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PIWI-interacting RNAs as novel regulators of pancreatic beta-cell function

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ABSTRACT

Aims/hypothesis: piRNAs are small non-coding RNAs that interact with PIWI proteins and guide them to silence transposable elements. They are abundantly expressed in germline cells and play key roles in spermatogenesis**.** There is mounting evidence indicating that piRNAs are also present in somatic cells where they may accomplish additional regulatory tasks**.** The aim of this study was to identify the piRNAs expressed in pancreatic islets and to determine whether they are involved in the control of beta-cell activities.

Methods: piRNA profiling of rat pancreatic islets was performed by microarray. The functions of piRNAs was investigated by silencing the two main *Piwi* genes or by modulating the level of selected piRNAs in islet cells.

Results: We detected about 18'000 piRNAs in rat pancreatic islets, many of which were differentially expressed throughout islet postnatal development. Moreover, we identified changes in the level of several piRNAs in the islets of Goto-Kakizaki rats, a well-established model of Type 2 diabetes. Silencing of *Piwil2* or *Piwil4* genes in adult rat islets caused a reduction in the level of several piRNAs and resulted in defective insulin secretion and in increased resistance of the cells to cytokine-induced cell death. Furthermore, overexpression in the islets of control animals of two piRNAs that are up-regulated in diabetic rats, led to a selective defect in glucose-induced insulin release.

Conclusions/interpretation: Our results provide evidence for a role of PIWI proteins and their associated piRNAs in the control of beta-cell functions and suggest a possible involvement in the development of Type 2 diabetes.

Keywords

Diabetes, Insulin secretion, Pancreatic islets, piRNAs, *Piwil* genes

Data have been deposited in Gene Expression Omnibus repository under the accession number GSE93792

The reviewers can access to the data via the following link:

https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ojklueugdzehpkv&acc=GSE93792

Abbreviations

PIWI : P-element induced Wimpy testis

Piwil : Piwi-like gene

piRNA: PIWI-interacting RNA

qRT-PCR: Quantitative Reverse Transcription real-time PCR

GK: Goto-Kakizaki

INTRODUCTION

PIWI-interacting RNAs (piRNAs) are small non-coding RNAs that are very abundant in animal gonads. They were named because of their ability to associate with Argonaute proteins of the PIWI subfamily [1-4]. piRNAs share common biochemical features, including the phosphorylation of the 5'end and the 2' O-methylation of the 3'end. Mature piRNAs are processed through a maturation pathway involving the cleavage by PIWI proteins of long single-stranded RNAs transcribed from 'piRNA clusters' present in both intra- and inter-genic regions of the genome [5, 6].

piRNAs are believed to maintain genome integrity by guiding PIWI proteins to repress transposon activity, to regulate the assembly of the telomere protection complex and to be involved in RNA silencing and in the epigenetic control of gene expression [5, 7-9]. Until recently, the piRNA pathway was perceived as germline-specific but piRNAs have also been detected in stem cells and other cell types, suggesting that these small non-coding RNAs may accomplish additional tasks [10-12]. Despite the poorly understood role of piRNAs in somatic cells, there is rising evidence indicating that these non-coding RNAs control gene expression by acting at genomic but also at transcriptional and post-transcriptional levels [13-17]. Indeed, recent studies have highlighted the involvement of the PIWI-piRNA pathway in both physiological and pathological processes, including development [18], memory [19], liver regeneration [20] and cancer [21-26]. However, nothing is known about their role in metabolic disease such as diabetes.

Pancreatic islets play key roles in the regulation of metabolism and energy homeostasis by secreting hormones in response to changes in the nutritional status. Pancreatic beta-cell dysfunction and loss are critical determinants for type 2 diabetes development. There is strong evidence indicating that altered expression of both protein-coding and non-coding genes is associated with beta-cell dysfunction under prediabetes and diabetes conditions [27]. Several studies have implicated miRNAs and lncRNAs as key players in diabetes [28-30]. So far, no information is available about the presence of PIWI-piRNAs complexes in beta-cells and their possible involvement in islet physiology and in diabetes development. The goal of this study was to analyze the piRNA expression patterns of rat pancreatic islets under both physiologic and pathological conditions and to assess their contribution to the maintenance of beta-cell function.

METHODS

Animals

Male Sprague-Dawley and Wistar rats and pregnant Sprague-Dawley rats, were obtained from Janvier Laboratories (Le Genest-Saint-Isle, France) and were housed on a 12-hour light, 12-hour dark cycle in climate-controlled and pathogen-free facilities. The detailed sources of Goto-Kakizaki rats have been described previously [31]. All animal procedures were performed in accordance with the National Institutes of Health guidelines and were approved by the Swiss Research Councils and Veterinary Offices or by the local ethics committee in Malmö.

Islet isolation, organ collection and cell culture

Rat pancreatic islets were isolated by collagenase digestion (Roche,), followed by purification on a Histopaque density gradient (Sigma-Aldrich, St Louis, MO, USA) [32] and final cleaning by hand-picking. The islets were cultured in RPMI1640 Glutamax medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% FCS (Gibco), 100 μg/mL streptomycin, 100 IU/mL penicillin , 1 mmol/l sodium pyruvate and 10 mmol/l HEPES, pH 7.4. Human islets obtained from the Cell Isolation and Transplantation Center (University of Geneva, Switzerland) were cultured in CMRL1066 medium (Invitrogen) supplemented with 10% FCS, 100 μg/mL streptomycin, 100 IU/ml penicillin, 2 mmol/l L-glutamine, and 250 μmol/l HEPES. The use of human islets was approved by the Geneva local ethical committee. Dissociated islet cells were obtained by digestion with 5 mg/ml of trypsin (Gibco). Adipose tissue, brain, liver and skeletal muscle were collected from Wistar rats aged of 12 weeks.

Fluorescence Activated Cell Sorting of islet cells

Dissociated islet cells from newborn and adult rats were sorted by Fluorescence Activated Cell Sorting (FACS) based on beta-cell autofluorescence [33]. Immunocytochemistry analysis using anti-insulin antibodies (Dako: #A0564) revealed that 94±1% of the cells in the purified fraction were insulin-positive.

Cell transfection

Dissociated islet cells were transfected with pre-designed siRNAs (Life technologies), using as control a siRNA directed against GFP (Eurogentec, Seraing, Belgium). Synthetic RNA oligonucleotides with a 5' phosphorylation and 3' 2-O-methylation (Integrated DNA technologies) were used as piRNA mimics. Transfection was carried out using Lipofectamine RNAiMax (Invitrogen). To overexpress the piRNAs we used following sequences: (DQ732700): 5'UAUGAAGAAUGACUUGGGGUACAUGACC-3'; (DQ746748): 5'-ACUGGAAACGGAAAACUCAGAGCGCCC-3'. Control oligonucleotide: 5'-GUGUAACACGUCUAUACGCCCA-3'. Functional assays were performed 48h after transfection.

Insulin secretion

Islet cells were preincubated in KREBS buffer containing 25mM HEPES, pH 7.4, 0.1% BSA (Sigma-Aldrich) and 2 mmol/l glucose for 30 minutes at 37°C. The medium was then replaced by KREBS buffer containing 0.1% BSA supplemented with 2 mmol/l glucose, 20 mmol/l glucose or 2 mmol/l glucose and 35mmol/l KCl. After 45 min, the supernatants were collected and centrifuged at 1200xg for 5min to remove cell debris. Total cellular insulin contents were recovered in EtOH acid (75% EtOH, 0.55% HCl) and protein contents were collected on ice in a lysis buffer containing 50 mmol/l Tris-HCl, pH 7.5, 5 mM EDTA, 0.5 % Triton-X-100 and protease inhibitors (Roche, Rotkreuz, Switzerland). The amount of insulin in the samples was determined by ELISA (Mercodia, Uppsala, Sweden). Protein content was evaluated using the Bradford protein Assay (Bio-Rad, Reinach, Switzerland).

Proliferation assay

Dissociated islet cells were cultured on poly-L-lysine–coated glass coverslips and treated, or not, for 48h with 500ng/ml Prolactin (Sigma-Aldrich) to stimulate proliferation. The cells were fixed in 4% PFA and incubated with PBS supplemented with 0.5% saponin (Sigma-Aldrich) for 20 min. The coverslips were incubated for 30 min in PBS supplemented with 0.5% saponin and 1% BSA and exposed for 1h to the following antibodies: 1:1'500 rabbit anti-Ki67 (Abcam, Cambridge, UK); 1:100 guinea pig anti-insulin (Millipore, Zug, Switzerland). They were then washed and incubated for 1h with goat anti-rabbit Alexa Fluor 488 or goat anti–guinea pig Alexa Fluor 594 diluted at 1:400 (Invitrogen). Finally, the coverslips were incubated for 10 min with

Hoechst 33342 (Invitrogen) and mounted on microscope glass slides. The cells were visualized with a Zeiss Axiovision fluorescence microscope. At least 600 cells were analyzed for each condition. The total number of cells was assessed using the ImageJ software, while Ki67-positive cells were counted manually.

Cell death assessment

Cell death was triggered by exposing the cells to pro-inflammatory cytokines (30 ng/ml IFNγ, 10 ng/ml TNFα and 0.1 ng/ml IL-1β) for 24 h. The cells were incubated for 5 min at 37°C with Hoechst 33342 (Invitrogen). About 400 cells per condition were analyzed by fluorescence microscopy (Axiovert 25; Zeiss, Feldbach, Switzerland) to score the fraction of them displaying pycnotic nuclei.

RNA extraction and measurement

Total RNA from islets or from other rat organs was extracted using the miRNeasy kit (Qiagen, Basel, Switzerland). For microarray analysis and qRT-PCR, pancreatic islets from two to five P10 pups (without sex distinction) were pooled while islets from adult male rats were extracted individually. piRNA levels were measured using specific custom primers and the miScript Plant RT kit (Qiagen). Measurement of mRNAs levels was performed by qPCR (Bio-Rad, Sso Advanced Universal SYBR Green Supermix) using custom primers (Microsynth, Balgach, Switzerland) (see [ESM] Methods). All PCR products where validated by sequencing (Microsynth). piRNA expression was normalised to U6, while mRNA expression was normalised to α-tubulin mRNA using the ΔCt method.

piRNA profiling

Global piRNA profiling was carried out using a Rat piRNA Array (Arraystar, Rockville MD). Rat piRNA sequences from the NCBI database were mapped to the RN4 genome using UCSC Blat. Probes for about 40’000 piRNAs were spotted on an Agilent array platform. Samples were labeled using a RNA ligase method and were hybridized onto the piRNA Array in Agilent’s SureHyb Hybridization Chambers. Quantile normalization and data processing were performed using the GeneSpring GX v11.5.1 software (Agilent Technologies, Santa Clara, CA, USA).

Differentially expressed piRNAs with statistical significance between the two groups were identified through Volcano Plot filtering. Hierarchical Clustering was performed to show the distinguishable piRNA expression pattern among samples.

Prediction of piRNA target genes

The putative binding sites of DQ732700 and DQ746748 present in the 3'UTR sequences of all the genes expressed in rat islets [34] were identified with the PITA algorithm using default parameters [35]. For each piRNA, the top 300 putative targets based on the PITA score were annotated with their gene ontology (GO) using biomaRt [36]. Pathway enrichment analysis was performed using DAVID with KEGG and Reactome pathway annotations.

Statistical analysis

Statistical differences were tested using Student's *t* test or, for multiple comparisons, one-way ANOVA followed by a post hoc Dunnett's test, with a discriminating *p*-value of 0.05 (GraphPad Software, San Diego, CA). Differences in piRNAs expression between purified beta-cells of p10 and adult rats were assessed using 1-sample *t*-test, with a discriminating *p*-value of 0.05 using Statistical Package for Social Sciences (IBM SPSS version 23).

RESULTS

The PIWI/piRNA pathway is active in rat pancreatic islets

There is mounting evidence suggesting that, in addition to their well-established role in germinal cells, piRNAs may contribute also to the regulation of somatic cell activities [11]. To investigate the potential involvement of piRNAs in the control of pancreatic beta-cell functions, we first assessed whether insulin-secreting cells express some of the key components of the piRNA pathway. Although to a much lower level compared to rat testis (Fig.1a), qRT-PCR analysis revealed the expression in rat pancreatic islets of the *PIWI-like* genes *Piwil2* and *Piwil4* but not *Piwil1* (Fig.1b). The expression of these two *PIWI-like* genes was confirmed in FACS-sorted beta-cells from adult (three-month old) (Fig.1c) and newborn (10-days old) rats (ESM Fig.1). Similar findings were also obtained with human pancreatic islets that were found to express the human orthologues of these PIWI genes, *Hili* and *Hiwi2* (Fig.1d). The expression of *PIWI-like* genes in islets was comparable to the level measured in other somatic tissues such as the adipose tissue, brain, liver and skeletal muscle (ESM Fig.1). Beside *Piwil2* and *Piwil4*, qRT-PCR analysis unveiled also the presence in rat pancreatic islets of the mRNAs of several other genes involved in piRNA biogenesis [5, 6, 37], including *Henmt1*, *Mael*, *Ddx4*, *Pld6* and *Prmt5* which are main players in the pathway as well as some of the TUDOR domain-containing genes [38] *Tdrd1*, *Tdrd2* and *Tdrd6* (Fig.1e,f).

The presence of several components of the piRNA pathway prompted us to assess whether pancreatic islets express a specific pool of piRNAs. Indeed, analysis of islet RNA isolated from adult and newborn rats using a dedicated microarray, permitted to detect the presence of 18’540 of the 40’000 tested piRNAs (GSE93790). Hierarchical clustering summarizes the top differentially regulated piRNAs (Fig.2a).

We then assessed whether islet piRNA expression is modulated in response to physiological or pathological conditions. Newborn beta-cells display major functional differences compared to fully mature beta-cells. In fact, neonatal beta-cells show a much higher proliferation rate compared to adult beta-cells [34]. Moreover, newborn beta-cells are unable to secrete insulin in response to glucose and acquire this property only after undergoing a postnatal maturation process that involves a major rearrangement in the gene expression profile [34]. We observed that the functional maturation of beta-cells is associated with changes in the level of numerous

piRNAs. Indeed, by comparing the levels in newborn (10-days old) and adult (three-month old) rat islets by microarray, we found that the expression of 735 piRNAs is reduced, while that of 1056 piRNAs is increased upon functional beta-cell maturation (nominal p-value ≤ 0.05, fold change ≥ 2) (GSE93790). The observed changes in the level of selected piRNAs were confirmed by qRT-PCR in whole islets (Fig.2B) and in highly purified beta-cell fractions (Fig.2c). These findings suggest that piRNAs may possibly contribute to the functional maturation of beta-cells.

We next assessed whether altered piRNA expression can contribute to beta-cell dysfunction observed under diabetic conditions. For this purpose, we compared the piRNA expression profile of the islets of Wistar and Goto-Kakizaki (GK) rats, a well-established model of non-obese Type 2 diabetes characterized by impaired glucose-stimulated insulin secretion [31, 39]. By microarray, we identified 347 piRNAs differentially expressed (p-value ≤ 0.05, fold change ≥ 2) in the islets of diabetic GK rats (128 up-regulated and 219 down-regulated) (GSE93791). Hierarchical clustering summarizes the top differentially regulated piRNAs (Fig.3a). The changes in the level of three of these piRNAs was confirmed by qRT-PCR (Fig.3b). Thus, our results indicate that the piRNA expression profile is modified under diabetic conditions, potentially contributing to the development of the disease.

PIWI/piRNAs impact on beta-cell functions

We next investigated whether global changes in piRNA activity impact on beta-cell functions. For this purpose, we used RNA interference to silence *Piwil2* and *Piwil4* genes in pancreatic islet cells. si*Piwil2* and si*Piwil4* reduced the expression of their target mRNAs by about 80 and 50%, respectively (Fig.4a-b). Silencing of *Piwil2* and *Piwil4* genes resulted in a reduction in the level of several piRNAs (Fig.4c). To investigate if the impaired piRNA expression affects the activities of beta-cells, we measured insulin secretion in response to glucose or to depolarizing KCl concentrations upon silencing of *Piwil2* or *Piwil4* in islet cells. We found that knockdown of *Piwil2* or *Piwil4* does not affect insulin content (Fig.5a,c). However, in the absence of *Piwil2* or *Piwil4*, both glucose- and KCl-induced insulin secretion were significantly decreased (Fig.5b,d), suggesting a functional impairment in the secretory machinery that could also involve a defect in glucose metabolism. We then tested cell survival and proliferation. This revealed that upon silencing of *Piwil2* or *Piwil4,* beta-cells become more resistant to cytokine-induced cell death

(Fig.5e). In contrast, beta-cell proliferation both in the presence or absence of the mitogenic hormone prolactin was not significantly affected (Fig.5f).

The observed changes in the expression of piRNAs occurring during the acquisition of the mature beta-cell phenotype and in diabetic conditions, suggest that deregulation of specific piRNAs may potentially contribute to beta-cell dysfunction under disease states. To test this hypothesis, we overexpressed DQ732700 and DQ746748 piRNAs in islet cells of normoglycemic Wistar rats, in order to mimic the increase observed in the islets of diabetic GK rats, and assessed the impact on insulin secretion. We found that overexpression of DQ732700 or DQ746748 does not affect cellular insulin content (Fig.6a,c). However, the increase in the level of these two piRNAs resulted in a decrease in insulin release in the presence of elevated glucose concentrations (Fig.6b). In contrast, insulin secretion in response to depolarizing concentrations of KCl was not affected (Fig.6d). Since Type 2 diabetes is in some cases associated with beta-cell apoptosis, we also assessed whether the overexpression of these two piRNAs affects the survival of the insulin-secreting cells. As shown in Fig.6e, the increase of these two piRNAs did not modify the survival of the cells both in the presence or absence of a mix of pro-inflammatory cytokines including IL-1β, TNFα and IFNγ.

piRNAs have been proposed to exert a translational repression through mechanisms similar to those of miRNAs [14]. Thus, we used a computational approach to search for the potential targets of DQ732700 and DQ746748. Interestingly, the putative targets of these piRNAs were significantly enriched for genes involved in insulin secretion and insulin action (GSE93792; ESM Tables 1-2).

DISCUSSION

Type 2 diabetes is characterized by a diminished sensitivity of insulin target tissues and defective insulin secretion from beta-cells. The mechanisms underlying these phenomena are not fully understood, but appear to involve alterations in the expression of mRNAs and non-coding RNAs [28-30]. piRNAs constitute an abundant class of non-coding RNAs and contribute to genome stability. In germ cells, they interact with PIWI proteins during early embryogenesis enabling the silencing of transposable elements in the genome [5]. In addition to their well-established role in the germline, there is emerging evidence for an involvement of these small RNAs in the regulation of gene expression in somatic cells [10-12, 40]. However, the presence of piRNAs in beta-cells and their possible contribution to the regulation of gene expression have so far not been explored. In this study, we used a microarray approach to obtain a comprehensive picture of the piRNAs expressed in rat pancreatic islets and to evaluate the modifications taking place during the acquisition of a fully mature beta-cell phenotype. This led to the identification of a large number of piRNAs, many of which display changes in their level occurring in association with the functional maturation of beta-cells. These findings suggest that piRNAs may potentially contribute to the development of pancreatic beta-cells. Additional studies will be needed to delineate the precise role of piRNAs in the acquisition of specific properties of beta-cells, such as the capacity to secrete insulin in response to glucose.

PIWI proteins are essential for the biogenesis and the activity of piRNAs and have been suggested to be involved in the transcriptional, post-transcriptional and epigenetic regulation of gene expression [13-17, 40, 41]. We found that rat and human pancreatic islet cells express two *PIWI* genes. These observations were confirmed in highly purified beta-cell fractions, rendering unlikely the possibility that the expression of these genes is confined exclusively in other cells present in the islets. Interestingly, down-regulation of *Piwil2* and *Piwil4* mRNAs in rat islets resulted in a significant decrease in insulin secretion upon glucose or KCl stimulation, suggesting that the activity of these PIWI proteins is important to preserve the secretory capacity of beta-cells. The silencing of *Piwil2* and *Piwil4* was associated with a decrease in the level of several piRNAs, suggesting that the effect may be linked to changes in the biogenesis, the stability and/or the activity of these small non-coding RNAs.

The involvement of piRNAs in pathological processes is starting to emerge. Indeed, the levels of some of these small RNAs have been associated with different clinical conditions and have been proposed as prognostic markers for different types of cancer [42]. However, the understanding of the contribution of these non-coding RNAs to diseases is still rudimentary and necessitates further exploration. *Piwil* mRNA levels have been reported to be deregulated in cancerous tissues and to be correlated with clinico-pathological features of the tumors [43-45]. In certain human cancers, *Piwil2* has been reported to affect the survival of the cells by interacting with signal transducer and activator of transcription 3 and by regulating the p53 signaling pathway [46]. Moreover, *Piwil4* was shown to promote cell proliferation and to inhibit apoptosis in human cervical cancer tissues by down-regulating the p14ARF/p53 pathway [47]. A decrease in the level of these *Piwil* genes was also found to be associated with tumor progression and with a reduced survival of renal carcinoma patients [43]. Thus, the role of *Piwil* genes may vary according to the cellular context. In our study, we observed that down-regulation of *Piwil2* and *Piwil4* mRNAs in beta-cells has a protective effect against cytokine-induced cell death and has no significant impact on proliferation. Further investigations will be needed to precisely delineate the signaling pathways through which *Piwil2* and *Piwil4* affect the survival of beta-cells.

In this study, we identified several piRNAs that are differentially expressed in the islets of diabetic GK rats, a Wistar substrain obtained by selective breeding of a colony displaying high blood glucose levels. The genetic determinants responsible for the development of diabetes in GK rats are not yet fully established. Thus, the observed changes in piRNA expression may be due either to genetic differences between the GK and Wistar substrains or be the consequence of chronic hyperglycemia. To investigate the potential role of these piRNAs in beta-cell dysfunction and in Type 2 diabetes development, we selected two piRNAs that are up-regulated in the islets of GK rats and we overexpressed them in the islets of control Wistar rats. Interestingly, this resulted in a selective defect in glucose-induced insulin secretion, providing initial evidence for a contribution of piRNA deregulation in the diabetic phenotype of GK rats. The overexpression of these two piRNAs did not affect cell survival, indicating that the defect in insulin secretion does not result from a toxic effect of these small RNAs. The mechanism through which DQ732700 and DQ746748 affect insulin secretion remains to be determined. Since KCl-induced insulin secretion is not impaired, the overexpression of these two piRNAs is unlikely to perturb the expression of general components of the exocytotic machinery.

Our findings would rather suggest that the overexpression of DQ732700 and DQ746748 interferes with the generation of metabolic factors coupling glucose sensing to insulin release. Alternatively the piRNAs may affect granular recruitment, a process that is dependent on metabolic factors [48, 49]. piRNAs have been suggested to control gene expression via mechanisms analogous to those of microRNAs [14]. Computational prediction of the potential targets of these two piRNAs highlighted an enrichment in genes involved in insulin secretion, insulin action or carbohydrate digestion. Future studies will have to experimentally validate these targets and to assess whether their silencing can indeed contribute to the effect of the piRNAs.

The unexpected discovery of the existence of thousands of non-coding transcripts with regulatory properties opens new perspectives in the understanding of the mechanisms that govern the activities of mammalian cells. Our data provide initial evidence for an involvement of piRNAs in the control of beta-cell functions under both physiological and pathological conditions. These findings add an additional layer of control to the regulation of gene expression in insulin-secreting cells. A better understanding of the role and the mode of action of piRNAs in beta-cells will help elucidate the molecular events driving the acquisition of a mature beta-cell phenotype and the causes of beta-cell dysfunction in diabetes conditions. This knowledge will be instrumental for the engineering of beta-cell surrogates for the replacement of insulin-secreting cells and to promote the development of new therapeutic strategies for the treatment of Type 2 diabetes.

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Disclosure Summary: The authors have nothing to disclose.

Contribution statement

IH, CJ, IGM, CG and JS generated and analyzed the data. CJ, IGM, CG and JS critically revised the manuscript and approved its final version. LE contributed to the interpretation of the data, critically revised the manuscript and approved its final version. RR designed the experiments and interpreted the data. IH and RR wrote the manuscript and approved its final version. RR is the guarantor of this work.

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FIGURE LEGENDS

Fig.1) Pancreatic islets express several components of the *Piwil*-piRNA pathway

qRT-PCR measurement of the level of *Piwil* mRNAs in rat testis **(a)** rat pancreatic islets (**b)** FACS-sorted rat beta-cells **(c)** and human islets (d). qRT-PCR analysis of the mRNAs of selected components of the *Piwil*-piRNA pathway in and rat testis (e) and rat islets (f). The results are means ± SD, n=3-4. ND: Not Detected.

Fig.2) Changes in the level of piRNAs during beta-cell maturation

A) Hierarchical clustering of 236 piRNAs differentially regulated (p-value<0.05, absolute fold change > 2, normalised expression > 6) between pancreatic islets of adult and newborn rats. Yellow tones highlight piRNAs displaying an up-regulation and blue tones a downregulation compared to the other group. Dark tones represent no significant change. The level of the indicated piRNAs in the whole islets (**b)** and purified beta-cells **(c)** of newborn (black bars) and adult rats (white bars) was analyzed by qRT-PCR. The data are means ± SD of the ΔCt values. Statistical differences were assessed by Student's t test *= $p \leq 0.05$, ** = $p \leq 0.01$, n=3.

Fig.3) Changes in piRNA expression in a type 2 diabetes model

a) Hierarchical clustering of 99 piRNAs (p-value<0.05, absolute fold change> 2, normalised expression > 6) differentially expressed between GK and Wistar rat islets. Dark tones represent no significant change, yellow tones an up-regulation and blue tones a downregulation. **b)** The expression of the three indicated piRNAs in the islets of GK (white bars) and Wistar rats (black bars) was measured by qRT-PCR. The data are means ± SD of the ΔCt values**.** Statistical differences were assessed by Student's *t* test *= $p \leq 0.05$, n=3.

Fig.4) Down-regulation of *Piwil2* and *Piwil4* expression in rat islet cells using siRNAs

a, b, c) The expression of the indicated mRNAs and piRNAs was measured by qRT-PCR. **a, b)** Dissociated rat islet cells were transfected with a control siRNA or with siRNAs directed against *Piwil2* **(a)** or *Piwil4* **(b)**. The expression of the indicated mRNAs was measured 48h after transfection by qRT-PCR. **c)** The level of the indicated piRNAs was measured by qRT-PCR, 48h after transfection with a control siRNA (black bars), si*Piwil2* (white bars) or si*Piwil4* (grey bars).

Results are presented as means ± SD. Statistical differences were assessed by Student's *t* test versus control, * = p≤0.05, ** = p≤0.01, n=3-5.

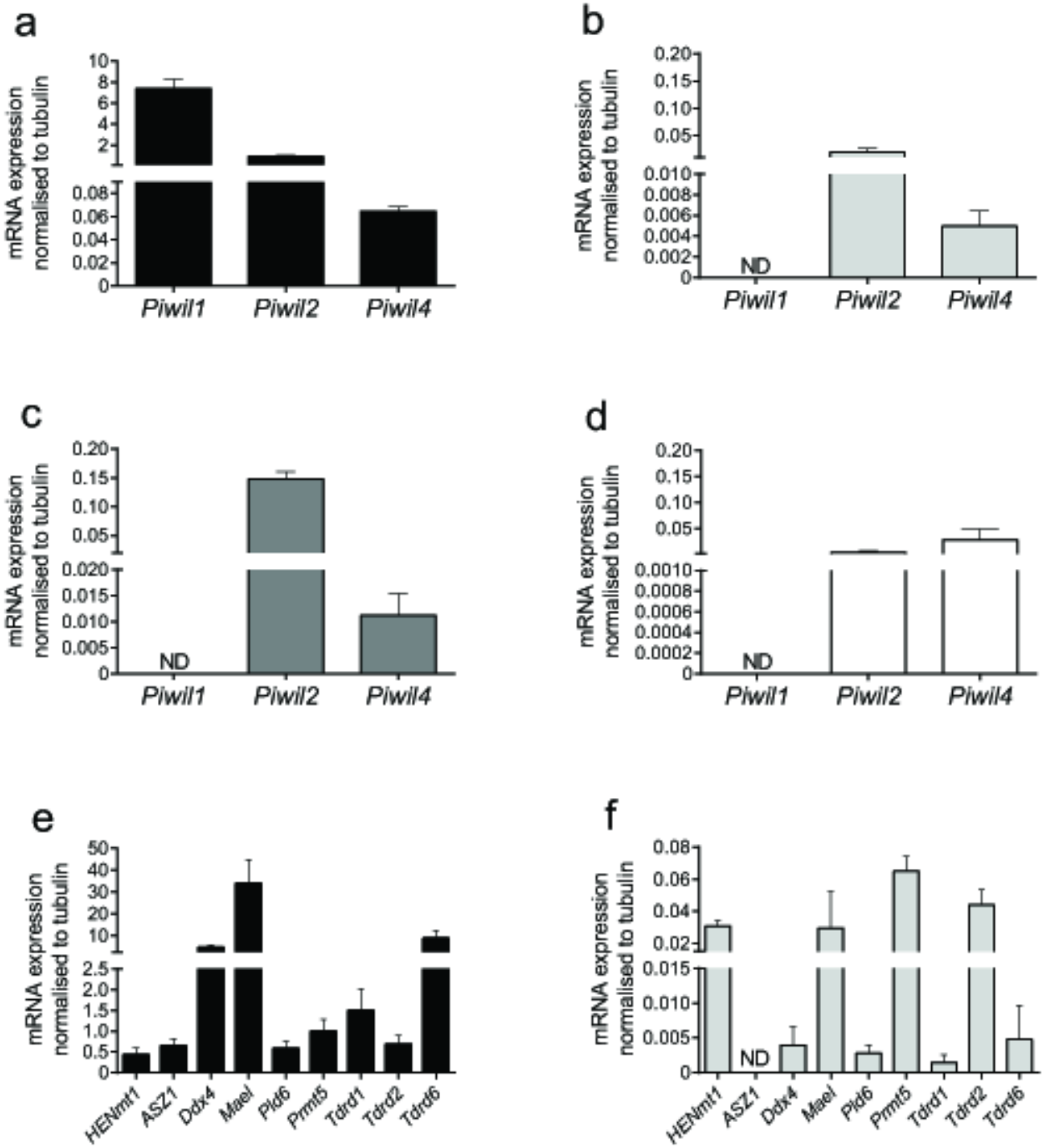
Fig.5) Functional impact of *Piwil2* and *Piwil4* silencing

Dissociated rat islet cells were transfected with a control siRNA or with siRNAs directed against *Piwil2* or *Piwil4*. The secretory properties of the cells were assessed 48h later. **a)** Insulin content and **b)** insulin secretion in the presence of 2 mmol/l (black bars) or 20 mmol/l glucose (white bars) were measured by ELISA. **c)** Insulin content and **c)** insulin secretion at 2mmol/l glucose in the absence (black bars) or in the presence of 35 mmol/l KCl (grey bars). **e)** Adult rat islet cells were transfected with the indicated siRNAs. One day later, the cells were incubated in the presence (white bars) or in the absence (black bars) of proinflammatory cytokines (IL-1β, TNFα and IFNγ). Cell death was assessed 24h later by counting the number of cells displaying pyknotic nuclei. **f)** Proliferation of beta-cells transfected with the indicated siRNAs was assessed by counting the fraction of cells positive for insulin and Ki67 in the presence (white bars) or absence (black bars) of 500ng/ml prolactin. Results are means ± SD. Statistical differences were assessed by One-Way ANOVA test, * = p≤ 0.05, ** = p≤0.01, n=4-5.

Fig.6) Effect of the overexpression of DQ732700 and DQ746748 piRNAs

Dissociated rat islet cells were transfected with a control oligonucleotide or with oligonucleotides mimicking the sequence of DQ732700 or DQ746748 piRNAs. Two days later, the cells were incubated for 45 min with 2 mmol/l glucose, 20 mmol/l glucose or 35 mmol/l KCl. **a)** Insulin contents and **b)** insulin secretion at 2 mmol/l (black bars) and 20 mmol/l glucose (white bars). **c)** Insulin contents and **d)** insulin secretion at 2mmol/l glucose in the absence (black bars) or in the presence of 35 mmol/l KCl (grey bars). **e)** Rat islet cells were transfected with the indicated piRNAs. The day after, the cells were incubated in the presence (white bars) or in the absence (black bars) of IL-1β, TNFα and IFNγ. Cell death was assessed 24h later by counting the number of cells displaying pyknotic nuclei. Results are means ± SD. Statistical differences were assessed by One-Way ANOVA test, * = p≤0.05, ** = p≤0.01, n=5-6.

Fig.1


a
mRNA expression normalised to tubulin
10
8
6
4
2
0.08
0.06
0.04
0.02
0
Piwil1
Piwil2
Piwil4
b
mRNA expression normalised to tubulin
0.20
0.15
0.10
0.05
0.010
0.008
0.006
0.004
0.002
0
ND
Piwil1
Piwil2
Piwil4
c
mRNA expression normalised to tubulin
0.20
0.15
0.10
0.05
0.020
0.015
0.010
0.005
0
ND
Piwil1
Piwil2
Piwil4
d
mRNA expression normalised to tubulin
0.20
0.15
0.10
0.05
0.0010
0.0008
0.0006
0.0004
0.0002
0
ND
Piwil1
Piwil2
Piwil4
e
mRNA expression normalised to tubulin
50
40
30
20
10
2.5
2.0
1.5
1.0
0.5
0
HENmt1
ASZ1
Ddx4
Mael
Pld6
Prmt5
Tdrd1
Tdrd2
Tdrd6
f
mRNA expression normalised to tubulin
0.08
0.06
0.04
0.02
0.015
0.010
0.005
0
ND
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ASZ1
Ddx4
Mael
Pld6
Prmt5
Tdrd1
Tdrd2
Tdrd6

Fig.2

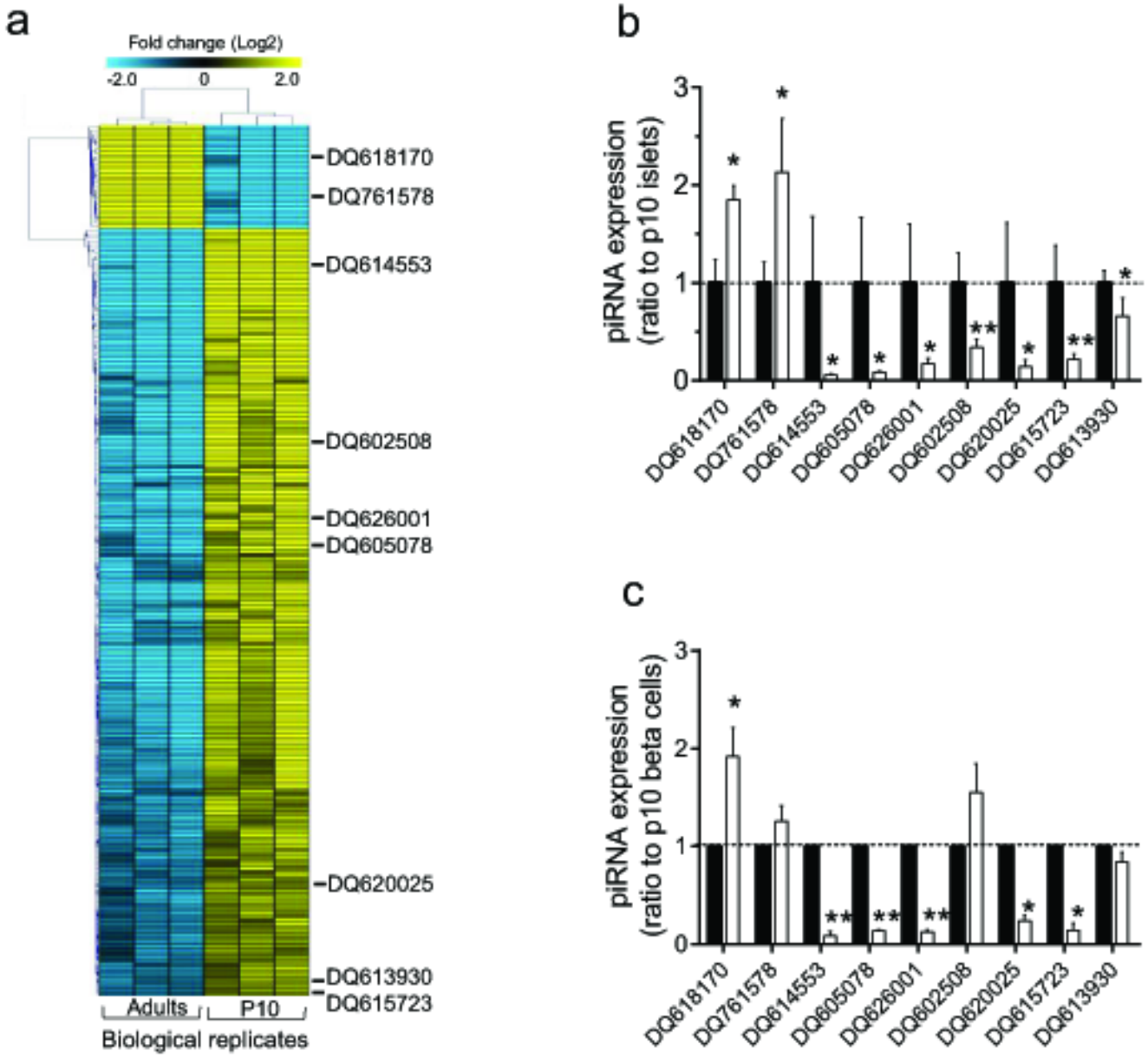

a
Fold change (Log2)
-2.0
0
2.0
DQ618170
DQ761578
DQ614553
DQ602508
DQ626001
DQ605078
DQ620025
DQ613930
DQ615723
Adults
P10
Biological replicates
b
piRNA expression
(ratio to p10 islets)
DQ618170
DQ761578
DQ614553
DQ605078
DQ626001
DQ602508
DQ620025
DQ615723
DQ613930
c
piRNA expression
(ratio to p10 beta cells)
DQ618170
DQ761578
DQ614553
DQ605078
DQ626001
DQ602508
DQ620025
DQ615723
DQ613930

Fig.3

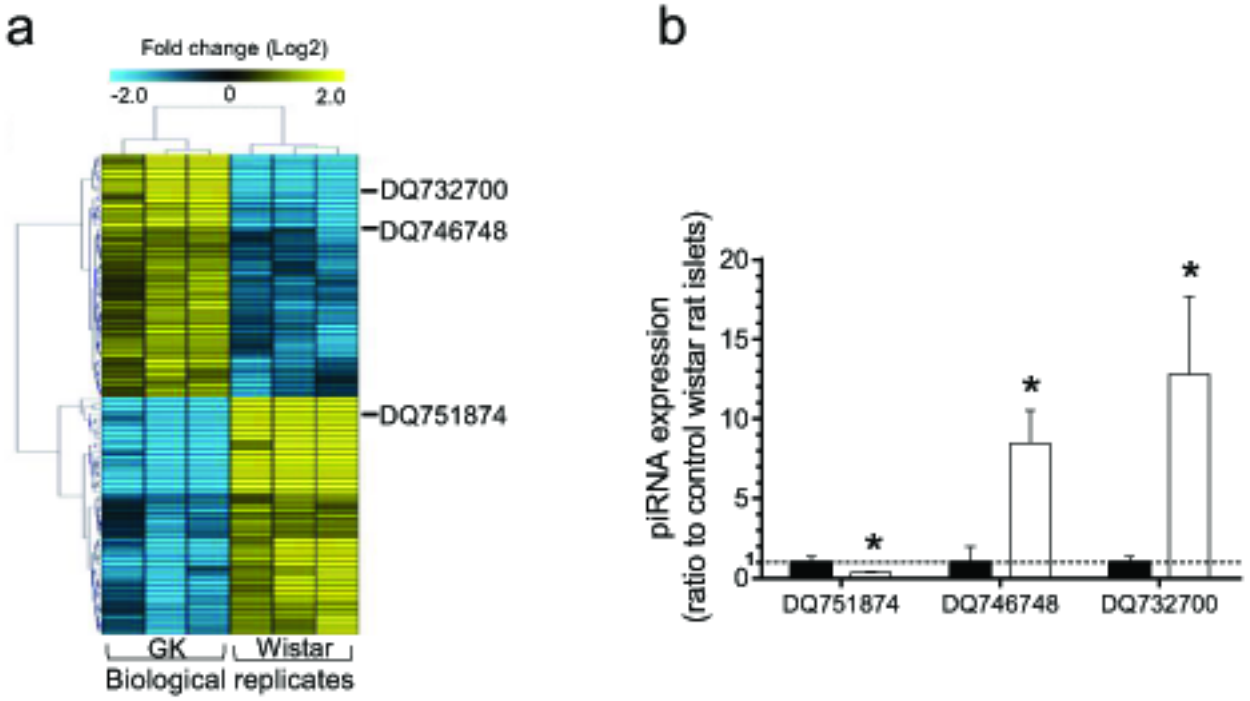

a
Fold change (Log2)
-2.0
0
2.0
DQ732700
DQ746748
DQ751874
GK
Wistar
Biological replicates
b
piRNA expression
(ratio to control wistar rat islets)
20
15
10
5
1
0
*
*
*
DQ751874
DQ746748
DQ732700

Fig.4

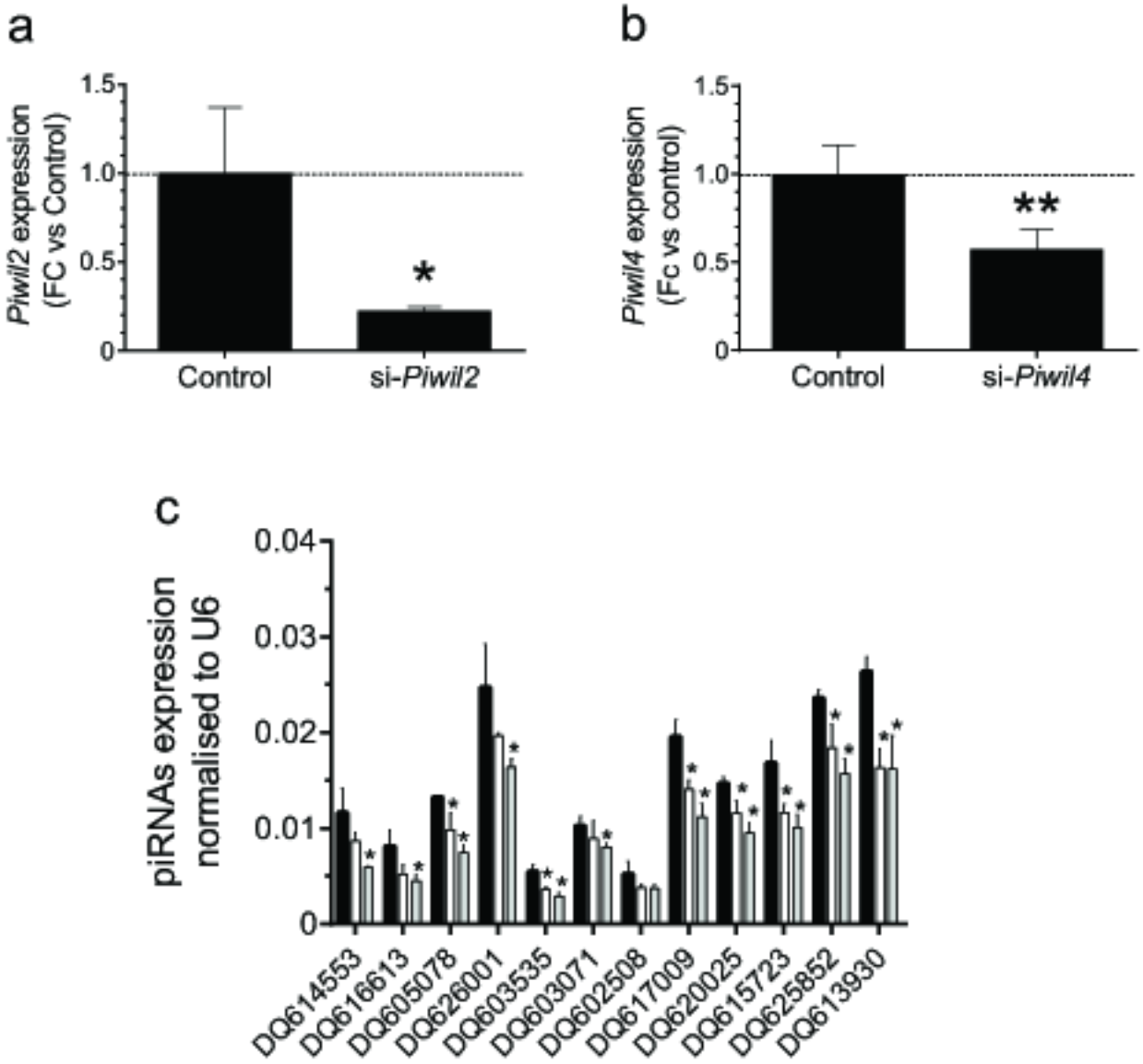

a
Piwil2 expression
(FC vs Control)
1.5
1.0
0.5
0
*
Control
si-Piwil2
b
Piwil4 expression
(Fc vs control)
1.5
1.0
0.5
0
**
Control
si-Piwil4
c
piRNAs expression
normalised to U6
0.04
0.03
0.02
0.01
0
DQ614553
DQ616613
DQ605078
DQ626001
DQ603535
DQ603071
DQ602508
DQ617009
DQ620025
DQ615723
DQ625852
DQ613930

Fig.5

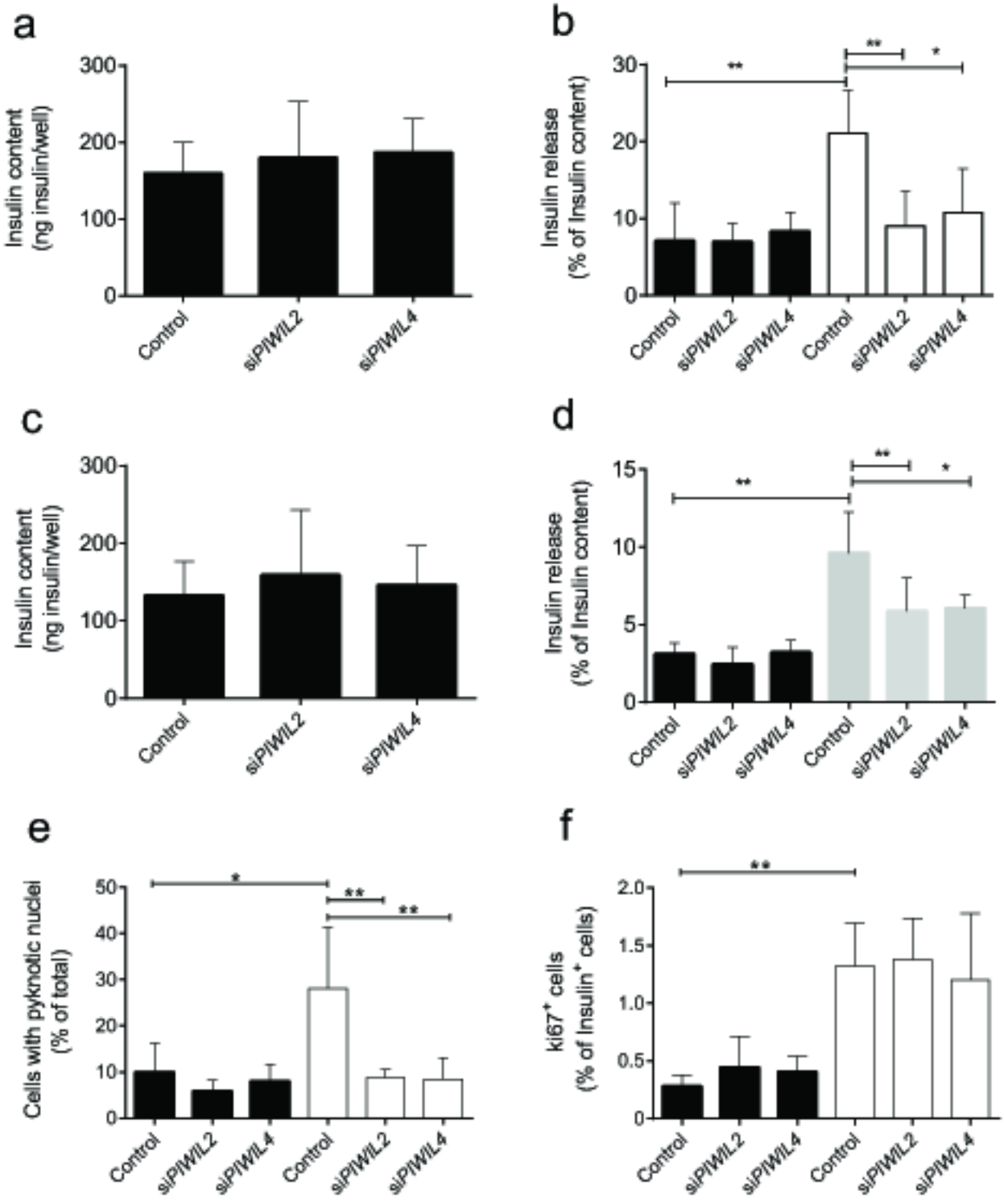
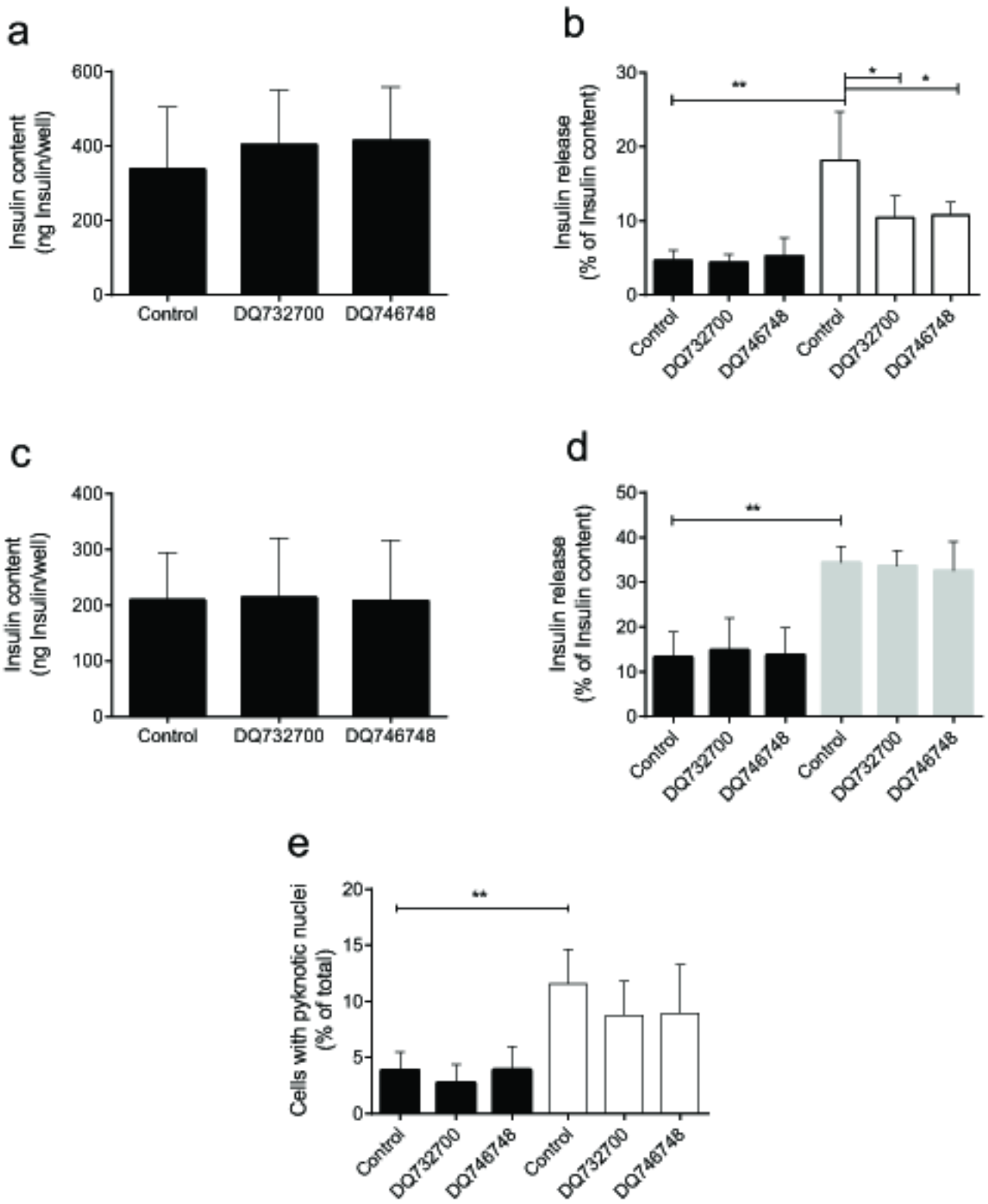

a
Insulin content (ng insulin/well)
300
200
100
0
Control
siPIWIL2
siPIWIL4
b
Insulin release (% of Insulin content)
30
20
10
0
**
**
*
Control
siPIWIL2
siPIWIL4
Control
siPIWIL2
siPIWIL4
c
Insulin content (ng insulin/well)
300
200
100
0
Control
siPIWIL2
siPIWIL4
d
Insulin release (% of Insulin content)
15
10
5
0
**
**
*
Control
siPIWIL2
siPIWIL4
Control
siPIWIL2
siPIWIL4
e
Cells with pyknotic nuclei (% of total)
50
40
30
20
10
0
*
**
**
Control
siPIWIL2
siPIWIL4
Control
siPIWIL2
siPIWIL4
f
ki67+ cells (% of Insulin+ cells)
2.0
1.5
1.0
0.5
0
**
Control
siPIWIL2
siPIWIL4
Control
siPIWIL2
siPIWIL4

Fig.6


a
Insulin content
(ng Insulin/well)
600
400
200
0
Control
DQ732700
DQ746748
b
Insulin release
(% of Insulin content)
30
20
10
0
**
*
*
Control
DQ732700
DQ746748
Control
DQ732700
DQ746748
c
Insulin content
(ng Insulin/well)
400
300
200
100
0
Control
DQ732700
DQ746748
d
Insulin release
(% of Insulin content)
50
40
30
20
10
0
**
Control
DQ732700
DQ746748
Control
DQ732700
DQ746748
e
Cells with pyknotic nuclei
(% of total)
20
15
10
5
0
**
Control
DQ732700
DQ746748
Control
DQ732700
DQ746748

ESM Methods

Sequence of the primers used for qRT-PCR

Gene name	Forward primer : 5` → 3`	Reverse primer : 5` → 3`
Piwil2	CATTGTCATCACACGCTACAAC	CTCCTTCCCATCCGACATTAC
Piwil4	GCGGTTGCTATGCTTTGTTC	ACCCTCTCCTTCCCAATACTTA
HENmt1	AAAACGGTGGGAAGCTCTCT	TCTCCTGCTGTAAACTCGGG
ASZ1	GTACTGGAGTGGGCTTCACC	TCCCGTTCATTCTGCATCTTCT
Ddx4	GATGCACCACCGGCAATTTT	ACTTCTGCACAGGAGTGAGC
Mael	GGCATGACCAAGCAACTGTG	GCCTTATTTCTGATGCCCGC
Pld6	CGCAAGGCAGGTATACAGGT	TGTGGTCCAATTGAGGGAGC
Prmt5	TTCTTCCCCATCAAGCAGCC	GCGACCAGTAGGGTTGTGAA
Tdrd1	TGGAACCGAGCCGTAGTAGA	TTATGGCGGAAGGGGGAAAC
Tdrd2	TCCTGTTCAGGTGTGCAAGG	TCACCGCCTCTCCCTATGAT
Tdrd6	GGCACACGACGAAATGAACC	TCAACTGCTGGTACTTCGCT
Hiwi/Piwil1	GACTCCGTTCAGCTCTTCTTT	TCTCCATTCCGGGTCTTACT
Hili/Piwil2	TGGGTTTGGTCTCCATGTTC	CTGTCCTTGCGTACCAGATTAG
Hiwi2/Piwil4	TCAGCCCATGCTTGTTAGTC	GTCAGTCAGCCCTGTTAGAAAG

ESM Table 1

Pathway analysis of the targets of DQ732700 predicted with the PITA algorithm using default parameters [35]. The top 300 putative targets based on the PITA score were annotated with their gene ontology using biomaRt [36]. Pathway enrichment analysis was performed using DAVID with KEGG and Reactome pathway annotations.

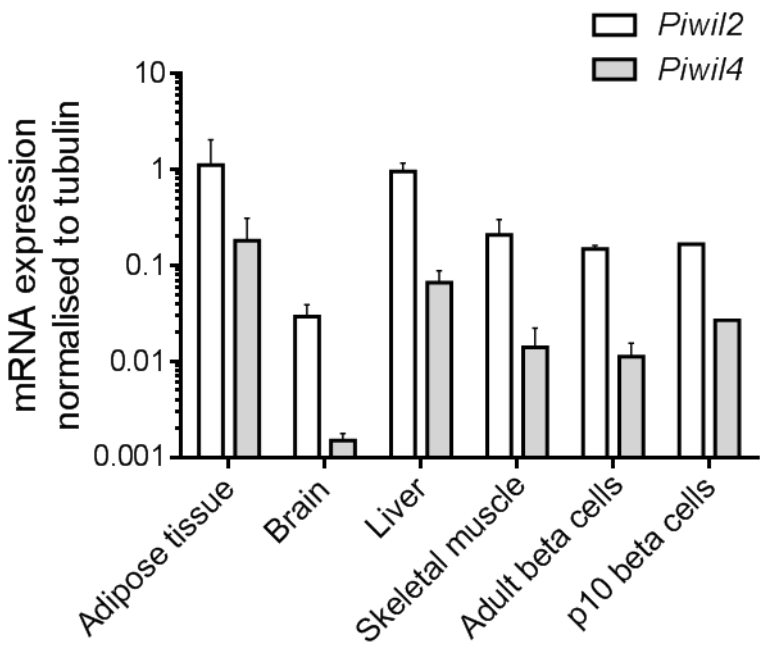
Category	Term	Count	p-Value	Genes	Fold Enrichment
KEGG_PATHWAY	rno04964:Proximal tubule bicarbonate reclamation	4	0.0043	ATP1B3, GLUD1, ATP1A2, AQP1	11.79
REACTOME_PATHWAY	R-RNO-2028269:Signaling by Hippo	4	0.0052	YWHAB, AMOTL2, STK4, LATS2	11.06
KEGG_PATHWAY	rno04931:Insulin resistance	7	0.0066	CRTC2, MLX, PPP1R3A, OGT, IRS1, AKT2, PTPN11	4.13
KEGG_PATHWAY	rno04911:Insulin secretion	6	0.0100	ATP1B3, CAMK2D, VAMP2, ATP1A2, PCLO, CACNA1D	4.52
REACTOME_PATHWAY	R-RNO-5578775: Ion homeostasis	5	0.0147	ATP2B2, ATP1B3, CAMK2D, AHCYL1, ATP1A2	5.217
KEGG_PATHWAY	rno04022:cGMP-PKG signaling pathway	8	0.0152	ATP2B2, ATP1B3, GATA4, GUCY1B3, ATP1A2, IRS1, CACNA1D, AKT2	3.05
KEGG_PATHWAY	rno05031:Amphetamine addiction	5	0.0163	DDC, ARC, MAOB, CAMK2D, CACNA1D	5.07
KEGG_PATHWAY	rno05206:MicroRNAs in cancer	7	0.0221	WNT3, SLC7A1, ERBB2, SOS2, RDX, IRS1, DDIT4	3.17
KEGG_PATHWAY	rno04261:Adrenergic signaling in cardiomyocytes	7	0.0242	ATP2B2, ATP1B3, CAMK2D, CACNB2, ATP1A2, CACNA1D, AKT2	3.11
KEGG_PATHWAY	rno04973:Carbohydrate digestion and absorption	4	0.0244	ATP1B3, ATP1A2, CACNA1D, AKT2	6.33
KEGG_PATHWAY	rno04920:Adipocytokine signaling pathway	5	0.0275	TNFRSF1B, ACSL1, IRS1, AKT2, PTPN11	4.32
KEGG_PATHWAY	rno04970:Salivary secretion	5	0.0287	ATP2B2, ATP1B3, GUCY1B3, VAMP2, ATP1A2	4.27

ESM Table 2

Pathway analysis of the targets of DQ746748 predicted with the PITA algorithm using default parameters [35]. The top 300 putative targets based on the PITA score were annotated with their gene ontology using biomaRt [36]. Pathway enrichment analysis was performed using DAVID with KEGG and Reactome pathway annotations.

Category	Term	Count	p-Value	Genes	Fold Enrichment
KEGG_PATHWAY	rno04910:Insulin signaling pathway	9	6.55E-04	PPP1R3D, CRKL, SOCS4, PPP1R3A, PPP1CB, CRK, LIPE, G6PC3, PYGB	4.63
KEGG_PATHWAY	rno04931:Insulin resistance	8	7.61E-04	PPP1R3D, GFPT2, PPP1R3A, PPP1CB, G6PC3, CPT1A, PYGB, PTPN11	5.24
KEGG_PATHWAY	rno04152:AMPK signaling pathway	7	0.0079	MAP3K7, AKT1S1, ADIPOR2, ADIPOR1, LIPE, G6PC3, CPT1A	3.97
KEGG_PATHWAY	rno04920:Adipocytokine signaling pathway	5	0.0195	ADIPOR2, ADIPOR1, G6PC3, CPT1A, PTPN11	4.80
KEGG_PATHWAY	rno04270:Vascular smooth muscle contraction	6	0.0261	EDNRA, RAMP3, MRVI1, PRKCH, PLA2G2D, PPP1CB	3.54
REACTOME_PATHWAY	R-RNO-170968: Frs2-mediated activation	3	0.0262	CRKL, FRS2, CRK	11.66
REACTOME_PATHWAY	R-RNO-163560: Hormone-sensitive lipase (HSL)-mediated triacylglycerol hydrolysis	3	0.0292	FABP7, PPP1CB, LIPE	11.01
KEGG_PATHWAY	rno01130:Biosynthesis of antibiotics	8	0.0309	TPI1, BCAT2, HMGCS2, GFPT2, GGPS1, AASS, TKT, PAPSS2	2.63

ESM Fig.1



qRT-PCR measurement of the level of *Piwil2 and Piwil4* mRNAs in rat tissues and purified beta-cells. Adipose tissue, brain, liver and skeletal muscle were collected from Wistar rats aged of 12 weeks. Highly enriched fractions of adult (3 month-old) and newborn (10 days-old) rats containing about 95% of insulin-positive cells were obtained by FACS-sorting. The results are means ± SD of the ΔCt values, n=3. Newborn beta-cells represent a pool of 5 pups.