RNA-binding protein CUGBP1 regulates insulin secretion via activation of phosphodiesterase 3B in mice

Kui Zhai¹ · Lei Gu¹ · Zhiguang Yang¹ · Yang Mao¹ · Meng Jin¹ · Yan Chang¹ · Qi Yuan¹ · Veronique Leblais² · Huiwen Wang^{1,3} · Rodolphe Fischmeister² · Guangju Ji¹

Received: 28 February 2016 / Accepted: 16 May 2016 / Published online: 2 June 2016
© Springer-Verlag Berlin Heidelberg 2016

Abstract

Aims/hypothesis CUG-binding protein 1 (CUGBP1) is a multifunctional RNA-binding protein that regulates RNA processing at several stages including translation, deadenylation and alternative splicing, as well as RNA stability. Recent studies indicate that CUGBP1 may play a role in metabolic disorders. Our objective was to examine its role in endocrine pancreas function through gain- and loss-of-function experiments and to further decipher the underlying molecular mechanisms. **Methods** A mouse model in which type 2 diabetes was induced by a high-fat diet (HFD; 60% energy from fat) and mice on a standard chow diet (10% energy from fat) were compared. Pancreas-specific CUGBP1 overexpression and knockdown mice were generated. Different lengths of the phosphodiesterase subtype 3B (*PDE3B*) 3' untranslated region (UTR) were cloned for luciferase reporter analysis. Purified CUGBP1 protein was used for gel shift experiments.

Kui Zhai, Lei Gu and Zhiguang Yang contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00125-016-4005-5) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

✉ Rodolphe Fischmeister
rodolphe.fischmeister@inserm.fr

✉ Guangju Ji
gj28@ibp.ac.cn

¹ National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, People's Republic of China

² Inserm, UMR-S 1180, Faculté de Pharmacie, Université Paris-Sud, 5 rue J.-B. Clément, 92296 Châtenay-Malabry, France

³ State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, People's Republic of China

Results CUGBP1 is present in rodent islets and in beta cell lines; it is overexpressed in the islets of diabetic mice. Compared with control mice, the plasma insulin level after a glucose load was significantly lower and glucose clearance was greatly delayed in mice with pancreas-specific CUGBP1 overexpression; the opposite results were obtained upon pancreas-specific CUGBP1 knockdown. Glucose- and glucagon-like peptide1 (GLP-1)-stimulated insulin secretion was significantly attenuated in mouse islets upon CUGBP1 overexpression. This was associated with a strong decrease in intracellular cAMP levels, pointing to a potential role for cAMP PDEs. CUGBP1 overexpression had no effect on the mRNA levels of *PDE1A*, *1C*, *2A*, *3A*, *4A*, *4B*, *4D*, *7A* and *8B* subtypes, but resulted in increased *PDE3B* expression. CUGBP1 was found to directly bind to a specific ATTTGTT sequence residing in the 3' UTR of *PDE3B* and stabilised *PDE3B* mRNA. In the presence of the PDE3 inhibitor cilostamide, glucose- and GLP-1-stimulated insulin secretion was no longer reduced by CUGBP1 overexpression. Similar to CUGBP1, *PDE3B* was overexpressed in the islets of diabetic mice.

Conclusions/interpretation We conclude that CUGBP1 is a critical regulator of insulin secretion via activating *PDE3B*. Repressing this protein might provide a potential strategy for treating type 2 diabetes.

Keywords cAMP · CUGBP1 · Insulin secretion · *PDE3B* · RNA-binding protein · Type 2 diabetes

Abbreviations

CUGBP1	CUG-binding protein 1
GLP-1	Glucagon-like peptide 1
GSIS	Glucose-stimulated insulin secretion
HFD	High-fat diet

KD	Knockdown
OE	Overexpression
PDE	Phosphodiesterase
qPCR	Quantitative PCR
RIP-Chip	RNA-binding protein immunoprecipitation profiling
SpCas9	<i>Streptococcus pyogenes</i> clustered, regularly interspaced, short palindromic repeat (CRISPR)-associated endonuclease 9
UTR	Untranslated region

Introduction

Insulin is secreted by pancreatic beta cells of the islets of Langerhans; it is the only hormone capable of lowering plasma glucose levels [1, 2]. Glucose-stimulated insulin secretion (GSIS) occurs via glucose metabolism in beta cells [3]. Glucose metabolism leads to an increased cytosolic ATP/ADP ratio and the closure of ATP-sensitive K^+ channels in the plasma membrane. The resulting depolarisation of the plasma membrane triggers Ca^{2+} influx through voltage-dependent Ca^{2+} channels, leading to an increased cytosolic Ca^{2+} concentration which is required for insulin granule exocytosis. However, GSIS also occurs via metabolic coupling factors other than ATP [3]. In addition, insulin secretion can be mediated by various hormones, including leptin, growth hormone and glucagon-like peptide 1 (GLP-1) [2].

Accumulating evidence indicates that cAMP is an integral component of GSIS [4, 5]. The intracellular level of cAMP is tightly controlled by its synthesis through adenylyl cyclases and its degradation through phosphodiesterases (PDEs). PDEs have been classified into 11 families according to their amino acid sequence, catalytic characteristics, substrate preference and regulatory properties [6]. Several PDE subtypes and isoforms, including PDE1 [7], PDE3B [8, 9], PDE4 [7] and PDE8B [7, 10], are known to mediate insulin secretion by pancreatic islets and/or beta cells. Through a feedback mechanism, insulin can reduce intracellular cAMP levels via activation of PDEs either by phosphorylation [11, 12] or in the longer term by regulating their protein levels [13, 14].

CUG-binding protein 1 (CUGBP1) is a member of the CUGBP and embryonic lethal abnormal vision-like factor (CELF) family of RNA-binding proteins. It preferentially binds to GU-rich elements located in the 5' and 3' untranslated regions (UTRs), as well as in mRNA coding regions [15, 16]. The association of CUGBP1 with its mRNA targets influences their alternative splicing, translation and turnover [17]. CUGBP1 is ubiquitously expressed and plays a critical role in various physiological and pathological cellular processes, including skeletal muscle differentiation and atrophy [18–21], cell proliferation [22], heart development and disease [23–25], and tumour growth [26]. Recently, several studies indicate that

CUGBP1 may be involved in metabolic disorders. CUGBP1 has been reported to mediate alternative splicing of the insulin receptor [27] and to contribute to insulin resistance [28]. Verma et al showed that CUGBP1 is upregulated in the hearts of diabetic mice [29]. A recent genome-wide association study analysis suggested that genetic variation at the *CUGBP1* locus is associated with obesity [30].

Here, we show that CUGBP1 is present in rodent islets and beta cells lines and is overexpressed in the islets of diabetic mice. We thus examined its potential role in endocrine pancreas function through gain- and loss-of-function experiments and further deciphered the underlying molecular mechanisms.

Methods

For detailed methods, please see the electronic supplementary material (ESM) [methods](#).

Animals All animal protocols described in this study were performed according to the Guidance for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the Institute of Biophysics Committee on Animal Care. Mice were housed under a 12 h:12 h light:dark cycle at a constant temperature (22–24°C), and had free access to water and food. Eight-week-old wild-type and *db/db* mice were kindly provided by W. Jin from the Institute of Zoology, Beijing, China. Adult male C57BL/6 mice were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China). As previously reported, seven-week-old mice were randomly divided into two groups [31]: one group received standard rodent chow (10% energy from fat; Beijing HFK Bioscience, Beijing, China) and the other group received a high-fat diet (HFD; 60% energy from fat; rodent diet D12492, Research Diets, New Brunswick, NJ, USA). Animals were fed a HFD or chow diet for 30 weeks.

Reagents and plasmids Cilostamide was purchased from Tocris Bioscience (Bristol, UK). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. The pcDNA3.1-CUGBP1 plasmid was a generous gift from H. Lou, Case Western Reserve University, Cleveland, OH, USA. The pLenti-OC-IRES-BSD plasmid and lentiviral sgRNA vector were kindly provided by W. Wei, Peking University, Beijing, China. The firefly and renilla luciferase vectors were provided by Y. Wu from the Institute of Biophysics, Chinese Academy of Sciences, Beijing, China. The pcDNA3.1 and pDsRed2-N1 plasmids were obtained from Addgene (Cambridge, MA, USA).

Adenovirus generation Adenoviruses were prepared using the AdEasy XL Adenoviral Vector System (Stratagene, La Jolla, CA, USA). Schematic diagrams are shown in ESM Fig. 1.

IPGTTs GTTs were performed by i.p. injection of 1.5 g/kg glucose into mice after fasting for 16 h. Glucose levels were measured using an automatic glucometer (Accu-Chek; Roche Diagnostics, Mannheim, Germany). In some experiments, the AUC for glucose during the IPGTT (120 min) was calculated with GraphPad Prism 6 (La Jolla, CA, USA).

Insulin secretion and content analysis Islets were isolated and cultured as previously described [32]. Islets were infected by Ad-DsRed or Ad-DsRed-*Cugbp1* and incubated for 48 h. Insulin secretion and total insulin content were then measured.

Western blotting Western blotting was performed as previously described [33]. Briefly, protein samples from HeLa cells or mouse islets were resolved by SDS-PAGE and immunoblotted with anti-PDE3A, anti-PDE3B, anti-CUGBP1 or anti- β -actin antibody.

Immunofluorescence analysis Immunofluorescence was used to determine the expression of CUGBP1 in mouse pancreases or MIN6 cells.

Plasma insulin measurement Eight-week-old male mice were infected with Ad-DsRed or Ad-DsRed-CUGBP1 (control mice vs CUGBP1 OE mice) or an adenovirus expressing the gene encoding clustered, regularly interspaced, short palindromic repeats (CRISPR)-associated endonuclease from *Streptococcus pyogenes* (SpCas9; Ad-SpCas9) plus Ad-GFP/Ad-SpCas9 plus Ad-SpGuide (control mice vs CUGBP1 KD mice) by direct injection into the pancreas, as previously published [34]. After infection, plasma insulin levels were measured.

cAMP assay Islets were extracted in lysis buffer provided in the cAMP XP assay kit (no. 4339; Cell Signalling Technology, Danvers, MA, USA). Intracellular cAMP concentrations were determined and normalised to protein concentration, according to the manufacturer's instructions.

RNA isolation and PCR RNA was extracted from HeLa cells or mouse islets using the Trizol RNA purification system (Invitrogen, Carlsbad, CA, USA), and reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). PCR and quantitative (q)PCR were performed as previously reported [35].

RNA-binding protein immunoprecipitation RNA-binding protein immunoprecipitation (RIP-CHIP) was performed with HeLa cell lysates, as previously described [36].

Luciferase reporter assay Different lengths of the *PDE3B* 3' UTR were amplified and cloned into the firefly vector. Sewing PCR was used to generate mutant *PDE3B* 3' UTR sequences, as previously reported [37].

Gel shift assay CUGBP1 protein was purified as previously reported [15]. The RNA oligonucleotide (5'-AUUUGUU-3') was synthesised by GenScript (Nanjing, China).

***PDE3B* mRNA stability assay** *PDE3B* mRNA stability assay was performed as previously reported [38].

Statistical analysis Significant differences were determined between two groups using the Student's *t* test or among multiple groups using ANOVA. Data are shown as mean $\pm$ SEM, and statistical significance was set at $p < 0.05$.

Results

CUGBP1 is overexpressed in the islets of diabetic mice To understand the function of CUGBP1, we first examined its expression in the whole murine pancreas. As shown in ESM Fig. 2a, we found that CUGBP1 was evenly distributed in the islets and exocrine areas. Western blotting showed that CUGBP1 was abundantly expressed in rodent islets and in two pancreatic beta cell lines (INS1 and MIN6), at similar levels as in the mouse extensor digitorum longus and soleus (ESM Fig. 2b). Immunostaining revealed that CUGBP1 was mainly located in the cytoplasm of MIN6 cells (ESM Fig. 2c).

Two classic mouse models of type 2 diabetes (*db/db* and HFD-fed mice) were used to investigate whether type 2 diabetes modifies the expression of CUGBP1. The *db/db* mice had a higher body weight ($p < 0.001$; Fig. 1a) and impaired glucose clearance compared with control mice (Fig. 1b). CUGBP1 protein was upregulated 2.2-fold in the islets of *db/db* mice compared with control mice ($p < 0.001$; Fig. 1c). As shown in Fig. 1d, the average body weights of HFD-fed and chow-fed mice were 52.6 ± 1.1 g and 36.4 ± 1.2 g ($p < 0.001$), respectively. IPGTTs revealed that glucose tolerance was markedly impaired in HFD-fed mice compared with chow-fed mice (Fig. 1e). As for *db/db* mice, the level of CUGBP1 protein was also significantly higher in the islets of HFD-fed mice compared with chow-fed mice ($p < 0.05$; Fig. 1f). Taken together, our results indicate that CUGBP1 is overexpressed in the islets of diabetic mice.

CUGBP1 is a negative regulator of insulin secretion Overexpression (OE) of *Cugbp1* in the islets of diabetic mice

Fig. 1 CUGBP1 is overexpressed in the islets of diabetic mice. **(a)** Body weight and **(b)** IPGTTs of wild-type (WT) and *db/db* mice (8-week-old; $n=4$). Black circles, WT; white squares, *db/db*. **(c)** The CUGBP1 protein level was significantly elevated in islets isolated from *db/db* mice. **(d)** Body weight and **(e)** IPGTTs of chow- and HFD-fed mice ($n=4$ –5).

led us to speculate that CUGBP1 may play a role in insulin secretion. To test this hypothesis, we generated mice overexpressing pancreas-specific *Cugbp1* by directly injecting Ad-DsRed-CUGBP1 into the mouse pancreas (ESM Fig. 3a). Control mice were injected with Ad-DsRed. We observed DsRed throughout the pancreas, indicating that the exogenous protein (DsRed or DsRed-CUGBP1) was strongly expressed (ESM Fig. 3b). CUGBP1 was overexpressed by about 2.5-fold in the islets of CUGBP1 OE mice (ESM Fig. 3c). As shown in Fig. 2a, the fasting plasma insulin level was not significantly different between control and CUGBP1 OE mice. However, the glucose-stimulated plasma insulin level was significantly lower in CUGBP1 OE mice than in control mice (Fig. 2a). Therefore, CUGBP1 OE mice cleared glucose at a significantly slower rate compared with control mice (Fig. 2b).

We further investigated the function of CUGBP1 by generating a mouse model with pancreas-specific CUGBP1 knockdown (KD) which was induced using SpCas9. As shown in ESM Fig. 4a, Ad-Sp-Cas9 and Ad-Sp-GuideGFP (1:1 ratio) were directly injected into the pancreas to disrupt the *Cugbp1* gene (CUGBP1 KD mice). Control mice were injected with Ad-Sp-Cas9 and Ad-GFP (1:1 ratio). A T7 endonuclease I cleavage assay and genomic DNA sequencing revealed no evidence of mutagenesis in control mice, while CUGBP1 KD mice displayed mutations at multiple sites (ESM Fig. 4b,c). Western blot analysis demonstrated that CUGBP1 protein content was reduced by about 50% in pancreatic islets (ESM Fig. 4d). Although the fasted plasma insulin level was only moderately enhanced, glucose-stimulated plasma insulin levels were significantly increased in CUGBP1 KD mice compared with control mice (Fig. 2c). IPGTT results showed that glucose clearance was faster in CUGBP1 KD mice than in control mice (Fig. 2d).

To test whether CUGBP1 is involved in *in vitro* insulin secretion, isolated islets were infected with Ad-DsRed-

Black circles, chow; white squares, HFD. **(f)** The CUGBP1 protein level was significantly elevated in the islets isolated from HFD-fed mice. Seven-week-old male mice were fed an HFD or chow diet for 30 weeks. Data are the mean $\pm$ SEM. * $p<0.05$ and *** $p<0.001$ vs control

CUGBP1; control islets were infected with Ad-DsRed. Ad-DsRed-CUGBP1 infection led to a 3.8-fold upregulation of CUGBP1 (ESM Fig. 5). As shown in Fig. 2e, secreted insulin levels were similar in control and CUGBP1 OE islets in the presence of 3.3 mmol/l glucose; however, upon 16.7 mmol/l glucose stimulation insulin levels in CUGBP1 OE islets were significantly lower than in control islets ($p<0.05$). GLP-1 is known to stimulate insulin secretion through binding to and activating the GLP-1 receptor, and thereby inducing cAMP production [39]. We therefore tested whether CUGBP1 has a role in GLP-1-induced insulin secretion. We found that GLP-1 (10 nmol/l) stimulated insulin secretion from 13.9 ± 0.7 to 50.9 ± 8.1 ng/mg in control islets and from 11.7 ± 0.8 to 27.3 ± 0.8 ng/mg in CUGBP1 OE islets (Fig. 2f). Although basal insulin levels were similar in CUGBP1 OE islets and control islets (13.9 vs 11.7 ng/mg), GLP-1-stimulated insulin secretion was markedly decreased in CUGBP1 OE islets (50.9 vs 27.3 ng/mg, $p<0.05$; Fig. 2f). However, the total insulin content (Fig. 2g) and *Ins1* and *Ins2* mRNA levels (Fig. 2h) were similar in control and CUGBP1 OE islets, suggesting that CUGBP1 has no effect on insulin production.

cAMP, but not intracellular Ca^{2+} mediates CUGBP1 function

We next investigated the mechanisms by which CUGBP1 negatively regulates insulin secretion. Given the importance of Ca^{2+} in GSIS and the fact that Ca^{2+} signalling can be modulated by CUGBP1 in the heart [25], we hypothesised that the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) might modulate CUGBP1-induced GSIS impairment. To test this, $[\text{Ca}^{2+}]_i$ was recorded in isolated islets, as previously reported [40]. We found that glucose-induced Ca^{2+} release was similar in control and CUGBP1 OE islets (Fig. 3a,b), indicating that Ca^{2+} signalling is not involved in CUGBP1-mediated effects.

cAMP is an important regulator of GSIS [4, 5, 41]. We therefore investigated whether the intracellular cAMP

Fig. 2 CUGBP1 is a negative regulator of insulin secretion. **(a)** Plasma insulin levels and **(b)** IPGTTs for control and CUGBP1 OE mice ($n=4-9$). White bars/squares, control; black bars/squares, CUGBP1 OE. **(c)** Plasma insulin levels and **(d)** IPGTTs for control and CUGBP1 KD mice ($n=4-10$). White bars/squares, control; grey bars/squares, CUGBP1 KD. **(e)** Glucose (Glu)-stimulated and **(f)** GLP-1-stimulated

insulin secretion and **(g)** total insulin content in control and CUGBP1 OE islets ($n=4$ groups). **(h)** *Ins1* and *Ins2* mRNA levels were measured in control and CUGBP1 OE islets ($n=4$ groups). **(e-g)** Each group contains 40–60 islets isolated from at least two mice. White bars, control; black bars, CUGBP1 OE. NS, not significant. Data are the mean $\pm$ SEM. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ vs control

concentration ($[Ca^{2+}]_i$) was modified upon CUGBP1 OE. As seen in Fig. 3c, the $[Ca^{2+}]_i$ stimulated by 16.7 mmol/l glucose was 2.4 ± 0.3 nmol/mg in control islets but only 1.4 ± 0.2 nmol/mg in CUGBP1 OE islets

($n=4$ groups, $p<0.05$), indicating that CUGBP1 decreases cAMP levels upon glucose stimulation.

CUGBP1 induces PDE3B expression As PDEs are the only enzymes that specifically hydrolyse cAMP [6], we speculated that PDE activation might be responsible for the CUGBP1-dependent decrease in cAMP content. We therefore measured the mRNA levels of most PDE family genes under control and CUGBP1 OE conditions in HeLa cells and mouse islets. CUGBP1 OE had no effect on the mRNA levels of all PDE genes with the exception of *PDE3B* which was clearly induced (Fig. 3d,e and ESM Fig. 6). Owing to their lack of gene expression in HeLa cells and mouse islets, the effects of CUGBP1 on *PDE4C*, *PDE10A* and *PDE11A* mRNA levels were not determined. In isolated islets, CUGBP1 OE increased the *Pde3b* mRNA level by about twofold (Fig. 4a) and upregulated PDE3B protein by threefold (Fig. 4b). Consistent with this, PDE3B expression was markedly decreased in islets from CUGBP1 KD mice compared with control mice (Fig. 4c). Unlike for PDE3B, PDE3A protein level was not changed upon CUGBP1 OE (ESM Fig. 7).

PDE3B inhibition rescues CUGBP1-mediated GSIS impairment So far, our results suggest that inhibition of GSIS by CUGBP1 is due to activation of PDE3B. The selective PDE3 inhibitor, cilostamide (10 μ mol/l), was used to further examine this hypothesis. Both glucose- and GLP-1-stimulated

Fig. 3 Effects of CUGBP1 on $[Ca^{2+}]_i$, $[cAMP]_i$ and PDE expression. **(a)** $[Ca^{2+}]_i$ was recorded in control (grey line) and CUGBP1 OE (black line) islets. **(b)** Summary data show the amplitude of the $[Ca^{2+}]_i$ response of control (white bar; $n=12$) and CUGBP1 OE (black bar; $n=11$) islets. $[Ca^{2+}]_i$ is expressed as ratio F/F0 (F0 is the average fluorescence intensity in the presence of 3.3 mmol/l glucose). **(c)** Glucose-stimulated $[cAMP]_i$ was measured in control (white bar) and CUGBP1 OE (black bar) islets ($n=4$ groups). Each group contains about 100 islets isolated from three mice. **(d, e)** Representative RT-PCR shows the effects of CUGBP1 OE on the mRNA levels of various PDE family genes in **(d)** HeLa cells or **(e)** mouse islets. Data are the mean $\pm$ SEM. * $p<0.05$ vs control

Fig. 4 PDE3B is activated by CUGBP1 and upregulated in the islets of diabetic mice. **(a, b)** PDE3B mRNA **(a)** and protein **(b)** levels were elevated in CUGBP1 OE islets ($n=3-6$ groups). Each group contains 40–60 islets isolated from at least two mice. White bars, control; black bars, CUGBP1 OE. **(c)** PDE3B protein was decreased in the islets isolated from CUGBP1 KD mice ($n=4-6$). White bars, control; grey bars, CUGBP1 KD. **(d, e)** Effect of 10 $\mu\text{mol/l}$ cilostamide on glucose-stimulated **(d)** and GLP-1-stimulated **(e)** insulin secretion in control and CUGBP1 OE islets ($n=4$ groups). Each group contains 40–60 islets isolated from at least two mice. White bars, control; black bars, CUGBP1 OE. **(f, g)** PDE3B protein level was elevated in **(f)** *db/db* and **(g)** HFD-fed mice ($n=4-5$). Cil, cilostamide; CTL, control; Glu, glucose. Data are the mean $\pm$ SEM. $*p<0.05$ and $**p<0.01$ vs control. Exo-CUGBP1, exogenous CUGBP1; endo-CUGBP1, endogenous CUGBP1

insulin secretion were strongly reduced by CUGBP1 OE (Fig. 4d,e). We found that cilostamide markedly enhanced insulin secretion by both control and CUGBP1 OE islets, but abolished the inhibitory effects of CUGBP1 (Fig. 4d). We also showed that GLP-1 induced insulin secretion was no longer reduced by CUGBP1 OE in the presence of cilostamide (Fig. 4e). These results confirm that PDE3B activation is involved in CUGBP1 inhibition of insulin secretion.

Based on these results, we speculated that PDE3B should be overexpressed in the islets of diabetic mice. This was indeed the case: PDE3B protein expression was increased by 11-fold ($p<0.01$; Fig. 4f) and 2.5-fold ($p<0.05$; Fig. 4g) in islets from *db/db* and HFD-fed mice, respectively.

CUGBP1 directly binds to the *PDE3B* 3' UTR and promotes mRNA stabilisation To gain insight into the molecular mechanisms underlying PDE3B activation by CUGBP1, we

performed RIP-CHIP, luciferase reporter, gel shift and mRNA stability assays. RIP-CHIP experiments showed that *PDE3B* mRNA was enriched in the RNA population pulled down by CUGBP1 but not by control IgG, indicating that CUGBP1 binds to *PDE3B* mRNA (Fig. 5a). We further demonstrated that most PDE mRNAs were not pulled down by CUGBP1; an exception was *PDE4B* mRNA (data not shown). As *PDE4B* expression was not affected by CUGBP1 OE (Fig. 3d), the interaction between the two proteins was not further investigated. A luciferase reporter assay was then conducted to determine whether CUGBP1 binds to the *PDE3B* 3' UTR. As CUGBP1 regulates the PDE3B level in both HeLa cells (Fig. 3d, ESM Fig. 6) and mouse islets (Fig. 4a–c), we reasoned that the mechanisms responsible for this regulation may be conserved. Thus, we aligned the 3' UTRs of human, rat and mouse *PDE3B* genes and found that about 1000 bp were conserved among these species. We therefore chose 1144 bp of the human *PDE3B* 3' UTR as the potential CUGBP1 target. As shown in Fig. 5b, luciferase activity was modestly increased in constructs containing the 1–1144 and 1–938 bp 3' UTR sequences but not in constructs containing the 1–557 and 1–756 bp sequences. Moreover, luciferase activity was not significantly different between constructs containing the 1–1144 and 1–938 bp sequences. These results indicate that the CUGBP1-interacting sequence is located in the region from 756 bp to 938 bp. Using a bioinformatics method (www.introni.it/splicing.html; accessed September 2013), two potential binding sites for CUGBP1 were found in this region: one is TTGTTGTTTTACTCT, the other is ATTTGTT. We therefore constructed two luciferase reporters containing a mutated 1–938 bp sequence in which either the TTGTTGTTTTACTCT (mutant-1) or ATTTGTT (mutant-2) sequence was deleted. The luciferase activity of the mutant-1 reporter was still increased by CUGBP1, whereas that of mutant-2 was unchanged, suggesting that CUGBP1 binds to the ATTTGTT sequence. A gel shift assay further demonstrated that CUGBP1 can directly bind to the ATTTGTT sequence in a dose-dependent manner (Fig. 5c). Finally, we found that the rate of *PDE3B* mRNA decay was much slower in CUGBP1 OE cells than in control cells, suggesting that CUGBP1 can stabilise *PDE3B* mRNA (Fig. 5d).

Discussion

In this study, we report that: (1) CUGBP1 is present in both rodent islets and beta cell lines, and is overexpressed in the islets of diabetic mice; (2) CUGBP1 acts as a negative regulator of insulin secretion upon glucose and GLP-1 stimulation; (3) the effects of CUGBP1 on insulin secretion are mediated by PDE3B activation; (4) CUGBP1 directly regulates *PDE3B* expression via binding to the ATTTGTT sequence within its 3'

Fig. 5 CUGBP1 directly interacts with the *PDE3B* 3' UTR and promotes mRNA stabilisation. **(a)** Representative *PDE3B* RT-PCR product after RIP-CHIP. **(b)** Luciferase activity of *PDE3B* 3' UTR constructs. White bars, control; black bars, different *PDE3B* 3' UTR constructs, as indicated. **(c)** PAGE shows that CUGBP1 interacts with the 5'-AUUUGUU-3' sequence. **(d)** HeLa cells were treated with actinomycin D to inhibit gene

transcription and the percentage *PDE3B* mRNA expression (relative to $t=0$) was quantified using qPCR at the indicated time points ($n=4$). White squares, control; black squares: CUGBP1 OE. CUG, CUGBP1; IP, immunoprecipitation; Luc, luciferase. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs control or as indicated.

UTR; and (5) *PDE3B* is overexpressed in the islets of diabetic mice.

In vivo genome editing in adult animals is a topic of intense interest and has had a profound impact on both basic and translational research. Recently, SpCas9 has been proven to be a powerful tool for making precise genomic perturbations in vivo. It has been used in vivo to study the function of genes in the liver and brain [42, 43] and to cure metabolic and genetic diseases in animal models [44]. Here, we demonstrated that SpCas9 can be used to disrupt the genes in adult mouse islets. To our knowledge, this is the first report of the use of SpCas9 to edit genes in adult mouse islets in vivo. Moreover, we found that SpCas9-mediated *Cugbp1* mutation had a strongly increased insulin secretion, reduced glucose homeostasis and significantly downregulated *PDE3B* expression, indicating that in vivo genome editing with SpCas9 is feasible in adult mouse islets.

We have demonstrated for the first time that CUGBP1 is critical for controlling insulin secretion by pancreatic islets. Importantly, CUGBP1 is overexpressed in the islets of diabetic mice, and this presumably leads to insufficient insulin secretion. A previous study indicated that CUGBP1 contributes to insulin resistance in skeletal muscle [28]. Moreover, a recent genome-wide association study suggested that genetic variation at the *CUGBP1* locus is associated with obesity [30], which is a major risk factor for the development of type 2 diabetes. Collectively, these results suggest that CUGBP1 may be an important regulator of type 2 diabetes. Thus, CUGBP1 inhibition might be a potential strategy for the treatment of type 2 diabetes.

In a mechanistic analysis, we identified cAMP and *PDE3B* as the molecules responsible for the effects of CUGBP1. Indeed, accumulating evidence indicates that intracellular cAMP is an important regulator of insulin secretion either dependent or independent of Ca^{2+} [4, 5, 41]. Although several PDE proteins regulate insulin secretion [7–10, 45], only *PDE3B* can be activated by CUGBP1. Actually, *PDE3B* OE results in reduced insulin secretion by islets and beta cells [9, 46, 47]; conversely, GSIS is enhanced in islets from *Pde3b*-null mice [8]. In addition, various hormones including IGF-1 [48] and leptin [49] regulate insulin secretion through phosphorylation of *PDE3B*.

PDE3A and *PDE3B* are well known to be involved in different cellular processes and to participate in distinct diseases [6], but there is no specific inhibitor for each isoform. In this study, we found that the ATTTGTT sequence residing in the 3' UTR of *PDE3B* is required to stabilise *PDE3B* mRNA. We are therefore now in a position to design specific molecular disruptors of *PDE3B* activation using a strategy similar to the one used to inhibit another RNA-binding protein, the Hu antigen R [50].

In summary, our data demonstrate that CUGBP1 is a critical regulator of insulin secretion via activation of *PDE3B*. As CUGBP1 is overexpressed in the islets of diabetic mice, its repression might provide a potential strategy for the treatment of type 2 diabetes.

Acknowledgements We thank D. Zhang, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China, for his help in CUGBP1 protein purification and gel shift experiments. We thank W. Jin, Institute

of Zoology, Beijing, China, for providing *db/db* mice and control mice, H. Lou, Case Western Reserve University, Cleveland, OH, USA, for providing the pcDNA3.1-CUGBP1 plasmid, W. Wei, Peking University, Beijing, China, for providing the pLenti-OC-IRES-BSD plasmid and lentiviral sgRNA vector, Y. Wu, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China, for the firefly and renilla luciferase vectors. We are grateful to Z. Chen, Northeast Normal University, for critically reading this manuscript and for helpful discussion.

Funding This work was supported by grants from the National Basic Research Programme of China (2011CB809104 to GJ), the National Foundation of Sciences and Technology (31371430 to HW) and the State Key Laboratory of Drug Research (SIMM1501KF-12).

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement KZ, VL, HW, RF and GJ conceived and designed the study; KZ, LG, ZY, YM, MJ, YC, QY and HW contributed to data acquisition, analysis and interpretation; KZ, RF, and GJ drafted the article; and KZ, LG, ZY, YM, MJ, YC, QY and HW revised the article critically for important intellectual content. All authors edited the manuscript and approved the final version. GJ is the guarantor of this work and is responsible for the integrity of the work as a whole.

References

- Ashcroft FM, Rorsman P (2012) Diabetes mellitus and the beta cell: the last ten years. *Cell* 148:1160–1171
- Fu Z, Gilbert ER, Liu D (2013) Regulation of insulin synthesis and secretion and pancreatic Beta-cell dysfunction in diabetes. *Curr Diabetes Rev* 9:25–53
- Prentki M, Matschinsky FM, Madiraju SR (2013) Metabolic signaling in fuel-induced insulin secretion. *Cell Metab* 18:162–185
- Dyachok O, Idevall-Hagren O, Sagetorp J et al (2008) Glucose-induced cyclic AMP oscillations regulate pulsatile insulin secretion. *Cell Metab* 8:26–37
- Tian G, Sol ER, Xu Y, Shuai H, Tengholm A (2014) Impaired cAMP generation contributes to defective glucose-stimulated insulin secretion after long-term exposure to palmitate. *Diabetes* 64:904–915
- Omori K, Kotera J (2007) Overview of PDEs and their regulation. *Circ Res* 100:309–327
- Tian G, Sagetorp J, Xu Y, Shuai H, Degerman E, Tengholm A (2012) Role of phosphodiesterases in the shaping of sub-plasma-membrane cAMP oscillations and pulsatile insulin secretion. *J Cell Sci* 125:5084–5095
- Choi YH, Park S, Hockman S et al (2006) Alterations in regulation of energy homeostasis in cyclic nucleotide phosphodiesterase 3B-null mice. *J Clin Invest* 116:3240–3251
- Hamdahl L, Wierup N, Enerback S et al (2004) Beta-cell-targeted overexpression of phosphodiesterase 3B in mice causes impaired insulin secretion, glucose intolerance, and deranged islet morphology. *J Biol Chem* 279:15214–15222
- Dov A, Abramovitch E, Warwar N, Nesher R (2008) Diminished phosphodiesterase-8B potentiates biphasic insulin response to glucose. *Endocrinology* 149:741–748
- Marchmont RJ, Houslay MD (1980) Insulin trigger, cyclic AMP-dependent activation and phosphorylation of a plasma membrane cyclic AMP phosphodiesterase. *Nature* 286:904–906
- Ahmad F, Lindh R, Tang Y, Weston M, Degerman E, Manganiello VC (2007) Insulin-induced formation of macromolecular complexes involved in activation of cyclic nucleotide phosphodiesterase 3B (PDE3B) and its interaction with PKB. *Biochem J* 404:257–268
- Oknianska A, Zmuda-Trzebiatowska E, Manganiello V, Degerman E (2007) Long-term regulation of cyclic nucleotide phosphodiesterase type 3B and 4 in 3T3-L1 adipocytes. *Biochem Biophys Res Commun* 353:1080–1085
- Ke B, Zhao Z, Ye X et al (2015) Inactivation of NF-kappaB p65 (RelA) in liver improved insulin sensitivity and inhibited cAMP/PKA pathway. *Diabetes* 64:3355–3362
- Teplava M, Song J, Gaw HY, Teplav A, Patel DJ (2010) Structural insights into RNA recognition by the alternate-splicing regulator CUG-binding protein 1. *Structure* 18:1364–1377
- Masuda A, Andersen HS, Doktor TK et al (2012) CUGBP1 and MBNL1 preferentially bind to 3' UTRs and facilitate mRNA decay. *Sci Rep* 2:209
- Dasgupta T, Ladd AN (2012) The importance of CELF control: molecular and biological roles of the CUG-BP, Elav-like family of RNA-binding proteins. *Wiley Interdiscip Rev RNA* 3:104–121
- Timchenko NA, Patel R, Iakova P, Cai ZJ, Quan L, Timchenko LT (2004) Overexpression of CUG triplet repeat-binding protein, CUGBP1, in mice inhibits myogenesis. *J Biol Chem* 279:13129–13139
- Lee JE, Cooper TA (2009) Pathogenic mechanisms of myotonic dystrophy. *Biochem Soc Trans* 37:1281–1286
- Ward AJ, Rimer M, Killian JM, Dowling JJ, Cooper TA (2010) CUGBP1 overexpression in mouse skeletal muscle reproduces features of myotonic dystrophy type 1. *Hum Mol Genet* 19:3614–3622
- Tang Y, Wang H, Wei B et al (2015) CUG-BP1 regulates RyR1 ASI alternative splicing in skeletal muscle atrophy. *Sci Rep* 5:16083
- Iakova P, Wang GL, Timchenko L et al (2004) Competition of CUGBP1 and calreticulin for the regulation of p21 translation determines cell fate. *EMBO J* 23:406–417
- Kalsotra A, Xiao X, Ward AJ et al (2008) A postnatal switch of CELF and MBNL proteins reprograms alternative splicing in the developing heart. *Proc Natl Acad Sci U S A* 105:20333–20338
- Koshelev M, Sarma S, Price RE, Wehrens XH, Cooper TA (2010) Heart-specific overexpression of CUGBP1 reproduces functional and molecular abnormalities of myotonic dystrophy type 1. *Hum Mol Genet* 19:1066–1075
- Giudice J, Xia Z, Wang ET et al (2014) Alternative splicing regulates vesicular trafficking genes in cardiomyocytes during postnatal heart development. *Nat Commun* 5:3603
- House RP, Talwar S, Hazard ES, Hill EG, Palanisamy V (2015) RNA-binding protein CELF1 promotes tumor growth and alters gene expression in oral squamous cell carcinoma. *Oncotarget* 6:43620–43634
- Sen S, Talukdar I, Webster NJ (2009) SRp20 and CUG-BP1 modulate insulin receptor exon 11 alternative splicing. *Mol Cell Biol* 29:871–880
- Savkur RS, Philips AV, Cooper TA (2001) Aberrant regulation of insulin receptor alternative splicing is associated with insulin resistance in myotonic dystrophy. *Nat Genet* 29:40–47
- Verma SK, Deshmukh V, Liu P et al (2013) Reactivation of fetal splicing programs in diabetic hearts is mediated by protein kinase C signaling. *J Biol Chem* 288:35372–35386
- Hinney A, Albayrak O, Antel J et al (2014) Genetic variation at the CELF1 (CUGBP, elav-like family member 1 gene) locus is genome-wide associated with Alzheimer's disease and obesity. *Am J Med Genet B* 165:283–293
- Fujii N, Ho RC, Manabe Y et al (2008) Ablation of AMP-activated protein kinase alpha2 activity exacerbates insulin resistance induced by high-fat feeding of mice. *Diabetes* 57:2958–2966

32. Brissova M, Fowler M, Wiebe P et al (2004) Intraislet endothelial cells contribute to revascularization of transplanted pancreatic islets. *Diabetes* 53:1318–1325
33. Zhai K, Chang Y, Wei B et al (2014) Phosphodiesterase types 3 and 4 regulate the phasic contraction of neonatal rat bladder smooth myocytes via distinct mechanisms. *Cell Signal* 26:1001–1010
34. Bindom SM, Hans CP, Xia HJ, Boulares H, Lazartigues E (2010) Angiotensin I-converting enzyme type 2 (ACE2) gene therapy improves glycemic control in diabetic mice. *Diabetes* 59:2540–2548
35. Zhai K, Hubert F, Nicolas V, Ji G, Fischmeister R, Leblais V (2012) beta-Adrenergic cAMP signals are predominantly regulated by phosphodiesterase type 4 in cultured adult rat aortic smooth muscle cells. *PLoS One* 7:e47826
36. Lee JE, Lee JY, Wilusz J, Tian B, Wilusz CJ (2010) Systematic analysis of cis-elements in unstable mRNAs demonstrates that CUGBP1 is a key regulator of mRNA decay in muscle cells. *PLoS One* 5:e11201
37. Lee J, Shin MK, Ryu DK, Kim S, Ryu WS (2010) Insertion and deletion mutagenesis by overlap extension PCR. *Methods Mol Biol* 634:137–146
38. Lu JY, Sewer MB (2015) p54(nrb)/NONO Regulates cyclic AMP-dependent glucocorticoid production by modulating phosphodiesterase mRNA splicing and degradation. *Mol Cell Biol* 35:1223–1237
39. Meloni AR, DeYoung MB, Lowe C, Parkes DG (2013) GLP-1 receptor activated insulin secretion from pancreatic beta-cells: mechanism and glucose dependence. *Diabetes Obes Metab* 15: 15–27
40. Chen Z, Li Z, Wei B et al (2010) FKBP12.6-knockout mice display hyperinsulinemia and resistance to high-fat diet-induced hyperglycemia. *FASEB J* 24:357–363
41. Charles MA, Fanska R, Schmid FG, Forsham PH, Grodsky GM (1973) Adenosine 3',5'-monophosphate in pancreatic islets: glucose-induced insulin release. *Science* 179:569–571
42. Xue W, Chen SD, Yin H et al (2014) CRISPR-mediated direct mutation of cancer genes in the mouse liver. *Nature* 514:380–384
43. Swiech L, Heidenreich M, Banerjee A et al (2015) In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9. *Nat Biotechnol* 33:102–106
44. Bakondi B, Lv W, Lu B et al (2015) In vivo CRISPR/Cas9 gene editing corrects retinal dystrophy in the S334ter-3 rat model of autosomal dominant retinitis pigmentosa. *Mol Ther* 24:556–563
45. Pyne NJ, Furman BL (2003) Cyclic nucleotide phosphodiesterases in pancreatic islets. *Diabetologia* 46:1179–1189
46. Harndahl L, Jing XJ, Ivarsson R et al (2002) Important role of phosphodiesterase 3B for the stimulatory action of cAMP on pancreatic beta-cell exocytosis and release of insulin. *J Biol Chem* 277: 37446–37455
47. Walz HA, Wierup N, Vikman J et al (2007) Beta-cell PDE3B regulates Ca^{2+} -stimulated exocytosis of insulin. *Cell Signal* 19:1505–1513
48. Zhao AZ, Zhao H, Teague J, Fujimoto W, Beavo JA (1997) Attenuation of insulin secretion by insulin-like growth factor 1 is mediated through activation of phosphodiesterase 3B. *Proc Natl Acad Sci U S A* 94:3223–3228
49. Zhao AZ, Bornfeldt KE, Beavo JA (1998) Leptin inhibits insulin secretion by activation of phosphodiesterase 3B. *J Clin Invest* 102: 869–973
50. Wu X, Lan L, Wilson DM et al (2015) Identification and validation of novel small molecule disruptors of HuR-mRNA interaction. *ACS Chem Biol* 10:1476–1484