Brief communication

MOLECULAR METABOLISM

# Increased biogenesis of glucagon-containing secretory granules and glucagon secretion in BIG3-knockout mice

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## ABSTRACT

**Objective:** Although both insulin and glucagon are intimately involved in the regulation of glucose homeostasis, the intrinsic control of glucagon secretion, including the biogenesis and exocytosis of glucagon-containing granules, is far less understood compared with that of insulin. As Brefeldin A-inhibited guanine nucleotide exchange protein 3 (BIG3) is a negative regulator of insulin-granule biogenesis and insulin secretion, we investigated whether BIG3 plays any role in alpha-cells and glucagon secretion.

**Methods:** We examined the expression of BIG3 in islet cells by immuno-fluorescence and confocal microscopy, and measured glucagon production and secretion in BIG3-depleted and wild-type mice, islets and cells.

**Results:** BIG3 is highly expressed in pancreatic alpha-cells in addition to beta-cells, but is absent in delta-cells. Depletion of BIG3 in alpha-cells leads to elevated glucagon production and secretion. Consistently, BIG3-knockout (BKO) mice display increased glucagon release under hypoglycemic conditions.

**Conclusions:** Together with our previous studies, the current data reveal a conserved role for BIG3 in regulating alpha- and beta-cell functions. We propose that BIG3 negatively regulates hormone production at the secretory granule biogenesis stage and that such regulatory mechanism may be used in secretory pathways of other endocrine cells.



**Keywords** Alpha-cell; BIG3; Diabetes; Exocytosis; Glucagon; Glucose homeostasis

## 1. INTRODUCTION

Pancreatic islets produce several hormones to regulate glucose production, uptake and utilization, and impaired islet function is a hallmark of both type 1 and type 2 diabetes [1—4]. Among the islet hormones, insulin from beta-cells and glucagon from alpha-cells are more prominent and better studied. The two hormones act oppositely to maintain glucose homeostasis in response to changes in energy states in the body. At fed state, insulin levels are elevated and glucagon levels suppressed to promote glucose uptake by the peripheral tissues. Conversely, at fasted state, insulin levels are reduced and glucagon levels elevated to enhance hepatic glucose output and fat mobilization. Although it is commonly believed that insulin deficiency and insulin resistance are the causal factors in diabetes development, glucagon secretion dysregulation is necessary in the hyperglycemic development [5—8].

There have been extensive studies on the mechanisms of insulin secretion by beta-cells. In contrast, much fewer studies have been performed to understand glucagon secretion by alpha-cells. The fact that under physiological conditions insulin and glucagon secretion is stimulated at opposite ends of glucose levels indicates that alpha- and beta-cells must possess distinct regulatory pathways to increase $Ca^{2+}$-levels, a common triggering signal for secretory granule exocytosis. However, at the molecular level, the two cell types share many common features, including the use of the same secretory machineries and a $Ca^{2+}$-sensing protein [9—14].

In our previous studies, we have identified that Brefeldin A-inhibited guanine nucleotide exchange protein 3 (BIG3) is a negative regulator of insulin granule biogenesis in pancreatic beta-cells, and that absence of BIG3 in beta-cells leads to elevated insulin production and secretion, which contribute to the dysregulation of glucose homeostasis in animals [15,16]. Here we investigated the role of BIG3 in alpha-cells and glucagon release. Our findings support a conserved BIG3 function in the regulation of hormone release in alpha- and beta-cells.

## 2. MATERIALS AND METHODS

### 2.1. Animal welfare

All experiments involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of A*STAR (Agency

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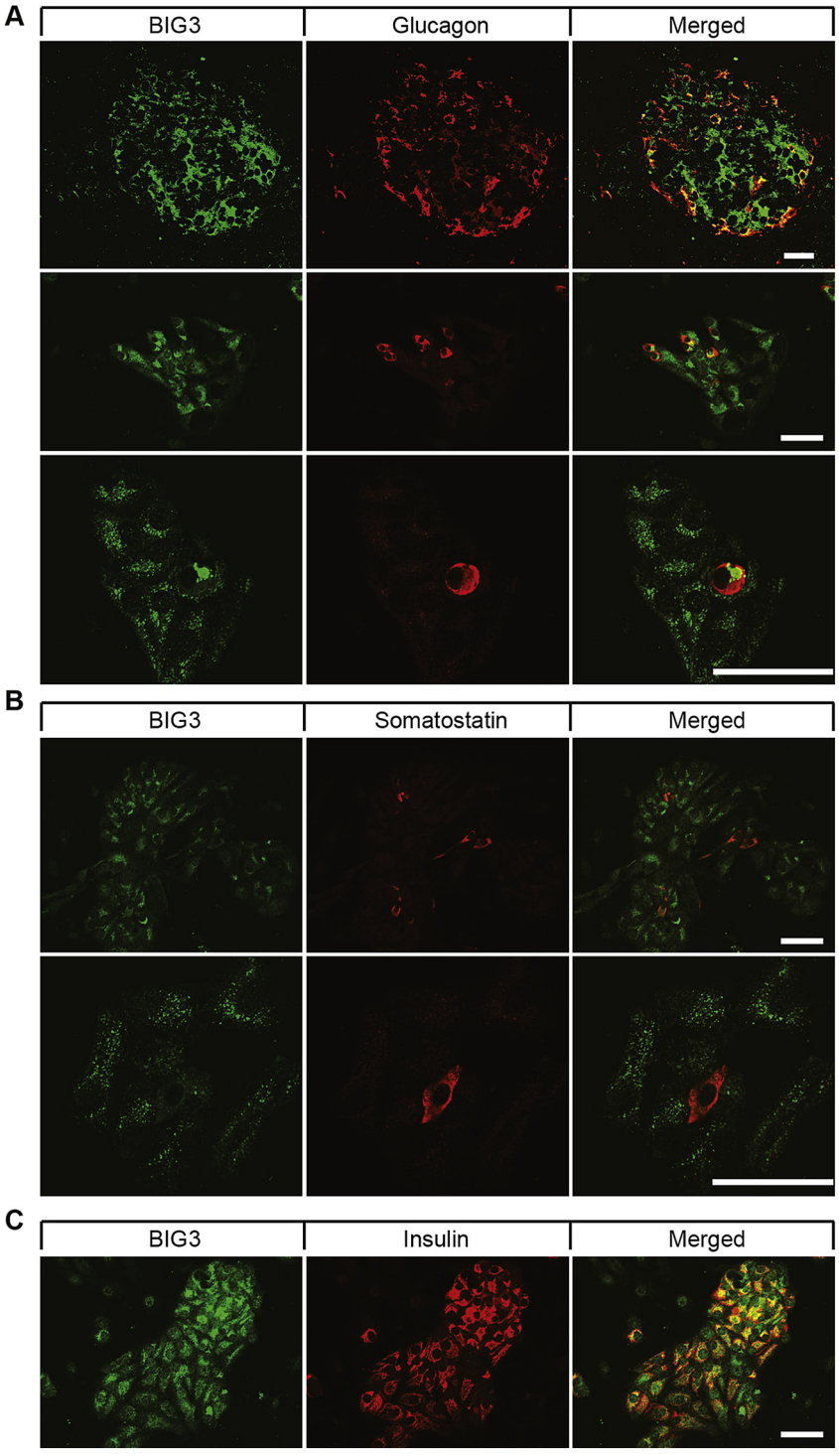


**Figure 1:** BIG3 is highly expressed in pancreatic alpha-cells. (A) Immunostaining of mouse islet cells showing BIG3 expression in glucagon-positive cells. The top panel is a frozen section of mouse pancreas; the middle and bottom panels are cultured islets. Scale bars are 100, 50 and 25 μm for top, middle and bottom panels, respectively. (B) Immunostaining of cultured mouse islet cells showing absence of BIG3 marked granular puncta in somatostatin positive cells. Scale bars are 50 and 25 μm for top and bottom panels, respectively. (C) Immunostaining of cultured mouse islet cells showing BIG3 expression in insulin-positive cells. Scale bar = 50 μm.

for Science, Technology and Research). All mice used in this study were bred and housed in the animal facility of Biological Resource Centre (A*STAR) under specific pathogen-free conditions with a 12 h light—dark cycle and free access to water and food. Heterozygous BIG3 global knockout (BIG3$^{+/-}$) mice generated as previously described [15] were back-crossed with wildtype C57BL/6 mice eight times to purify the genetic background. Male littermates between 10 and 20 weeks of age were used in all studies. N referred to the number of pairs of BKO and control littermates unless specified otherwise.

  

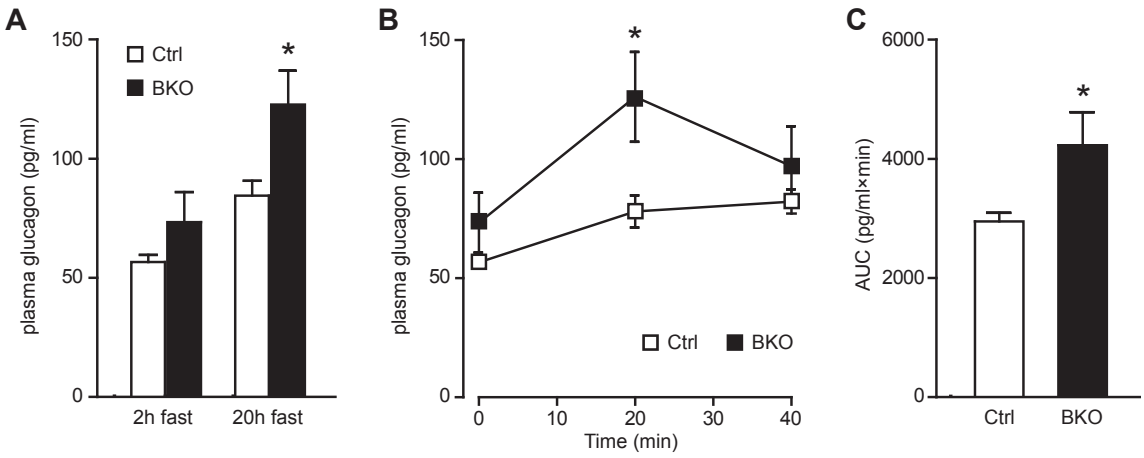


**Figure 2:** Enhanced glucagon secretion in BKO mice. (A) Plasma glucagon levels after 2-hr and 20-hr fasting. N = 8 pairs. (B) Plasma glucagon levels during insulin tolerance tests. N = 10 pairs. (C) Total glucagon secretion during ITT as calculated from area-under-curve in B. N = 10 pairs. Data are presented as mean ± SEM. Statistical difference was determined by two tailed student t-test. * indicates $p < 0.05$.

### 2.2. Physiology tests and measurements

Physiology tests were performed essentially as previously described [17]. Briefly, fasted and fed glucose measurements and insulin tolerance tests (ITT) were performed between 09:00 to 12:00. Glucose levels were determined by using a glucometer (Accu-Chek, Roche Diagnostics, Singapore). For ITT, 1 unit of insulin per kg of body weight diluted in saline was injected intraperitoneally after a 2 h fast. Blood samples were collected before, 20 and 40 min after injection. For islet isolation and histochemistry, mice were sacrificed by cervical dislocation.

### 2.3. Islet isolation and glucagon secretion measurement

Pancreatic islets were isolated from 14 to 20 weeks old male C57BL/6 mice as previously described [11]. For histological analysis, mouse pancreas tissues were fixed in 4% PFA at 4 °C before frozen embedding, followed by sectioning and immunostaining. Islets from BKO and control mice were isolated after collagenase P (1.5 mg/5 ml, Roche) digestion. For secretion assay, islets were cultured for 24 h at 11.2 mM glucose in RPMI medium. Subsequent secretion assays were performed with serum free RPMI medium supplemented with 1 mg/ml BSA, 10 mM HEPES pH7.4 and indicated glucose levels. Similar-sized islets from a single mouse (10 islets per group) were first incubated at 37 °C for 60 min with 6 mM glucose, followed by fresh medium containing 0.7 mM glucose for 60 min. The media were collected for ELISA to determine released glucagon (ALPCO Diagnostics, Salem, NH). For estimation of islet glucagon content, islets were snap frozen after harvest and lysed in 100 μl of passive lysis buffer (Cell Signaling Technology, Beverley, MA). Total protein of lysed islets was quantified by the BCA method (Pierce, Rockford, IL). For immunofluorescence staining, isolated islets were treated with trypsin briefly with gentle pipetting to obtain islet cell clumps, and cultured on matrigel-coated glass slides for 2 days before fixation and staining. For electrophysiology study, isolated islets were treated with trypsin with pipetting to get single cell suspension and cultured on matrigel slides over night.

### 2.4. Quantitative PCR

Quantitative PCR was performed essentially as previously described [18]. Total mRNA from overnight cultured islets was extracted by mini column kit (Qiagen, Hilden, Germany), and reverse-transcribed to generate cDNA (Fermentas, Life Sciences, Germany). Quantitative PCR was performed on a StepOnePlus RT-PCR system (Applied Biosystems, Foster, CA) with SYBR Green kits (Invitrogen, Carlsbad CA). Primers used for qPCR were beta-actin: ATG CTC CCC GGG CTG TAT and CAT AGG AGT CCT TCT GAC CCA TTC; proglucagon: GAT TTT GTG CAG TGG TTG AT and ACT TCT TCT GGG AAG TCT TCG; and SNAP25: CAA CTG GAA CGC ATT GAG GAA and GGC CAC TAC TCC ATC CTG ATT AT.

### 2.5. Electrophysiology

Patch clamp recording and membrane capacitance recording $C_m$ were performed using a software lock-in module of the Pulse software together with an EPC10/2 amplifier (HEKA Elektronik, Bellmore NY) as described previously [10,19]. Briefly, cells were bathed in extracellular solution containing (in mM) 118 NaCl, 20 tetraethylammonium-Cl, 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 5 HEPES, and 5 glucose, pH adjusted to 7.4 by NaOH. Forskolin (10 μM; Tocris Bioscience, Ellisville, MO) was included in extracellular solution in all experiments to enhance alpha-cell exocytosis [20]. Alpha-cells were identified based on their size (basal membrane capacitance <5 pF) and the discernible outward voltage-gated $K^+$-current [21]. Voltage clamp in the whole cell configuration was used to record voltage-gated membrane current. Intracellular solution contained (in mM) 125 Glutamic Acid, 10 NaCl, 10 KCl, 1 $MgCl_2$, 3 MgATP, 0.1 cAMP, and 5 HEPES, pH adjusted to 7.15 using KOH. Pipette resistance was 3–4 MΩ. All experiments were performed at 29–30 °C (Warner Instruments, Hamden, CT) with continuous bath perfusion (ISMATEC).

### 2.6. Immunostaining and imaging

For immunostaining, the following antibodies were used: primary antibodies against BIG3 (generated as described [15]), glucagon (Sigma–Aldrich, St. Luis, MO), insulin (Dako, Tokyo, Japan), somatostatin (Santa Cruz Biotechnology, Santa Cruz, CA); and Alexa Fluor 488-, 555- and 647-conjugated secondary antibodies (Invitrogen, Carlsbad CA). Images were acquired by using an A1R+ confocal microscope (Nikon, Tokyo, Japan).

### 2.7. Electron microscopy

Electron microscopy of isolated islets was performed as described previously [15]. In all cases, a single section from each block was used

 

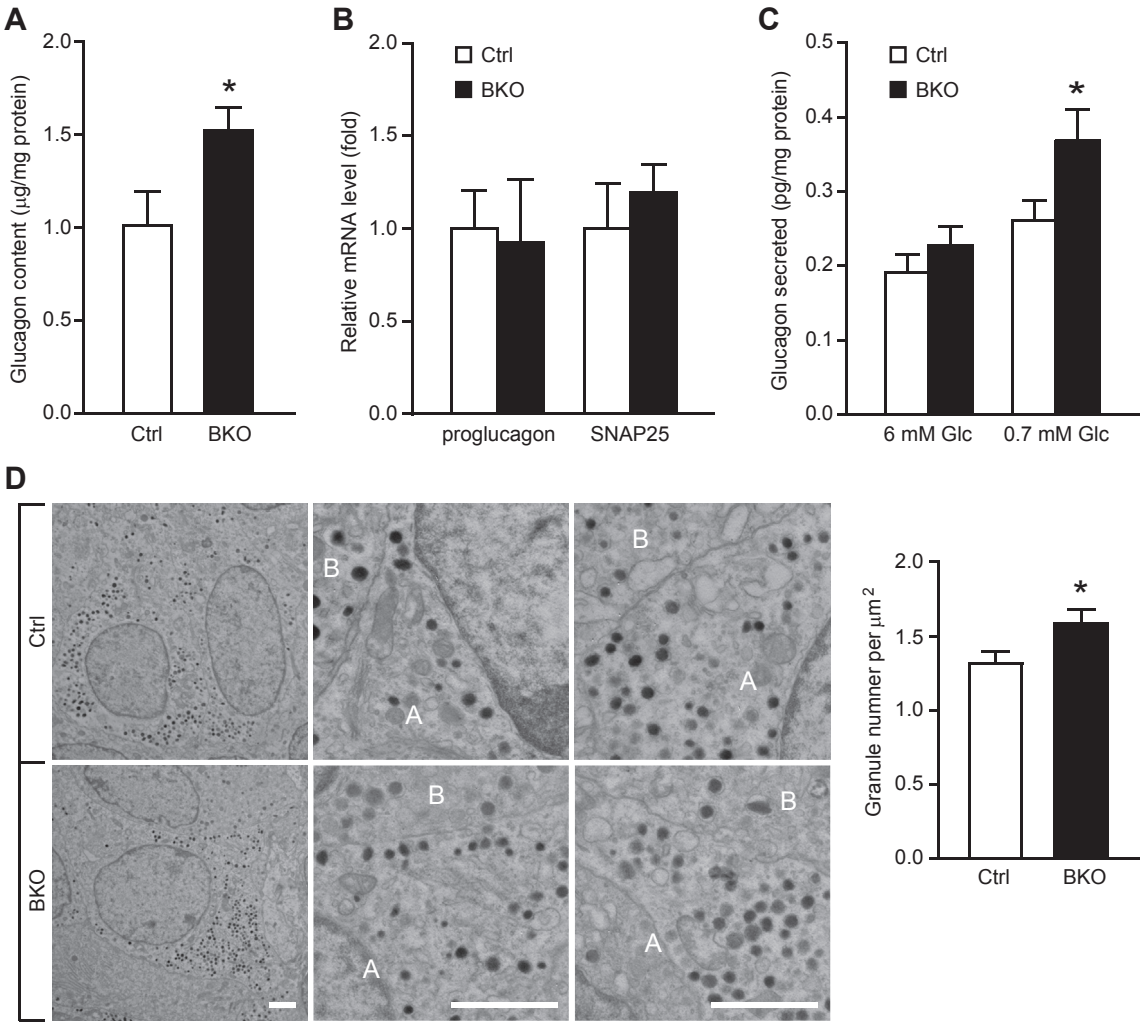


**Figure 3:** Increased glucagon content and secretion in isolated BKO islets. (A) Glucagon content normalized to total protein level. N = 6 pairs of BKO and control littermates from 3 independent islet isolations. (B) Relative mRNA levels of proglucagon and SNAP25. N = 4 pairs of BKO and control littermates from 2 independent islet isolations, each performed in triplicates. (C) Glucagon secretion in 1 h at indicated glucose level. N = 9 pairs of BKO and control littermates from 3 independent islet isolations. (D) Images of randomly selected alpha-cells with complete nucleus (left panels) and alpha- and beta-cell boundary (middle and right panels). Granule density (number per area of cytoplasm) was quantified. N = 42 for control and 34 for BKO cells from 2 independent islet isolations. Data are presented as mean ± SEM. Statistical difference was determined by two tailed student t-test. * indicates $p < 0.05$. Scale bar = 2 µm.

for cells quantification so that possible overlap between sections would be avoided. Cells were identified by ultrastructural specializations.

### 2.8. Data analysis and statistics

Data were presented as mean ± SEM. All electrophysiological data were analyzed with IGOR software (Wavemetrics, Lake Oswego, OR). Imaging data were analyzed with ImageJ (NIH) and Adobe Photoshop (Adobe Systems). Statistical significance of difference was determined by using two-tailed Student's t test. A value of $p < 0.05$ was considered to be statistically significant.

## 3. RESULTS

### 3.1. BIG3 is expressed in both pancreatic alpha- and beta-cells

We previously showed that BIG3 was highly expressed in pancreatic islets. To further understand BIG3 expression in this micro-organ of heterogenous cell populations, we performed immuno-staining in intact and dispersed islet cells. BIG3 was highly expressed in glucagon-positive alpha-cells (Figure 1A), while at barely detectable level in somatostatin-labeled delta-cells (Figure 1B). As expected from our previous studies that show BIG3 in the regulation of insulin granule biogenesis and insulin secretion, BIG3 was expressed in insulin-expressing beta-cells (Figure 1C). In contrast to the broad distribution in beta-cells, BIG3 was exclusively concentrated to a compact perinuclear area (Figure 1A), suggesting possible cell type specific divergence of BIG3 action.

### 3.2. Enhanced glucagon secretion in BKO mice

To examine the functional role of BIG3 in alpha-cells, we first measured plasma glucagon levels in BIG3-knockout (BKO) and control mice, and found that BKO mice had significantly higher glucagon levels after 20 h fasting than their control (Figure 2A). To test whether glucagon secretion was affected in BKO mice, we determined glucagon levels at various time points during insulin tolerance tests (ITT). Although there was no difference in the glucose levels between BKO and control mice during the insulin-induced hypoglycemia (control

  

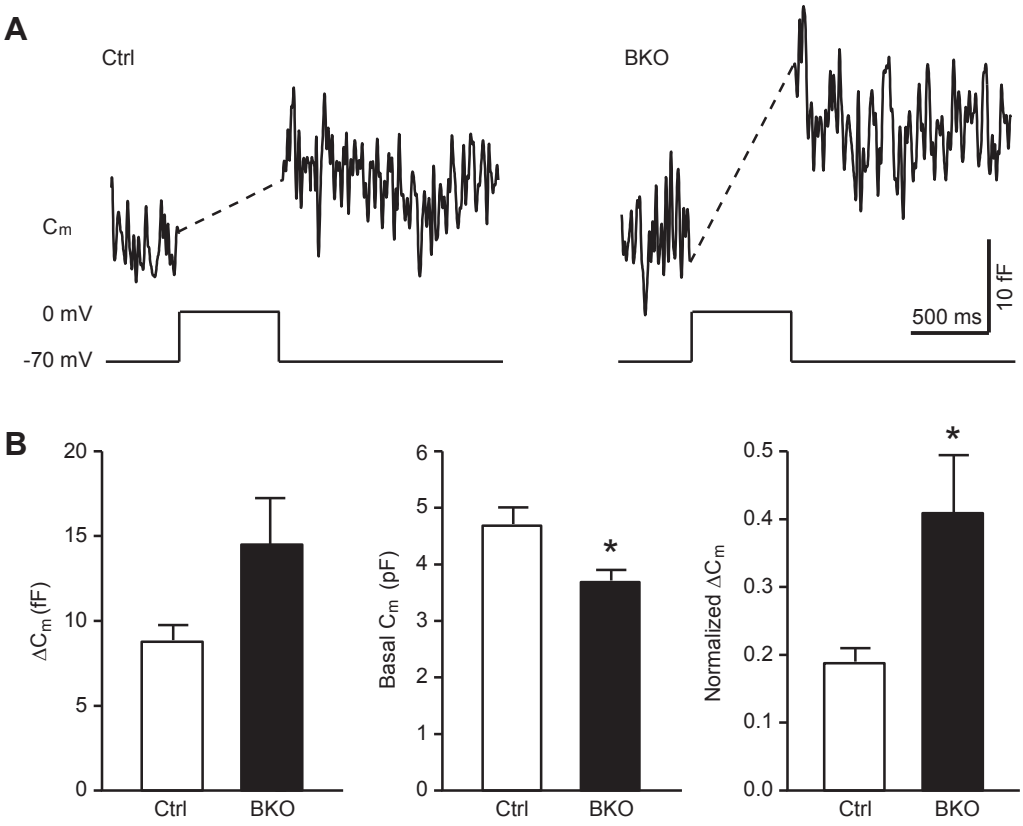


**Figure 4:** Enhanced granule exocytosis upon stimulation in single BKO alpha-cells. (A) Representative recordings of membrane capacitance by patch clamp. (B) Calculated exocytosis events in control and BKO alpha-cells elicited by 500-ms depolarization pulses from −70 to 0 mV. N = 6 for control and 7 cells for KO from 2 independent islet isolations. Data are presented as mean ± SEM. Statistical difference was determined by two tailed student t-test, * indicates $p < 0.05$.

4.5 ± 0.3 mM vs. BKO 4.9 ± 0.3 mM at 40 min, N = 10 pairs of littermates), glucagon levels were much higher in BKO mice (Figure 2B–C), indicating increased glucagon secretion in these mice. The finding of enhanced glucagon secretion in BKO mice suggests that BIG3 may promote glucagon production and glucagon granule biogenesis, similar to its role in beta-cells [15,16].

### 3.3. Elevated glucagon content and release in BKO islets

To ascertain the effect of BIG3 in regulating glucagon production and secretion free from confounding physiological factors, such as insulin sensitivity, we investigated glucagon transcription, production and release from isolated islets. Similar to the findings in BKO beta-cells, glucagon content was increased by ~50% (Figure 3A) in BKO islets, while no difference was found in proglucagon mRNA levels by qPCR analysis (Figure 3B). Moreover, glucagon release induced by low glucose (0.7 mM) was increased by ~40% (Figure 3C). Glucagon granule density (the number of glucagon granules per cytoplasmic area) in BKO alpha-cells was increased by ~20% (Figure 3D). As reported in our previous study, BKO mice exhibited no difference in islet number or size. Together, these results support a similar role of BIG3 in both alpha- and beta-cells in the regulation of secretory granule biogenesis, i.e. depletion of BIG3 leads to elevated hormone production and secretion in pancreatic islets.

### 3.4. Enhanced secretory granule exocytosis in alpha-cells

Considering that paracrine regulation may contribute to the above analysis of glucagon release from isolated islets, we prepared dissociated single-cell cultures from trypsin-treated islets, and analyzed glucagon granule exocytosis by using patch clamp recording of membrane capacitance. Alpha-cells were distinguished from beta-cells by their size and electrophysiological differences (refer to the methods section for details). Depolarization-induced membrane capacitance change trended higher in BKO alpha-cells (Figure 4A). Basal membrane capacitance measurements indicated that BKO alpha-cells appeared to be smaller than control alpha-cells (Figure 4B). This observation was further supported by morphological analysis of alpha-cells, which showed that BKO alpha-cells were ~15% smaller in cytoplasmic area ($p = 0.09$, N = 42 WT and 34 KO cells). In contrast, we did not observe any size difference in beta-cells [15,16]. After normalization of capacitance change to basal capacitance, BKO alpha-cells showed significant elevation of stimulated exocytosis compared with control alpha-cells (Figure 4B). The electrophysiology data at single cell level provide further support that BIG3 regulates glucagon granule production and glucagon secretion upstream of exocytosis machinery.

## 4. DISCUSSION

In the current study, we report that BIG3 is highly expressed in both alpha- and beta-cells. Depletion of BIG3 in alpha-cells results in enhanced glucagon release at the whole animal, islet and single-cell levels. Our findings suggest a conserved mechanism of secretory granule biogenesis involving BIG3 action in both alpha- and beta-cells. We notice that BIG3 is expressed at a barely detectable level in delta-cells, in contrast to alpha- and beta-cells. During mouse

 

embryogenesis [22,23], glucagon-positive cells appear as early as E9.5, a subset of which starts to express insulin a day later, often co-expressing glucagon during proliferation. At E14, fully differentiated beta-cells start to form organized aggregates. Somatostatin-positive delta-cells as well as PP-cells are developed from the same sub-population of progenitor cell pool as beta-cells but occur at a later time point. Thus, it is conceivable that BIG3 expression is highest in alpha-cells, reduced in beta-cells and lost during differentiation into delta- and PP-cells.

Even though alpha-cells are smaller in size when compared with beta-cells, the distribution of BIG3 in alpha-cells is disproportionally concentrated to a compact perinuclear area. As seen in the EM images, BIG3 granules in alpha-cells tend to present a more condensed pattern than in adjacent beta-cells. We have previously reported that the distribution pattern of BIG3 at the perinuclear region of MIN6 cells reflects the status of granule biogenesis, and BIG3 tends to accumulate to active granule biogenesis sites to serve as a mediator in coupling granule trafficking to the time-limiting pro-hormone maturation step [15,16]. The different distribution patterns of BIG3 in alpha- and beta-cells may imply a difference in the granule biogenesis status, which may also reflect the distinct response by the two cell types to extracellular glucose levels.

Although insulin and glucagon secretion responds to distinct physiological regulators, insulin secretion at high glucose levels while glucagon at low glucose levels, the molecular mechanisms, especially those downstream of $Ca^{2+}$ influx and secretory granule-plasma membrane fusion are likely very similar. Indeed, both alpha- and beta-cells use the same synaptotagmin-7 as the $Ca^{2+}$-sensor to trigger secretory granule exocytosis [10,11,13], and SNARE proteins for fusion [24–27]. The mechanisms, however, from glucose sensing to the generation of elevated $Ca^{2+}$ levels appear to differ between alpha- and beta-cells, although multiple lines of evidence support a shared role of ATP-sensitive $K^{+}$-channels in the two cell types [14]. Our current study demonstrates another important shared mechanism between alpha- and beta-cells: regulation of secretory granule biogenesis by BIG3. Functional assays on BKO have shown a consistent role of BIG3 in the negative regulation of hormone production and release in both alpha- and beta-cells, while imaging data have shown differed membrane recruitment status in two cell types. It suggests that although the function of BIG3 may be identical in both cell types, the regulation of BIG3 itself depends on cell-specific context. Though the exact signaling pathway remains to be identified, the regulated secretory pathway upstream of exocytosis could be one of the intrinsic differences between alpha- and beta-cell. Moreover, as BIG3 is absent or at very low levels in delta-cells, identification of the BIG3-equivalent in delta-cells is necessary to understand somatostatin-containing granule biogenesis. Future detailed cell and molecular biology studies are needed to address these questions.

Although there is no difference in islet number or size between BKO and control mice [15], we did not examine whether there is any difference in alpha-cell number in this study, and thus cannot rule out a potential contribution of increased alpha-cell number to the observed glucagon content increase.

Our study shows that BIG3 is highly expressed in alpha-cells, and the loss of BIG3 leads to elevated glucagon production and its enhanced secretion at single alpha-cell, islet, as well as the whole organism level. Together with our previous studies, a conserved function of BIG3 in regulating secretory granule biogenesis and hormone secretion in pancreatic islets has emerged.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest associated with this manuscript.

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