The Rab-GTPase activating protein, TBC1D1, is critical for maintaining normal glucose homeostasis and β-cell mass

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Abstract: Tre-2/USP6, BUB2, cdc16 domain family, member 1 (TBC1D1), a Rab-GTPase activating protein, is a paralogue of AS160, and has been implicated in the canonical insulin-signaling cascade in peripheral tissues. More recently, TBC1D1 was identified in rat and human pancreatic islets; however, the islet function of TBC1D1 remains not fully understood. We examined the role of TBC1D1 in glucose homeostasis and insulin secretion utilizing a rat knockout (KO) model. Chow-fed TBC1D1 KO rats had improved insulin action but impaired glucose-tolerance tests (GTT) and a lower insulin response during an intraperitoneal GTT compared with wild-type (WT) rats. The in vivo data suggest there may be an islet defect. Glucose-stimulated insulin secretion was higher in isolated KO rat islets compared with WT animals, suggesting TBC1D1 is a negative regulator of insulin secretion. Moreover, KO rats displayed reduced β-cell mass, which likely accounts for the impaired whole-body glucose homeostasis. This β-cell mass reduction was associated with increased active caspase 3, and unaltered Ki67 or urocortin 3, suggesting the induction of apoptosis rather than decreased proliferation or dedifferentiation may account for the decline in islet mass. A similar phenotype was observed in TBC1D1 heterozygous animals, highlighting the sensitivity of the pancreas to subtle reductions in TBC1D1 protein. An 8-week pair-fed high-fat diet did not further alter β-cell mass or apoptosis in KO rats, suggesting that dietary lipids per se, do not lead to a further impairment in glucose homeostasis. The present study establishes a fundamental role for TBC1D1 in maintaining in vivo β-cell mass.

Key words: insulin sensitivity, pancreas, AS160, GAP, glucose-stimulated insulin secretion.

Résumé: TBC1D1, une protéine activant la Rab-GTPase, est paralogue d’AS160 impliqué, selon des études, dans la cascade de signalisation canonique au sein des tissus périphériques. Très récemment, on a identifié TBC1D1 dans les îlots pancréatiques d’humains et de rats; cependant, on ne connaît pas très bien le rôle de TBC1D1 dans les îlots. On examine le rôle de TBC1D1 dans l’homéostasie du glucose et la sécrétion de l’insuline dans le modèle de rat dépourvu (KO) de TBC1D1. Comparativement à des rats du type sauvage (WT), les rats TBC1D1 KO recevant une alimentation standard présentent une meilleure action de l’insuline, une tolérance défectueuse au test de glucose (GTT) et une plus faible réponse insulinique lors d’un ipGTT. Les données in vivo suggèrent une défectuosité des îlots. La sécrétion d’insuline stimulée par le glucose est plus élevée dans les îlots isolés de rats KO comparativement aux animaux WT, ce qui suggère que TBC1D1 exerce une régulation négative de la sécrétion de l’insuline. En outre, les rats KO présentent une réduction de la masse des cellules β, ce qui explique probablement une dysfonction de l’homéostasie du glucose dans l’organisme en entier. La diminution de la masse des cellules β est associée à une plus grande activité de la caspase 3 et à l’absence de modification de Ki67 ou de l’urocortine 3, indiquant une probable induction de l’apoptose au lieu de la diminution de la prolifération ou de la dédifférenciation pour expliquer la diminution de la masse des îlots. Un phénotype similaire est observé chez les animaux hétérozygotes avec TBC1D1, mettant en évidence la sensibilité du pancréas à de subtiles diminutions de la protéine TBC1D1. Un régime riche en graisses administré à des rats appariés n’exacerbe pas davantage la masse des cellules β ou l’apoptose chez les rats KO; on peut donc penser que les lipides alimentaires per se ne dérèglat pas davantage l’homéostasie du glucose. La présente étude attribue un rôle fondamental à TBC1D1 dans le maintien de la masse des cellules β in vivo. [Traduit par la Rédaction]

Mots-clés : sensibilité de l’insuline, pancréas, AS160, GAP, sécrétion d’insuline stimulée par le glucose.

Introduction
Type 2 diabetes is associated with impaired insulin-signaling in insulin-sensitive tissues, including pancreatic β cells (Gunton et al. 2005), indicating an inverse association between insulin sensitivity and β-cell function (Porte and Kahn 1995). This is supported by the findings that ablation of the insulin receptor (Kulkarni et al. 1999), insulin receptor substrate 2 (Withers et al. 1998), or PDK1 (Hashimoto et al. 2006) in mice results in insulin resistance and loss of β-cell mass. Distal modulators of the insulin signaling cascade include 2 Rab-GTPase activating proteins (GAPs),...
TBC1 (tre-2/USP6, BUB2, cdc16) domain family, member 1 (TBC1D1) and TBC1D4 (or AS160) (Cartee 2015). Upon insulin stimulation, AS160 and TBC1D1 are phosphorylated by Akt and capped by a molecular adapter protein, 14-3-3, to render AS160 and TBC1D1 in an inactive state. Inhibition of these GAPs removes a “brake” on Rab-GTPase GDP-to-GTP cycling (Roach et al. 2007). In skeletal muscle, AS160 and TBC1D1 are thought to regulate GLUT4 vesicle translocation to the plasma membrane, and therefore are considered to have important roles in skeletal muscle glucose handling (Chadt et al. 2008; Chavez et al. 2008; Taylor et al. 2008). More recent studies have determined that skeletal muscle TBC1D1 is necessary for exercise-regulated glucose metabolism (Szekeres et al. 2012; Stockli et al. 2015).

AS160 and TBC1D1 are expressed at both the mRNA and protein level in human and rodent pancreatic β cells (Bouzakri et al. 2008; Rutti et al. 2014); however, to date only one study has specifically examined TBC1D1 in isolated islets (Rutti et al. 2014). Knockdown experiments in isolated wild-type (WT) rat pancreatic cells suggest that TBC1D1 is required for β-cell proliferation (Rutti et al. 2014), ultimately influencing insulin secretion rates. However, discrepant results between cell and animal models have made it difficult to understand the role of TBC1D1 in insulin signaling within the pancreas, as whole-body TBC1D1- and AS160-deficient mice have been reported to have normal, reduced, or increased plasma insulin concentrations during a glucose tolerance test (GTT) (Szekeres et al. 2012; Dokas et al. 2013; Chadt et al. 2015; Hargett et al. 2016). Therefore, the in vivo functional influence of TBC1D1 within the pancreas remains under debate.

In the present study, we evaluated the consequence of TBC1D1 deficiency in a rat knockout (KO) model on glucose homeostasis under normal chow and high-fat fed conditions. We demonstrate that impaired glucose clearance in TBC1D1 KO rats was not attributed to insulin resistance but due to a blunted plasma insulin response to a glucose challenge and reduced β-cell mass. In addition, a marker of apoptosis was enhanced in TBC1D1 KO islets, providing a plausible mechanism to account for the observed reduction in β-cell mass. This suggests TBC1D1 ablation manifests with a prominent phenotype within pancreatic islets. Interestingly isolated TBC1D1-deficient islets had enhanced insulin secretion in response to glucose as compared with control WT islets. Altogether, these studies highlight a fundamental role for TBC1D1 in maintaining β-cell mass and function.

Material and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

Rats

Two TBC1D1 heterozygous (HET) rats on a Sprague Dawley background were provided by Dr. Hamra (University of Texas Southwestern Medical Center, Dallas, Texas, USA), and generated with transposon technology as described elsewhere (Izsvak et al. 2010). WT Sprague Dawley rats (purchased from Charles River, Wilmington, MA) were bred with these TBC1D1 HET rats to build a colony of Wistar Sprague Dawley rats (purchased from Charles River, Wilmington, MA) and generated with transposon technology as described elsewhere (Izsvak et al. 2010). Two TBC1D1 heterozygous (HET) rats on a Sprague Dawley background were provided by Dr. Hamra (University of Texas Southwestern Medical Center, Dallas, Texas, USA), and generated with transposon technology as described elsewhere (Izsvak et al. 2010). WT Sprague Dawley rats (purchased from Charles River, Wilmington, MA) were bred with these TBC1D1 HET rats to build a colony of WT, HET, and KO animals for TBC1D1; subsequently HET animals were bred together. All animals were genotyped as follows: DNA was extracted from ear snips using the REDExtract-N-AMP tissue PCR kit (Sigma-Aldrich) according to manufacturing instructions. DNA was amplified using Quanta Biosciences Accusart II PCR Supremix, as per the manufacturer’s instructions, using the following primers: Forward: 5′-GAACGAGGTCCTGGTTTTT-3′ and Reverse: 3′-TAATCCACCCCCAAAGGAAT-5′. After genotyping, animals were housed based on genotype. At least 5 generations were bred for the current study. All rats were housed in a temperature-regulated room on a 12–12 h light–dark cycle. Adult male (12–20 weeks old) WT, HET, and KO rats were maintained on a chow diet (4% kcal fat, Harlan Laboratories Inc., Indianapolis, IN) with ad libitum access to food and water. During the pair-feeding study, both adult male (n = 5 per genotype) and female (n = 7 per genotype) animals were placed on either a control diet (10% kcal fat, 20% kcal protein, and 70% kcal carbohydrate, Research Diets Inc., New Brunswick, NJ, Cat. #D12450J) or high-fat diet (HFD; 60% kcal fat from lard and 20% from both protein and carbohydrate, Research Diets Inc. Cat. #D12492) for 8 weeks. WT and TBC1D1 KO rats on the control diet (Research Diets Inc.) were given unrestricted access to the diet and HFD rats within each genotype were pair fed (PF) to match caloric intake. All protocols were approved by and performed in accordance with the Committee on Animal Care guidelines at the University of Guelph. All efforts were made to minimize animal suffering.

Glucose and insulin tolerance tests

Four-hour fasted rats (n = 8–12) underwent intraperitoneal (ip) insulin tolerance tests (ITT; 1 U/kg body weight, Humulin, Lilly) and GTT (2 g/kg body weight). Blood glucose was measured using a glucometer (Freestyle Lite, Abbott, Chicago, IL) before and up to 90 min postinjection. Plasma insulin was measured by a commercially available kit (Millipore, Billerica, MA) in samples collected before (basal) and 10 min after an intraperitoneal glucose injection.

Insulin secretion assay

Islets were harvested, cultured, and glucose-stimulated insulin secretion was carried out as previously described (Huyppens et al. 2011; Pillai et al. 2011). Glucose-stimulated insulin secretion assays were carried out over a 2 h static incubation period at 37 °C.

Western blots

Tissue samples were homogenized and processed as previously described (Herbst et al. 2014). Standard running, blocking, and incubation procedures were followed. Blots were incubated for 48 h with antibodies raised against TBC1D1 and total and phosphorylated AS160 or for 24 h with antibodies for Akt2, GLUT4, and α-tubulin as a loading control, see Supplementary Table S1 for antibody concentrations.

Immunohistochemistry

Bouin’s fixed pancreata were cut into 5 μm thick sections separated 100 μm apart and mounted on glass slides. Sections were stained for insulin (red; β cells) with an anti-insulin antibody and glucagon (brown; α cells) with an anti-glucagon antibody, and slides were detected by DAB (3,3’-diaminobenzidine) using the EnVision G/2 Doublestain System (Dako, Glostrup, Denmark). Anti-Ki67 and anti-active caspase 3 were visualized with DAB staining using the EnVision+ System-HRP kit (Dako). Hematoxylin was used as a counter stain (purple) (Joseph et al. 2002). For α- and β-cell mass, slides were imaged at 4.2x magnification with an Olympus (Tokyo, Japan) microscope and analyzed with CellSens software. Three levels of sections at least 100 μm apart were stained for each pancreata. The stained area (mm2) and percent area for each dye were determined and the average α- and β-cell mass was calculated by multiplying the percent pancreata area stained by total pancreata wet weight (Lillard-Wetherell 2008). For Ki67 and active caspase 3 stains, 10 islets per sample, were analyzed using an Aperio Scancope. Immunohistochemistry for GLUT2, insulin, and urocortin 3 (Ucn3) was conducted as previously described (van der Meulen et al. 2014). Images were acquired on a Nikon (Tokyo, Japan) A1R+ confocal microscope using a 20×

Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/apnm-2016-0585.
are glucose intolerant TBC1D1 KO rats have improved insulin sensitivity, yet they the curve (AUC) (WT rats (Fig. 2A), as indicated by a 30% decrease in the area under during an ipITT were lower in male KO rats compared with male

Statistics

Results are expressed as mean ± SEM and were analyzed by Student’s t test and 1- or 2-way ANOVA with a Fisher’s least significant difference posthoc where appropriate. Significance was set at p < 0.05, where NS indicates not significant. No statistical sex difference was observed during the pair-feed study; therefore, combined male and female results are shown. All graphs and statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA).

Results

Rat tissue expression of TBC1D1

Since TBC1D1 has been mainly studied in mouse and human tissues, we first determined the protein expression of TBC1D1 in a number of insulin-sensitive tissues in WT rats. TBC1D1 protein was abundantly expressed in spleen and in skeletal muscles (EDL, red and white gastrocnemius) with little to no detectable protein in soleus muscle, kidney, or adipose tissue (Fig. 1A).

TBC1D1 deficient rat characterization

We next characterized a rat model deficient in TBC1D1. KO rats were devoid of TBC1D1 protein in muscle as shown for red gastrocnemius, but they expressed similar protein levels of AS160 and total GLUT4 compared with WT rats (Fig. 1B). In addition, HET rats were devoid of TBC1D1 protein in muscle as shown for red gastrocnemius muscle. To address the role of TBC1D1 in glucose homeostasis, we first evaluated glucose handling at the whole-body level. Glucose levels during an ipITT were lower in male KO rats compared with male WT rats (Fig. 2A), as indicated by a 30% decrease in the area under the curve (AUC) (p < 0.01, Fig. 2B). Meanwhile HET rats displayed similar results to WT rats (Figs. 2A and 2B). The increase in glucose clearance observed in KO rats suggests greater insulin sensitivity. On the other hand, during an ipGTT, KO rats displayed impaired glucose clearance (Fig. 2C) and an ~2-fold increased AUC (p < 0.01, Fig. 2D) compared with WT rats indicating impaired insulin sensitivity. While HET rats demonstrated a trend in increased AUC (53%, p = 0.06, Fig. 2D). Impaired glucose tolerance in concert with enhanced peripheral insulin sensitivity suggests a dysfunction at the level of the pancreatic islets.

Next, plasma insulin concentrations were determined during a glucose challenge. Upon glucose stimulation, WT insulin levels rose ~3-fold, whereas this was severely blunted in the TBC1D1-deficient rats (p < 0.01, Fig. 3A). Circulating insulin levels were 67% and 73% (p < 0.01) lower in HET and KO rats, respectively, compared with WT glucose-stimulation (Fig. 3A). Taken together, the in vivo data suggest there is altered pancreatic β-cell function when TBC1D1 is ablated or decreased.

Increased basal and reduced fold islet glucose-stimulated insulin secretion

Since TBC1D1-deficient rats display impaired glucose tolerance as a result of attenuated plasma insulin concentrations, we proceeded to examine in vitro β-cell function. While Rutti et al. (2014) demonstrated TBC1D1 protein expression was confirmed in rat primary islets sorted for β cell, we previously found that TBC1D1 RNA is expressed in α, β, and δ mouse cell; however, it is highest in beta cells (DiGruccio et al. 2016); see supplemental Fig. S1). Now, we demonstrate that both TBC1D1 and AS160 proteins are highly expressed in WT pancreata homogenate (Fig. 3B), and in a pancreas that lacks TBC1D1 there is no compensation in AS160 protein levels (Fig. 3B). Glucose-stimulated insulin secretion (GSIS) in isolated islets was determined at low (2 mM) and high (10 mM) glucose concentrations. Surprisingly, insulin secretion rates were significantly elevated in islets harvested from KO rats compared with WT rats at all glucose concentrations tested (2 mM: +58%, p < 0.01; 10 mM: +76%, p < 0.05, Fig. 3C). To evaluate KATP channel-independent GSIS, a process involved in the ‘amplifying phase’ of β-cell insulin secretion, islets were incubated with two anaplerotic substrates (dimethylmalate and dimethyl α-ketoglutarate) in the presence of 10 mM glucose. Both WT and KO islets showed similar response, and the
KO islets did not reach as high a stimulation with the addition of anaplerotic substrates compared with high glucose alone (Fig. 3C). Next, GSIS was examined in the presence of KCl and diazoxide; these substrates work to open KATP channels and clamp cytosolic calcium at an elevated level. KO islet insulin secretion in the presence of low glucose (2 mM) and KCl+diazoxide was significantly reduced compared with WT (−35%, p < 0.05, Fig. 3C). The fold insulin secretion response in the presence of KCl+diazoxide was significantly lower in KO rats at both low- and high-glucose concentrations (Fig. 3D). Altogether, these data suggest that TBC1D1 may inhibit glucose-stimulated insulin exocytosis, since there is an enhancement in insulin secretion in the KO animal at both low- and high-glucose levels and that this effect involves both the KATP channel-dependent and independent pathways.

Reduced β-cell mass in TBC1D1-deficient rats

Given the apparent disconnect between the observed attenuation in vivo plasma insulin concentrations and the enhanced capacity of isolated β cells to secrete insulin in KO animals, we next evaluated β-cell mass. While α-cell mass was unaffected, β-cell mass in KO and HET rats was reduced by 34% and 39%, respectively (Figs. 4A and 4B). In addition, the overall number of islets isolated from KO pancreata was also reduced compared with WT rats (data not shown). Altogether, these data indicate that in vivo, impaired glucose tolerance in TBC1D1 KO and HET rats is associated with reduced β-cell mass. Combined with an increased compensatory GSIS, this rat model is reminiscent of a prediabetes phenotype. Therefore we sought to explore the effect of a HFD in TBC1D1 KO rats at the level of the whole-body and pancreas.

High-fat pair-feeding study

Male and female WT and KO rats were PF a HFD for 8 weeks to match total caloric content with control diet-fed rats of the same genotype. Since there was no difference between the 2 sexes for glucose homeostasis or histology assessments, results were pooled (data not shown). The PF high-fat modality was employed to avoid confounding effects of increased body weight, to minimize hypoinsulinemic adverse events, and to examine the effect of dietary fatty acids per se on β-cell mass. Overall, the weight gain between WT and KO rats on either the control or HFD were similar (final body weight, WT control: 328.0 ± 31.4 g and KO control: 327.5 ± 32.3 g, p = NS; WT PF-HFD: 352.3 ± 40.4 g and KO PF-HFD: 355.6 ± 37.5 g, p = NS).

Similar to the results shown in Fig. 2A, control diet KO rats were more insulin sensitive than control diet WT rats as shown by a reduced ITT AUC (−18%, p < 0.05, Fig. 5B). On the other hand, PF-HFD KO rats had a greater diet-induced insulin resistance compared with their control diet littermates as determined by an increase in the ITT AUC (Figs. 5A and 5B). Moreover, both PF-HFD groups demonstrated impaired glucose tolerance as compared with chow diet fed animals regardless of genotype, as shown by a delay in glucose clearance and an increase in the GTT AUC (Figs. 5C and 5D). Again, a similar trend of increased GTT AUC is observed in KO controls animals, as described above.

Since the PF-HFD induced a glucose-impaired condition reminiscent of a prediabetes state, we next examined α- and β-cell mass. Again α-cell mass was similar between WT and KO rats and was not affected by the HFD (Figs. 5E and 5G). In contrast, β-cell mass was significantly reduced by −30% (p < 0.05) in KO rats regardless of diet (Figs. 5F and 5G). Therefore, PF high-fat feeding does not lead to further derangements in β-cell mass in KO rats.
Fig. 3. TBC1D1 KO and HET rats have a blunted insulin response to a glucose challenge. (A) Insulin levels before (Pre) and after (10 min, Post) an intraperitoneal glucose injection in WT, HET, and KO rats, n = 6–8. (B) Representative Western blots of TBC1D1 and AS160 in WT and KO pancreata. (C) Isolated islets from WT and TBC1D1 KO male rats were cultured for glucose-stimulated insulin secretion assay. Islets were incubated with 2 mM, 10 mM glucose, or 10 mM glucose + anaplerotic (Anapl., dimethylmalate (5 mM) and dimethyl α-ketoglutarate (5 mM)) substrates, or 2 mM or 10 mM glucose with KCl (30 mM) and diazoxide (Dia, 100 μM). (D) KCl and diazoxide stimulated insulin secretion fold change. The dashed line indicates incubation with 2 mM and 10 mM glucose only. *p < 0.05, **p < 0.01, vs. WT and ††p < 0.01 vs. Pre for n = 10–15 from 3–6 rats. KO, knockout; HET, heterozygous; WT, wild-type.

Fig. 4. TBC1D1-deficiency results in reduced β-cell mass. (A) Immunohistochemical analysis of α- and β-cell mass in WT, HET, and KO male rats, n = 4. (B) Representative images taken at 20× magnification, where red = β cell, brown = α cells, purple = counter stain. *p < 0.05, **p < 0.01 vs. WT. HET, heterozygous; WT, wild-type; KO, knockout. [Colour online.]
TBC1D1 KO islets display enhanced apoptosis staining

To elucidate a potential mechanism responsible for the loss in β-cell mass in the TBC1D1 KO rats, we measured several markers of glucose signaling, insulin content, and cell proliferation by immunohistochemistry. GLUT2, the glucose transporter responsible for nutrient sensing, was found to be similarly expressed in islets from WT and KO rats regardless of diet (Figs. 6A and 6B). Likewise, insulin content did not change (Figs. 6C and 6D); therefore, deficiencies in nutrient sensing or islet insulin content were not associated with the reduction in β-cell mass observed in KO rats. Next, 2 markers for β-cell proliferation and maturation, Ucn3 and Ki67, were evaluated. In rodents, Ucn3 is highly expressed in adult β-cells and is a marker of maturation (van der Meulen et al. 2014). Ucn3 islet expression was similar between WT and KO rats and was not influenced by the HFD (Figs. 6E and 6F). On the other hand, Ki67 staining, a classical marker of cell proliferation, was significantly reduced by the HFD and this effect was independent of genotype suggesting that TBC1D1 does not affect β-cell proliferation (Figs. 7A and 7B). In contrast, active caspase 3 (a robust apoptosis marker) was increased by ~60% in KO islets, regardless of diet (p < 0.05, Figs. 7C and 7D). Interestingly, the active caspase 3 staining appeared to be localized to the surrounding α-cells.

Discussion

In the present study we examined a TBC1D1-deficient rat model to address the role of TBC1D1 in glucose homeostasis in vivo. While current murine models of TBC1D1 deficiency report a ~50% reduction in skeletal muscle GLUT4 content (Szekeres et al. 2012; Dokas et al. 2013; Chad et al. 2015; Hargett et al. 2016), this finding confounds insulin sensitivity results in mice. In the present study, we demonstrated that the rat model of TBC1D1 deficiency has normal GLUT4 protein expression, allowing for a discriminative investigation into the role of TBC1D1 in glucose homeostasis. We provided evidence that whole-body glucose tolerance was attenuated as a result of decreased plasma insulin levels, which was attributable to reduced nutrient-stimulated insulin secretion and diminished β-cell mass, suggesting that whole-body TBC1D1 deletion exerted a primary effect on pancreatic β-cells. This was especially observed in the TBC1D1 HET rats that demonstrated normal skeletal muscle function (glucose uptake) during an ITT; however, upon a glucose challenge when insulin secretion is required to control glucose clearance, glucose uptake was impaired. TBC1D1 HET rats displayed a reduction in glucose-stimulated plasma insulin levels and a significantly reduced β-cell mass, essentially “phenocopying” their KO littermates. These results suggest that TBC1D1 is an important regulator of pancreatic β-cell mass in vivo. We also demonstrated that high-fat feeding did not further impact β-cell mass in TBC1D1-deficient animals. Altogether, reduced β-cell mass and in vivo insulin secretion in the KO rats, associated with increased in vitro insulin secretion, may indicate that the KO islets never expanded to the extent as their WT littermates. Therefore, the present study has revealed a novel role for TBC1D1 in glucose homeostasis by regulating pancreatic β-cell mass and insulin secretion.

Glucose-stimulated insulin secretion

β-cell insulin secretion is dependent on nutrient-stimulated granule exocytosis. This is a highly regulated process involving the trafficking of insulin containing granules to the plasma membrane and release into circulation. Several Rab-GTPases are required for regulating insulin granule trafficking in β-cells, such as Rab3a, Rab27a, and Rab11 (Yaekura et al. 2003; Sugawara et al. 2009), and KO mouse models of these GTPases are associated with insulin secretion deficiencies (Yaekura et al. 2003). Moreover, ablating TBC1D1, a Rab-GTPase regulator, improved glucose-stimulated insulin secretion. Similar findings have been reported in rat islets by utilizing siRNA to transiently decrease TBC1D1 (Rutti et al. 2014). In addition, knockdown of TBC1D1 in rat islets blunted KCl-induced insulin secretion, suggesting an essential role for TBC1D1 in Ca2+-mediated insulin release upon depolarization with KCl (Rutti et al. 2014). Our studies also revealed impaired KCl-induced insulin secretion and show for the first time that there is also a defect in the KATP channel-independent pathway regulating insulin secretion in KO rats. Therefore, current evidence from our study and others suggests that TBC1D1 impacts β-cell function and inhibits insulin secretion. In contrast to TBC1D1, AS160 ablation impairs granule mobilization and insulin exocytosis (Bouzakri et al. 2008; Rutti et al. 2014). Interestingly, while TBC1D1 and AS160 are thought to...
be paralogues, they appear to have divergent functions with respect to insulin secretion.

**TBC1D1 is required to maintain pancreatic β-cell mass**

KO and HET rat models of TBC1D1 deficiency displayed blunted insulin levels upon a glucose challenge. Histological analysis suggests that β-cell mass as well as total islet numbers are altered implicating TBC1D1 as an important regulator in the maintenance of β-cell mass. It is of note that several members of the canonical insulin-signaling cascade are crucial proliferative β-cell factors. Specifically, IRS-2 (Bouzakri et al. 2008) and Akt (Butoa et al. 2006) are required for maintaining β-cell mass, as Akt regulates FoxO1 phosphorylation and nuclear exclusion, resulting in cell growth, survival, and proliferation (Zhang et al. 2011). Furthermore, targeted siRNA AS160 knockdown in cultured β cells increased apoptotic markers such as TUNEL and active caspase 3 (Bouzakri et al. 2008). Moreover, similar to AS160 knockdown experiments (Bouzakri et al. 2008), we demonstrated that ablation of TBC1D1 increases active caspase 3; however, this staining was visualized near the periphery of the islet cells, and therefore likely colocalized with α cells. Although we only observed a diet effect in Ki67 staining and no change in Ucn3 expression in KO rats, this is in line with blunted β-cell mass expansion. In agreement, Rutti et al. (2014) suggested that TBC1D1 is an important factor for β-cell proliferation, as cultured rat TBC1D1 knockdown islets revealed decreased proliferation. Altogether, the direct cause-and-effect relationship between TBC1D1, apoptosis, and reduced β-cell mass still remains unknown, but it may require an interaction between α and β cells. Regardless of the mechanism, the current data, along with the in vitro data previously published by Rutti et al. (2014), suggest that TBC1D1 is required for maintenance of normal β-cell mass. While this is in stark contrast to the original report in congeneric TBC1D1-deficient mice which displayed elevated fasting.

**Fig. 6.** Preserved markers of islet nutrient sensing and insulin content. Quantified (A) GLUT2, (C) insulin, and (E) Ucn3 staining determined by immunofluorescence analysis for control diet and HFD pancreas sections, n = 3. Representative (B) GLUT2, (D) insulin, and (F) Ucn3 images taken at 20× magnification. AU, arbitrary units; Ucn3, urocortin 3; HFD, high-fat diet; WT, wild-type; KO, knockout. [Colour online.]
insulin plasma levels (Szekeres et al. 2012), subsequent reports have not replicated this finding. Specifically, although not statistically different, the single KO of TBC1D1 appears to display the lowest insulin value during a GTT compared with WT, AS160 KO, or combined AS160–TBC1D1 double KO mice (Szekeres et al. 2012; Dokas et al. 2013; Chadt et al. 2015; Hargett et al. 2016). Moreover, it has recently been reported that combined AS160–TBC1D1 ablation decreases serum insulin concentrations during a GTT, whereas AS160 independent KO does not. Together with the present data, this suggests the reduction in insulin during a glucose challenge may be attributed to TBC1D1 ablation. Since it appears that a species-specific TBC1D1 effect exists in the pancreas, additional work may be required to delineate the role of TBC1D1 in humans and especially with regards to the development and progression of type 2 diabetes.

PF HFD-induced glucose intolerance

Under hyperglycemic or hyperlipidemic conditions, β cells will undergo a “compensation” phase to maintain glucose homeostasis when encountering such chronic nutrient-induced insults (Cerf 2013). When this compensation phase is exhausted, β-cell dysfunction occurs (Cerf 2013). Moreover, full-blown type 2 diabetes is characterized by insulin resistance as well as β-cell dysfunction and death, resulting in reduced insulin secretion (Chang-Chen et al. 2008). Since our initial analysis demonstrated hypoinsulinemic effects in KO rats, especially under a substrate challenge, we opted to examine β-cell mass in a prediabetes glucose-intolerant state, through a pair high-fat feeding scheme to minimize any potential hypoinsulinemic related adverse events. Indeed, 8 weeks of pair-feeding induced glucose intolerance mimicking a prediabetes state. Since insulin sensitivity, as evaluated by the ITT, was preserved in WT PF-HFD rats we did not anticipate any change in β-cell mass. However, we wanted to examine if the addition of lipids would lead to further β-cell diminishment in KO rats. Interestingly, PF high-fat feeding did not aggravate the decrease in β-cell mass in these animals. This indicates that reductions in β-cell mass in KO rats are likely independent of nutrient signals and are due to an inherent loss of TBC1D1 in the islets.

Limitations

A number of study limitations must be addressed. Since we examined a whole-body TBC1D1 ablated rat model, we cannot rule out systemic effects of losing the protein in insulin sensitive tissues, such as skeletal muscle, influenced pancreas function as a secondary event. Several mouse model studies have shown a role for TBC1D1 in muscle glucose uptake (Chadt et al. 2008; Chavez et al. 2008; Taylor et al. 2008) and fat metabolism (Maher et al. 2014). Therefore, regardless of the observed improvement in whole body insulin tolerance in the current study, it is possible that humoral changes at the level of the muscle in TBC1D1 KO rats may have impacted the results observed here in the islets. Nonetheless, the in vitro assays in isolated islets from KO rats does confirm a pancreas-specific effect; an upregulation of insulin secretion in the presence of glucose, therefore implicating TBC1D1 as a negative regulator of insulin release. Another limitation to the current study is that we only examined the rats in adulthood, and did not follow changes in β-cell function over time. Future studies should address the effect of islet dysfunction with age in this model.

Conclusion

The current study provides new insight on the role of TBC1D1. We demonstrated that glucose intolerance was associated with reduced total β-cell mass in TBC1D1-deficient rats, although islet function in vitro was normal or slightly improved. We pursued several mechanisms to identify the causal factor for altered β-cell mass, such as nutrient sensing, insulin content, proliferation, and...
apoptosis. Our results revealed a robust upregulation of active caspase 3, likely expressed in α cells. Further research is required to establish the link between α-cell apoptosis and compromised β-cell function. Nonetheless, the present study suggests that TBC1D1 is essential for maintaining normal β-cell mass, as well as a negative regulator of insulin secretion. This may be of clinical relevance as a polymorphism (R125W in TBC1D1) has been previously identified in obese, insulin-resistant individuals (Stone et al. 2006; Meyre et al. 2008). While type 2 diabetes is characterized by reduced β-cell mass and insulin secretion (Chang-Chen et al. 2008), developing therapies designed to improve β-cell function and stability are required to treat and prevent this chronic disease. In this context, TBC1D1 is an attractive therapeutic target.

Competing interest
The authors declare that there have no competing interests.

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