Loss of prohibitin induces mitochondrial damages altering β-cell function and survival and responsible for gradual diabetes development

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Short title: Prohibitin controls β-cell function and survival

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ABSTRACT
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 present 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 mtDNA copy number and respiratory chain complex IV levels, altered mitochondrial activity, cleavage of L-Opa1, 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.

INTRODUCTION
Canonical glucose-stimulated insulin secretion (GSIS) process 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 K-ATP channels, thereby promoting plasma membrane depolarization inducing elevation of
cytosolic Ca\textsuperscript{2+} 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 to 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 diabetics (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 \textit{in vitro} (6; 7), and recently documented \textit{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 evolutionary highly conserved proteins, which 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 homologues, 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 (MEFs) 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 to a concomitant β-cell loss, which induced severe diabetes in these animals.

RESEARCH DESIGN AND METHODS

Generation of β-cell specific Phb2 knockout mice. Phb2^{0/0} (18) and RipCre mice (19) were crossed to generate Phb2^{0/0}, RipCre^{+} mice designated as β-Phb2^{+/-}. In order to maintain single copy of the RipCre allele in homozygous β-Phb2^{+/-} knockout mice throughout the study, we systematically crossed heterozygous β-Phb2^{0/wt} (Phb2^{0/wt}, RipCre^{+}) males with Phb2^{0/fl} (control) females. Because Phb2^{0/fl}, RipCre^{+} mice (homozygous β-Phb2^{+/-} knockout) became diabetic at the age of 6 weeks, this strategy also avoided mating diabetic animals. As control mice, we used Phb2^{0/fl} littermates in order to optimize standardization of the genetic background between the groups. Cre-mediated excision of Phb2 was assessed by PCR on genomic DNA extracted from isolated pancreatic islets using the primers

5’-ATCGTATTGGTGCGTGACGA-3’ and 5’-AGGGAGGCTTGGTTTGAGGGGA-3’.

Mice were maintained on 12-hours dark-light cycle and were allowed free access to standard
laboratory chow (RM3-E-SQC #811181, SDS Diets, Essex, UK) and water. Mice were maintained 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 (2g/kg body weight) was administered intra-peritoneally in 6h-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 blood glucose >11.1mM 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 (Mercodia AB, Uppsala, Sweden). For plasma glucagon, blood was collected after 2h fasting and 1h re-feeding as well as after 6h fasting and 30 min after i.p. glucose (2g/kg body weight) injection and glucagon levels determined by RIA (GL-32K, Millipore, MA, USA). Where indicated, mice were treated either with long-acting insulin (Levemir, Novo Nordisk, Gentofte, Denmark) injected subcutaneously twice per day (0.15 and 0.20U in the morning and evening, respectively) or with leptin by using subcutaneous implantation of 14-day osmotic pump (Alzet Model 1002, Cupertino, CA, USA) 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 2h 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 anti-glucagon (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 (http://rsb.info.nih.gov/nih-image/), and α- and β-cell mass was calculated as previously described (22).

For mitochondrial morphology, dispersed islet cells were allowed to adhere in culture to poly-l-lysine coated slides before fixation and immuno-staining with primary antibodies anti-TOM20 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-insulin (1:400, Sigma-Aldrich, St. Louis, MO, USA), followed by Alexa-488 and -647 conjugated secondary antibodies, respectively. For each channel z-axis stacks separated by 0.36 µm were acquired using a Zeiss LSM 510 Meta microscope with a 63 x 1.4 NA Plan Apochromat objective (Zeiss). 3D-reconstruction as well as quantification of mitochondrial length was performed with the help of ImageJ software by manually measuring length of mitochondria in a randomly selected 2D z-stack.

For ultrastructural analysis, isolated islets and whole pancreas of Phb2^{0/} and β-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.
**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, 10mM HEPES, 1mM sodium pyruvate, 50µM 2-mercaptoethanol, and antibiotics in non-adherent petri dishes. For static incubation studies, overnight cultured islets were preincubated with Kreb’s-Ringer bicarbonate HEPES (KRBH) buffer supplemented with 2.8mM glucose and 0.1% BSA for 1h (23). Then, batches of 10 islets were hand-picked and incubated with basal 2.8mM and stimulatory 22.8mM glucose at 37°C for 1h. At the end of the incubation period, supernatants were collected and islets were resuspended in acid-ethanol for determination of insulin concentrations. For *in vitro* recombination experiments, islets isolated from adult *Phb2*<sup>fl/fl</sup> mice were treated overnight either with control (Ad-LacZ) or Cre-recombinase (Ad-RipCre) expressing adenoviruses as described previously (24). The genomic deletion of *Phb2* was confirmed 24h after transduction by PCR as described previously (18). GSIS was tested as mentioned earlier 72h after transduction. Additionally, *in situ* pancreatic perfusions were performed in anesthetized mice following previously published procedure (23). Insulin concentrations in supernatants, perfusates, extracted islets and pancreata were measured using RIA (Millipore). Total pancreatic glucagon contents were determined by glucagon RIA (Millipore).

**Measurements of ATP, mitochondrial membrane potential and Ca<sup>2+</sup> in isolated islets.**

Following overnight culture, islets were handpicked and preincubated in KRBH at 2.8mM glucose for 1h and then incubated at basal 2.8mM and stimulatory 22.8mM glucose for 15 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 (Fluostar Optima, BMG labtechnologies, Offenburg,
Germany), maintained in KRBH with 2.8 mM glucose before addition of stimulatory glucose concentration (final 22.8mM) according to published protocol (25).

For cellular Ca\textsuperscript{2+} measurements, isolated islets were allowed to adhere on poly-l-lysine coated coverslips during overnight culture. Then, islets were incubated with 5 µM Fura-2/AM in KRBH containing 2.8 mM glucose and 0.02% pluronic acid for 30 min at 37°C, washed and further incubated for 30 min in 2.8 mM glucose KRBH. Coverslips were placed in a thermostatic chamber (Harvard Apparatus, Holliston, MA, USA) maintained at 37°C and images were acquired using oil-immersion objective (40x) on an Axiovert microscope (Zeiss) as detailed previously (26).

**Immunoblotting.** Overnight cultured islets were washed once with PBS and lysed in standard RIPA buffer supplemented with protease inhibitor cocktail (Roche). Proteins were separated by SDS-PAGE and transferred onto nitro-cellulose membranes. Primary antibodies used were directed against Phb1 and Phb2 (1:1000, BioLegend, San Diego, CA, USA), cleaved caspase-3 (1:1000, Cell Signaling, Danvers, MA, USA), Opa1 (1:1000, BD Biosciences, San Jose, CA, USA), mitochondrial OXPHOS components (1:500, Mitosciences, Eugene, OR, USA), and 4-Hydroxynonenal (1:1000, Abcam, Cambridge, UK). Isotype-matched HRP-conjugated secondary antibodies were used and proteins were visualized by enhanced chemiluminescence (GE Healthcare, Glattbrugg, Switzerland). Densitometry analysis of different blots for mitochondrial complexes was performed with the help of Quantity One\textsuperscript{®} software (Bio-Rad Hercules, CA).

**TUNEL assay.** TUNEL assay was performed on 15 min proteinase K pretreated deparafinized pancreas sections according to the manufacturer’s protocol by using Apoptag Fluorescein apoptosis detection kit (S7110; Chemicon, Millipore, MA, USA). Anti-insulin
primary antibody (1:400) and its appropriate fluorochrome-coupled secondary antibody (1:500) were used to label β-cells. Nuclei were counter stained with DAPI.

**mtDNA copy number.** Quantification of mtDNA copy number was achieved by qPCR. Briefly, DNA was extracted from overnight cultured islets using DNeasy Blood and Tissue kit (Qiagen, Düsseldorf, Germany) according to manufacturer’s protocol. Nuclear and mitochondrial DNA copy number was assessed by qPCR using primers targeted towards COX1 gene (for mtDNA) and nuclear RNAse P (for nuclear DNA).

**RESULTS**

**Generation and characterization of β-Phb2−/− mice.** First, we documented the expression of prohibitins in human islets by immunoblotting (Fig. 1A). Next, we generated mice lacking β-cell Phb2 (β-Phb2−/) by crossing Phb2flo/flo animals carrying a floxed Phb2 allele (18) with Tg(Ins2-cre)1Herr 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 Phb2flo/flo littermate controls, showing efficient Cre-mediated recombination (Fig. 1B). Consistent with genomic deletion, there was a near total ablation of Phb2 protein 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 few β-cells which had escaped recombination. In islets isolated from β-Phb2−/− mice, loss of Phb2 was accompanied by reduced levels of its homologue Phb1 (Fig. 1C), as previously observed (18). This reveals the interdependence of these proteins and the existence of functional prohibitin complexes in β-cells. Mis-expression of Rip-Cre transgene in brain and hypothalamus has been noticed in a Tg(Ins2-cre)25Mgn transgenic line (27), although not particularly in the Tg(Ins2-cre)1Herr (19) used in the present study (28). Consistent with these
reports, Phb2 loss in β-Phb2<sup>−/−</sup> mice was restricted to pancreatic β-cells without apparent ablation in the brain, including the hypothalamus (Fig. 1D).

Monitoring of non-fasting glycemia in β-Phb2<sup>−/−</sup> and littermate control Phb2<sup>0/0</sup> mice, showed normoglycemia in 4-week old β-Phb2<sup>−/−</sup> males. However, at the age of 6 weeks, knockout mice became hyperglycemic (>11.1mM), thereafter progressing to strong hyperglycemia (>25mM) and severe diabetes (weight loss) over a 3-week period (Fig. 1E). Similar phenotypes were observed in female β-Phb2<sup>−/−</sup> mice (Supplementary Fig. 1). Recordings of body weight showed growth impairment starting at the age of 7 weeks, with no further weight gain compared to control littermates (Fig. 1F). Non-fasting hypoinsulinemia (54% less plasma insulin than control) displayed by 4-week old β-Phb2<sup>−/−</sup> mice (Fig. 1G) was further lowered at 10 weeks of age (88% less than age-matched control). Heterozygous Phb2 knockout (β-Phb2<sup>wt/−</sup>) mice grew similarly to Phb2<sup>0/0</sup> (data not shown) and had normal lifespan, whereas homozygous β-Phb2<sup>−/−</sup> 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<sup>−/−</sup> mice.** At 3 weeks of age, ipGTT resulted in similar glycemic excursions in β-Phb2<sup>−/−</sup> mice and control littermates (Fig. 2A). One week later, mice exhibited impaired blood glucose clearance along with normal fasting glycemia (Fig. 2B), indicating a pre-diabetic state. At 6 weeks of age, β-Phb2<sup>−/−</sup> mice became severely diabetic with fasting hyperglycemia around 15 mM (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 span of about 3 weeks. Heterozygous β-Phb2<sup>wt/−</sup> mice had normal glucose excursions even at the age of 10 weeks (Supplementary Fig. 2), indicating that the observed phenotype in β-Phb2<sup>−/−</sup> mice was not
mediated by the Rip-Cre allele present as a single allele in both heterozygous $\beta$-Phb2$^{wt}$- and homozygous $\beta$-Phb2$^{-/-}$ mice.

We further ascertained the possible impairment of insulin delivery suggested by low non-fasting plasma insulin levels (Fig. 1G). In 4-week old $\beta$-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, $\beta$-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 $\beta$-Phb2$^{+/}$ by glucose intolerance and lack of glucose-induced elevation of plasma insulin. Interestingly, 10-week old $\beta$-Phb2$^{+/}$ mice exhibited hyperglucagonemia following a moderate 2-hour fasting. Glucagon levels were further elevated in knockout animals upon refeeding (Fig. 2F), although not upon a glucose i.p. challenge (Supplementary Fig. 3).

**Reduced GSIS in $\beta$-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 $\beta$-cell response in islets maintained in their native pancreatic environment. This revealed a dramatic reduction of GSIS when pancreas of $\beta$-Phb2$^{-/-}$ were compared to those of Phb2$^{fl/fl}$ controls (Fig. 3A). In the former mice, first and second phases of insulin secretion were reduced by 76% and 78%, respectively when compared to the corresponding phases of Phb2$^{fl/fl}$ controls (Fig. 3B).

**Impaired metabolism-secretion coupling in Phb2$^{+/}$ $\beta$-cells.** Because the chronic hyperglycemia observed by the age of 5-6 weeks might induce secondary changes perturbing islet function, we studied islets isolated from normoglycemic mice at the age of 3-4 weeks (Fig. 1E, Fig. 2A-B). Despite normoglycemia, insulin content in $\beta$-Phb2$^{+/}$ islets was decreased by 46% when compared to that of control islets (Fig. 4A). Islet function tested in static
incubation revealed a markedly reduced GSIS in islets lacking Phb2 (Fig. 4B-C). When normalized per islet, the secretory response was 63\% lower in β-Phb2+/− islets compared to 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 inferior blunted glucose response. Accordingly, there was no significant difference between control and knockout islets when insulin secretion was stimulated by non-metabolic KCl-induced cell depolarization (data not shown). This indicates that the exocytotic machinery downstream of Ca^{2+} signaling was preserved in β-Phb2+/− islets. However, the Ca^{2+} rise secondary to glucose stimulation requires mitochondrial activation in terms of ATP generation. Hence, we measured intracellular Ca^{2+} ([Ca^{2+}]_i) in response to glucose stimulation in isolated islets. In comparison with littermate controls, β-Phb2+/− islets exhibited a lower [Ca^{2+}]_i rise upon glucose stimulation (Fig. 4D), while the response to KCl, used as Ca^{2+}-raising agent, was similar in the two groups (Supplementary Fig.4).

Additionally, we acutely depleted Phb2 in vitro in β-cells by treating non-recombined Phb2^{fl/fl} islets isolated from adult mice (age 10-14 weeks) with adenovirus expressing Cre recombinase (Fig. 5A,B). Compared to control islets transduced with Ad-LacZ virus, we observed impaired GSIS in recombined Ad-RipCre treated islets 72h 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 3-4 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,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 to control β-cells (Fig 6B). 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 Phb2fl/fl and knockout β-Phb2−/− mice, whether these cells were studied in isolated islets (Fig. 6D,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 & 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,H). Alterations in the electron transport chain might favor production of reactive oxygen species (30; 31). However, we recorded similar levels of 4-hydroxynonenal (4-HNE), an aldehydic product of lipid peroxidation commonly enhanced during oxidative stress (Supplementary Fig. S6A,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 to their control counterparts (Fig. 6I). This was translated into impaired ATP generation after glucose stimulation. At basal 2.8 mM glucose, Phb2fl/fl and β-Phb2−/− islets had similar concentrations of ATP (29.4±9.2 and 28.2±12.2 pmol per µg islet proteins, respectively). Upon 22.8mM glucose stimulation we measured a 30% rise in ATP
for control islets versus only 7% for β-Phb2<sup>+-</sup> islets (Fig. 6J), which might be sufficient for the blunted elevation of [Ca<sup>2+</sup>]<sub>i</sub> (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<sup>+-</sup> mice.** The defective GSIS observed in β-Phb2<sup>+-</sup> 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<sup>+-</sup> 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<sup>+-</sup> mice, with only few β-cells intermingled with α-cells (Fig. 7B). Quantification of islet β-cell mass in β-Phb2<sup>+-</sup> mice revealed that β-cell loss amounted to 35% at the age of 4 weeks and more than 90% at the age of 10 weeks compared to age-matched controls, consistent with the corresponding alterations in pancreatic insulin content (Fig. 7C, D). In 10-week old β-Phb2<sup>+-</sup> mice, we observed an expanded α-cell mass compared to that seen in controls (Fig. 7E). This was substantiated by higher pancreatic glucagon content in β-Phb2<sup>+-</sup> mice at 10 weeks of age compared to 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<sup>+-</sup> 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<sup>+-</sup> animals were treated with insulin. The daily administration of insulin to β-Phb2<sup>+-</sup> diabetic mice, which was initiated at 8 weeks of age, fully corrected glycemia (Fig. 7G) and provided for maintenance of control body weights...
(Fig. 7H) for the 10-week period of the treatment. Untreated β-Phb2−/− mice were sacrificed at 11 weeks of age because of 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-F), we tested leptin treatment in these animals. Fig. 7I 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 up-regulation of caspase-3 cleavage compared to age-matched controls (Fig. 8A). Additionally, these cells were more susceptible to apoptotic stimuli, such as staurosporine (Fig. 8A). On pancreas sections, we typically observed 1-3 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 about 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 present 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-Opa1 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 (34). 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 (34). Fragmented mitochondrial pattern has been documented in β-cells of human type 2 diabetic subjects and animal models for diabetes (8; 35; 36). 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 (34). 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 (34).

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 about 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 (37). Interestingly, a recent study in humans reported that diabetes appears after a reduction in β-cell mass of ~65%, while post-challenge glucose excursions in pre-diabetic subjects exhibit glucose intolerance before this critical threshold is reached (38). Therefore, within a 2-3 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 MEFs lacking Phb2 (18), as well as in Opa1-null β-cells (34), 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 about 10 weeks. Enhanced apoptosis mediated by cleavage of caspase-3 was a likely consequence of Phb2 deletion rather than Opa1 degradation, since caspase-3 is not activated in Opa1-null β-cells (34).

In this study we also observed the expansion of the α-cell mass and pancreatic glucagon content in 10-week old β-Phb2−/− mice. In diabetic patients, loss of β-cells accompanied by an increase in α-cell mass has been documented and might contribute to hyperglucagonemia, precipitating hyperglycemia because of increased hepatic glucose production (39). Interestingly, after 2 hours of fasting, 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
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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No potential conflicts of interest relevant to this article were reported.

S.S. conducted experiments, analyzed data, and wrote the manuscript. F.T., L.S. and P.Me. developed specific techniques and analyzed corresponding data. A.G. generated data. C.M., P.L.H. and T.L. generated transgenic animals essential for this study. P.Ma. supervised the project, analyzed data and wrote the manuscript.

P. Maechler is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis.
REFERENCES


FIGURE LEGENDS

**Figure 1** β-cell specific deletion of Phb2 renders mice diabetic. **A**: Abundance of PHB1 and 2 proteins in human islets revealed by immunoblotting. **B**: Recombination of Phb2 allele was assessed by PCR on DNA extracted from isolated islets of 4-week-old β-Phb2+/− and littermate control mice. **C**: Representative immunoblotting analysis showing expression of Phb1 and Phb2 proteins in islets of control and knockout mice. β-actin was used as loading control. **D**: Assessment of non-specific loss of Phb2 in other tissues such as skeletal muscle, brain, hypothalamus (Hypo), and liver by immunoblotting (representative of 3 immunoblottings performed on tissues isolated from 3 mice of each genotype). **E**: Non-fasting glycaemia recorded once every week in between 9.00 and 10.00am from the age 4 weeks in β-Phb2+/− (■) and littermate Phb2fl/fl (○) male mice (n=6 per group). **F**: Body weights of β-Phb2+/− (■) and littermate Phb2fl/fl (○) mice measured once a week (n=6 for each group). **G**: Non-fasting plasma insulin measured at 4 and 10 weeks of age in β-Phb2+/− (□) and littermate Phb2fl/fl (■) mice (n=4 for each genotype). Data are expressed as means ± SEM; *P<0.05, **P<0.01 and ***P<0.001; control Phb2fl/fl versus knockout β-Phb2+/−.

**Figure 2** Loss of Phb2 in β-cells induces development of diabetes over a 3-week period in β-Phb2+/− mice. **A-C**: Glucose tolerance test (2 g/kg) in 6h fasted β-Phb2+/− (■) and littermate Phb2lox/lox (○) mice. (A) 3-week old β-Phb2+/− (n=6) and Phb2fl/fl (n=4) mice, (B) 4-week old β-Phb2+/− (n=7) and Phb2fl/fl (n=6), and (C) 6-week old β-Phb2+/− (n=7) and Phb2fl/fl (n=7) mice. **D-E**: In addition to blood glucose, plasma insulin levels were determined before and 15 min after glucose injection. β-Phb2+/− (□) and littermate Phb2fl/fl (■) mice. **D**: At the age of 4 weeks, β-Phb2+/− (n=6) and Phb2fl/fl (n=5), (E) 6-week old β-Phb2+/− (n=3) and Phb2fl/fl (n=4) animals. **F**: At 10 weeks of age, plasma glucagon levels were determined after 2h fasting and subsequent 1h re-feeding. β-Phb2+/− (□) and littermate Phb2fl/fl (■) mice (n=4 for each group).
Data presented are means ± SEM; *P<0.05, **P<0.01 and ***P<0.001 between the two groups.

**Figure 3** Kinetics of GSIS is altered in 6-week old β-Phb2+/− mice as revealed by *in-situ* pancreatic perfusion. **A**: Glucose-induced insulin release determined by *in situ* pancreatic perfusions on 6-week old β-Phb2+/− (■) and littermate Phb20/0 (○) mice. Glucose was raised from basal 2.8 mM (2.8G) to stimulatory 22.8 mM (22.8G). **B**: Insulin released during 1st phase of the glucose response, *i.e.* 5 min after 22.8G stimulation, and 2nd phase, *i.e.* 40 min after completion of 1st phase of secretion. Traces show β-Phb2+/− (□, n=4) and littermate Phb20/0 (■, n=3) mice. All the data are expressed as means ± SEM; **P<0.01 between the two groups.

**Figure 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−/− (□) and littermate Phb20/0 (■) mice (n=5). **B**, **C**: GSIS tested as static incubation at basal 2.8 mM (2.8G) and stimulatory 22.8 mM (22.8G) glucose on islets isolated from 4-week old mice. Insulin secretion rate is expressed as insulin release normalized (**B**) per islet and (**C**) to total islet insulin content. β-Phb2−/− (□) and littermate Phb20/0 (■) mice (n=5). **D**: [Ca²⁺]i at basal 2.8G and in response to 22.8G in isolated islets from Phb20/0 (○) and β-Phb2−/− (□) mice. Traces show averages of recordings from 3 animals per genotype. Data are expressed as means ± SEM; *P<0.05, **P<0.01 and ***P<0.001 between the two groups.

**Figure 5** Acute *in vitro* knockout of Phb2 in β-cells. Islets isolated from Phb20/0 mice (10-14 weeks of age) were transduced with control (Ad-LacZ) or Cre-recombinase (Ad-RipCre) adenoviruses. **A**: Flow chart for *in vitro* recombination experiments. **B**: PCR on DNA
extracted from islets treated with respective viruses showing efficient recombination (699bp band) 24h after overnight incubation with Ad-RipCre. C: Insulin release during static incubation at basal 2.8 mM (2.8G) and stimulatory 22.8 mM (22.8G) glucose from islets after treatment with indicated viruses (n=4). Data are expressed as means ± SEM; *P<0.05.

**Figure 6** Mitochondrial morphology and function in β-cells from 3-4 week old β-Phb2−/− mice. A: Mitochondrial morphology was analyzed on dispersed pancreatic islet cells by immunofluorescence. Representative β-cells from control Phb2fl/fl and knockout β-Phb2−/− mice as indicated: Single plane showing mitochondrial network, three-dimensional (3D) reconstruction of z-stacks through entire β-cell, staining of insulin. Scale bar 2µm. B: Average mitochondrial length measured on 25-40 distinct mitochondria per cell in a randomly selected 2D z-stack. Phb2fl/fl (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 to controls. D, E: Electron microscopy of β-cells in islets isolated from 4-week old control Phb2fl/fl (D) and knockout β-Phb2−/− mice (E). The structure of mitochondria (arrows) appeared normal in the two mouse genotypes. Bar, 200 nm. F: Mitochondrial DNA copy number in islets isolated from respective genotypes. DNA copies of mitochondrial complex I was normalized to the DNA levels of nuclear RNAseP. n=3 and n=5 for Phb2fl/fl and β-Phb2−/−, respectively. G: Two independent representative immunoblots showing mitochondrial respiratory chain complexes (C) of isolated islets using antibody cocktail targeted towards different subunits (C-I, NDUFB8; C-II, Iron-Sulfur protein; C-III, Core protein 2; C-IV, Subunit-I; C-V, ATP synthase subunit-α). H: Densitometry analysis of three independent blots. β-Phb2−/− (□ n=4) and Phb2fl/fl (■ n=3). I: Mitochondrial membrane potential (∆ψm) in islets isolated from respective genotype was measured with rhodamine123. Fluorescence intensity was recorded.
during incubations at 2.8mM (basal) glucose and subsequently at 22.8mM (stimulated) glucose. Triplicates of 10 islets from each animal were used in independent experiment. \(\beta\)-\textit{Phb2}\(^{-/-}\) (■) and littermate \(\textit{Phb2}\(^{0/0}\) mice (○); \(n=3\). \textbf{J:} Cellular ATP levels were measured after incubating 50 islets each at 2.8mM glucose and stimulatory 22.8mM glucose for 15min. Percentage of rise in ATP production in response to stimulatory glucose concentration against basal glucose concentrations was calculated. \(\textit{Phb2}\(^{0/0}\) (■) \(n=5\) and \(\beta\)-\textit{Phb2}\(^{-/-}\) (□) \(n=4\). Data on panels \textbf{B, F, H, I} and \textbf{J} are means ± SEM; *\(P<0.05\), **\(P<0.01\) between the two groups. Immunofluorescence and immunoblotting images are representative of at least 3 independent analyzes performed on different mice.

\textbf{Figure 7} Lack of Phb2 in \(\beta\)-cells alters islet cell composition and architecture. \textbf{A, B:} Representative immunohistochemistry images of pancreatic sections from \(\beta\)-\textit{Phb2}\(^{-/-}\) and littermate \(\textit{Phb2}\(^{0/0}\) mice with insulin stained in green and glucagon in red. Upper panels show individual islets while lower panels show 2 islets in close proximity. Scale bar 20µm, (\textbf{A}) 4-week-old and (\textbf{B}) 10-week-old mice, representative of 3 independent analyzes performed on 3 different mice of each genotype per time point. \textbf{C:} Total pancreatic \(\beta\)-cell mass calculated at the age of 4 and 10 weeks from \(\beta\)-\textit{Phb2}\(^{-/-}\) (□) and littermate \(\textit{Phb2}\(^{0/0}\) (■) mice. \(n=3\) for each genotype. \textbf{D:} Total pancreatic insulin contents from \(\beta\)-\textit{Phb2}\(^{-/-}\) and littermate \(\textit{Phb2}\(^{0/0}\) mice at the age of 4 and 10 weeks; \(n=8\) and 3 per genotype for animals of 4 weeks (● \(\textit{Phb2}\(^{0/0}\), ■ \(\beta\)-\textit{Phb2}\(^{-/-}\)) and 10 weeks (▲ \(\textit{Phb2}\(^{0/0}\), ▼ \(\beta\)-\textit{Phb2}\(^{-/-}\)), respectively. \textbf{E:} Total pancreatic \(\alpha\)-cell mass at the age of 10 weeks from \(\beta\)-\textit{Phb2}\(^{-/-}\) (□) and littermate \(\textit{Phb2}\(^{0/0}\) (■) mice; \(n=3\) per genotype. \textbf{F:} Total pancreatic glucagon contents from \(\beta\)-\textit{Phb2}\(^{-/-}\) (■) and littermate \(\textit{Phb2}\(^{0/0}\) (●) mice at the age of 10 weeks; \(\textit{Phb2}\(^{0/0}\) (\(n=6\)) and \(\beta\)-\textit{Phb2}\(^{-/-}\) (\(n=5\)). \textbf{G, H:} Non-fasting glycemia (\textbf{G}) and body weights (\textbf{H}) of \(\beta\)-\textit{Phb2}\(^{-/-}\) mice measured once a week at 5.00 pm from age of 7 weeks onward. Long-acting insulin was injected subcutaneously twice a day (□ grey zone, \(n=3\)).
Non-treated $\beta$-$Phb2^{-/-}$ mice (●, $n=2$) were sacrificed at the age of 11 weeks due to end-stage diabetic state. *I*: Glycemia of 4hr-fasted $\beta$-$Phb2^{-/-}$ mice at indicated time points after subcutaneous implantation at the age of 8 weeks of 14-day osmotic pump releasing either 10 µg per day of leptin (□, $n=3$) or saline (●, $n=2$). All the data are expressed as means ± SEM; *$P<0.05$, **$P<0.01$ and ***$P<0.001$; control ($Phb2^{fl/fl}$) versus $\beta$-$Phb2^{-/-}$.

**Figure 8** Both apoptosis and proliferation of $\beta$-cells are induced in $\beta$-$Phb2^{+/+}$ mice at 4 weeks of age. *A*: Representative immunoblotting for apoptosis analysis showing increased levels of cleaved caspase-3 in islets of $\beta$-$Phb2^{-/-}$ versus control $Phb2^{fl/fl}$ mice. Islets were treated (+) or not (-) with 1 µM staurosporine for 4h before analysis. Representative of 3 independent analyzes performed on different mice. *B*: Representative images of 3 independent experiments of immunofluorescence on pancreatic sections showing labeling of TUNEL-positive $\beta$-cells in islets of $\beta$-$Phb2^{-/-}$ and littermate $Phb2^{fl/fl}$ mice at the age of 4 weeks. The TUNEL marker anti-digoxin 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 $\beta$-cell proliferation in islets of $\beta$-$Phb2^{+/+}$ and littermate $Phb2^{fl/fl}$ 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 arrow heads. *D*: $\beta$-cell proliferating index is expressed as % of proliferating $\beta$-cells after computing Ki-67 positive nuclei for 1,000 $\beta$-cells analyzed per mouse at the age of 4 weeks in both $\beta$-$Phb2^{+/+}$ (□) and control $Phb2^{fl/fl}$ (■). Data expressed as means ± SEM, *$P<0.05$, $n=3$ per genotype.
Fig. 1 β-cell specific deletion of Phb2 renders mice diabetic.
Fig. 2 Loss of Phb2 in β-cells induces development of diabetes over a 3-week period in β-Phb2-/- mice.
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190x254mm (96 x 96 DPI)
Fig. 6 A-E Mitochondrial morphology and function in β-cells from 3-4 week old β-Phb2-/- mice.

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Fig. 7 G-I Lack of Phb2 in β-cells alters islet cell composition and architecture.

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Fig. 8 Both apoptosis and proliferation of β-cells are induced in β-Phb2-/- mice at 4 weeks of age.
Supplementary Figure 1: Non-fasting glycemia in female mice. Non-fasting glycemia recorded once a week between 9.00 and 10.00 am starting at the age of 4 weeks until the age of 10 weeks in female β-Phb2/fl/fl(♀) and littermate Phb2/fl/fl(♂) mice, n = 5. Data expressed as means ± SEM; *p<0.05, **p<0.01 and ***p<0.001 between the two groups.
Supplementary Figure 2: Glucose tolerance test in heterozygous 10-week-old β-Phb2<sup>wt/-</sup> mice. Glucose tolerance test (2 g/kg body weight; i.p. injection) in 6h-fasted β-Phb2<sup>wt/-</sup> (---) and littermate control Phb2<sup>fl/fl</sup> (----) mice. Blood glucose was recorded by glucometer at indicated time points, n = 5. Data expressed as means ± SEM.
Supplementary Figure 3: Plasma glucagon levels in 10-week-old β-Phb2/- mice. Plasma glucagon before and 30 min after glucose (2 g/kg body weight) i.p. injection in 10 week-old β-Phb2/- (□) and littermate control Phb2fl/fl (■) mice fasted for 6h. Blood glucose was recorded by glucometer at indicated time points. Data expressed as means ± SEM; *p<0.05, **p<0.01 and ***p<0.001; NS: non-significant; n = 3.
Supplementary Figure 4: KCl-induced intracellular Ca\textsuperscript{2+} rise in islets of 4-week-old Phb2\textsuperscript{fl/fl} and β-Phb2\textsuperscript{-/-} mice. Representative traces of intracellular calcium levels in response to depolarization-induced calcium rise evoked by 30 mM KCl at basal glucose in isolated islets from 4-week old control Phb2\textsuperscript{fl/fl} (●) and knockout β-Phb2\textsuperscript{-/-} (□) mice. Recordings were performed on at least 2 islets each from 4 animals per genotype.
A. Mitochondrial network in Phb2<sup>fl/fl</sup> β-cells

B. Mitochondrial network in β-Phb2<sup>−/−</sup> β-cells

Supplementary Figure 5: Series of 7 successive Z-stacks separated by 0.36µm showing mitochondrial network β-cells of 3-week-old Phb2<sup>fl/fl</sup> and β-Phb2<sup>−/−</sup> mice. Representative images of β-cells from Phb2<sup>fl/fl</sup> (A) and β-Phb2<sup>−/−</sup> (B). Mitochondria are revealed with the help of immunofluorescence by using antibody against TOM20 shown in green and insulin is shown in blue.
Supplementary Figure 6: Peroxide levels in islets isolated from 4-week-old Phb2^0/0 and β-Phb2^{+/−} mice. A. Representative immunoblot showing lipid oxidation profile with 4-hydroxynonenal antibody. (B). Densitometric analysis of 4HNE labeled proteins normalized with loading control β-actin (n=4).
Supplementary Figure 7: No signs of insulitis in $\beta$-$Phb2^{+/v}$ mice. Hematoxylin and eosin (H&E) staining on sections of pancreas from $Phb2^{fl/fl}$ and $\beta$-$Phb2^{-/-}$ mice revealed absence of infiltration of immune cells in and around islets at the age of 4-weeks.