

Depletion of homeodomain-interacting protein kinase 3 impairs insulin secretion and glucose tolerance in mice

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Abstract

Aims/hypothesis Insufficient insulin secretion and reduced pancreatic beta cell mass are hallmarks of type 2 diabetes. Here, we focused on a family of serine-threonine kinases known as homeodomain-interacting protein kinases (HIPKs). HIPKs are implicated in the modulation of Wnt signalling, which plays a crucial role in transcriptional activity, and in pancreas development and maintenance. The aim of the present study was to characterise the role of HIPKs in glucose metabolism.

Methods We used RNA interference to characterise the role of HIPKs in regulating insulin secretion and transcription activity. We conducted RT-PCR and western blot analyses to analyse the expression and abundance of HIPK genes and proteins in the islets of high-fat diet-fed mice. Glucose-induced insulin secretion and beta cell proliferation were

measured in islets from *Hipk3*^{-/-} mice, which have impaired glucose tolerance owing to an insulin secretion deficiency. The abundance of pancreatic duodenal homeobox (PDX)-1 and glycogen synthase kinase (GSK)-3β phosphorylation in *Hipk3*^{-/-} islets was determined by immunohistology and western blot analyses.

Results We found that HIPKs regulate insulin secretion and transcription activity. *Hipk3* expression was most significantly increased in the islets of high-fat diet-fed mice. Furthermore, glucose-induced insulin secretion and beta cell proliferation were decreased in the islets of *Hipk3*^{-/-} mice. Levels of PDX1 and GSK-3β phosphorylation were significantly decreased in *Hipk3*^{-/-} islets.

Conclusions/interpretation Depletion of HIPK3 impairs insulin secretion and glucose tolerance. Decreased levels of HIPK3 may play a substantial role in the pathogenesis of type 2 diabetes.

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Keywords Glucose homeostasis · Homeodomain-interacting protein kinase · Insulin secretion

Abbreviations

ChIP Chromatin immunoprecipitation
GSK Glycogen synthase kinase
HIPK Homeodomain-interacting protein kinase
NK Neurokinin
PCNA Proliferating cell nuclear antigen
PDX Pancreatic duodenal homeobox
SF-1 Splicing factor 1
siRNA Small interfering RNA

Introduction

Type 2 diabetes is a complex disease characterised by chronic hyperglycaemia resulting from insulin resistance

and impaired beta cell function [1–3]. Accumulating evidence shows that Wnt signalling modulates beta cell function [4–9]. The Wnts are a family of secreted glycoproteins that influence cell development via autocrine and paracrine mechanisms [10, 11]. Activation of the canonical pathway occurs by binding of Wnt ligands to the frizzled receptor. This binding triggers an intracellular signalling cascade, which leads to serine/threonine phosphorylation, inactivation of glycogen synthase kinase (GSK)-3 β and destabilisation of the destruction complex, preventing GSK3 β phosphorylation of β -catenin. This mechanism enables the accumulation and nuclear translocation of β -catenin. Once inside the nucleus, β -catenin acts in combination with the T cell factor to stimulate transcription of Wnt-responsive genes.

GSK-3 was originally identified as a serine/threonine kinase that inactivates glycogen synthase. GSK3 affects many cellular processes, including transcription, cell cycle regulation and apoptosis. GSK3 β has been shown to affect beta cell mass, proliferation and apoptosis [12–14].

Homeodomain-interacting protein kinases (HIPKs) are implicated in the modulation of Wnt signalling in cultured cells [15], and in mouse [16, 17], *Drosophila* [18, 19] and *Xenopus* embryos [20, 21]. Members of the HIPK family (HIPK1, HIPK2, HIPK3, HIPK4) were originally identified as binding partners of the neurokinin (NK) homeodomain protein [22]. HIPKs have been reported to be essential for coordinated death in early developmental stages and for the regulation of proper cell number in diverse tissue types. Studies have shown that the HIPK family, and in particular its most studied member, HIPK2, interact with, phosphorylate and modulate the function of other homeodomain proteins, as well as other transcription factors, including p53 [23–25] and carboxy-terminal binding protein (CtBP)-1 [26, 27]. HIPK2 has been reported to modulate the transcriptional activity of pancreatic duodenal homeobox (PDX)-1 [28], which plays a crucial role in pancreas development [29–31].

Several studies have revealed important roles of HIPK3 in the regulation of transcription and phosphorylation in cultured cells [32–36]. HIPK3 overproduction has been reported to enhance androgen receptor-dependent transcription in various cell lines [32]. HIPK3 induced Fas-associated death domain (FADD) phosphorylation and inhibited Fas-mediated Jun NH2-terminal kinase activation [33]. HIPK3 also increased splicing factor 1 (SF-1) activity for steroidogenic gene transcription in response to cAMP through phosphorylation of death-associated protein 6 [35]. Interestingly, *HIPK1*, *HIPK2* and *HIPK3* expression in islets from patients with type 2 diabetes is decreased by 32% ($p=0.326$), 30% ($p=0.082$) and 46% ($p=0.064$), respectively, according to data from the Diabetes Genomic Anatomic Project [3], suggesting that HIPK genes play a role in the control of beta cell function. However, the question of whether HIPKs might be involved in the effects of glucose metabolism has not been addressed.

The current study was undertaken to define the mechanisms by which HIPKs regulate glucose metabolism. Here we show that *Hipk* transcripts are expressed in pancreatic beta cells, and modulate insulin secretion and insulin promoter activity. We found that a high-fat diet enhanced *Hipk3* expression. Moreover, our analysis of *Hipk3*^{-/-} mice revealed that they had impaired glucose tolerance due to a deficiency of insulin secretion. We also demonstrated that levels of PDX1 and phosphorylation of GSK3 β were significantly decreased in *Hipk3*^{-/-} islets. Hence, our data provide evidence of a new mechanism by which insulin secretion is regulated.

Methods

RT-PCR Total RNA was isolated from mouse islets and mouse pancreas using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RT-PCR was performed using reverse transcriptase (RevTraAce; Toyobo, Osaka, Japan). The RT-PCR products were amplified using ExTaq polymerase (Takara, Kyoto, Japan). The gene-specific primer pairs were used, as previously described [37].

Quantitative RT-PCR Quantification of the selected genes was performed using TaqMan gene expression assays with a commercially available system (7900HT; Applied Biosystems, Foster City, CA, USA). For each gene, an assay was selected to amplify the region corresponding to the location of the relevant probe. Gene expression analysis was performed using commercially available mouse assays as follows: *Hipk1*: Mm00501689_m1; *Hipk2*: Mm00439329_m1; *Hipk3*: Mm00468880_m1; *Pdx1*: Mm00435565_m1; *Gck*: Mm00439129_m1; *Slc2a2*: Mm00446229_m1; Cyclin D1: Mm00432359_m1; *Hnf4* (also known as *Hnf4a*): m00433964_m1; *Tcf7l2*: Mm00501505_m1; *Irs2*: Mm03038438_m1; *Ins1*: Mm01950294_s1; *Ins2*: Mm00731595_g1; *Hnf1a*: Mm00493434_m1; *Hnf1b*: Mm00447459_m1; *Irs1*: Mm01278327_m1; *Ucp2*: Mm00627599_m1; and *Gapdh*: Mm99999915_g1 (Applied Biosystems). The assays for *Gapdh* and *Pdx1* were performed in parallel as controls. Standard TaqMan cycling conditions were used and all reactions were performed in triplicate. Changes in expression levels in quantitative PCR were calculated as $2^{-\Delta\Delta C_t}$ (cycle threshold) values and presented relative to average changes.

Immunoprecipitation and immunoblotting analysis of mouse islets Polyclonal anti-PDX1, anti-hepatocyte nuclear factor 4 alpha, anti-GSK3 β , anti-phospho-GSK3 β (Ser9), anti- β -catenin and anti- α -tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal anti-IRS2 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Islets were sonicated, using

an ultrasound sonicator, in 300 to 500 μ l ice-cold buffer containing: 0.5% (vol./vol.) Triton X-100, 50 mmol/l TRIS-HCl (pH 8.0), 100 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l Na_3VO_4 , 10 mmol/l NaF and 1 mmol/l phenylmethylsulfonyl fluoride. Supernatant fractions were cleared at 12,000g for 5 min, and incubated with protein A-Sepharose and each specific antibody. Samples were separated by SDS-polyacrylamide gel electrophoresis and immunodetection performed with a kit (ECL; Amersham Bioscience, Arlington Heights, IL, USA). Protein was prepared from more than 100 islets pooled from several mice of identical genotype.

RNA interference and transient transfection Small interfering RNA (siRNA)-Lipofectamine 2000 complexes were prepared using 50 nmol/l siRNA for *Hipk* genes (Stealth Select RNAi; Invitrogen) and control siRNA (Invitrogen). Islets were precultured for 24 h, medium was changed to OptiMEM (Invitrogen) and siRNA-Lipofectamine 2000 complexes with or without DNA-Lipofectamine 2000 complexes were added. After 8 h incubation, the transfection medium was aspirated and replaced, for an additional 48 h, with fresh culture medium containing 2.8 mmol/l glucose.

Luciferase assays Insulin reporter constructs were constructed from rat pancreas cDNA and genomic DNA using Advantage2 Taq (Invitrogen). The luciferase reporter plasmid harbouring the 5'-flanking region of rat *Ins1* exon 1 (−450 to 51) was subcloned into the pGL4 vector (Promega Biosciences, Madison, WI, USA). Islets plated on to a 24 well plate were transfected with 0.5 μ g of each luciferase reporter plasmid and 0.02 μ g Renilla luciferase plasmid with the herpes simplex virus thymidine kinase promoter (pRL-TK) using Lipofectamine 2000. Cells were collected 48 h after transfection and luciferase assays were carried out (Promega).

Chromatin immunoprecipitation assay Assays were performed in mouse islets. A chromatin immunoprecipitation (ChIP) assay kit (Upstate, Lake Placid, NY, USA) was used according to procedures suggested by the manufacturer. After crossing and sonication, cell lysates were immunoprecipitated with the indicated antibodies. The eluted genomic DNA from immunoprecipitates was subjected to PCR amplification. Anti-HIPK1, anti-HIPK2, anti-HIPK3 and anti-PDX1 antibodies, as well as control rabbit IgG (Santa Cruz Biotechnology) were used in ChIP assays. For PCR primer sequences, see electronic supplementary material (ESM) Table 1.

Analysis of *Hipk3*^{−/−} mice Mice deficient for *Hipk3* were generated using the gene trapping method developed by Lexicon Genetics (Woodlands, TX, USA) and supplied by CLEA (Tokyo, Japan). The artificial DNA was designed such that it could be inserted randomly into any gene, preventing the RNA splicing mechanism from working

properly and thereby knocking out the gene's function. The inserted DNA was specifically engineered to disrupt the function of the trapped gene, activate a selectable marker, enable the generation of knockout mice and allow precise identification of the chromosomal insertion site (www.lexpharma.com/research/drug-discovery/gene-knockouts.html, last accessed 8 August 2012). Mice were 8 to 20 weeks of age at the time of the experiment. Male 8-week-old BKS. Cg-*Lepr*^{db}/*Lepr*^{db}/Jcl (*db/db*) mice and BKS. Cg-*m*⁺/*m*⁺/Jcl mice (control) were purchased (CLEA). We used a high-fat diet consisting of 32% fat, 25.5% (wt/wt) protein, 2.9% fibre, 4.0% ash, 29.4% carbohydrates and 6.2% water (CLEA). For all other experiments, the diet consisted of standard chow (CLEA) with the following composition: 25.6% (wt/wt) protein, 3.8% fibre, 6.9% ash, 50.5% carbohydrates, 4% fat and 9.2% water.

Mice were fasted for >16 h before the glucose tolerance test, then loaded with 1.5 mg/g body weight glucose by oral or intraperitoneal administration. Blood samples were collected from the orbital sinus at various time points and glucose measured using an automatic blood glucose meter (Glutest Ace; Sanwa Kagaku, Nagoya, Japan). Insulin and GLP-1 levels were determined using a mouse insulin ELISA kit (Shibayagi, Shibukawa, Japan) and GLP-1 ELISA kit (Wako, Osaka, Japan), respectively. Mice were intraperitoneally challenged with 0.75 mU/g body weight human insulin (Novolin R; Novo Nordisk, Bagsvaerd, Denmark) in an insulin tolerance test. Animal care and the procedures of the experiments were approved by the Animal Care Committee of the University of Tokyo.

Histological and immunohistochemical analysis of mouse islets Female mice ($n=4$) aged 8 to 20 weeks were used for each genotype and 20 sections of islets were evaluated for morphometry. We studied four animals each per genotype and diet. Analysis was based on cell counts ranging from 1,500 to 2,000 per control and transgenic mouse islet samples. Tissues were routinely processed for paraffin embedding, and 4 μ m sections were cut and mounted on silanised slides. Pancreatic sections were stained with anti-insulin, anti-HIPK3 anti-proliferating cell nuclear antigen (PCNA), anti-PDX1 and anti-phospho-GSK3 β antibodies. Non-specific staining was blocked by 2 mg/ml poly-L-lysine pre-absorption [38]. Images of pancreatic tissue and islet beta cells were viewed on the monitor of a computer through a microscope connected to a charge-coupled device camera (Olympus, Tokyo, Japan). The areas of the pancreas, beta cells and non-beta cells were traced manually and analysed with Win ROOF software (Mitani, Fukui, Japan).

Islet isolation and culture Mouse pancreas was incubated for 30 min at 37°C in 2.5 ml KRB buffer containing collagenase

(Sigma-Aldrich, St Louis, MO, USA). The pancreas was dispersed by pipetting and washed twice with KRB buffer. Mouse recombinant Wnt3a was purchased from Wako. Glibenclamide, LiCl and 1-azakenpaullone were purchased from Sigma-Aldrich. Static incubation was performed for 1 h at 37°C with ten islets per tube after 20 min of preincubation. Insulin levels were determined with a mouse insulin ELISA kit (Shibayagi). For the perfusion experiment, ten islets of similar size were maintained in KRB buffer delivered using a peristaltic pump. Perfusion was as follows: 30 min with KRB buffer containing 2.8 mmol/l glucose; 20 min with KRB buffer containing 20 mmol/l glucose; 40 min with KRB buffer containing 2.8 mmol/l glucose; 10 min with KRB buffer containing 2.8 mmol/l KCl; and 30 min with KRB buffer containing 2.8 mmol/l glucose. Perfusate was collected every 5 min. At the end of the perfusion, islets were collected by handpicking and extracted with 0.18 mol/l HCl in 70% ethanol for determination of insulin content.

ATP measurement ATP measurement of islets was performed as previously described with some modifications [39]. After preincubation, 1 ml fresh KRB buffer containing 2.8, 10 or 22 mmol/l glucose was introduced. Incubation was carried out for 1 h at 37°C and stopped by adding trichloroacetic acid. ATP content of the cells was assayed in duplicate by the luminometric method using an ATP bioluminescent assay kit (Sigma).

Two-photon excitation imaging Two-photon excitation imaging of islets was performed as previously described [40]. Ca^{2+} measurements were performed using fura-2 as the Ca^{2+} indicator. Fura-2 post-stimulation fluorescence (F) was normalised to the resting fluorescence (F_0). The increase of Ca^{2+} concentration was calculated as $(F - F_0)/F_0$.

Statistical analysis All data are presented as the mean \pm SEM of independent replicates. All statistical analyses were performed by one-way ANOVA or χ^2 analysis as indicated.

Results

The reduction of *Hipk1*, *Hipk2* and *Hipk3* expression by RNA interference attenuates insulin secretion We assessed the expression of Hipk family genes by RT-PCR using cDNA prepared from isolated mouse islets. Hipk family expression was detected in islets (Fig. 1a). Quantitative RT-PCR using TaqMan gene expression assays revealed that the expression of *Hipk2* and *Hipk3* was two- and 2.5-fold higher, respectively, than that of *Hipk1* (Fig. 1b,c). We estimated whether Hipk genes can also decrease insulin secretion from isolated islets. siRNA to *Hipk1*, *Hipk2* and *Hipk3* suppressed endogenous expression by 45%, 44% and

46%, respectively (Fig. 1d), leading upon stimulation with 20 mmol/l glucose to decreases in insulin secretion by 42%, 44% and 46%, respectively (Fig. 1d). We also found that simultaneous inhibition of *Hipk1* and *Hipk2*, *Hipk2* and *Hipk3*, and *Hipk1* and *Hipk3* inhibited insulin secretion by 52%, 54% and 56%, respectively (Fig. 1e). Simultaneous inhibition of *Hipk1*, *Hipk2* and *Hipk3* inhibited insulin secretion by 78% (Fig. 1e). These observations suggest that the additive inhibition of Hipk genes leads to impaired beta cell function. To evaluate whether Hipk genes also affect insulin promoter transcriptional activity, we conducted luciferase assays using RNA interference approaches. siRNA to *Hipk1*, *Hipk2* and *Hipk3* decreased insulin promoter transcriptional activity in isolated mouse islets by 38%, 44% and 52%, respectively (Fig. 1f). Endogenous insulin transcript levels were assessed by quantitative RT-PCR and correlated with the results obtained in luciferase reporter assays (Fig. 1g). ChIP assay revealed that HIPKs interacted with the insulin promoter (Fig. 1h).

***Hipk* family expression in the islets of mice fed a high-fat diet** mRNA expression of *Hipk1*, *Hipk2*, and *Hipk3* was increased in the extracts of islets isolated from B6 mice fed a high-fat diet for 12 weeks, compared with islets from control chow-fed B6 mice. Increases were by 44%, 78% and 109%, respectively (Fig. 2a). HIPK1, HIPK2 and HIPK3 levels were increased by 41%, 98% and 112%, respectively, in the extracts of islets isolated from B6 mice fed a high-fat diet (Fig. 2b), indicating that HIPK3 accumulation may correlate with glucose tolerance in this environmental model of type 2 diabetes.

***Hipk3* expression is enhanced in the islets of *db/db* mice** We examined *Hipk3* expression in *db/db* mice. *Hipk3* mRNA was increased by 96% and 54%, respectively, in the extracts of islets isolated from 8- and 20-week-old *db/db* mice compared with 8-week-old control mouse islets (Fig. 2c). HIPK3 protein was increased by 94% and 51%, respectively, in the extracts of islets isolated from 8- and 20-week-old *db/db* mice compared with 8-week-old control mouse islets (Fig. 2d). We also performed immunohistochemical analysis, finding that HIPK3 levels were increased in islets from *db/db* mice (Fig. 2e). This indicates that HIPK3 accumulation correlates with glucose tolerance in this genetic model of type 2 diabetes.

***Hipk3*^{-/-} mice had impaired insulin secretion** To elucidate the physiological role of HIPK3 in glucose metabolism, we examined the phenotypes of *Hipk3*^{-/-} mice. *Hipk3*^{-/-} mice showed normal weight gain on a standard and a high-fat diet (Fig. 3a). Their overall appearance at death after 12 weeks on a high-fat diet revealed no difference between *Hipk3*^{-/-} and wild-type mice on either a standard or a high-fat diet. We performed the OGTT on mice fed a standard diet. *Hipk3*^{-/-} mice fed a standard diet had significantly impaired

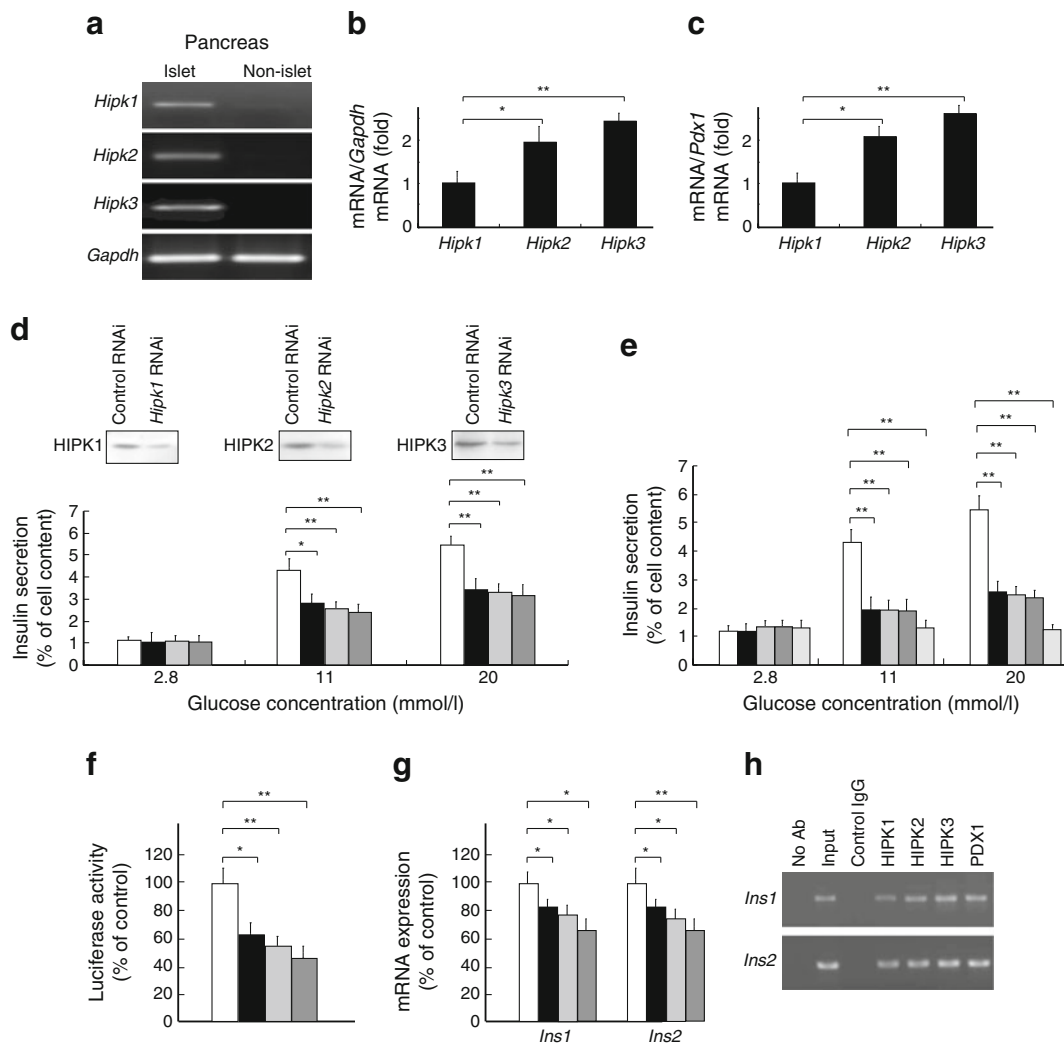


Fig. 1 (a) RT-PCR analysis of *Hipk* genes in islets isolated from a wild-type mouse. Quantitative RT-PCR using TaqMan gene expression assays was performed to validate the relative expression of *Hipk* genes. (b) Assays for *Gapdh* and (c) *Pdx1* were performed in parallel as control. (d) Blots showing *Hipk1*, *Hipk2* and *Hipk3* inactivation by RNA interference (RNAi). siRNA-Lipofectamine 2000 complexes were prepared ($n=4$), and suppression of glucose-stimulated insulin secretion by gene inactivation was quantified. White bars, control; black bars, *Hipk1*; light grey bars, *Hipk2*; dark grey bars, *Hipk3*. (e) Glucose-stimulated insulin secretion was decreased by a combination of *Hipk1*, *Hipk2* and *Hipk3* RNA interference treatment ($n=4$). White bars, control; black bars, *Hipk1*+*Hipk2*; mid-grey bars, *Hipk2*+*Hipk3*; dark grey bars, *Hipk1*+*Hipk3*; light grey bars, *Hipk1*+*Hipk2*+*Hipk3*. (f) Insulin promoter transcriptional activity was suppressed by *Hipk1*, *Hipk2* and *Hipk3* inactivation by RNA interference in mouse isolated islets. Islets were precultured for 24 h, and DNA-Lipofectamine 2000

complexes along with siRNA-Lipofectamine 2000 complexes were added ($n=4$). Isolated islets were transfected with 0.1 μg of the rat insulin I promoter luciferase reporter plasmid pINS (-450 to 51)-pGL4 and by siRNA to *Hipk1*, *Hipk2* and *Hipk3*, and control siRNA. Luciferase activity was measured 48 h after transfection. Key as above (d). (g) Endogenous insulin transcript levels were assessed by quantitative RT-PCR. Key as in (d). (h) The recruitment of HIPKs on the *Ins1* and *Ins2* promoter in mouse islets. ChIP assays were performed in islets using the indicated antibodies (Ab) and primer pairs. As controls, PCR reactions were performed with input DNA (lane 2), DNA immunoprecipitated in the absence of antibody (lane 1). Data represent the average results from three independent ChIP experiments. Data (a–g) are from three independent islet preparations and experiments, with measurements in duplicate, and are presented as mean \pm SEM; * $p<0.05$ and ** $p<0.01$ compared with control siRNA

glucose tolerance (Fig. 3b) and decreased insulin response to glucose (Fig. 3c) compared with wild-type mice. Under standard diet conditions, intraperitoneal glucose tolerance and glucose-dependent insulin secretion of *Hipk3*^{-/-} mice were similar to those of wild-type mice (Fig. 3b). *Hipk3*^{-/-} mice had significantly impaired GLP-1 secretion during an OGTT compared with that of wild-type mice (Fig. 3d).

Hipk3^{-/-} mice fed a high-fat diet for 4 and 12 weeks had significantly impaired glucose tolerance (Fig. 3e, f) and decreased insulin response to glucose (Fig. 3g, h, k) compared with wild-type mice. The glucose-lowering effect of insulin did not differ between *Hipk3*^{-/-} and wild-type mice on a standard or a high-fat diet (Fig. 3i, j, l), supporting the hypothesis that glucose intolerance in *Hipk3*^{-/-} mice was

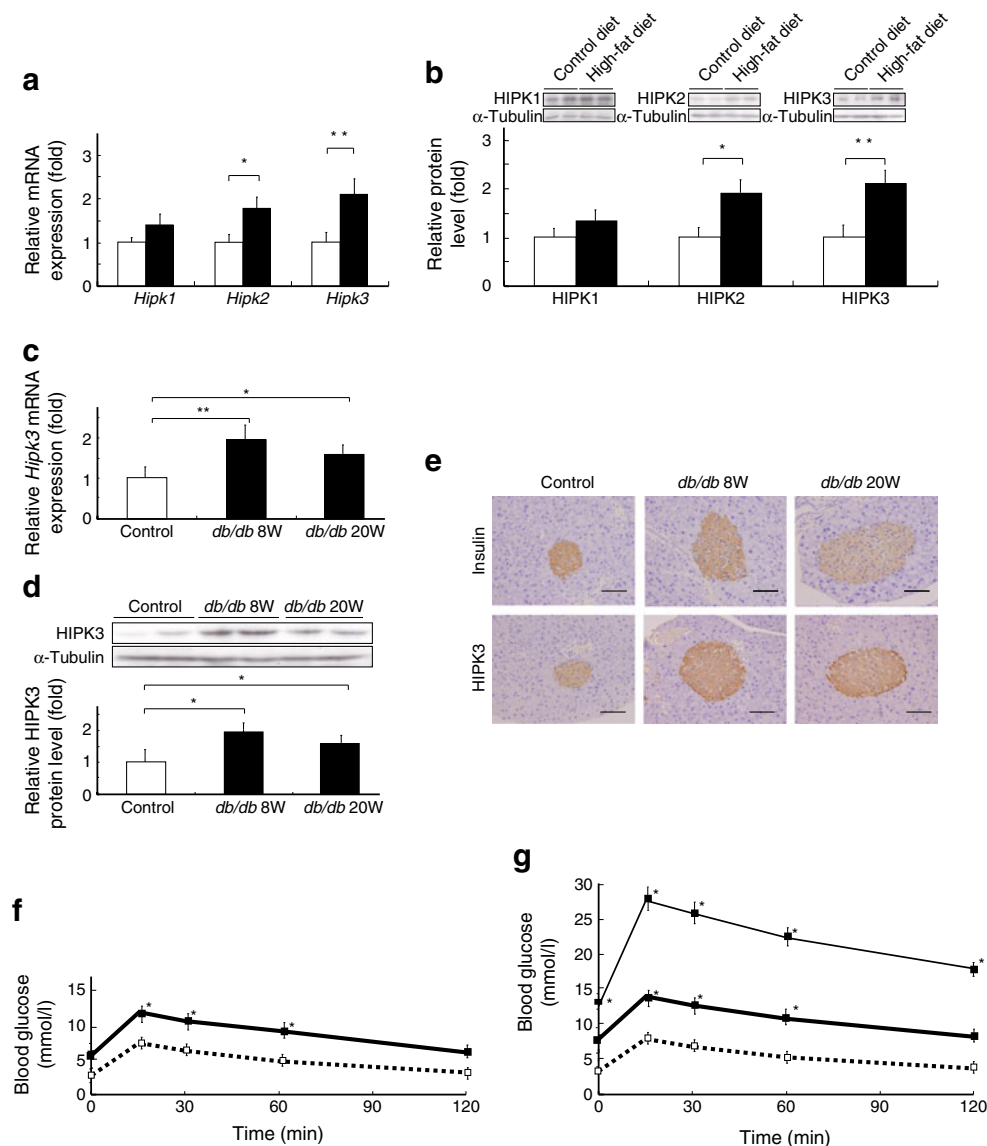


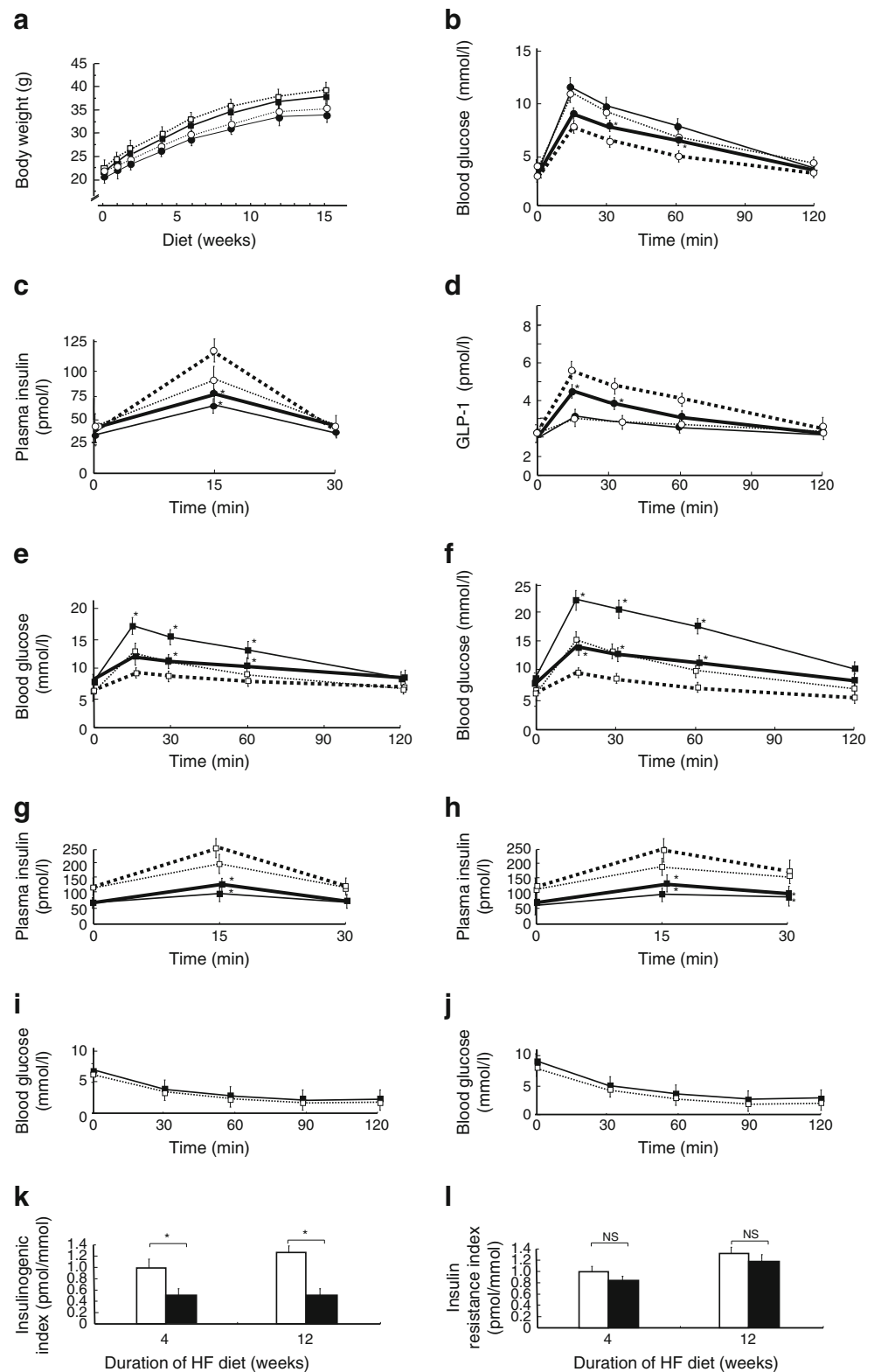
Fig. 2 Changes in *Hipk* genes mRNA expression (**a**) and protein (**b**) abundance in islets from mice fed a high-fat (black bars; white bars, control) diet ($n=4$). Quantitative RT-PCR analysis of *Hipk3* was done in islets isolated from wild-type mice. Protein was prepared from more than 100 islets pooled from several mice. Protein samples from lysates of isolated islets were immunoprecipitated using anti-HIPK1, -HIPK2 and -HIPK3 antibodies, and separated by SDS-PAGE. Western blot analyses were performed with the same antibodies. (**c**) Changes to *Hipk3* mRNA expression and (**d**) protein levels in islets from 8-week-old (8W) and 20-week-old (20W) *db/db* mice ($n=4$). RT-PCR analysis of *Hipk3* was done in islets isolated from wild-type mice.

not the result of major differences in insulin responsiveness to glucose.

Loss of *Hipk3* decreases glucose-induced insulin secretion We next determined glucose-induced insulin secretion in *Hipk3*^{-/-} mice by static incubation of the same numbers of islets. Whereas insulin secretion into the medium by islets from *Hipk3*^{-/-} and wild-type mice was similar at 2.8 mmol/l

glucose, insulin secretion by islets from *Hipk3*^{-/-} mice was significantly decreased at 11 and 20 mmol/l glucose compared with islets from wild-type mice (Fig. 4a). However, the secretory response in *Hipk3*^{-/-} mouse islets was restored by treatment with KCl or glibenclamide (Fig. 4b). To characterise the molecular defect in insulin secretion, we performed pancreas perfusion experiments. In *Hipk3*^{-/-} mice, the insulin secretory response to 20 mmol/l glucose was delayed and decreased

Fig. 3 Impaired glucose homeostasis and insulin secretion in *Hipk3*^{-/-} mice. **(a)** The body weight of *Hipk3*^{-/-} mice on a high-fat (black squares) and control (black circles) diet, and of wild-type mice on high-fat (white squares) and control (white circles) diet was similar. **(b)** Mice were loaded with 1.5 mg/g body weight glucose by oral (OGTT) (bold dotted line, wild-type; bold continuous line, *Hipk3*^{-/-}) or intraperitoneal (IPGTT) (thin dotted line, wild-type; thin continuous line, *Hipk3*^{-/-}) administration. The OGTT revealed impaired glucose homeostasis in *Hipk3*^{-/-} mice ($n=7$). **(c)** The OGTT revealed impaired insulin secretion in *Hipk3*^{-/-} mice. Insulin levels were determined using a mouse insulin ELISA kit. **(d)** Impaired GLP-1 secretion in *Hipk3*^{-/-} mice. **(e)** *Hipk3*^{-/-} mice fed a high-fat diet for 4 or **(f)** 12 weeks had significantly impaired glucose tolerance. Key, as above **(b)**. **(g)** The glucose intolerance observed in *Hipk3*^{-/-} mice fed a high-fat diet for 4 or **(h)** 12 weeks coincided with impaired insulin release. **(i)** The insulin tolerance test showed that wild-type (dotted line) and *Hipk3*^{-/-} (continuous line) mice fed a high-fat diet for 4 or **(j)** 12 weeks had similar rates of glucose clearance. **(k)** Insulino-genic index, defined as the ratio of insulin to glucose at 30 min after an oral glucose load in *Hipk3*^{-/-} (black bars) and wild-type (white bars) mice on a high-fat (HF) diet. **(l)** Values under the glucose curve and under the insulin curve during the OGTT were similar between *Hipk3*^{-/-} and wild-type mice. The insulin resistance index was calculated as AUC of insulin/AUC of glucose in the OGTT. Data represent means \pm SEM; * $p<0.05$ compared with wild-type control mice



(Fig. 4c). The ATP content was significantly decreased in *Hipk3*^{-/-} islets (Fig. 4d). The increase in intracellular calcium concentration in response to glucose was impaired in islets of *Hipk3*^{-/-} mice (Fig. 4e).

Loss of Hipk3 decreases beta cell proliferation and increases apoptosis Although the insulin content of islets from *Hipk3*^{-/-} mice was not decreased at week 4 of a high-fat diet, it was significantly decreased at week 12 of a high-

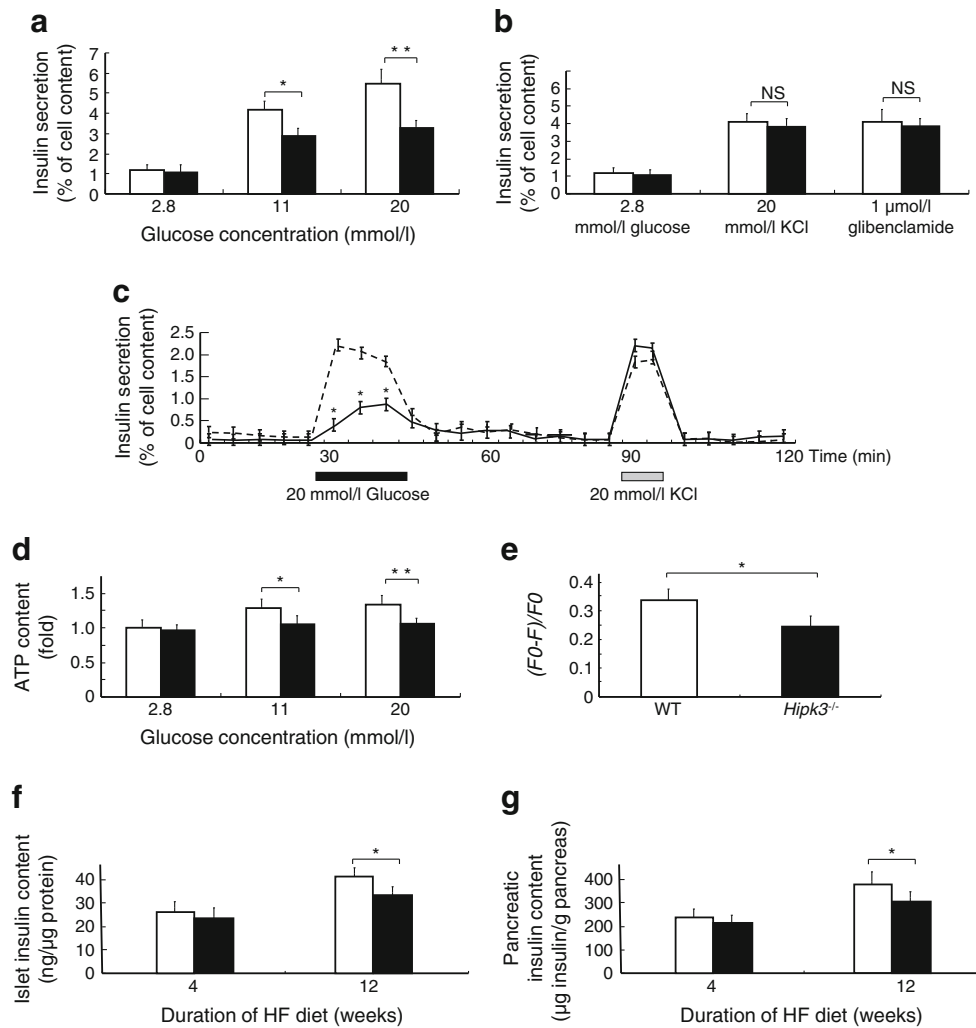


Fig. 4 Characteristics of islets from *Hipk3*^{-/-} mice. **(a)** Insulin secretion by islets from *Hipk3*^{-/-} mice (black bars) fed a high-fat diet for 4 weeks was significantly decreased at 11 and 20 mmol/l glucose vs wild-type mice (white bars) ($n=4$). **(b)** Insulin secretion of *Hipk3*^{-/-} and wild-type islets in the presence of KCl and glibenclamide. **(c)** Islets from *Hipk3*^{-/-} (continuous line) and wild-type (dashed line) mice were perfused with a medium containing 20 mmol/l glucose and 20 mmol/l KCl ($n=4$). **(d)** Changes in ATP content in islets from wild-type and *Hipk3*^{-/-} mice after glucose stimulation for 1 h ($n=4$). **(e)** The increase in calcium

concentration in response to glucose was impaired in *Hipk3*^{-/-} islets ($n=6$). Glucose-stimulated Ca^{2+} influx in single beta cells was measured with the Ca^{2+} indicator fura-2. Fura-2 post-stimulation fluorescence (F) was normalised to resting fluorescence (F_0). **(f)** Changes in beta cell mass during consumption of a high-fat (HF) diet. The insulin content of islets was decreased in *Hipk3*^{-/-} vs wild-type mice at week 12 of an HF diet. **(g)** Insulin content per pancreas was compared between *Hipk3*^{-/-} and wild-type mice ($n=4$). Data represent means \pm SEM; * $p<0.05$ and ** $p<0.01$ compared with wild-type control mice

fat diet (Fig. 4f, g). Similarly, the proportion of islets to total area of dissected pancreas from *Hipk3*^{-/-} mice was not decreased at week 4 of a high-fat diet, but was significantly decreased at week 12 of a high-fat diet (Fig. 5a, b). We estimated beta cell proliferation on the basis of PCNA staining. During high-fat diet conditions, significantly more PCNA-positive cells were observed in the islets of wild-type mice than in *Hipk3*^{-/-} mice (Fig. 5a, c). We estimated islet cell apoptosis on the basis of TUNEL staining. During high-fat diet conditions, significantly more TUNEL-positive cells were observed in the islets of wild-type mice than in *Hipk3*^{-/-} mice (Fig. 5a, d). In summary, HIPK3 has an effect on beta cell secretory function, beta cell proliferation and apoptosis.

Decreased PDX1 and GSK3 β phosphorylation in the absence of Hipk3 To identify the mechanism underlying the loss of glucose-stimulated insulin secretion and beta cell proliferation, we evaluated the mediators of HIPK3-regulated genes relative to islet function. Expression of *Pdx1*, a beta cell-specific transcription factor, was significantly decreased in the islets of *Hipk3*^{-/-} mice fed a high-fat diet for 12 weeks, falling by approximately 50% (Fig. 6a, m, n). Notably, we detected decreases of 34% and 30% in *Gck* and *Slc2a2* mRNA expression in *Hipk3*^{-/-} mice, indicating that loss of HIPK3 negatively modulates the expression of these genes that encode glucose-sensing proteins (Fig. 6b, c). Moreover, we observed significantly reduced mRNA levels of *Hnf4a* and *Tcf7l2* in *Hipk3*^{-/-}

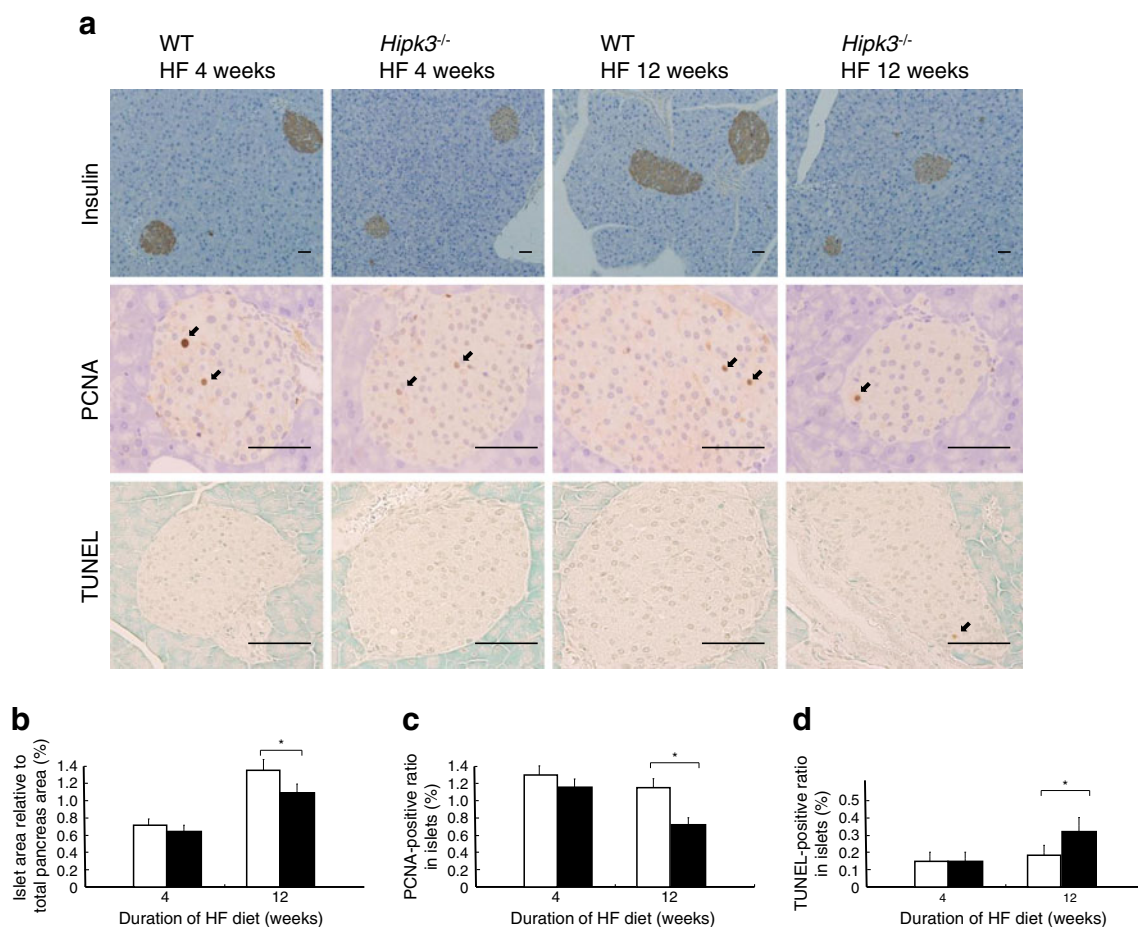


Fig. 5 (a) PCNA immune staining analysis was based on the counting of 1,662 and 1,879 cells, respectively, per islet sample from wild-type (WT) ($n=4$) and *Hipk3*^{-/-} ($n=4$) mice fed a high-fat diet for 4 weeks, and (high-fat diet, 12 weeks) on the counting of 1,674 and 1,518 cells, respectively, per islet sample from wild-type ($n=4$) and *Hipk3*^{-/-} ($n=4$) mice. Tissues were processed for paraffin embedding, and 4 μm sections were cut and mounted on silanised slides. Pancreatic sections were stained with anti-insulin and anti-PCNA antibodies as indicated.

We estimated islet cell apoptosis on the basis of TUNEL staining. Scale bars, 100 μm . (b) The proportion of islet to total area was decreased in *Hipk3*^{-/-} compared with wild-type mice fed a HF diet ($n=4$). (c) Replication rate of beta cells from *Hipk3*^{-/-} and wild-type mice ($n=4$). (d) The apoptosis rate of islet cells from *Hipk3*^{-/-} and wild-type mice ($n=4$). Data represent means \pm SEM; * $p<0.05$ compared with wild-type control mice

islets, suggesting that HIPK3 plays a role in influencing the expression of transcription factors. Additionally, cyclin D1 gene expression (Fig. 6d), GSK3 β phosphorylation (Fig. 6m, o) and β -catenin abundance (Fig. 6m, p) were decreased in the islets of *Hipk3*^{-/-} mice, indicating a role of HIPK3 in Wnt signalling. Histological analysis showed that nuclear PDX1 abundance was significantly decreased in the islets of *Hipk3*^{-/-} mice fed a high-fat diet for 12 weeks (Fig. 6q, r). Histological analysis also showed that GSK3 β phosphorylation was decreased in the islets of *Hipk3*^{-/-} mice (Fig. 6s).

Wnt signalling lessens the decrease in insulin secretion induced by loss of Hipk3 We investigated whether Wnt signalling can also increase glucose-stimulated insulin secretion in *Hipk3*^{-/-} islets. Wnt3a enhanced insulin secretion by 19% in *Hipk3*^{-/-} islets (Fig. 7a) upon 20 mmol/l glucose stimulation. We also found that the GSK3 inhibitor LiCl and

1-azakenpaullone treatment enhanced insulin secretion in *Hipk3*^{-/-} islets by 31% and 28%, respectively, upon 20 mmol/l glucose stimulation (Fig. 7b, c). These observations suggest that impaired Wnt signalling caused by *Hipk3* deficiency leads to impaired beta cell function.

Discussion

It is becoming increasingly clear that the diverse biological consequences of the loss of HIPK activity include the coordinated death of cells in earlier developmental stages and the dysregulation of proper cell number in diverse tissue types. Mice that are deficient for *Hipk1* and *Hipk2* singly have a normal overall appearance, a finding that is probably due to a functional redundancy between HIPK1 and HIPK2 [41]. However, *Hipk1*^{-/-}; *Hipk2*^{-/-} mice are progressively lost in

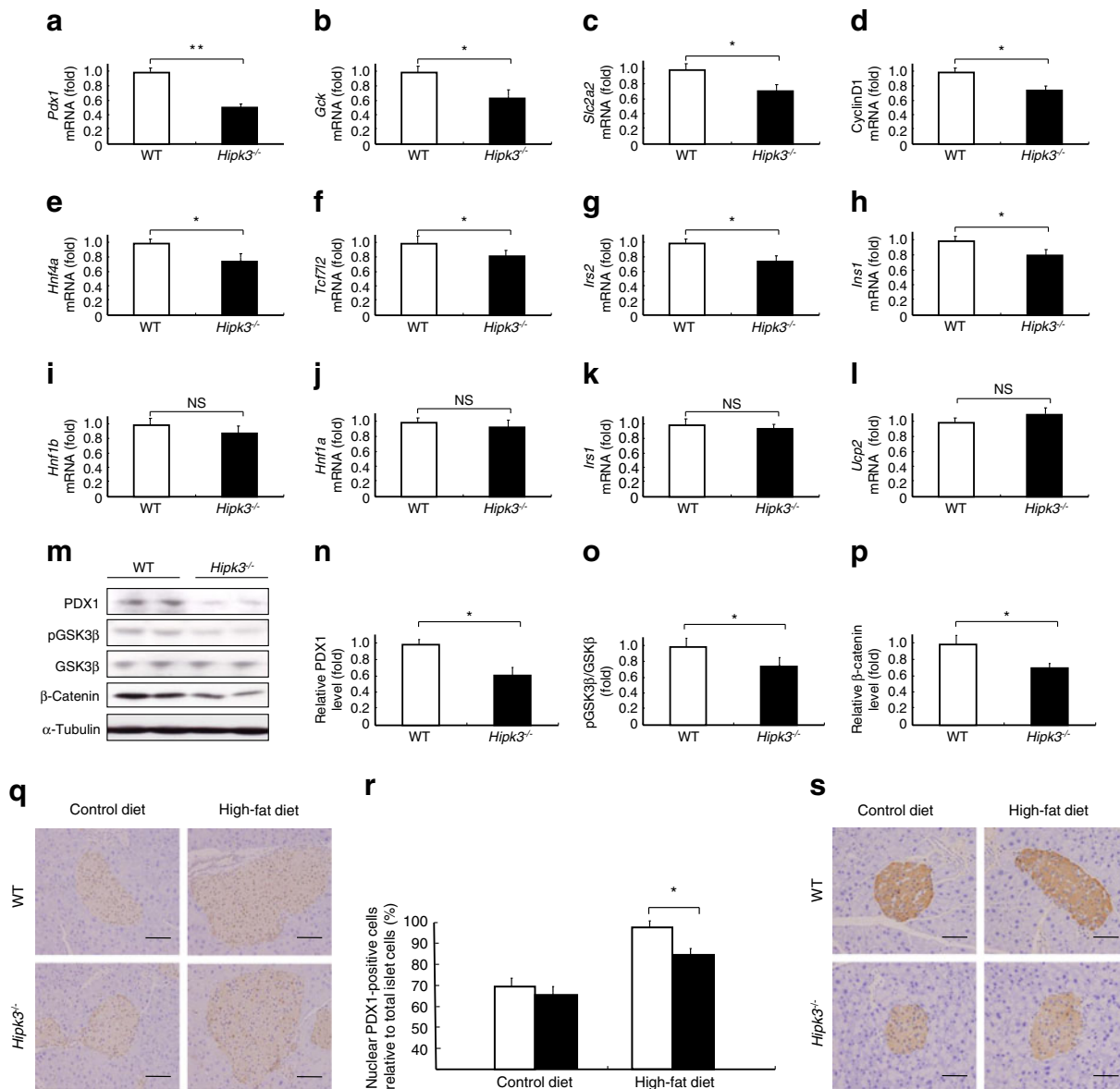


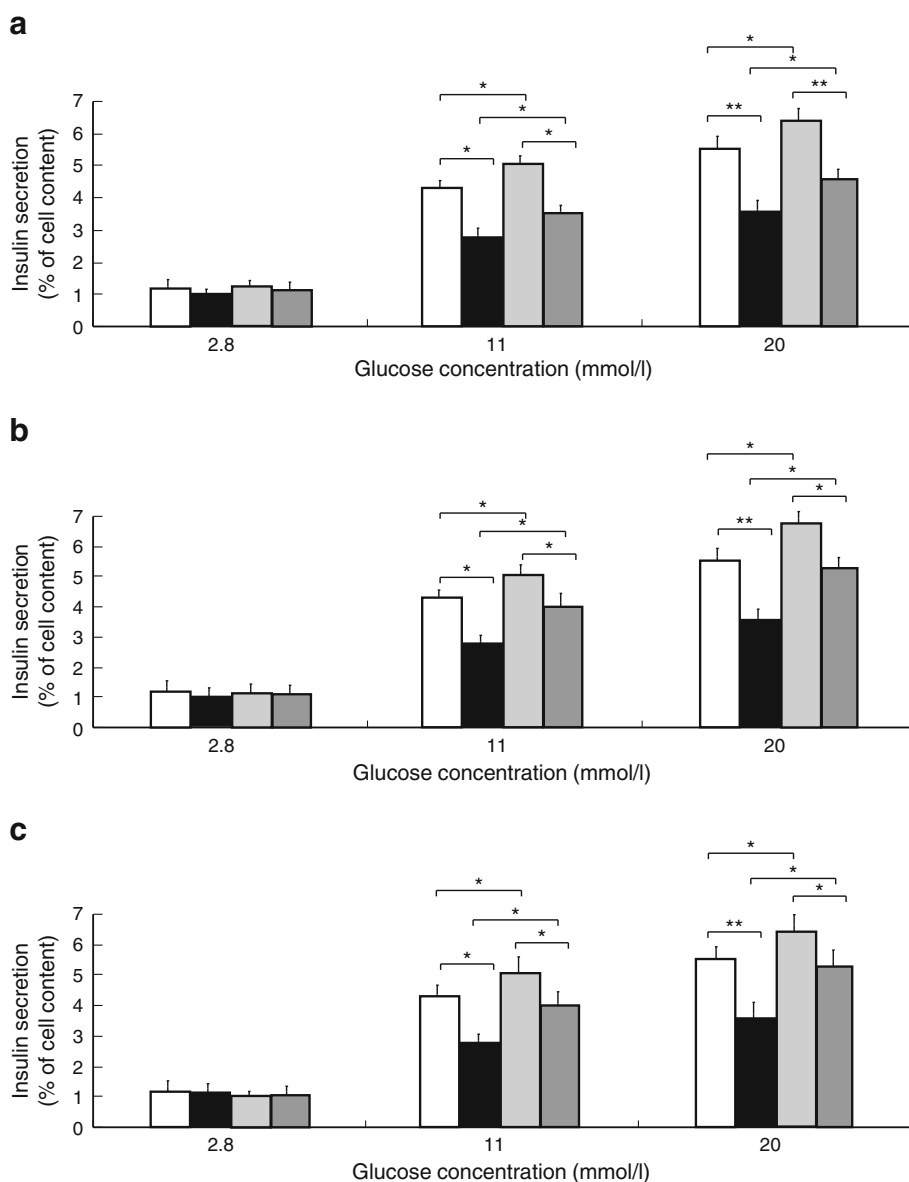
Fig. 6 (a) Quantitative RT-PCR analysis of islet-enriched transcription factors and glucose-sensing proteins *Pdx1*, (b) *Gck*, (c) *Slc2a2*, (d) Cyclin D1, (e) *Hnf4a*, (f) *Tcf7l2*, (g) *Irs2*, (h) *Ins1*, (i) *Hnf1b*, (j) *Hnf1a*, (k) *Irs1* and (l) *Ucp2*. Analysis was in islets isolated from wild-type (WT) and *Hipk3*^{-/-} mice ($n=4$) on a high-fat diet for 12 weeks. Samples were normalised to *Gapdh*. **m** Protein samples from lysates of isolated islets from *Hipk3*^{-/-} mice were separated by SDS-PAGE and western blot analyses performed using the antibodies as labelled ($n=4$). **n** Quantification of relative PDX1, **o** pGSK3 β and **p** β -catenin levels. **q** Pancreatic sections were stained with anti-PDX1 antibody. PDX1 immune staining analysis was based on the counting of 1,845 and 1,683 cells, respectively, per islet sample from wild-type ($n=4$) and *Hipk3*^{-/-} ($n=4$) mice fed a high-fat diet for 12 weeks, and

(control diet) on the counting of 1,545 and 1,609 cells, respectively, per islet sample from wild-type ($n=4$) and *Hipk3*^{-/-} ($n=4$) mice. **r** Ratio of the number of nuclear PDX1-positive cells to the total number of islet cells. **s** Histological analysis of pancreatic islet phosphorylated GSK3 β in wild-type and *Hipk3*^{-/-} mice. Pancreatic sections were stained with anti-phospho-GSK3 β (Ser9) antibodies. Analysis was based on the counting of 1,705 and 1,692 cells, respectively, per islet sample from wild-type ($n=4$) and *Hipk3*^{-/-} ($n=4$) mice fed a high-fat diet for 12 weeks, and (control diet) on the counting of 1,875 and 1,586 cells, respectively, per islet sample from wild-type ($n=4$) and *Hipk3*^{-/-} ($n=4$) mice. Scale bars (**q**, **s**), 100 μ m. Data represent means \pm SEM; * $p<0.05$ and ** $p<0.01$ compared with wild-type control mice

in utero, and exhibit exencephaly and severe defects in haematopoiesis, vasculogenesis and angiogenesis. In our experiments, expression of *Hipk* genes was detected in adult islets, and the HIPK family additively regulated insulin secretion. Our data also indicate that high-fat diet leads most

significantly to *Hipk3* upregulation. The other two *Hipk* genes correlate with glucose tolerance and may play roles in glucose homeostasis. Given the inability of the intact *Hipk3* gene to support early development in *Hipk1*^{-/-}; *Hipk2*^{-/-} mice and the contribution of HIPK3 to impaired

Fig. 7 Wnt3a regulates HIPK3-induced insulin secretion. **(a)** Insulin secretion was decreased by Wnt3a ($n=4$). Insulin secretion by islets from wild-type (white bars; light grey bars, wild-type+Wnt3a) and $Hipk3^{-/-}$ (black bars; dark grey bars, $Hipk3^{-/-}$ +Wnt3a) mice was measured in KRB with a glucose concentration of 2.8, 11 or 20 mmol/l. Static incubation was performed for 1 h on ten islets per tube at 37°C, after preincubation with the basal glucose concentration for 20 min. Insulin levels were determined with a mouse insulin ELISA kit. **(b)** The insulin secretion of isolated islets was decreased by $Hipk3$ deficiency and increased by the GSK3 inhibitor LiCl ($n=4$). Key, as above (a), except: light grey bars, wild-type+LiCl; dark grey bars, $Hipk3^{-/-}$ +LiCl. **(c)** Insulin secretion of isolated islets from $Hipk3^{-/-}$ mice was increased by Wnt signalling activation by the GSK3 inhibitor, 1-azakenpauillone ($n=4$). Key, as above (a), except: light grey bars, wild-type+1-azakenpauillone; dark grey bars, $Hipk3^{-/-}$ +1-azakenpauillone. Data represent means \pm SEM; * $p<0.05$ and ** $p<0.01$



insulin secretion, we anticipate that HIPK3 may serve unique physiological roles especially in diabetes-prone circumstances such as a high-fat diet.

We also analysed $Hipk3^{-/-}$ mice. Major changes in gene phenotype were found with very modest increases in glucose levels, such as impaired glucose tolerance. Changes in islet gene expression can be explained by $Hipk3$ deficiency and glucose toxicity.

HIPK2 positively influences PDX1 levels and transcriptional activity, and directly phosphorylates the C-terminal portion of PDX1 [28]. HIPK2 is a potential kinase for PDX1 Ser-269, with high glucose concentrations decreasing the degree of phosphorylation on PDX1 Ser-269 in beta cells [42]. We showed that the number of PDX1-positive cells was reduced by 20% in $Hipk3$ -knockout mice on a 12 week high-fat diet (Fig. 6r). The insulin content was reduced by

18% in the same conditions (Fig. 4f). Thus, we speculate that decreased PDX1 positivity is related to beta cell mass reduction. Considering the role of PDX1 in beta cells and non-beta cells, HIPK may affect beta cells and non-beta cells, and reduce beta cell mass. Further studies are needed to clarify the mechanisms of beta cell reduction that decreased PDX1 positivity. HIPK3 has been shown to augment transcription of androgen receptor [32], SF-1 [35] and runt-related transcription factor 2 [36]. In this study, we identified HIPK3 as a novel modulator of PDX1 abundance. Several transcription factors converge on regulatory areas within the *Pdx1* promoter [43–45], including NKx2.2, a member of the NK-2 family of homeobox genes [44, 45]. HIPKs possess a homeodomain-interacting domain that is important for enhancing the repressor activities of NK homeodomain transcription factors [22], allowing the

possibility that HIPK3 may affect PDX1 levels via interacting factors that promote PDX1 transcription.

We showed that insulin transcript levels are correlated with the results of luciferase assays. We used rat insulin promoter instead of mouse insulin promoter, and this difference in species may have affected our results. Concerning the effect of HIPK on insulin expression, HIPK may interact with the insulin promoter or act by modulating PDX1 levels. Further studies are needed to elucidate the mechanisms by which HIPK affects insulin expression.

This study also revealed the involvement of the Wnt–GSK3 β – β -catenin cascade in the action of HIPK3. Wnt signalling influences endocrine pancreas development and modulates mature beta cell functions, including insulin secretion, beta cell survival and beta cell proliferation [6–9]. Treatment with Wnt ligands increases glucose-induced insulin secretion in wild-type islets, but not in islets deficient in the Wnt co-receptor, lipoprotein receptor-related protein 5, suggesting a deficit in glucose sensing in islets when canonical Wnt signalling is impaired [46]. β -Catenin is sufficient to induce proliferation markers and to increase beta cell mass in transgenic mice [4]. Adipocyte-derived Wnt signalling molecules induce cyclin D1 transcription, and the proliferation of beta cell lines and primary murine beta cells [5]. Beta cell-specific *Gsk3b* knockout prevents diabetes in *Irs2*-knockout [13] and high-fat diet-fed mice [14] through expanded beta cell mass with increased beta cell proliferation. In line with these reports, we have shown that beta cell proliferation is decreased in *Hipk3*^{-/-} mouse islets upon feeding a high-fat diet. In contrast to our findings, pancreas-specific depletion of β -catenin has been shown to lead to acinar cell hypoplasia, but not to change the mass and function of islets [47]. This discrepancy might be explained by β -catenin-independent signal transduction downstream of HIPK3.

Previous reports indicate that β -catenin and transcription factor 7 like 2, T cell specific, HMG box regulate proglucagon gene expression and GLP-1 synthesis in enteroendocrine cells [48, 49]. In this study, we obtained evidence that HIPK3 deficiency lowered GLP-1 concentrations and impaired glucose tolerance. It can thus be speculated that HIPK3 activates the Wnt signalling pathway for GLP-1 synthesis and secretion. Furthermore, HIPK3 may alter the response of intestinal K cells to known secretagogues, including nutrients, fatty acids and related molecules. These hypotheses deserve further examination.

In conclusion, HIPK3 has a role in insulin secretion, affecting the levels of pancreatic PDX1 and GSK3 β phosphorylation. Thus, targeting HIPK3 with novel agonists could lead to new therapeutic strategies for preserving beta cell function. The present results provide new insight into the pathogenesis of type 2 diabetes.

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Contribution statement NS, KH and TK planned and designed the study. NS, KH, KU and TK conceived and designed the experiments, and wrote the manuscript. HF and MH contributed to the conception and design, the analysis and interpretation of the data, and drafting the article. NT carried out the two-photon excitation imaging and contributed to drafting the article. IT, MO, HA, MN, NK and TY provided advice, and contributed to the conception and interpretation of the data and drafting the article. All authors approved the final version.

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