**Perk Gene Dosage Regulates Glucose Homeostasis by Modulating Pancreatic β-Cell Functions**

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**Abstract**

**Background:** Insulin synthesis and cell proliferation are under tight regulation in pancreatic β-cells to maintain glucose homeostasis. Dysfunction in either aspect leads to development of diabetes. PERK (EIF2AK3) loss of function mutations in humans and mice exhibit permanent neonatal diabetes that is characterized by insufficient β-cell mass and reduced proinsulin trafficking and insulin secretion. Unexpectedly, we found that Perk heterozygous mice displayed lower blood glucose levels.

**Methodology:** Longitudinal studies were conducted to assess serum glucose and insulin, intracellular insulin synthesis and storage, insulin secretion, and β-cell proliferation in Perk heterozygous mice. In addition, modulation of Perk dosage specifically in β-cells showed that the glucose homeostasis phenotype of Perk heterozygous mice is determined by reduced expression of PERK in the β-cells.

**Principal Findings:** We found that Perk heterozygous mice first exhibited enhanced insulin synthesis and secretion during neonatal and juvenile development followed by enhanced β-cell proliferation and a substantial increase in β-cell mass at the adult stage. These differences are not likely to entail the well-known function of PERK to regulate the ER stress response in cultured cells as several markers for ER stress were not differentially expressed in Perk heterozygous mice.

**Conclusions:** In addition to the essential functions of PERK in β-cells as revealed by severely diabetic phenotype in humans and mice completely deficient for PERK, reducing Perk gene expression by half showed that intermediate levels of PERK have a profound impact on β-cell function and glucose homeostasis. These results suggest that an optimal level of PERK expression is necessary to balance several parameters of β-cell function and growth in order to achieve normoglycemia.

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**Introduction**

The endocrine pancreatic β-cells have an exclusive and singular function to synthesize and secrete insulin. While insulin is essential for maintaining glucose homeostasis, hyperinsulinemia can result in hypoglycemic shock and death. Therefore insulin synthesis and secretion must be tightly regulated to provide the appropriate level of circulating insulin in response to episodic input of dietary carbohydrates and release of glucose stores. Pancreatic insulin output is controlled by a combination of regulating β-cell mass in the endocrine pancreas [1–4] and by regulating insulin synthesis and secretion in β-cells [5–9]. Although a large number of genes have been shown to influence β-cell growth and insulin synthesis and secretion, a small number of genes (ca. 20) including Perk have been identified in humans that are absolutely essential for β-cell growth or insulin production [10,11]. The consequence of the loss of function mutations in these genes is permanent neonatal diabetes (PND). Among these PND genes, the function of the Perk (EIF2AK3) gene has been the most controversial and perplexing [12–15]. Perk was initially identified as one of the three regulatory arms of the ER stress response pathway in cultured mammalian cells [16,17]. Shortly after its discovery [18] and characterization in cell culture, mutations in Perk were found to be the cause of the Wolcott-Rallison syndrome (WRS) in humans [19] that featured permanent neonatal diabetes, exocrine pancreas deficiency, growth retardation, and osteopenia. Perk knockout (KO) mouse strains were generated by us [15] and by Harding and Ron [12], which exhibited a nearly identical phenotype to that seen in human WRS patients, including permanent neonatal diabetes. By generating and analyzing tissue-specific Perk KO and transgenic rescue strains, we showed that the neonatal diabetes was caused by deficient β-cell growth and multiple problems in proinsulin synthesis and trafficking and insulin secretion [13,14,20].

An extensive analysis of PERK function by us has failed to support the initial hypothesis that the β-cell defects seen in Perk deficiency are due to misregulation of the ER stress response pathway [13,14]. Moreover, mutations in the other two regulatory arms of the ER stress pathway, ATF6 and IRE1, do not cause major β-cell dysfunctions or diabetes [21,22]. This demonstrates that dysfunction in the ER stress response generally does not result in permanent neonatal diabetes. Some of these β-cell dysfunctions seen in Perk KO mice can be attributed to the lack of...
phosphorylation of eIF2α, the primary substrate of PERK, because mutations that block the Ser51 phosphorylation site either in whole animals or in just the β-cells also result in diabetes [23,24]. However, other PERK-dependent β-cell functions may be independent of eIF2α phosphorylation including regulation of secretagogue stimulated calcium influx and insulin secretion [25].

Humans and mice that are heterozygous for a loss-of-function Perk mutation do not exhibit overt abnormal phenotypes[15,19,26]. However, we found that Perk heterozygous Perk+/− mice exhibit significantly lower serum blood glucose levels among several hundred litters of mice analyzed over the past ten years, opposite to the Perk KO mice which are severely hyperglycemic. To determine the underlying reasons for this shift in glucose homeostasis of Perk+/− mice, we conducted a postnatal developmental analysis of β-cell growth and function in Perk+/− mice compared to their homozygous wild-type littermates. We found that Perk+/− mice first exhibited enhanced insulin synthesis and secretion during neonatal and juvenile development followed later at the adult stage by enhanced β-cell proliferation and a substantial increase in β-cell mass. These findings support the hypothesis that PERK dynamically regulates β-cell growth, insulin synthesis and secretion during postnatal development.

Methods and Materials

Genetic Strains

Perk global KO allele, floxed Perk allele were generated as previously described [15]. A Perk transgene under the control of the rat insulin promoter was introduced into the wild-type mice to generate mice with overexpression of Perk specifically in β-cells with an otherwise wild-type background (Perk+/−/Perk[27]). Perk+/− mice that carried one Perk KO allele were in congenic C57Bl/6, 129 SvEvTac, or in a mixed background. To generate pancreatic specific Perk+/− mice, pds1-cre transgenic mice were crossed with mice homozygous for floxed Perk allele. Mice were sacrificed by CO2 euthanasia. All animal studies were approved by the Institutional Animal Care and Use Committee of Pennsylvania State University, and all efforts were made to minimize suffering.

Islet isolation

Mouse islets were isolated using a modified Histopaque-1077 separation method [28]. Before experiments, islets were allowed overnight recovery in fresh RPMI1640 medium with 10% fetal bovine serum, 1% Antibiotic Antimycotic Solution (Sigma) and 5.5 mM glucose at 5% CO2, 95% air.

Cell culture

INS1 832/13 cells containing a short-hairpin RNA directed against the rat Perk mRNA (shPerk) were obtained from Dr. Fumihiko Urano (University of Massachusetts). The shPerk is stably integrated into the genome of INS1 832/13 β-cell lines and under the inducible regulation of doxycycline. The INS1 832/13 shPerk cells were cultured in a tetracycline-free environment to avoid leaky expression of shPerk.

Determination of serum glucose and glucose tolerance test

Blood samples were obtained from the tails and glucose were measured using OneTouch Ultra glucose meters. Glucose tolerance tests were performed on mice fasted 4 hours (for P17 mice) or overnight (for P50 mice) and injected intraperitoneally with 2 mg glucose/g of body weight.

Insulin measurement

Insulin concentrations were determined by immunoassay (Meso Scale Discovery, MSD). For serum insulin measurement, serum was obtained by centrifugation of blood samples at 10,000 g for 5 min. For islet and pancreatic insulin measurement, islets or pancreata were sonicated in 1 ml of cold acid ethanol (1.5% volume HCl in 75% ethanol). Insulin concentrations were further normalized to total protein concentration (determined by Bio-RAD Protein Assay). For studies of glucose stimulated insulin secretion, isolated islets or cultured β-cell line were firstly cultured overnight at 37°C (5% CO2) in RPMI1640 medium containing 10% fetal bovine serum and 5.5 mM glucose. Samples were then incubated, at 37°C in KRB-HEPES buffer (pH 7.4) with 1% bovine serum albumin and 2.8 mM glucose for 1 hour before insulin stimulation with 2.8 or 20 mM glucose. At the end of the 30 min stimulation, the supernatant was assayed for secreted insulin (by MSD), and cells/islets were assayed for total insulin and total protein.

RNA isolation and gene expression measurement

RNA was extracted from pancreas, islets or cultured cells using RNeasy Mini Kit (Qiagen) and quantified by RiboGreen RNA Assay Kit (Invitrogen). Reverse transcription was performed using qScript cDNA supermix (Quanta). Quantitative mRNA measurement was carried out by using qPCR core kit for SYBR Green I (Quanta) with the StepOne Plus detection system (Applied Biosystems). Levels of Xbp-1 (spliced form) were normalized to Xbp-t (total levels). Other gene expression levels were normalized to Gapdh and Actin levels of the same sample. Mouse primer sequences were listed as follows:

Actin 5′-GCCCTGAGGCTCTTTTTC-3′, 5′-TGGCCAGAAGGATTCTATCCAG-3′.
Gapdh 5′-GGGAGCCCTCATATCCACAGT-3′, 5′-ACATACTCAGCACCAGGCTC-3′.
Perk 5′-TCCTGCTTGGATGTAGACC-3′, 5′-GATGGAAGACCTGGC-3′.
Insulin II exon, 5′-TTCGCAGATTCATCGTGC-3′, 5′-GATCGATGATGTCATCG-3′.
Insulin II intron, 5′-CTTCATGGGACAGGTTTC-3′, 5′-GATCGATGATGTCATCG-3′.
Gclc 5′-TGCCTTATGATGTCATCG-3′, 5′-GATCGATGATGTCATCG-3′.
Mafa 5′-GGCTGGATGATCAGGTTTCG-3′, 5′-GATCGATGATGTCATCG-3′.
Pdx-1, 5′-GAGGCTTCTCAATTACGCACAG-3′, 5′-TACGCCCCGATGCATGGT-3′.
Hdh, 5′-TGGGCTTATGATGTCATCG-3′, 5′-GATCGATGATGTCATCG-3′.
MafA, 5′-GGCCCCCTATCAGGGTG-3′, 5′-GATCGATGATGTCATCG-3′.
Cdt1, 5′-GGAGGCTCTCTTCGGTAC-3′, 5′-GATCGATGATGTCATCG-3′.
BiP, 5′-CTTTGGGCTTTCTTCCG-3′, 5′-GATCGATGATGTCATCG-3′.
Golgi-MAP, 5′-GGTAGCTGAATCTGTCTGC-3′, 5′-GATCGATGATGTCATCG-3′.
Chop, 5′-GGGGAGGTACCAGCTCAGACACC-3′, 5′-GATCGATGATGTCATCG-3′.
BiP, 5′-CTTTGGGCTTTCTTCCG-3′, 5′-GATCGATGATGTCATCG-3′.
Cdt1, 5′-GGAGGCTCTCTTCGGTAC-3′, 5′-GATCGATGATGTCATCG-3′.
MafA, 5′-GGCCCCCTATCAGGGTG-3′, 5′-GATCGATGATGTCATCG-3′.
Cdt1, 5′-GGGCTTCTCCATGCTCG-3′, 5′-GATCGATGATGTCATCG-3′.
Hdh, 5′-TGGGCTTATGATGTCATCG-3′, 5′-GATCGATGATGTCATCG-3′.
doxycycline, 0.1% SDS, 1 per sample with RIPA buffer (1% Nonidet P40, 0.5% sodium SUnSET method was used [29]. Briefly, 10 anti-ERp72 (1:1000, Stressgen, Inc), anti-GRP78/BiP (1:500, Cell Signaling), western blot analysis were: anti-PERK (1:500, Cell Signaling), anti-rabbit secondary antibodies (1:20000, LI-COR) for visualiza-
primary antibodies, followed by IR800 anti-mouse and IR700 Millipore) and rabbit anti-proinsulin C-peptide (1:500, Bio Vision) b
b expressed in mRNA in whole pancreata was first determined and then divided to single cell level can be used to

Western Blot Analysis

Total cellular protein was extracted from 60 islets or 10^6 cells per sample with RIPA buffer (1% Nonidet P40, 0.5% sodium doxycycline, 0.1% SDS, 1× PBS, pH 8.0) containing 1× protease and phosphatase inhibitor cocktails (Sigma) and were boiled in 2× SDS sample buffer and then loaded onto 4–15% gels for Western blots. To measure newly synthesized proinsulin the SUNSET method was used [29]. Briefly, 10 µg/ml puromycin was added 15 minutes before harvest. Western blot was performed and proteins were probed with mouse anti-puromycin (1:5000, Millipore) and rabbit anti-proinsulin C-peptide (1:300, Bio Vision) primary antibodies, followed by IR800 anti-mouse and IR700 anti-rabbit secondary antibodies (1:20000, LI-COR) for visualization of newly synthesized proteins co-localized with proinsulin using LI-COR Odyssey scanner. Other primary antibodies used in western blot analysis were: anti-PERK (1:500, Cell Signaling), anti-ERp72 (1:1000, Stressgen, Inc), anti-GRP78/BiP (1:500, Santa Cruz, Inc), anti-ERp57 (1:500, Santa Cruz), anti-PDI (1:500, Stressgen, Inc).

Measurement of β-cell proliferation and volume

To measure β-cell proliferation, all experimental mice received BrdU at a concentration of 0.8 mg/ml in drinking water. Mice ≤ P17 days were treated with BrdU water for 3 days, and mice ≥ 30 days were treated for 7 days. After BrdU administration, whole pancreata were harvested for immunohistochemistry following the standard procedure as previously described [30]. Guinea pig Anti-insulin (1:500, Abcam) and mouse anti-BrdU (1:150, DAKO) were applied overnight at 4°C. Anti-guinea pig and anti-mouse secondary antibodies conjugated with Alexa Fluor488 and 555 dye (Molecular Probes) were used (1:400 dilution) to visualize the labeled cells. Anti-fade reagent with Dapi (Life technologies) was used to mount slides and label the nucleus. Fluorescence images were captured with a Nikon Eclipse E1000 and Image-Pro Plus (Phase 3 Imaging Systems, GE Healthcare, Inc.). To calculate β-cell daily proliferation, ratios of BrdU-positive to total β-cells were counted from a total of 4 tissue sections per mouse and further divided by total days of BrdU treatment. The same images were used for β-cell volume estimates. Insulin-positive cells were traced by using imageJ software, and single cell volume was estimated by dividing the total area of β-cells by cell number.

Estimation of β-cell number

Total β-cell number was estimated by a method we used previously [14,31]. The total amount of Glut2 mRNA or Insulin II mRNA in whole pancreata was first determined and then divided by the estimated amount of Glut2 or Insulin II mRNA, respectively, per β-cell. Since these two genes are exclusively expressed in β-cells of the endocrine pancreatic islets, the ratio of their total pancreatic level to single cell level can be used to estimate total β-cell number [14,31]. Similarly, β-cell number was estimated by dividing insulin protein level in whole pancrea by insulin content per β-cell.

Statistical Analysis

All numerical data were represented as mean ± SE. Statistical significance was determined using Student’s t testing.

Results

Perk heterozygous mice exhibit increased levels of circulating insulin and decreased glucose

Mice and humans that are completely deficient for PERK exhibit severe hyperglycemia (>400 mg/dl) even in the fasted state [12,14,15,19,26]. Therefore we expected that half dosage of PERK associated with Perk heterozygosity would either be recessive with no effect on glucose homeostasis or would be semi-dominant with elevated blood glucose. Surprisingly we found that adult Perk+/− mice exhibited significantly lower random-fed serum glucose levels than mice homozygous for the normal (Perk+/+) wild-type allele (Fig 1A). The mouse Perk KO allele used in this study was generated by us [13] and utilized in several previous studies [13-15,20,25,27,30,32-37]. Our Perk KO allele was made by Cre-mediated deletion of exons 5–7 that encoded part of the luminal domain, the transmembrane domain, and part of the catalytic domain of PERK yielding a loss-of-function null allele. Consistent with this, Perk+/− mice exhibited the expected reduction in Perk mRNA and protein (ca. 60% normal, P<0.0001) (Fig 1B–C) levels compared to wild-type Perk+/+ mice. Moreover, reduction in PERK activity was observed in Perk+/− β-cells as phosphorylation of eIF2α, a downstream substrate of PERK, was significantly reduced by 30% (P<0.01) (Fig 1D). Although circulating glucose levels and PERK activity are reduced, the islet composition and β-cell morphology in the pancreata of Perk+/− is indistinguishable from Perk+/+ and these observations are in contrast to radical changes seen in Perk KO mice, which include reduced β-cell number (Fig 1E). Perk+/− β-cells also show normal enrichment of proinsulin in the Golgi (not shown) in contrast to ER retention seen Perk KO β-cells [38].

To determine when in development Perk+/- mice first display mild hypoglycemia, we analyzed a large cohort of mice at various postnatal stages (Fig 2A). The genetic background of these mice was congenic C57Bl/6. Perk+/- and Perk+/- littermates were used throughout this study to reduce inter-litter variation. Blood glucose levels of neonatal (postnatal day 5) Perk+/- mice were indistinguishable compared to Perk+/-, but as mice approached the weaning stage of development (postnatal day 17) a substantial reduction in blood glucose levels was seen in Perk+/- mice. Curiously, the difference in blood glucose between Perk+/- and Perk+/- mice was reduced with age. By the time the mice reached postnatal 75 days, the difference in blood glucose was no longer statistically significant. However, male Perk+/- mice showed a persistent trend towards reduced blood glucose as seen in 9–12 month old mice in three genetic backgrounds (Fig 2B) including a highly statistically significant difference for Perk+/- in a 129 SvEvTac genetic background (Fig 2B, P<0.01). Serum insulin levels were inversely proportional to serum glucose levels with Perk+/- mice (C57Bl/6 genetic background) showing significantly higher levels than Perk+/- at P17 (P<0.05, Fig 2C) and a non-significant trend towards higher insulin levels in older mice.

To determine if Perk dosage would impact serum glucose and insulin under glucose stimulation, a glucose tolerance test (GTT) was performed in juvenile (P17) and adult mice (P50), Perk+/- mice trended towards being more glucose tolerant (P=0.1) than WT in P17 mice (Fig 2D) and were significantly more glucose tolerant in P50 mice (P<0.05, Fig 2F). Serum insulin exhibited a strong trend towards being higher in Perk+/- mice injected with glucose (Fig 2E) but was not quite statistically significant.

Perk Gene Dosage Regulates Pancreatic β-Cell Functions
Insulin content and β-cell are modulated by Perk gene dosage during postnatal development

To determine whether insulin synthesis and storage contribute to PERK-dependent regulation of glucose and insulin homeostasis, whole pancreatic insulin content was measured. Compared to Perk+/+, Perk−/− mice exhibited 42.0% and 92.8% increase of pancreatic insulin at P17 and P50 (P<0.05 at both ages) (Fig 3A). In summary, neonatal Perk−/− mice exhibit higher insulin content per β-cell, enhanced insulin synthesis but fewer β-cells, whereas in mature adult Perk−/− mice insulin concentration is reduced, while β-cell number is increased.

β-cell number is increased in Perk heterozygotes due to elevated β-cell proliferation

Unlike P17 mice, Perk−/− mice at P50 did not show increased expression of insulin mRNA level (Fig 4B) or protein level per β-cell (Fig 4C). However, P50 Perk−/− mice had substantially higher β-cell number (Fig 3D). To confirm this observation, β-cell number was estimated using the expression of mRNA of two genes, insulin II and Glut2, after previously published methods [38,39]. Since both genes are exclusively expressed in β-cells, their mRNA levels in whole pancrea are directly proportional to β-cell number [38,39]. Perk−/− mice at P50 had higher total insulin (P<0.05, Fig 4A) and total Glut2 (p = 0.08) mRNA in the total pancreas compared to wild-type mice whereas expression levels of these two genes in islets were not different between genotypes (Fig 4A), reflecting a 56%–69% increase in total β-cells in Perk−/− (Fig 4A) with equivalent level of expression of insulin II and Glut2 per β-cell.

To further investigate the reason for increased β-cell number in P50 Perk−/− mice, β-cell proliferation was determined by BrdU incorporation. β-cell proliferation was found to be significantly increased, reflecting a developmental complexity. We also estimated pancreatic β-cell number by dividing the total amount of insulin in whole pancrea by the estimated insulin content per β-cell. We found that Perk−/− mice initially had fewer β-cells during neonatal development, but this trend was reversed in mature adult mice (Fig 3D). In summary, neonatal Perk−/− mice exhibit higher insulin content per β-cell, enhanced insulin synthesis but fewer β-cells, whereas in mature adult Perk−/− mice insulin concentration is reduced, while β-cell number is increased.

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β-cell number is increased in Perk heterozygotes due to elevated β-cell proliferation

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Figure 2. Glucose and insulin homeostasis were impacted by modulation of Perk during development. A. Random fed serum glucose of Perk<sup>−/−</sup> mice relative to Perk<sup>+/−</sup> during postnatal development. Age of mice was shown as indicated, except that mice belonging to P75 group were from P70 to P90. Mice are in C57BL/6 background. Shown are means ± SEM (n for Perk<sup>−/−</sup>, Perk<sup>+/−</sup> = 18, 16 (P5); 16, 17 (P17); 13, 11 (P30); 12, 16 (P50); 17, 18 (P75). *P<0.05, **P<0.01, ***P<0.001). B. Random fed serum glucose of Perk<sup>−/−</sup> mice relative to Perk<sup>+/−</sup> at 9–12 month. Mice are in C57BL/6, 129 SveEvTac or mixed background. Shown are means ± SEM. (n for Perk<sup>−/−</sup>, Perk<sup>+/−</sup> = 4, 5 (B6); 23, 20 (129); 5, 3. **P<0.01). C. Random fed serum insulin of Perk<sup>−/−</sup> mice relative to Perk<sup>+/−</sup> during postnatal development. Age of mice was shown as indicated, except that mice belonging to P75 group were from P70 to P90. Shown are means ± SEM (n for Perk<sup>−/−</sup>, Perk<sup>+/−</sup> = 28, 28 (P5); 19, 17 (P17); 25, 18 (P30); 56, 75 (P50); 29, 32 (P75). *P<0.05). D. Glucose tolerance test of P17 mice after 4 hour fasting. Serum glucose was measured at various time points after glucose injection as indicated above. GTT traces are shown as means ± SEM of each time point and quantified by calculating area under the curve (AUC) in relative to Perk<sup>+/−</sup> (n for Perk<sup>−/−</sup>, Perk<sup>+/−</sup> = 5, 4). E. Serum insulin collected in P17 mice 30 minutes after injection with glucose or vehicle (n for Perk<sup>−/−</sup>, Perk<sup>+/−</sup> = 5, 4, P = 0.16 for stimulated insulin levels). F. Glucose tolerance test of P50 mice after 16 hour fasting. Experiment and analysis were as in 2D (n for Perk<sup>−/−</sup>, Perk<sup>+/−</sup> = 18, 18, *P<0.05). doi:10.1371/journal.pone.0099684.g002
increased in P50 Perk+/− mice compared to WT controls (Fig 4B).

We also examined β-cell proliferation at four other developmental time points and found that Perk+/− exhibited elevated proliferation at P30 and P50 but not earlier or later time points (Fig 4B), indicating that enhanced proliferation was transient and corresponded to the time period when β-cell number was increased in Perk+/− mice. In addition, β-cell death was estimated using TUNEL assay and found to be negligible and not different between Perk genotypes (data not shown).

Insulin transcription and proinsulin synthesis were up-regulated in Perk+/− mice at postnatal day 17

Despite a lower number of β-cells, P17 Perk+/− mice exhibited higher pancreatic insulin due to a significant increase in insulin content per β-cell (Fig 3A-D). To probe the mechanism underlying the increased β-cell insulin content in Perk+/− P17 mice, Insulin mRNA was measured in Perk+/− islets and found to be 21% higher (P<0.05) than WT. To determine if increased Insulin gene transcription was responsible for the increased steady-state levels of Insulin mRNA, Insulin pre-mRNA, which has a much shorter half-life and is less abundant than the mature mRNA, was measured using primers detecting the Insulin intron after methods of Evans-Molina and coworkers [40]. Insulin pre-mRNA was elevated 52% in Perk+/− β-cells (P<0.05), whereas Glucagon mRNA was not impacted by modulation of Perk (Fig 5A), suggesting that Perk-dependent difference in Insulin gene transcription contributed to the difference in insulin content. Unlike stage P17 mice, no change of mature mRNA or pre-mRNA of insulin was seen in Perk+/− β-cells at other developmental time points (Fig 5B).

To determine if proinsulin protein synthesis in Perk+/− at P17 reflected the higher levels of insulin mRNA, new proinsulin synthesis was estimated by puromycin incorporation [29]. Perk+/−
exhibited a 15% higher proinsulin synthesis (Fig 6A), which was nearly statistically significant (p = 0.06). Consistently, pancreatic mature proinsulin was found to be 41% higher in P17 Perk+/2 mice (P, 0.05) than Perk+/+ mice (Fig 6B). In contrast, no genotypic difference in mature proinsulin level was observed in mice at other developmental time points (Fig 6B). Moreover, proinsulin to insulin ratio was significantly elevated in P17 Perk+/2 mice (Fig 6C). Taken together, our data suggest that the increased insulin content per β-cell seen in the Perk+/2 mice at P17 is the result of an increase in all aspects of insulin biosynthesis, including insulin gene transcription, proinsulin synthesis and maturation. It is also possible that genotypic differences in proinsulin and/or insulin stability could contribute to the observed differences in proinsulin and insulin.

ER chaperones are differentially regulated by Perk gene dosage

To determine if the expression of other genes associated with insulin biosynthesis exhibited Perk genotypic differences in mice at P17, mRNA levels were determined in isolated islets for MafA, Pdx1, Hrd1, ERp57, BiP, and ERp72. MafA mRNA was increased by 25% (p = 0.06) in Perk+/2 whereas Pdx1 was not changed (Fig 7A). The expression of the mRNAs encoding the ER chaperones HRD1, BIP, and ERp72 levels were significantly elevated in Perk+/2 β-cells, while ERp57 mRNA was reduced (Fig 7A). The expression of the same genes was measured in isolated islets of mice at P30 (Fig 7B) and P50 (Fig 7C) but none showed a genotypic difference. The protein levels of BIP, ERp72, ERp57 and another protein disulfide isomerase family protein PDI

Figure 5. Insulin transcription was up-regulated in P17 Perk+/2 mice. A. Gene expression levels of mouse islets relative to Perk+/+. Mice are postnatal 17 days. Shown are means ± SEM. (n for Perk+/+, Perk+/+ = 11, 9. *P<0.05). B. Gene expression level of mouse islets relative to Perk+/+. Islets were isolated from mice at various ages as indicated. Shown are means ± SEM. (n for Perk+/+, Perk+/+ = 8, 8 (P35); 3, 4 (P50); 6, 6(1 year). No significant difference was seen for any gene at any age). doi:10.1371/journal.pone.0099684.g005

Figure 4. P50 Perk+/2 mice exhibit higher β-cell number due to elevated β-cell proliferation. A. Gene expression levels of islets or whole pancreata from P50 Perk+/2 mice relative to Perk+/+ control. Shown are means ± SEM. Below the bar graph shows the estimated fold-increase of β-cell number of P50 Perk+/2 mice relative to Perk+/+ control using each β-cell specific gene (islet data: n = 3, 4; pancreas data: n = 6, 4. *P<0.05). B. Daily BrdU incorporation rate of β-cells in mice at various ages. Shown are means ± SEM (n for Perk+/+, Perk+/+ = 5, 5 (P5); 3, 4 (P17); 10, 10 (P30); 4, 4 (P50); 3, 3 (P70). *P<0.05). doi:10.1371/journal.pone.0099684.g004
were assessed in pancreatic islets of P17 mice. ERp72 was elevated \( \text{Perk}^{+/–} \) islets (Fig 7D), whereas PDI was decreased and BIP and ERp57 were not different from levels seen in \( \text{Perk}^{+/+} \) islets. In addition, we also measured mRNA level of Chop, Atf4 and Xbp-1 splicing in P17 mouse islets, which are sensitive indicators of ER stress. None of the ER stress markers showed \( \text{Perk} \) genotype differences (Fig 7A), suggesting that regulation of \( \beta \)-cell functions by \( \text{Perk} \) dosage was not mediated through ER stress pathway.

**Perk** gene dosage specifically in the pancreatic \( \beta \)-cells regulates glucose homeostasis

Although our analysis of \( \beta \)-cell functions suggests that the \( \text{Perk} \) genotype specific differences in growth homeostasis are due to differences in expression levels of PERK in \( \beta \)-cells, other organs that are known to regulate glucose homeostasis, including the liver, may also participate in this regulation. To pinpoint the responsible organ/cell type, we generated mouse strains in which \( \text{Perk} \) gene dosage was altered in specific organs and/or cell types. Examination of liver specific \( \text{Perk} \) KO (\( \text{shPKO} \)) mice revealed no differences in random fed glucose levels (Fig 8A). By contrast, we previously reported that pancreatic specific \( \text{Perk} \) KO (\( \text{pcPKO} \)) rapidly developed severe hyperglycemia similar to global \( \text{Perk} \) KO mice [14]. In addition, we now report that \( \text{pcPKO} \) heterozygotes exhibit 21% (\( P<0.01 \)) lower random fed glucose levels than corresponding wild-type control in mice 3-5 weeks old (Fig 8B), suggesting that reducing \( \text{Perk} \) gene dosage in half specifically in the pancreas recapitulates the reduced serum glucose seen in the \( \text{Perk} \) heterozygous mice. Consistent with these observations, mice expressing an extra copy of \( \text{Perk} \) specifically in \( \beta \)-cells and the otherwise wild-type background (\( \text{Perk}^{+/+};\beta\text{Perk} \)) exhibited significantly elevated serum glucose (\( P<0.05 \), Fig 8C) and reduced serum insulin (\( P<0.001 \), Fig 8D). Therefore, the effect of \( \text{Perk} \) gene dosage on insulin and glucose homeostasis is likely to be \( \beta \)-cell specific.

We sought to reduce \( \text{Perk} \) expression in cultured \( \beta \)-cells to confirm the importance of \( \text{Perk} \) gene dosage in \( \beta \)-cells and the difference in insulin synthesis and secretion we observed in \( \text{Perk}^{+/+} \) mice. To accomplish this we modulated \( \text{Perk} \) mRNA in INS1 832/13 \( \beta \)-cells through regulating the expression of a stably integrated \( \text{shPerk} \) transgene under the control of doxycycline (denoted as \( \text{INS1 832/13 shPerk} \) cells). After 24-hour administration of various concentration of doxycycline ranging from 0 to 2 \( \mu \)g/ml, \( \text{Perk} \) mRNA level was modulated within a range of 39.7%–100% of normal (Fig 8E). Maximum knockdown of \( \text{Perk} \) mRNA was achieved by using 2 \( \mu \)g/ml doxycycline. After 24-hour treatment of 2 \( \mu \)g/ml doxycycline, cells exhibited impaired GSIS and significantly elevated \( \text{ERp72} \) expression (Fig 8F and 8G), which were consistent with previous observations in mice or culture cells with total ablation of PERK by other means [14,36]. By examining the dose-response curve, we found that the application of 0.002 \( \mu \)g/ml doxycycline for 24 hours provided a 40% reduction in \( \text{Perk} \) mRNA (\( P<0.001 \), Fig 8E) that mimicked the levels observed in \( \text{Perk}^{+/–} \) mice (Fig 1B). Using this strategy, we found that both glucose stimulated insulin secretion and \( \text{ERp72} \) gene expression were significantly elevated in \( \text{shPerk} \) cells treated with 0.002 \( \mu \)g/ml doxycycline for 24 hours (Fig 8F and 8G), which was consistent with our observations in \( \text{Perk}^{+/–} \) \( \beta \)-cells.

**Discussion**

A complete deficiency of PERK results in the severest form of insulin-dependent diabetes [12,13,19], and therefore we expected that \( \text{Perk} \) heterozygosity would either be recessive with no effect on glucose homeostasis or would be semi-dominant with reduced insulin and elevated blood glucose. Unexpectedly, we found that \( \text{Perk} \) heterozygous mice exhibit an over-dominant phenotype in early postnatal development characterized by elevated insulin and elevated blood glucose. Unexpectedly, we found that \( \text{Perk} \) heterozygous mice exhibit an over-dominant phenotype in early postnatal development characterized by elevated insulin and elevated blood glucose. Unexpectedly, we found that \( \text{Perk} \) heterozygous mice exhibit an over-dominant phenotype in early postnatal development characterized by elevated insulin and elevated blood glucose. Unexpectedly, we found that \( \text{Perk} \) heterozygous mice exhibit an over-dominant phenotype in early postnatal development characterized by elevated insulin and elevated blood glucose.
showed that wild-type *Perk* transgene exclusively targeted to be expressed in β-cells could reverse the diabetes of the *Perk* KO mouse [14,27]. However, when this transgene is present in an otherwise wild-type (*Perk*+/+) background it results in the reduction of serum insulin and the elevation of blood glucose. Thus, circulating insulin and blood glucose levels are negatively and positively correlated, respectively, with *Perk* gene dosage in the pancreatic β-cells. The effect of *Perk* gene dosage on glucose homeostasis is amplified in combination with the dominant Akita insulin mutant, which progressively develops diabetes postnatally.

Lower *Perk* gene dosage slows the progression of diabetes in the Akita mouse whereas overexpression of *Perk* specifically in β-cells hasten it [20]. The stark exception to this rule is when *Perk* gene dosage is equal to zero.

The expression of PERK in the liver has been suggested to play an important role in glucose homeostasis in the first few days of life when gluconeogenesis plays a crucial role in providing glucose to the neonates [15,23]. However, we found that glucose homeostasis was unaffected by genetically deleting the *Perk* gene in the adult liver. Consequently we assert that the effect of *Perk* gene dosage on
insulin and glucose homeostasis is unlikely to be dependent upon liver functions and is only dependent upon the relative expression of Perk in the insulin-secreting β-cells as also supported by direct manipulation of Perk gene dosage therein.

Comparison of serum insulin and glucose levels throughout postnatal development shows a simple inverse relationship (Fig 9A). Given that we found no evidence for differences in peripheral insulin sensitivity before six-months of age, we conclude that Perk genotypic differences in blood glucose are directly determined by the amount of insulin secreted by the pancreatic β-cells. However the underlying reasons for elevated insulin secretion in Perk heterozygous mice change during postnatal development. Initially, as seen at postnatal day 17, total pancreatic insulin is elevated despite a reduced β-cell number indicating that each β-cell has substantially more stored insulin (Fig 9B). Later β-cell proliferation is accelerated in Perk heterozygotes. Although this acceleration is modest and transient, the compounding effect of increased proliferation over three weeks leads to a significant accrual of β-cell number in Perk +/+ mice. The relative large β-cell number in Perk heterozygotes is maintained thereafter. However, as β-cell number increases insulin content per β-cell drops resulting in no genotypic difference in total pancreatic insulin in mice beyond 7 weeks. One constant observed across all ages is an elevation in the amount of insulin secreted per β-cell in Perk heterozygotes. Recently we showed that PERK acutely regulates calcium dynamics and insulin secretion in human and rodent β-cells [25] independently of the eIF2α pathway. These experiments were performed using a PERK inhibitor that allowed us to determine
the immediate effect of PERK on the crucial steps in calcium mobilization and insulin secretion. We concluded that PERK acts to regulate calcium dynamics and insulin secretion independently of its well-known role in phosphorylating the translation initiation factor eIF2α [25]. PERK has also been shown to regulate proinsulin quality control and trafficking in the endoplasmic reticulum [20], which is dependent on the phosphorylation of eIF2α by PERK. In the absence of PERK, proinsulin and ER client proteins eventually accumulate to extremely high levels in the ER and the ER ceases to function. The function of PERK in regulating ER quality control and trafficking is likely to be associated with its phosphorylation of eIF2α, as mutants of the regulatory phosphorylation site of eIF2α results in the same cellular phenotypes in β-cells [23]. These findings support the hypothesis that PERK has multiple functions in the pancreatic β-cells including immediate regulation of calcium dynamics and insulin secretion and long term regulation of the ER chaperones that orchestrate quality control, protein folding, and anterograde trafficking to distal compartments of the secretory pathway. In addition, PERK regulates β-cell proliferation as first demonstrated in Perk KO mice and now shown in Perk heterozygous mice [14]. It is unclear, whether the transient increase in β-cell proliferation in Perk heterozygotes is a primary function of PERK or is in response to changes in other β-cell functions.

The complex behavior of β-cells in Perk heterozygotes over the first few months of postnatal development can be subdivided into direct effects of PERK expression differences and adaptive responses to primary effects. Insulin secretion is likely to be a direct effect of PERK expression changes because it has been shown to be acutely regulated by PERK [25]. The relationship between insulin secretion and PERK expression exhibits a biphasic, inverted U-shaped dose response with half-dosage (Perk+/−) defining the maximum. This unexpected biphasic relationship between PERK expression and insulin secretion can account for the unexpected lower blood glucose levels seen in Perk heterozygotes. A similar inverted dose response relationship was noted between PERK expression and regulation of intermediary metabolism genes in a cell culture system [41]. More mysterious is the transient elevation of β-cell proliferation in Perk heterozygotes, which is unlikely to be due to a normal compensatory response in response to hyperglycemia and an increase demand for insulin similar to that seen in the progression to type 2 diabetes [42]. Previous studies suggested that intracellular Ca2+ positively regulates β-cell proliferation [43–45]. Given the fact that elevated insulin secretion continues to be observed in P30 and P50 Perk+/− mice, the increased β-cell proliferation in P30 and P50 Perk+/− mice might be due PERK-dependent regulation of Ca2+ signaling and insulin secretion. Regardless of the reason, the enhanced β-cell proliferation results in an increase in β-cell mass in Perk−/− mice, which is maintained indefinitely in the mature adult. The decrease in stored insulin that is seen in older adult mice (>2 months old) is strongly correlated with the increased β-cell mass and is therefore likely to be a compensatory response to avoid hyperinsulinemia. Among the five postnatal stages studied, P17 exhibited the largest array of differences including insulin gene transcription, proinsulin synthesis, insulin content, insulin secretion, hypoglycemia, β-cell mass, and changes in the expression of ER chaperones. It is likely that subsequent changes represent adaptive response in order to maintain glucose homeostasis.

Harding and Ron [12] independently generated a Perk KO mouse, which exhibited the same phenotype as our Perk KO strain including diabetes. Harding and Ron reported that older Perk

Figure 9. Developmental summary and model of PERK-dependent regulation of β-cell development and glucose homeostasis. A. Genotypic difference of serum glucose and insulin throughout development. Figure 9A was generated based on the data in Fig 2A and Fig 2C. Serum glucose and insulin are inversely correlated during postnatal development of Perk−/− mice. B. Genotypic difference of total pancreatic insulin content (based on data in Fig 3A), insulin content per β-cell (Fig 3C), β-cell number (Fig 3D), and β-cell proliferation rate (Fig 7B). Initially β-cell number is relatively low in Perk−/−, but β-cell and total pancreatic insulin are high. In response to low β-cell number, β-cell proliferation is accelerated between postnatal 17–50 resulting in increased β-cell number in Perk−/−. However, as β-cell number rises, insulin content per β-cell drops resulting in a balance between cell number and insulin concentration in each cell and a return to equivalent levels of total pancreatic insulin content.
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heterozygous mice (6 months old) exhibited mild glucose intolerance in three different genetic backgrounds and this defect was stable from 6 months to 1 year old [12]. Although our Perk heterozygous mice show the opposite phenotype in terms of glucose tolerance in younger mice, they exhibit mild glucose intolerance at 6 months of age (data not shown). Moreover, the highly significant difference in random fed glucose levels between Perk genotypes observed in younger mice gradually diminishes with age and becomes non-significant. Taken together we speculate that the higher level of circulating insulin that we observed in Perk heterozygous mice during early postnatal development eventually lead to insulin resistance in older mice analogous to the progression of type 2 diabetes from compensation to decompensation [42].

In conclusion, the complex and dynamic regulation of β-cell functions revealed by investigating Perk heterozygotes strongly argues that PERK has important physiological and postnatal developmental functions in β-cells. These functions are not likely to entail the well-known function of PERK to regulate the ER stress response in cultured cells as markers for ER stress were not differentially expressed.

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Author Contributions
Conceived and designed the experiments: RW BCM DRG. Performed the experiments: RW EEM SZ. Analyzed the data: RW DRG. Contributed reagents/materials/analysis tools: DRG. Wrote the paper: RW DRG.

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