Loss of Prohibitin Induces Mitochondrial Damages Altering β-Cell Function and Survival and Is Responsible for Gradual Diabetes Development

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Prohibitins are highly conserved proteins mainly implicated in the maintenance of mitochondrial function and architecture. Their dysfunctions are associated with aging, cancer, obesity, and inflammation. However, their possible role in pancreatic β-cells remains unknown. The current study documents the expression of prohibitins in human and rodent islets and their key role for β-cell function and survival. Ablation of Phb2 in mouse β-cells sequentially resulted in impairment of mitochondrial function and insulin secretion, loss of β-cells, progressive alteration of glucose homeostasis, and, ultimately, severe diabetes. Remarkably, these events progressed over a 3-week period of time after weaning. Defective insulin supply in β-Phb2−/− mice was contributed by both β-cell dysfunction and apoptosis, temporarily compensated by increased β-cell proliferation. At the molecular level, we observed that deletion of Phb2 caused mitochondrial abnormalities, including reduction of mitochondrial DNA copy number and respiratory chain complex IV levels, altered mitochondrial activity, cleavage of L-optic atrophy 1, and mitochondrial fragmentation. Overall, our data demonstrate that Phb2 is essential for metabolic activation of mitochondria and, as a consequence, for function and survival of β-cells. Diabetes 62:3488–3499, 2013

Canonical glucose-stimulated insulin secretion (GSIS) involves three main components associated with mitochondria (1). First, upstream of mitochondria, glucose enters the cell and is phosphorylated by glucokinase, initiating glycolysis to generate pyruvate. Second, mitochondrial metabolism of pyruvate leads to the generation of ATP along with metabolic coupling factors. Finally, downstream of mitochondrial activation, ATP closes KATP channels, thereby promoting plasma membrane depolarization inducing elevation of cytosolic Ca2+ and insulin exocytosis. Lack of insulin secretory response to glucose by mitochondrial DNA (mtDNA) depleted β-cells provided compelling evidence that mitochondrial activation is not dispensable in metabolism-secretion coupling (2). Likewise, β-cell-specific ablation of mitochondrial transcription factor Tfam results in mtDNA loss, mitochondrial dysfunction, and diabetes in mice (3). To date, several mitochondrial defects have been associated with β-cell dysfunction, including oxidative stress, nutrient toxicity, or altered mitochondrial morphology (4).

β-Cell mitochondria are interconnected and dynamic (5–7). Functional and morphological impairments of β-cell mitochondria have been associated with insulin secretory defects in type 2 patients with diabetes (8). In addition, animal models of diabetes have shown altered mitochondrial morphology, suggesting possible implication of mitochondrial dynamics in maintaining β-cell function (4). This was studied in vitro (6,7) and recently documented in vivo after deletion of the mitochondrial fusion protein optic atrophy 1 (Opa1) in β-cells, leading to defective mitochondrial activation and GSIS, which made mice hyperglycemic (9).

Prohibitins are evolutionarily highly conserved proteins that are ubiquitously expressed in eukaryotic organisms, mostly in tissues with high mitochondrial metabolism (10). These proteins have been implicated in pleiotropic functions, such as cell-cycle progression, transcriptional regulation, cell signaling, apoptosis, and mitochondrial biogenesis (11–13). Prohibitins have also been associated with pathological conditions such as inflammation, obesity, and cancer (11,14,15). The prohibitin family comprises two functionally and physically interdependent homologs, Prohibitin-1 (Phb1) and Prohibitin-2 (Phb2), forming heterodimers assembled in ring-shaped complexes within the mitochondrial inner membrane (16). These complexes have been proposed to serve as molecular scaffolds maintaining the integrity of the mitochondrial inner membrane (16). Silencing of Phb1 in endothelial cells reduces mitochondrial membrane potential and complex I activity (17). Moreover, deletion of Phb2 in mouse embryonic fibroblasts impairs their proliferation and alters mitochondrial morphology (18). These effects have been mainly attributed to excessive proteolysis of Opa1 long isoforms secondary to Phb2 loss (18).

In order to investigate the role of Phb2 in an endocrine cell type, we have generated β-cell-specific Phb2 knockout mice. Our results demonstrate that loss of Phb2 caused accelerated proteolysis of Opa1, associated with altered mitochondrial network and function. Furthermore, we observed lower mtDNA copy number and reduced complex IV levels. These events led to β-cell dysfunction and a concomitant β-cell loss, which induced severe diabetes in these animals.

RESEARCH DESIGN AND METHODS

Generation of β-cell-specific Phb2 knockout mice. Phb2Δfl/fl (18) and RipCre mice (19) were crossed to generate Phb2Δfl/Δfl, RipCre mice designated as β-Phb2−/−. In order to maintain single copy of the RipCre allele in...
homozygous $\beta$-Phb2$^{+/+}$ knockout mice throughout the study, we systematically crossed heterozygous $\beta$-Phb2$^{+/+}$ (Phb2$^{+/+}$, RipCre$^{-/-}$) males with Phb2$^{−/−}$ (control) females. Because Phb2$^{−/−}$, RipCre$^{-/−}$ mice (homozygous $\beta$-Phb2$^{−/−}$ knockout) became diabetic at the age of 6 weeks, this strategy also avoided mating diabetic animals. As control mice, we used heterozygous (Phb2$^{+/−}$, RipCre$^{−/−}$) and knockout (Phb2$^{−/−}$, RipCre$^{−/−}$) mice throughout the study. PCR of genomic DNA extracted from isolated diabetic islets using the primers 5′-ATCGATTGTTGGCGTGAGCA-3′ and 5′-AGGGAGTTGTGTTGAGGAA-3′. Mice were maintained on a 12-h dark/light cycle and were allowed free access to standard laboratory chow (PMI, St. Louis, MO) and water. Mice were housed in our animal facility according to procedures approved by the animal care and experimentation authorities of the Canton of Geneva.

Glucose tolerance test and hormone levels. Glucose (2 g/kg body weight) was administered intraperitoneally in 6-h-fasted mice before measurements of glucose levels on blood collected from tail vein at indicated times using a glucometer (Accu-Check, Roche Diagnostics, Rotkreuz, Switzerland). Hyperglycemia and diabetes were defined as glucose >11.1 mmol according to the criteria published by the American Diabetes Association (20). Plasma insulin levels from blood sampled by retro-orbital puncturing at time 0 and 15 min after glucose administration were determined using an ultrasensitive mouse insulin ELISA (Merckida, AB, Uppsala, Sweden). For plasma glucagon, blood was collected after 2-h fasting and 1-h refeeding as well as after 6-h-fasting at 0 min and 15 min after i.p. glucose (2 g/kg body weight) injection and glucagon levels determined by radioimmunoassay (RIA; GL-2K, Millipore, Billerica, MA).

Where indicated, mice were treated either with long-acting insulin (Levemir; Novo Nordisk, Gentofte, Denmark) injected subcutaneously twice per day (0.15 and 0.20 U in the morning and evening, respectively) or without leptin by subcutaneous implantation of a 14-day osmotic pump (Alzet Model 1002; Alzet, Cupertino, CA) releasing 10 µg/day human leptin (Bachem, Bubendorf, Switzerland).

Islet morphology, α- and β-cell mass, and mitochondrial morphology. Pancreata were excised, weighed, fixed for 2 h in 4% paraformaldehyde, and finally embedded in paraffin. Sections of 5 µm separated by at least 250 µm were stained for insulin and glucagon using guinea pig anti-insulin (1:400) and mouse antiguacagon (1:500) primary antibodies as described (21). Fluorochrome-linked secondary antibodies were used for visualization, and images were captured by confocal microscopy (LSM 510 Meta; Carl Zeiss, Feldbach, Switzerland).

For assessment of α- and β-cell mass, sections at an interval of 250 µm throughout the pancreas were stained for glucagon and insulin, respectively, with the aforementioned primary antibodies. Horseradish peroxidase-conjugated secondary antibodies were used in order to reveal α- and β-cells by diaminobenzidine staining, and hematoxylin was used for counterstaining (21). Sections were scanned by digital microscopy (Nikon Coolscope; Nikon, Egg, Switzerland), quantification was achieved using ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij/), and α- and β-cell mass was calculated as previously described (22).

For mitochondrial morphology, dispersed islet cells were allowed to adhere in 3-D cell culture matrices for ultraviolet light excision and immunostaining with primary antibodies anti-TOM20 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-insulin (1:400; Sigma-Aldrich, St. Louis, MO), followed by Alexa 488- and 647-conjugated secondary antibodies, respectively. For each channel, z-axis sections separated by 0.36 µm were acquired using a Zeiss LSM 510 Meta microscope with a 63 × 1.4 NA Plan Apochromat objective (Zeiss). Three-dimensional reconstruction as well as quantification of mitochondrial length was performed with the help of ImageJ software (National Institutes of Health) by manually measuring length of mitochondria in each section using the help of ImageJ software (National Institutes of Health) by manually measuring length of mitochondria in each section.

For ultrastructural analysis, isolated islets and whole pancreas of Phb2$^{+/+}$ and Phb2$^{−/−}$ mice were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, by either immersion (islets, pancreas) or in situ perfusion (pancreas). After post-fixation in 1% osmium tetroxide, all samples were processed for transmission electron microscopy as per standard procedures. Sections were analyzed in a CM10 Philips electron microscope (Philips).

Isolation of islets and measurements of their hormones. Pancreatic islets were isolated by collagenase digestion as described (23) and cultured overnight in RPMI 1640 medium supplemented with 5% heat-inactivated FCS, 10 mM Hepes, 10 µM 2-mercaptoethanol, and 0.05% Penicillin/Streptomycin. After 16 h incubation, islets were washed once with PBS and further incubated in 96-well plates for determination of insulin concentrations. For in vitro antibiotic incubations, media were incubated with 5 µg/ml Fura-2/AM in KRBH containing 2.8 mmol/L glucose for 30 min at 37°C before measurements of ATP levels as described previously (23).

For mitochondrial membrane potential, isolated islets were placed in 96-well plates for recording of rhodamine-123 signal (Phostar Optima; BMG Labtech, Offenburg, Germany), maintained in KRBH with 2.8 mmol/L glucose before addition of stimulatory glucagon concentration (final 22.8 mmol) according to published protocol (25).

Results

Generation and characterization of β-Phb2$^{−/−}$ mice. First, we documented the expression of proteins in human islets by immunoblotting (Fig. 1A). We next generated mice lacking β-cell Phb2 (β-Phb2$^{−/−}$) by crossing Phb2$^{−/−}$ animals carrying a floxed Phb2 allele (18) with Tg(Ins2-cre)1Ehre mice, expressing Cre-recombinase under the control of an insulin promoter (19). PCR amplification across the Phb2 locus generated a Phb2-deleted fragment in islets of β-Phb2$^{−/−}$ mice, but not in those of Phb2$^{+/−}$ littermate controls, showing efficient Cre-mediated recombination (Fig. 1B). Consistent with the genomic deletion, there was no residual expression of Phb2 (18) in β-Phb2$^{−/−}$ islets (Fig. 1C). The residual Phb2 expression observed in knockout islets is presumably accounted for by expression of the protein in non-β-cells and/or the persistence of the few β-cells that had escaped recombination. In islets isolated from β-Phb2$^{−/−}$ mice, loss of Phb2 was accompanied by reduced levels of its homolog Phb1 (Fig. 1C), as previously observed (18). This reveals the interdependence...
of these proteins and the existence of functional prohibitin complexes in β-cells. Misexpression of Rip-Cre transgene in brain and hypothalamus has been noticed particularly in the 4-week-old β-Phb2−/− mice (27), although not consistently in the Tg(Ins2-cre)1Herr (19) used in the current study (28). Consistent with these reports, Phb2 loss in β-Phb2−/− mice was restricted to pancreatic β-cells without apparent ablation in the brain, including the hypothalamus (Fig. 1D).

Monitoring of nonfasting glycemia in β-Phb2−/− and littermate control Phb2+/+ mice showed normoglycemia in 4-week-old β-Phb2+/+ males. However, at the age of 6 weeks, knockout mice became hyperglycemic (>11.1 mmol/L), thereafter progressing to strong hyperglycemia (>25 mmol/L) and severe diabetes (weight loss) over a 3-week period (Fig. 1E). Similar phenotypes were observed in female β-Phb2−/− mice (Supplementary Fig. 1). Recordings of body weight showed growth impairment starting at the age of 7 weeks, with no further weight gain compared with control littermates (Fig. 1F). Nonfasting hypoinsulinemia (54% less plasma insulin than control) displayed by 4-week-old β-Phb2−/− mice (Fig. 1G) was further lowered at 10 weeks of age (88% less than age-matched control). Heterozygous Phb2 knockout (β-Phb2+−/−) mice grew similarly to Phb2+/+ (data not shown) and had a normal life span, whereas homozygous β-Phb2−/− mice died at the age of 12–15 weeks suffering of overt diabetes.

Phb2 loss in β-cells gradually altered glucose homeostasis in β-Phb2−/− mice. At 3 weeks of age, an intraperitoneal glucose tolerance test resulted in similar glycemic excursions in β-Phb2−/− mice and control littermates (Fig. 2A). One week later, mice exhibited impaired blood glucose clearance along with normal fasting glycemia (Fig. 2B), indicating a prediabetic state. At 6 weeks of age, β-Phb2−/− mice became severely diabetic with fasting hyperglycemia at ~15 mmol/L (Fig. 2C). This timing of sequential alterations (normoglycemia followed by glucose intolerance and ultimately diabetes) was consistently observed in various litters. Thus, in this mouse model, the etiology of diabetes could be followed within a short time.
At the age of 6 weeks, glucose injection. Plasma insulin levels were determined before and 15 min after glucose challenge (Fig. 2). Reduced GSIS in β-Phb2−/− pancreas at 6 weeks. At the age of 6 weeks, when Phb2 knockout animals became diabetic, we performed in situ pancreatic perfusions to test the β-cell response in islets maintained in their native pancreatic environment. This revealed a dramatic reduction of GSIS when pancreas of β-Phb2−/− were compared with those of Phb2lox/lox controls (Fig. 3A). In the former mice, first and second phases of insulin secretion were reduced by 76 and 78%, respectively, when compared with the corresponding phases of Phb2lox/lox controls (Fig. 3B).

**Impaired metabolism-secretion coupling in Phb2−/− β-cells.** Because the chronic hyperglycaemia observed by the age of 5 to 6 weeks might induce secondary changes perturbing islet function, we studied islets isolated from normoglycemic mice at the age of 3 to 4 weeks (Figs. 1E, 2A and B). Despite normoglycaemia, insulin content in β-Phb2−/− islets was decreased by 46% when compared with that of control islets (Fig. 4A). Islet function tested in static incubation revealed a markedly reduced GSIS in islets span of ~3 weeks. Heterozygous β-Phb2wt/− mice had normal glucose excursions even at the age of 10 weeks (Supplementary Fig. 2), indicating that the observed phenotype in β-Phb2−/− mice was not mediated by the floxed-cre allele present as a single allele in both heterozygous β-Phb2wt/− and homozygous β-Phb2−/− mice.

We further ascertained the possible impairment of insulin delivery suggested by low nonfasting plasma insulin levels (Fig. 1G). In 4-week-old β-Phb2−/− mice, the plasma insulin levels observed 15 min after a glucose challenge were markedly lower (~50%) than in control mice (Fig. 2D). At the age of 6 weeks, β-Phb2−/− mice exhibited very low fasting as well as glucose-induced plasma insulin levels (Fig. 2E). Therefore, diabetes development is first revealed in 4-week-old β-Phb2−/− by glucose intolerance and lack of glucose-induced elevation of plasma insulin. Interestingly, 10-week-old β-Phb2−/− mice exhibited hyperglucagonemia following a moderate 2-h fasting. Glucagon levels were further elevated in knockout animals upon refeeding (Fig. 2F), although not upon a glucose i.p. challenge (Supplementary Fig. 3).
lacking Phb2 (Fig. 4B and C). When normalized per islet, the secretory response was 63% lower in β-Phb2−/− islets compared with controls. Normalized per insulin content, the difference was −50%, indicating that, at this stage, the lower insulin content of β-Phb2−/− islets did not account for their blunted glucose response. Accordingly, there was no significant difference between control and knockout islets when insulin secretion was stimulated by nonmetabolic KCl-induced cell depolarization (data not shown). This indicates that the exocytotic machinery downstream of Ca²⁺ signaling was preserved in β-Phb2−/− islets. However, the Ca²⁺ rise secondary to glucose stimulation requires mitochondrial activation in terms of ATP generation. Hence, we measured intracellular Ca²⁺ concentration ([Ca²⁺]i) in response to glucose stimulation in isolated islets. In comparison with littermate controls, β-Phb2−/− islets exhibited a lower [Ca²⁺]i rise upon glucose stimulation (Fig. 4D), while the response to KCl, used as a Ca²⁺-raising agent, was similar in the two groups (Supplementary Fig. 4).

Additionally, we acutely depleted Phb2 in vitro in β-cells by treating nonrecombined Phb2fl/fl islets isolated from adult mice (age 10–14 weeks) with adenovirus expressing Cre recombinase (Fig. 5A and B). Compared with control islets transduced with Ad-LacZ virus, we observed impaired GSIS in recombinant Ad-RipCre treated islets 72 h after viral transduction (Fig. 5C). These data demonstrate that Phb2 is required for proper metabolism-secretion coupling in β-cells.

**Perturbed mitochondrial morphology and function in Phb2-null β-cells.** We next studied mitochondrial morphology in animals aged 3 to 4 weeks. Three-dimensional reconstructions of confocal microscopy z-stacks images of mitochondrial reticulum through entire β-cells are shown in Fig. 6A and Supplementary Fig. 5A and B. Normal mouse β-cell displayed tubular interconnected mitochondria. On the contrary, absence of Phb2 in β-cells resulted in fragmented mitochondrial network, revealing an average mitochondrial length ~50% shorter compared with

![**FIG. 4.** Stimulus-secretion coupling is already deficient in β-cells from 4-week-old β-Phb2−/− mice. A: Insulin contents of islets isolated from 4-week-old β-Phb2−/− (white bars) and littermate Phb2fl/fl (black bars) mice (n = 5). B and C: GSIS tested as static incubation at basal 2.8 mmol/L (2.8G) and stimulatory 22.8 mmol/L (22.8G) glucose on islets isolated from 4-week-old mice. Insulin secretion rate is expressed as insulin release normalized per islet (B) and to total islet insulin content (C). β-Phb2−/− (white bars) and littermate Phb2fl/fl (black bars) mice (n = 5). D: [Ca²⁺]i at basal 2.8G and in response to 22.8G in isolated islets from Phb2fl/fl (●) and β-Phb2−/− (□) mice. Traces show averages of recordings from three animals per genotype. Data are expressed as means ± SEM. *P < 0.05 between the two groups.](http://diabetesjournals.org/diabetes/article-pdf/62/10/3488/570257/3488.pdf)
In spite of these changes, electron microscopy revealed mitochondrial inner membrane fusion protein Opa1, resulting in the accumulation of the short isoform S5 (Fig. 6). Proteolytic cleavage of the long isoforms (L1 and L2) of the mitochondrial network, we observed excessive maintenance of mtDNA, probably requiring Opa1-dependent mitochondrial ultrastructure. Islets from diabetic mice, whether these cells were studied in islets (Fig. 6D and E) or intact pancreas (not shown), revealed a 47% reduction of the mitochondrial genome in Phb2 null islets versus controls (Fig. 6F). This was substantiated by higher pancreatic glucagon content in Phb2 null mice compared with that seen in controls (Fig. 7). At the age of 10 weeks, however, islets were completely disorganized in Phb2 null mice, with only few β-cells intermingled with α-cells (Fig. 7B). Quantification of islet β-cell mass in Phb2 null mice revealed that β-cell loss amounted to 35% at the age of 4 weeks and >90% at the age of 10 weeks compared with age-matched controls, consistent with the corresponding alterations in pancreatic insulin content (Fig. 7C and D). In 10-week-old β-Phb2 null mice, we observed an expanded α-cell mass compared with that seen in controls (Fig. 7E). This was substantiated by higher pancreatic glucagon content in Phb2 null mice at 10 weeks of age compared with control animals (Fig. 7F).

Deficient β-cell function at 4 weeks of age, followed by loss of β-cell mass at 10 weeks in β-Phb2 null mice, might explain the development of diabetes. In order to test if the lack of insulin delivery was a key contributor to the disease, β-Phb2 null animals were treated with insulin. The daily administration of insulin to β-Phb2 null diabetic mice, which was initiated at 8 weeks of age, fully corrected glycemia (Fig. 7G) and provided for maintenance of pancreatic insulin content (Fig. 7H) and δ-cell function, which was impaired in control β-cells (Fig. 6D). In accordance with this fragmented mitochondrial network, we observed excessive proteolytic cleavage of the long isoforms (L1 and L2) of the mitochondrial inner membrane fusion protein Opa1, resulting in the accumulation of the short isoform S5 (Fig. 6C). In spite of these changes, electron microscopy revealed a comparable ultrastructural appearance of mitochondria in the β-cells of 4-week-old control Phb2 null and knockout β-Phb2 null mice, whether these cells were studied in isolated islets (Fig. 6D and E) or intact pancreas (not shown). Similar observations were made in β-cells of newborn, 2-week, and 6-week-old mice (not shown). These pictures (Fig. 6A, D, and E) show that mitochondrial fragmentation is not necessarily associated with alteration of mitochondrial ultrastructure.

In neuronal mitochondria, Phb2 is necessary for the maintenance of mtDNA, probably requiring Opa1-dependent mitochondrial fusion (29). In this context, we quantified mtDNA copy number normalized to nuclear DNA. This revealed a 47% reduction of the mitochondrial genome in Phb2 null islets versus controls (Fig. 6F), which was accompanied by lower levels of mtDNA-encoded subunit of complex IV of the respiratory chain at the protein level (Fig. 6G and H). Alterations in the electron transport chain might favor production of reactive oxygen species (30,31). However, we recorded similar levels of 4-hydroxynonenal, an aldehydic product of lipid peroxidation commonly enhanced during oxidative stress (Supplementary Fig. 6A and B). The mitochondrial function was assessed by measuring mitochondrial membrane potential and ATP. Upon glucose stimulation, mitochondria of Phb2 null β-cells exhibited weak hyperpolarization compared with their control counterparts (Fig. 6F). This was translated into impaired ATP generation after glucose stimulation. At basal 2.8 mmol/L glucose, Phb2 null and β-Phb2 null islets had similar concentrations of ATP (29.4 ± 9.2 and 28.2 ± 12.2 pmol/μg islet proteins, respectively). Upon 22.8 mmol glucose stimulation, we measured a 30% rise in ATP for control islets versus only 7% for β-Phb2 null islets (Fig. 6J), which might be sufficient for the blunted elevation of [Ca2+]i (Fig. 4D). These data show that loss of Phb2 induced alterations in mitochondrial morphology and function, resulting in an impaired metabolic response.

**Progressive decline in β-cell mass and islet architecture in β-Phb2 null mice.** The defective GSIS observed in β-Phb2 null mice might be caused by physical or functional loss of β-cells or both. Hematoxylin and eosin staining of pancreas of 4-week-old knockout mice revealed absence of infiltration of immune cells within and around islets (Supplementary Fig. 7). At the same age, immunohistochemistry showed that most islets of β-Phb2 null mice exhibited reduced size with preserved architecture (i.e., glucagon-positive α-cells at the periphery and insulin-producing β-cells forming the core of the islet) (Fig. 7A). At the age of 10 weeks, however, islets were completely disorganized in β-Phb2 null mice, with only few β-cells intermingled with α-cells (Fig. 7B). Quantification of islet β-cell mass in β-Phb2 null mice revealed that β-cell loss amounted to 35% at the age of 4 weeks and >90% at the age of 10 weeks compared with age-matched controls, consistent with the corresponding alterations in pancreatic insulin content (Fig. 7C and D). In 10-week-old β-Phb2 null mice, we observed an expanded α-cell mass compared with that seen in controls (Fig. 7E). This was substantiated by higher pancreatic glucagon content in β-Phb2 null mice at 10 weeks of age compared with control animals (Fig. 7F).
control body weights (Fig. 7D) for the 10-week period of the treatment. Untreated β-Phb2⁻/⁻ mice were killed at 11 weeks of age because of a severe diabetic state with dramatic loss of body weight. Incidentally, the rescue by insulin treatment of multiple alterations of the β-Phb2⁻/⁻ mice shows the absence of a hypothalamic contribution to the observed phenotype. Leptin therapy in diabetic mice has been shown to normalize glycemia through suppression of hyperglucagonemia (32). In view of the higher α-cell mass and pancreatic glucagon of β-Phb2⁻/⁻ mice (Fig. 7E and F), we tested leptin treatment in these animals. Fig. 7D shows that subcutaneous delivery of leptin over a 10-day period partially corrected glycemia in 8-week-old diabetic β-Phb2⁻/⁻ mice.

**β-Cell apoptosis and proliferation in β-Phb2⁻/⁻ mice.** Islets isolated from 4-week-old β-Phb2⁻/⁻ mice exhibited a marked upregulation of caspase-3 cleavage compared with age-matched controls (Fig. 8A). Additionally, these cells were more susceptible to apoptotic stimuli, such as staurosporine (Fig. 8A). On pancreas sections, we typically

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**FIG. 6.** Mitochondrial morphology and function in β-cells from 3- to 4-week-old β-Phb2⁻/⁻ mice. A: Mitochondrial morphology was analyzed on dispersed pancreatic islet cells by immunofluorescence. Representative β-cells from control Phb2⁺/⁺ and knockout β-Phb2⁻/⁻ mice as indicated. Single plane showing mitochondrial network, three-dimensional (3D) reconstruction of z-stacks through entire β-cell, and staining of insulin. Scale bars, 2 μm. B: Average mitochondrial length measured on 25–40 distinct mitochondria per cell in a randomly selected two-dimensional z-stack. Phb2⁺/⁺ (n = 27) and Phb2⁻/⁻ (n = 21) β-cells from different mice (n = 3) of each genotype were analyzed. C: Representative immunoblotting showing increased proteolysis of long isoforms of mitochondrial fusion protein Opa1 in Phb2⁻/⁻ islets compared with controls. Electron microscopy of β-cells in islets isolated from 4-week-old control Phb2⁺/⁺ (D) and knockout β-Phb2⁻/⁻ mice (E). The structure of mitochondria (arrows) appeared normal in the two mouse genotypes. Scale bars, 200 nm. F: mtDNA copy number in islets isolated from respective genotypes. DNA copies of mitochondrial complex I was normalized to the DNA levels of nuclear RNaseP (aDNA). n = 3 and n = 5 for Phb2⁺/⁺ and β-Phb2⁻/⁻, respectively. G: Two independent representative immunoblots showing mitochondrial respiratory chain complexes (C) of isolated islets using antibody cocktail targeted toward different subunits (C-I, NDUF8; C-II, iron-sulfur protein; C-III, core protein 2; C-IV, subunit-I; and C-V, ATP synthase subunit-α). H: Densitometry analysis of three independent blots. β-Phb2⁻/⁻ (white bar; n = 4) and Phb2⁺/⁺ (black bar; n = 3). I: Mitochondrial membrane potential (∆Ψm) in islets isolated from respective genotype was measured with rhodamine123. Fluorescence intensity was recorded during incubations at 2.8 mmol (basal) glucose and subsequently at 22.8 mmol (stimulated) glucose. Triplicates of 10 islets from each animal were used in independent experiments. β-Phb2⁻/⁻ (□) and littermate Phb2⁺/⁺ mice (○); n = 3. J: Cellular ATP levels were measured after incubating 50 islets each at 2.8 mmol glucose and stimulatory 22.8 mmol glucose for 15 min. Percentage of rise in ATP production in response to stimulatory glucose concentration against basal glucose concentrations was calculated. Phb2⁺/⁺ (black bar) n = 5 and β-Phb2⁻/⁻ (white bar) n = 4. Data on panels B, F, H, I, and J are means ± SEM. Immunofluorescence and immunoblotting images are representative of at least three independent analyses performed on different mice. *P < 0.05, **P < 0.01 between the two groups. a.u., arbitrary units.
observed one to three apoptotic β-cells by TUNEL assay in β-Phb2−/− islets, while we detected none in control islets (Fig. 8B). Given that β-Phb2−/− mice maintained a sufficient β-cell mass up to the age of 4 weeks, before the rapid development of diabetes, data suggest the involvement of some compensatory mechanism.

Preservation of ~65% of the β-cell mass in 4-week-old β-Phb2−/− mice (Fig. 7C) despite active apoptosis (Fig. 8A and B), prompted us to investigate β-cell proliferation. Quantification of Ki-67–positive β-cells in control mice showed the expected 1% proliferating β-cells at the age of 4 weeks (Fig. 8C). Surprisingly, we observed an increased number of Ki-67–positive nuclei within insulin-positive cells of β-Phb2−/− mice (Fig. 8C). This resulted in a 2.5-fold β-cell proliferation increment in β-Phb2−/− mice versus control (Fig. 8D). However, at the age of 10 weeks, the 90% reduction in β-cell mass of β-Phb2−/− mice (Fig. 7C) suggested loss of such a compensatory proliferation.

**DISCUSSION**

The current study documents the expression of prohibitins and their importance for β-cell function and survival. Ablation of Phb2 in mouse β-cells sequentially resulted in impaired mitochondrial function and insulin secretion, loss of β-cells, progressive alteration of glucose homeostasis, and ultimately severe diabetes. Defective insulin supply was contributed by both β-cell dysfunction and apoptosis, suggesting a pivotal role for Phb2 in maintenance of the β-cell integrity. At the molecular level, we observed that deletion of Phb2 caused mitochondrial abnormalities such as reduction of mtDNA copy number and complex IV levels. Our β-Phb2−/− mice share some phenotypic similarities with β-cell–specific frataxin knockout. Frataxin is located in the mitochondrial matrix, controlling iron-sulfur cluster assembly (33). Mice lacking frataxin in β-cells are born healthy but subsequently develop glucose intolerance and then diabetes by the age of 9 months, explained by
oxidative stress, apoptosis, and then reduced islet mass (31). Phb2 deficiency caused more rapid development of diabetes across the age of 3 to 6 weeks, also accompanied by β-cell loss but without apparent oxidative stress. Phb2-null β-cells exhibited short, fragmented, and globular mitochondria, which, however, retained a normal ultrastructural appearance, as judged by the persistence of a double membrane boundary and numerous thin elongated cristae. This was nevertheless associated with mitochondrial dysfunction, as shown by reduced glucose-induced ATP production. Phb2 knockout β-cells had accelerated proteolytic degradation of L-Opal isoforms.
Indeed, we observed the accumulation of the short Opa1 isoform S5, when a balanced proportion of short and long forms is necessary for the proper function of this protein (16). Deletion of Opa1 in mouse β-cells decreases the activity of electron transport chain complex IV and alters ATP generation (9). The present data indicate that a selective loss of L-Opa1, and/or the accumulation of its short isoform, results in mitochondrial pattern distinct from complete ablation of Opa1 (9). Fragmented mitochondrial pattern has been documented in β-cells of human type 2 diabetic subjects and animal models for diabetes (8,34,35). However, it was unclear from these studies whether the alterations in mitochondrial morphology were the cause or consequence of diabetes. Strikingly, alterations of mitochondria appeared well before the onset of diabetes in β-Phb2−/− mice. Therefore, our data favor a role for Phb2 in Opa1-dependent mitochondrial fusion for β-cell function and morphology.

We measured lower mtDNA copy number and complex IV levels in β-Phb2−/− islets. In Opa1-null islets, mtDNA copies are unchanged (9). However, in neurons lacking Phb2, destabilization of L-Opa1 is associated with progressive loss of the mitochondrial genome (29), pointing to a complex equilibrium between the Opa1 isoforms for the maintenance of mtDNA integrity. In β-Phb2−/− islets, degradation of L-Opa1 accompanied by a decline in mtDNA might explain the marked reduction in the glucose response, which was more severe than in the complete absence of Opa1 (9).

Mitochondrial abnormalities were present in the β-cells of β-Phb2−/− mice, rendering these animals severely diabetic by the age of 6 weeks. The lower insulin release of β-Phb2−/− mice was also contributed by reduced β-cell mass, although at the age of 4 weeks, the secretory response was decreased independently of insulin content and specifically for mitochondrion-dependent secretagogue. Glucose intolerance appeared at this age of 4 weeks when β-cell mass in β-Phb2−/− mice was still ∼65% of control animals, whereas hyperglycemia developed 2 weeks later. Clinical data have documented the maintenance of glucose tolerance in healthy donors of functional islets who underwent partial pancreatectomy (36). Interestingly, a recent study in humans reported that diabetes appears after a reduction in β-cell mass of ∼65%, while postchallenge glucose excursions in prediabetic subjects exhibit glucose intolerance before this critical threshold is reached (37). Therefore, within a 2- to 3-week period, the β-Phb2−/− mouse model recapitulates the progressive stages of human diabetes, with progression from β-cell impairment associated with glucose intolerance to diabetes with fasting hyperglycemia.

In mouse embryonic fibroblasts lacking Phb2 (18), as well as in Opa1-null β-cells (9), apoptosis is not increased (unless stimulated by extrinsic stimuli), although the proliferative capacity of β-cells is lost. In marked contrast, the 4-week-old β-Phb2−/− mice exhibited a higher β-cell proliferation, which partially compensated the enhanced apoptosis during the first weeks of life. Apoptotic TUNEL-positive β-cells were regularly observed in islets of β-Phb2−/− mice, while such events were extremely rare in control mice. This suggests that β-Phb2−/− mice gradually lost β-cells by apoptosis over a period of

**FIG. 7.** Continued.
β-Phb2−/− mice exhibited hyperglucagonemia, which was further enhanced following food ingestion. Moreover, leptin treatment partially corrected glycemia in diabetic β-Phb2−/− mice, in accordance with a glucagon-suppressor effect of leptin (38). These findings support the growing interest in α-cell as a critical therapeutic target for the treatment of diabetes.

β-Phb2−/− mice represent a unique model of spontaneous diabetes development, through a series of molecular events appearing over a 3-week period, not requiring the administration of toxic diets or chemicals. Overall, our data demonstrate that Phb2 is essential for the function and survival of β-cells. Phb2 regulates mitochondria by preserving some of their key components such as mtDNA, respiratory chain subunits, and the morphology regulator L-Opa1. Phb2 ablation impairs mitochondrial activation, rendering β-cells unresponsive to glucose and ultimately leading to apoptosis, which promotes β-cell loss and diabetes.

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S.S. conducted experiments, analyzed data, and wrote the manuscript. F.T., L.S., and P.Me. developed specific techniques and analyzed corresponding data. C.M., P.L.H., and T.L. generated transgenic animals essential for this study. A.G. generated data. P.Ma. supervised the project, analyzed data, and wrote the manuscript. P.Ma. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES


FIG. 8. Both apoptosis and proliferation of β-cells induced in β-Phb2−/− mice at 4 weeks of age. A: Representative immunoblotting for apoptosis analysis showing increased levels of cleaved caspase-3 in islets of β-Phb2−/− versus control Phb2fl/fl mice. Islets were treated (+) or not (−) with 1 μmol/L staurosporine for 4 h before analysis. Representative of three independent analyses performed on different mice. B: Representative images of three independent experiments of immunofluorescence on pancreatic sections showing labeling of TUNEL-positive β-cells in islets of β-Phb2−/− and littermate Phb2fl/fl mice at the age of 4 weeks. The TUNEL marker antidiogoxin was stained in pseudo-color red, appearing in pink due to overlapping with blue-colored DAPI staining showing nucleus. Insulin was stained in green. TUNEL-positive nuclei are highlighted by yellow arrows. Pancreatic sections from control mice that were treated with DNase show extensive TUNEL labeling in endocrine and exocrine region. C: Representative images of immunofluorescence on pancreatic sections showing β-cell proliferation in islets of β-Phb2−/− and littermate Phb2fl/fl mice at the age of 4 weeks. The proliferation marker Ki-67 was stained in red, appearing in pink due to DAPI staining overlap and revealing nuclear localization. Insulin was stained in green and DAPI in blue. Ki-67-positive nuclei are highlighted by yellow arrowheads. D: β-Cell proliferating index is expressed as percent of proliferating β-cells after computing Ki-67−positive nuclei for 1,000 β-cells analyzed per mouse at the age of 4 weeks in both β-Phb2−/− (white bar) and control Phb2fl/fl (black bar). Data expressed as means ± SEM. *P < 0.05; n = 3 genotype.


