siRNA targeting *Ggpps* or scrambled RNA. Islets were pretreated with (L) 10 μM DGBP; (M) 20 μM GGPP; (N) 20 μM GGTI or (O) DMSO; the islets were transfected with siRNA targeting *RGGT*β or scrambled RNA; $^{*}p < 0.05$; $^{**}p < 0.01$

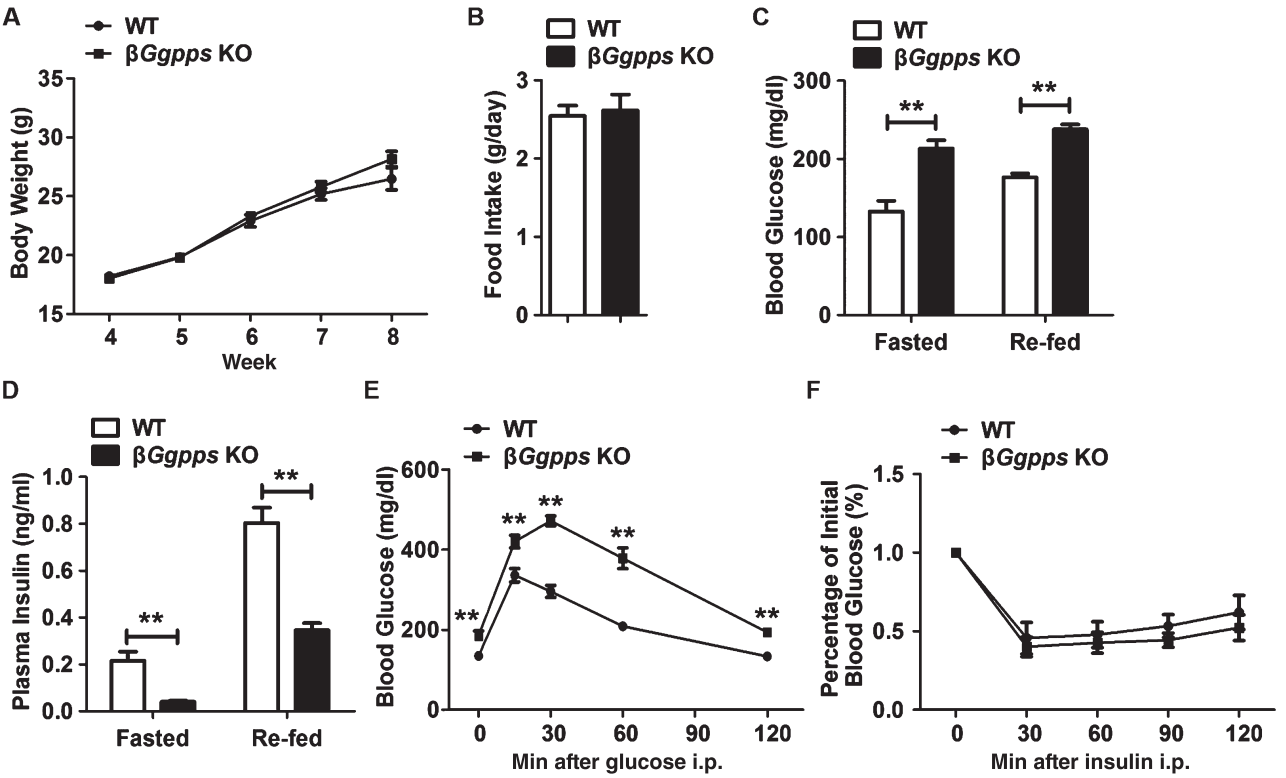

Figure 3. GGPPS deficiency in β cells is related to β cell dysfunction during the development of T2DM. The metabolic parameters were measured in 8 week-old WT ($n = 8–10$) and β*Ggpps* KO ($n = 8–10$) mice. Profiles of (A) body weight and (B) daily food intake. (C, D) 16 h fasting and 6 h re-feeding blood glucose and plasma insulin levels. (E) GTT was performed by i.p. injection of 1.2 g/kg glucose into WT and β*Ggpps* KO mice. (F) ITT was performed by i.p. injection of 1 IU/kg insulin into WT and β*Ggpps* KO mice; data were normalized to the basal blood glucose levels; $^{*}p < 0.05$; $^{**}p < 0.01$

generally preserved (Figure 4A) and isolated islets from these mice showed no discernable differences compared to WT mice (Figure 4B). Likewise, β cell mass was not changed in β*Ggpps* KO mice (Figure 4C). Serial sections and H&E staining indicated that *Ggpps* deletion in β cells did not influence islet morphology (Figure 4D), size (Figure 4E), number (Figure 4 F) or architecture, as indicated by insulin and glucagon immunostaining (Figure 4G) and the calculated ratio of glucagon-expressing α-cells to insulin-expressing β cells (Figure 4H). The number of β cells positive for Ki-67 was equivalent between β*Ggpps* KO and WT mice and we found no TUNEL-positive β cells in β*Ggpps* KO or WT islets (Figure 4I–K; see also supplementary material, Figure S4, positive and negative controls). These results suggest that *Ggpps* deletion in β cells had no effect on islet morphology or development. Thus, insulin deficiency in β*Ggpps* KO mice might derive from attenuated insulin biosynthesis or defects in insulin secretion.

GGPPS deficiency in β cells impairs glucose-responsive biphasic insulin secretion

Surprisingly, we found that there were no differences in the expression of insulin biosynthesis-related genes in the islets from β*Ggpps* KO and WT mice (Figure 5A). This was further confirmed by the detection of total cellular insulin content, which was not altered in β*Ggpps* KO mouse islets compared to WT mice (Figure 5B). Next, we assessed the insulin-release capability of islets. In static *ex vivo* GSIS tests, *Ggpps*-null islets showed impaired insulin release in response to glucose challenge (Figure 5C). This was also seen in the presence of 50 mM KCl, in which *Ggpps*-null islets secreted less insulin than WT islets (Figure 5C). In addition, the dynamic determination of insulin release revealed that β*Ggpps* KO mice failed to respond to glucose infusion by secreting adequate insulin, which resulted in a total decrease in plasma insulin levels of 41% relative to WT mice (Figure 5D). When we normalized the insulin level to the basal levels, β*Ggpps* KO mice displayed impaired biphasic insulin secretion (see supplementary material, Figure S5). These findings indicate that the insulin deficiency in β*Ggpps* KO mice has resulted from failure of insulin exocytosis, even though their islets contained sufficient insulin. Moreover, *Ggpps* deficiency in β cells impairs insulin secretion in a manner that resembles the β cell dysfunction observed in T2DM [2].

Ggpps deletion impairs docked granule pool formation without affecting reserve pool via blocking Rab27A geranylgeranylation

The biphasic secretion of insulin is determined by the size of the docked granule pool inside the β cell. Using TEM on isolated islets, we found no obvious difference in the appearance (dense-core), number and

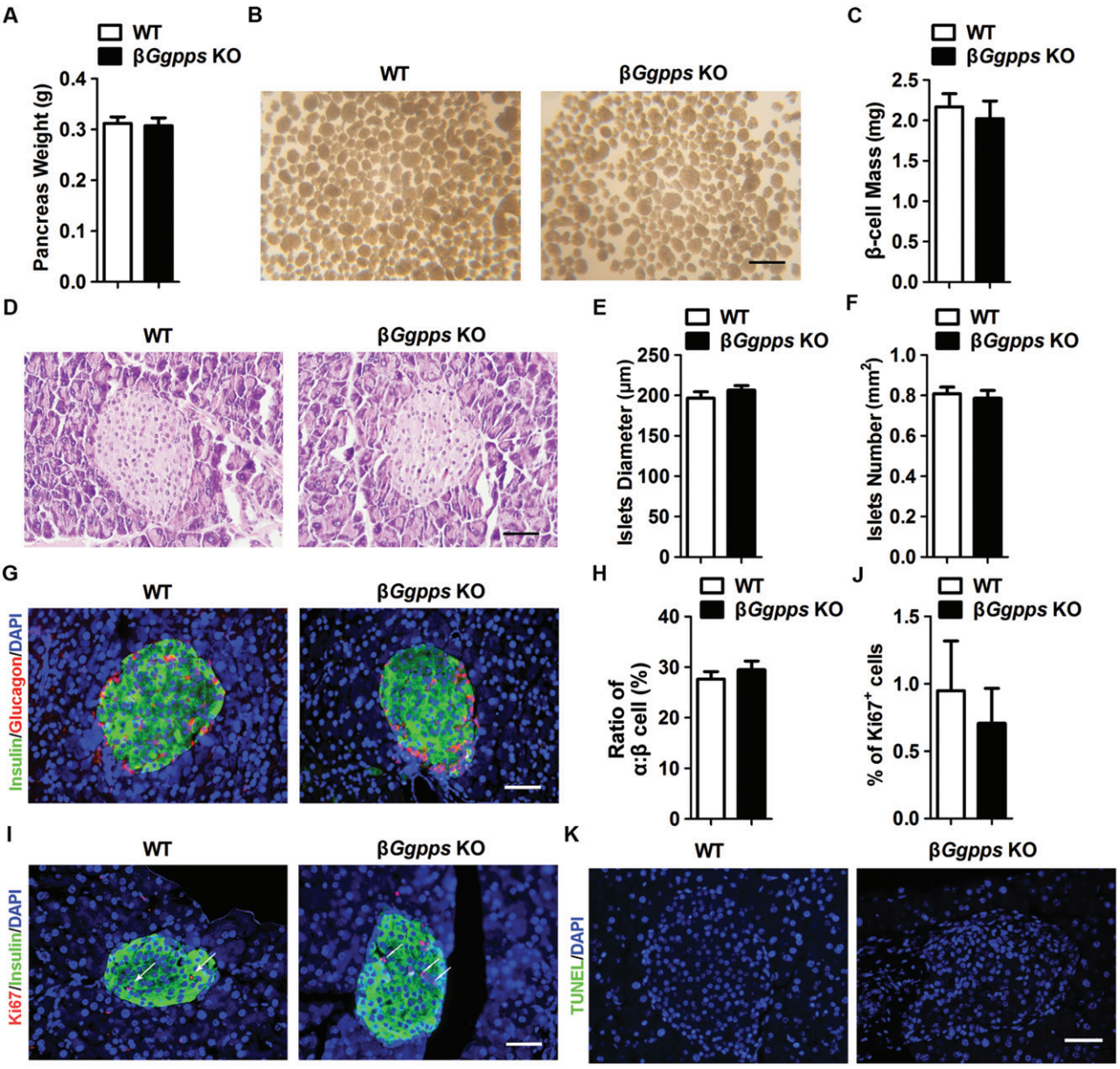

Figure 4. GGPPS deficiency does not affect the development of islets in 10 week-old WT ($n = 8-10$) and β*Ggpps* KO ($n = 8-10$) mice. (A) Pancreas weight. (B) Morphology of isolated islets; scale bar = 500 μm. (C) β cell mass, calculated by multiplying the total area occupied by the insulin-positive cells in the pancreatic sections by the weight of the pancreas for each mouse. (D) Representative microphotographs of H&E-stained pancreatic sections from WT and β*Ggpps* KO mice; scale bar = 100 μm. (E) Islet diameters were calculated from the islet areas, measured using Image Pro Plus ($n > 100$ each genotype). (F) Number of islets/mm^2. (G) Representative immunofluorescence staining of insulin and glucagon in pancreatic sections (DAPI counterstain). (H) Quantification of α:β cells ratio/islet. (I) Representative images and (H) quantitation of Ki67-positive β cells as the percentage of β cells/islet; arrows, Ki67-positive β cells. (K) Representative images of TUNEL staining; scale bars = 100 μm

size of insulin-containing vesicles in *Ggpps*-null β cells versus WT β cells (Figure 6A–C), which implied that the reserve pool was not affected by *Ggpps* deletion. Intriguingly, there were fewer docked granules beneath the plasma membrane in *Ggpps*-null β cells evaluated according to the distance from dense-core centre to the plasma membrane (Figure 6A, D), which suggested that the formation of the docked granule pool was blocked.

Rab27A has been reported to control docked pool formation and insulin exocytosis [13,15], activation of which is mediated by post-translational geranylgeranylation [17,18]. We found that Rab27A expression and geranylgeranylation were inhibited in 16 week-old diabetic *db/db* mice islets (Figure 6E) [37], implying that impaired Rab27A function might associate with β cell dysfunction in T2DM. Intracellular GGPP levels were lower in *Ggpps*-null islets than WT according to HPLC–MS/MS (Figure 6F). Moreover, *Ggpps* deletion inhibited Rab27A geranylgeranylation and membrane association without affecting H-Ras prenylation, which can only be farnesylated with FPP (Figure 6G; see also supplementary material, Figure S6). These data indicate that GGPPS deficiency abrogates protein geranylgeranylation and membrane anchoring of proteins

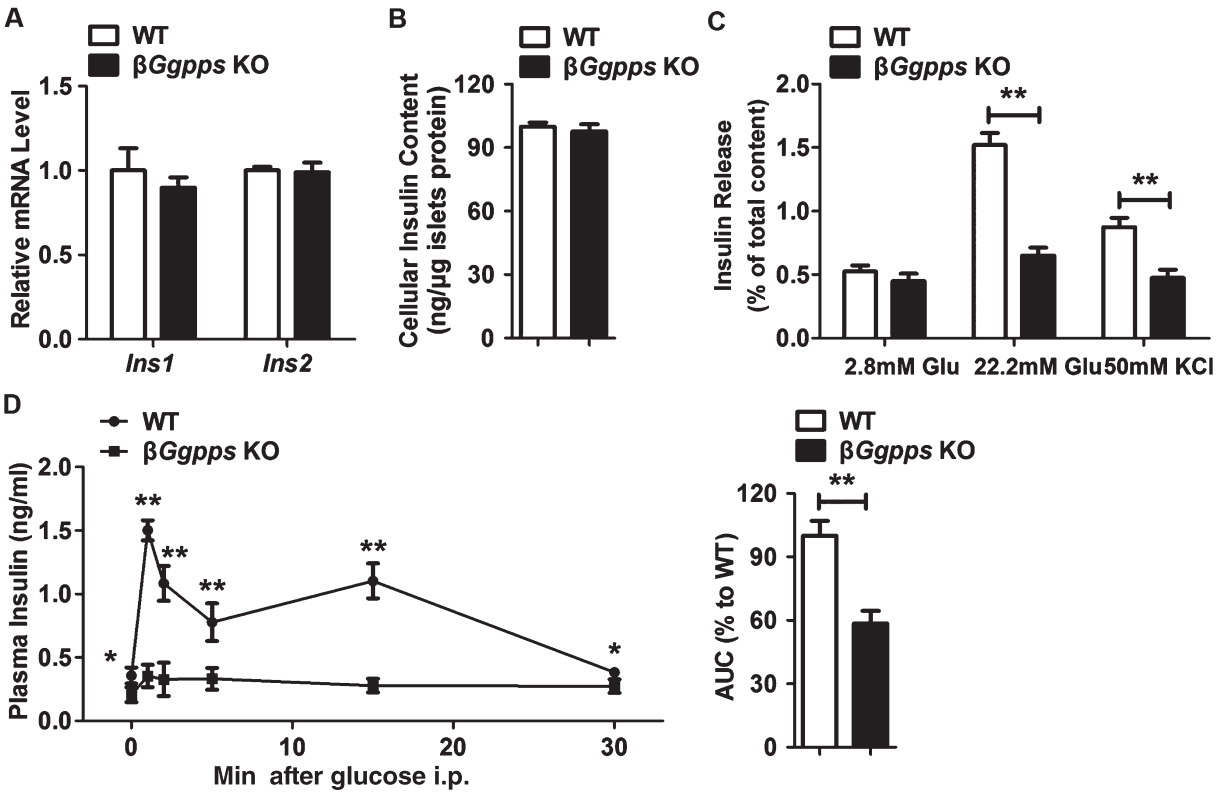

Figure 5. GGPPS deficiency impairs glucose-responsive biphasic insulin secretion. (A–C) Islets were isolated from 10 week-old WT and β*Ggpps* KO mice: (A) real-time PCR for insulin biosynthesis-related gene expression in WT and KO mouse islets; (B) adherent islets were lysed to measure the total cellular insulin; (C) islets were incubated for 1 h in medium containing 2.8 or 22.2 mM glucose or 50 mM KCl and the insulin content in the supernatant was evaluated; insulin release was normalized to the total cellular insulin content. (D) Dynamic determination of insulin release was performed in 10 week-old WT ($n = 10$) and β*Ggpps* KO ($n = 11$) mice; mice fasted for 16 h were subjected to 1.2 g/kg i.p. injection of glucose and the plasma insulin was measured at the indiated times; AUC, area under the curve; $^{*}p < 0.05$, $^{**}p < 0.01$

such as Rab27A in β cells, which resembles β cell dysfunction in T2DM. Moreover, when we infected islets with adenovirus expressing *Ggpps*, Rab27A geranylgeranylation was increased (see supplementary material, Figure S7) with a restoration of insulin secretion in *Ggpps*-null islets (Figure 6H). This was also seen in *Ggpps*-null islets treated with GGPP, where insulin release after glucose stimulation was partially rescued (Figure 6I).

Discussion

The present study identifies GGPPS as a critical regulator of β cell function during the development of T2DM. GGPPS influences the β cell function of glucose-responsive insulin secretion by controlling formation of the docked granule pool. The underlying mechanism involves GGPPS deficiency, which blocks the protein geranylgeranylation and membrane-anchoring of Rab27A required for insulin granule docking behaviour. These findings suggest that GGPPS is essential for maintaining physiological insulin secretion from pancreatic β cells and that deregulation of GGPPS may contribute to β cell dysfunction during T2DM development.

In compensatory hyperinsulinaemia, pancreatic β cells respond to gradually elevated blood glucose to increase insulin release. This process is related to enhanced insulin biosynthesis and exocytosis and altered gene expression in β cells [1]. For instance, the transcription factor early growth response 1 (*EGR1*) is required for islet development and function [38,39], is induced by glucose in islets [40] and is responsive to insulin stimulation [41]. Intriguingly, we have previously shown that GGPPS is a direct target of *EGR1* in adipocytes under conditions of hyperinsulinaemia [31,42]. In the present study, we observed an up-regulation of GGPPS in the islets of *db/db* mice during the compensatory hyperinsulinaemia period (Figure 1D, E). This up-regulation was also observed in islets under glucose-stimulated and HFD-induced conditions (Figure 2A–F). EGR1 might contribute to the elevation of GGPPS under these conditions, which may be required for building the insulin docked pool and maintaining insulin release. However, prolonged excessive insulin mobilization leads to β cell exhaustion and dysfunction, which finally develops into T2DM [11,12]. Here, we also observed a progressive decrease in GGPPS in accordance with the age-dependent impairment of insulin secretion in the islets of diabetic *db/db* mice (Figure 1D, E). The reason for reduced expression of GGPPS during β cell dysfunction is unknown, although it might result from oxidative stress, ER stress or β cell exhaustion due to prolonged excessive

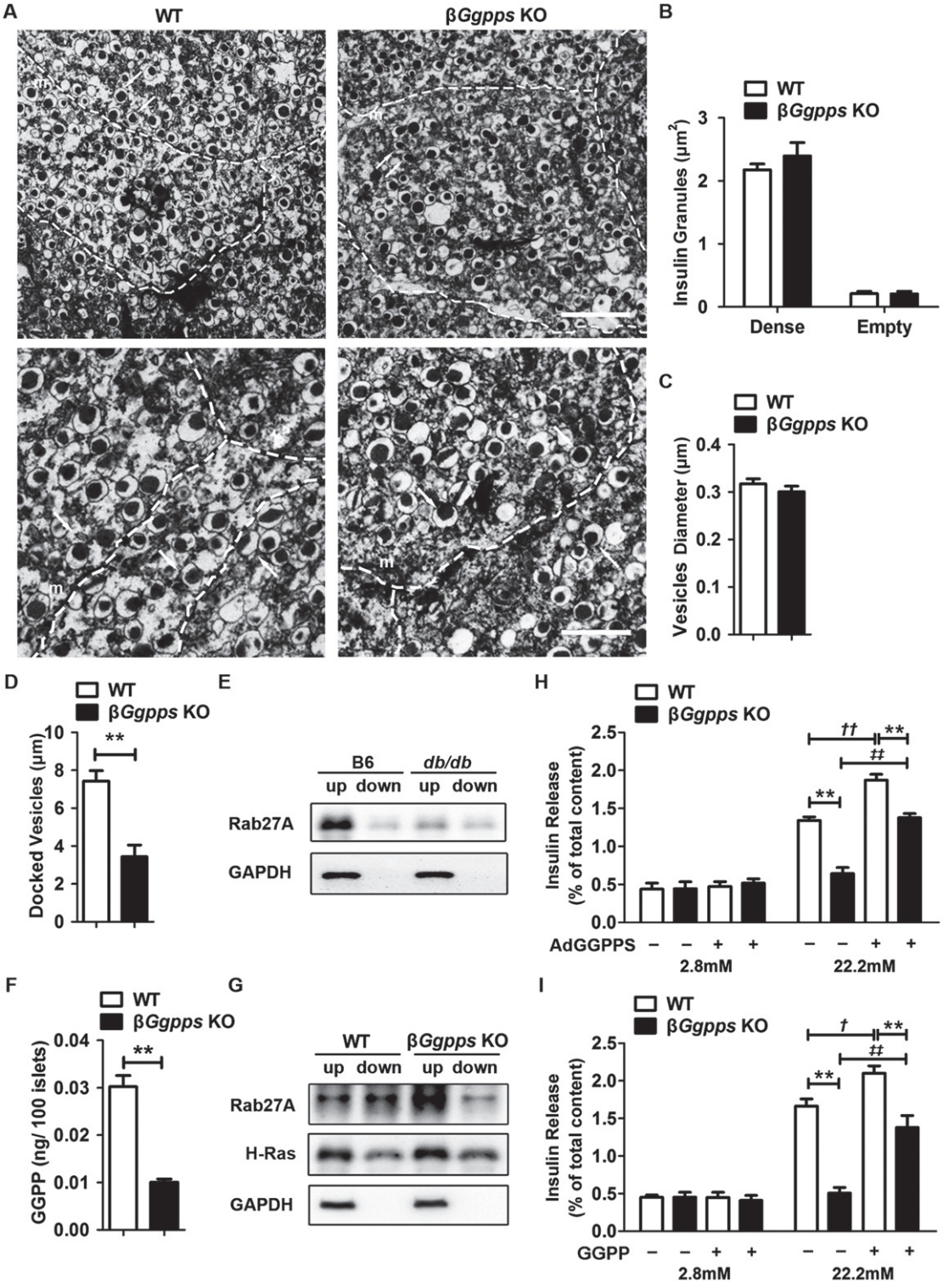

Figure 6. GGPPS deletion impairs insulin granules docked pool formation without affecting reserve pool through Rab27A geranylgeranylation. (A–D) Islets were isolated from 10 week-old WT and β*Ggpps* KO mice and subjected to TEM: (A) insulin granule morphology; white dashed lines, β cell membranes (m, membrane); arrows, insulin granules; scale bars = (upper) 2 μm; (lower) 1 μm; (B) number of mature (dense-core) and empty insulin granules/μm^2; (C) insulin vesicle diameters, calculated from the vesicle area; (D) number of docked vesicles/1 μm plasma membrane; a distance < 400 nm from the centre of the vesicle to the plasma membrane was considered to be a docked granule. (E) Rab27A geranylgeranylation in the islets of 16 week-old *db/db* mice: subcellular fractionation was performed using the Triton X-114 partition method; the aqueous upper phase (up) contains water-soluble and the organic lower phase (down) contains lipid-soluble small GTPases. (F) Intracellular GGPP content, detected by HPLC–MS/MS in WT and *GGPPS*-null islets; $^{**}p < 0.01$. (G) Rab27A geranylgeranylation and H-Ras farnesylation in subcellular fractions of islets. (H, I) Insulin release from isolated WT and β*Ggpps* KO mouse islets with (H) GGPPS over-expression or (I) the addition of either 20 μM GGPP or vehicle in medium containing basal glucose (2.8 mM) or stimulated medium (22.2 mM glucose); $^{**}p < 0.01$, WT versus β*Ggpps* KO; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, WT versus WT (*AdGGPPS* or *GGPP*); $^{\ddagger\ddagger}p < 0.01$, β*Ggpps* KO versus β*Ggpps* KO (*AdGGPPS* or *GGPP*)

use [11]. The altered expression pattern of GGPPS is closely correlated with β cell function, which was further evidenced by over-expression and knockdown of GGPPS (Figure 2 J–L). Therefore, we propose that GGPPS plays a crucial role in physiological insulin release and that the decline in its expression causes β cell dysfunction as observed in T2DM.

The mevalonate pathway has been implicated in glucose-responsive insulin secretion in laboratory settings [19,23]. Our current data provide the first evidence that GGPPS-controlled protein geranylgeranylation is required for β cell function in terms of insulin release. *Ggpps* deletion in β cells markedly suppressed insulin release, resulting in impaired glucose tolerance with an absolute insulin insufficiency (Figure 3D, E). The lack of GGPPS, and therefore GGPP depletion (Figure 6 F), resulted in a decrease of Rab27A geranylgeranylation (Figure 6G; see also supplementary material, Figure S6), which is required for maintaining the hydrophobicity and membrane-anchoring properties of Rab27A that allow it to form the insulin vesicle docked pool [13,43]. In addition to Rab proteins, Cdc42 and proteins from the Rho and Rac families also undergo geranylgeranylation and reportedly have roles in mediating insulin secretion [24]. Thus, although the GGPPS-mediated synthesis of GGPP is insulinotropic in β cells, target proteins beyond Rab27A have yet to be identified.

Another indication of our research is that inhibition of protein geranylgeranylation blocks GSIS. Thus, the use of mevalonate pathway inhibitors such as statins (used in the treatment of hypercholesterolaemia) and nitrogenous bisphosphonates (NBPs; used for osteoporosis and metastatic bone disease) [44,45] should be cautiously evaluated before being applied in clinical settings, because they both abrogate protein prenylation and may impair insulin secretion.

In conclusion, we have identified a critical role for GGPPS in β cell function during the development of T2DM. GGPPS correlates with islet insulin secretion during the compensatory period and with defective insulin exocytosis during β cell dysfunction. GGPPS exerts its function through the synthesis of GGPP, which is required for protein geranylgeranylation and membrane anchoring that controls the formation of the docked pool of insulin granules. These findings indicate that decreased expression of GGPPS is responsible for β cell dysfunction during the development of T2DM.

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Author contributions

SJ and CJL designed the research; SJ, DS, WJJ, NS, WWT researched the data; XH and XG edited the manuscript and provided reagents; and SJ, BX and CJL wrote the manuscript.

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SUPPLEMENTARY MATERIAL ON THE INTERNET

The following supplementary material may be found in the online version of this article:

Figure S1. *GGPPS* expression in the islets of type 2 diabetes patients

Figure S2. Gene expression in isolated islets

Figure S3. Construction of β cell-specific *Ggpps* KO mice and metabolic parameters in *RIP–Cre* mice

Figure S4. Negative and positive controls in the TUNEL assay

Figure S5. Normalized glucose-stimulated insulin secretion in WT and β*Ggpps* KO mice

Figure S6. Rab27A membrane anchoring is significantly inhibited after *GGPPS* deletion

Figure S7. Rab27A geranylgeranylation is enhanced after over-expression of *GGPPS*

Table S1. siRNA sequences

Table S2. Primer sequences