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RNA-binding protein HuD reduces triglyceride production in pancreatic β cells by enhancing the expression of insulin-induced gene 1

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Abstract

Although triglyceride (TG) accumulation in the pancreas leads to β-cell dysfunction and raises the chance to develop metabolic disorders such as type 2 diabetes (T2DM), the molecular mechanisms whereby intracellular TG levels are regulated in pancreatic β cells have not been fully elucidated. Here, we present evidence that the RNA-binding protein HuD regulates TG production in pancreatic β cells. Mouse insulinoma βTC6 cells stably expressing a small hairpin RNA targeting HuD (shHuD) (βTC6-shHuD) contained higher TG levels compared to control cells. Moreover, downregulation of HuD resulted in a decrease in insulin-induced gene 1 (INSIG1) levels but not in the levels of sterol regulatory element-binding protein 1c (SREBP1c), a key transcription factor for lipid production. We identified *Insig1* mRNA as a direct target of HuD by using ribonucleoprotein immunoprecipitation (RIP) and biotin pulldown analyses. By associating with the 3′-untranslated region (3′UTR) of *Insig1* mRNA, HuD promoted INSIG1 translation; accordingly, HuD downregulation reduced while ectopic HuD expression increased INSIG1 levels. We further observed that HuD downregulation facilitated the nuclear localization of SREBP1c, thereby increasing the transcriptional activity of SREBP1c and the expression of target genes involved in lipogenesis; likewise, we observed lower INSIG1 levels in the pancreatic islets of

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Transparency document

The Transparency document associated with this article can be found, in online version.

Appendix A. Supplementary data

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HuD-null mice. Taken together, our results indicate that HuD functions as a novel repressor of lipid synthesis in pancreatic β cells.

Keywords

RNA-binding protein; HuD; Triglyceride; INSIG1; SREBP1c

1. Introduction

Elevated serum levels of free fatty acids (FFAs) are closely related to the development of metabolic diseases including type 2 diabetes and heart disease [1,2]. Fatty acids (FAs) are mainly stored in triglyceride (TG) form in lipid droplets in adipocytes; however, non-adipose tissues such as myocytes and pancreatic β cells also have limited capacity for lipid storage. TG accumulation in pancreatic β cells in response to lipid overload represents an initial cellular defense mechanism against lipotoxicity [2]. However, excessive FFA levels facilitate intracellular TG accumulation, increase intracellular free fatty acyls, and induce lipotoxicity of pancreatic β cells, leading to dysregulation of insulin secretion and apoptotic cell death [3–5]. The amounts of pancreatic TGs in patients with type 2 diabetes mellitus (T2DM) are higher than those in non-diabetic populations, suggesting that lipid content may contribute to the development of T2DM [6]. Although TG accumulation in the pancreas is closely related with β cell dysfunction and T2DM pathogenesis, the specific mechanisms governing the intracellular accumulation of TGs in pancreatic β cells are unknown.

The sterol regulatory element binding proteins (SREBPs) are lipogenic transcription factors which critically maintain lipid homeostasis by activating gene expression necessary involved in the biosynthesis of various cellular lipids [7–9]. In particular, SREBP1c mediates lipogenic gene expression in insulin-sensitive tissues and organs such as the adipose tissues, the liver, and the pancreas [10–12]. Moreover, aberrant levels or activities of SREBP1c in pancreatic β cells contribute to β-cell dysfunction and the pathogenesis of T2DM [12–14]. The transcriptional activity of SREBPs is regulated via insulin-induced gene 1 (INSIG1)-SREBP cleavage activation protein (SCAP)-SREBP pathway in response to cellular glucose or FA levels [15]. INSIG1 is an important upstream regulator of lipogenic gene expression by binding to SCAP-SREBPs in the endoplasmic reticulum (ER), thus prohibiting the activation of SREBPs [15]. Overexpression of INSIG1 inhibits lipid accumulation and FFA synthesis and protects β cells against glucolipotoxicity via SREBP1c [16].

Regulation of gene expression at the RNA level underlies several aspects of biological and pathological events. RNA-binding proteins (RBPs) and non-coding RNAs (ncRNAs) including microRNAs (miRNAs) have been implicated in post-transcriptional gene regulatory processes: splicing of pre-mRNA, as well as mRNA transport, editing, stability, translation and localization [17,18]. In particular, RBPs recognize a wide range of RNAs via various RNA-binding modules such as the RNA-recognition motif (RRM), the K-homology (KH) domain, RNA-binding zinc-finger (ZnF) domain, and the double-stranded RNA-binding domain (dsRBD). Through interaction with target mRNAs, RBPs regulate target gene expression post-transcriptionally and thus modulate cell fate by controlling

proliferation, death, differentiation and senescence [19,20]. HuD belongs to the human antigen (Hu) family and regulates gene expression by affecting the stability and translation of the mRNAs with which it interacts [21]. HuD associates with cis elements of target mRNAs and plays essential roles not only in brain development but also in neuronal outgrowth in the brain [22–26]. In addition, our previous studies revealed that HuD is essential for insulin biosynthesis and autophagosome formation in pancreatic β cells [27–28].

Here, we describe a novel function of HuD in the regulation of cellular triglyceride levels in pancreatic β cells. We observed that downregulation of HuD promotes the accumulation of triglycerides by decreasing INSIG1 expression at the post-transcriptional level, thereby enhancing the nuclear translocation of SREBP1c, a critical factor for lipogenesis.

2. Materials and methods

2.1. Cell culture, treatment, and transfection of siRNAs and plasmids

Mouse insulinoma βTC6 cells were cultured in Dulbecco's modified essential medium (DMEM, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS) and antibiotics. βTC6 cells stably expressing shCtrl or shHuD were was established by screening with 2 μg/ml puromycin (Invitrogen, Carlsbad, CA, USA). Control small interfering RNA (siCtrl), HuD siRNA, shCtrl (pMX-puro), shHuD plasmids were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All plasmids, including myc-tagged HuD (pHuD), enhanced green fluorescent protein (EGFP) reporter plasmids, and luciferase reporter vectors (pGL3-promoter-Luc and pGL3-SREX3-Luc), were transfected with Lipofectamine™ RNAiMAX or Lipofectamine™ 2000 (Invitrogen). EGFP reporters were cloned by inserting 3′UTR-3 (3U-3) fragments of the *Insig1* mRNA into the pEGFP-C1 (BD Bioscience, Heidelberg, Germany). Palmitate was prepared in 0.1 N NaOH/70% ethanol [29].

2.2. Western blot analysis

Whole-cell lysates were prepared using RIPA buffer [10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1 mM EDTA, and 0.1% SDS], separated by SDS-PAGE, and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Belfor, MA, USA). Incubations with primary antibodies to detect INSIG1 (Abcam, Cambridge, MA, USA, ab70784, rabbit polyclonal), β-actin (Abcam, ab3280, mouse monoclonal), HuD (Santa Cruz Biotechnology, sc-28299, mouse monoclonal), SREBP1c (Santa Cruz Biotechnology, sc-366, rabbit polyclonal), lamin B (Santa Cruz Biotechnology, sc-6216, goat polyclonal), GAPDH (Santa Cruz Biotechnology, sc-32233, mouse monoclonal), and EGFP (Santa Cruz Biotechnology, sc-9996, mouse monoclonal) were followed by incubations with the appropriate secondary antibodies conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotechnology) and by detection using enhanced luminescence (Clarity™ Western ECL Substrate Kit, Bio-Rad, Hercules, California, USA).

2.3. RNA analysis and ribonucleoprotein (RNP) immunoprecipitation (RIP) analysis

Total RNA was prepared from whole cells using TRIzol® Reagent (Life Technologies, Inc., Carlsbad, CA, USA). After reverse transcription (RT) using ReverTra Ace® qPCR RT Kit (Toyobo, Japan), the abundance of transcripts was assessed by real-time quantitative (q)PCR analysis using KAPA SYBR® FAST qPCR Kits (Kapa Biosystems, Inc., Woburn, MA, USA) and gene-specific primer sets (Table 1). RT-qPCR analysis was performed on StepOnePlus™ (Applied Biosystems, Foster City, CA, US). The efficiency of primer pairs was investigated using StepOnePlus™ Software v2.3 (data not shown). RIP analysis was performed using primary antibodies anti-HuD or control IgG (Santa Cruz Biotechnology) [27]. In brief, RNP complexes were immunoprecipitated using anti-HuD or control IgG antibodies, and incubated with DNase I and Proteinase K; RNA in the IP samples was isolated and further analyzed by RT-qPCR using the primers listed in Table 1.

2.4. Biotin pull down analysis

To synthesize biotinylated transcripts, PCR fragments were prepared using forward primers that contained the T7 RNA polymerase promoter sequence [(T7), CCAAGCTTCTAATACGACTCACTATAGGGAGA]. Primers used to prepare biotinylated transcripts spanning the *Insig1* mRNA (NM_153526.5) are listed in Table 1. After purification of the PCR products, biotinylated transcripts were synthesized using MaxiScript® T7 kit (Ambion, Austin, TX, USA) and biotin-CTP (Enzo Life Sciences, Inc., Farmingdale, NY, USA). Whole-cell lysates (200 μg per sample) were incubated with 1 μg of purified biotinylated transcripts for 30 min at room temperature, and then complexes were isolated with Streptavidin-coupled Dynabeads® M-280 Streptavidin (Invitrogen). The interaction between HuD and biotin-labeled transcripts were analyzed by Western blot analysis [28].

2.5. Oil-Red-O staining and TG assay

Cells were fixed with 10% (*v*/*v*) formalin and were stained with Oil-Red-O (Sigma–Aldrich, St. Louis, MO, USA) [16]. Stained lipid droplets in βTC6 cells were observed and photographed under a microscope (Axiovert 200; Carl Zeiss, Oberkochen, Germany). Intracellular TG content was determined on total cell extracts using a serum TG determination kit (Sigma-Aldrich) following the manufacturer's protocols. Briefly, cells were rinsed three times with phosphate buffered saline (PBS) and lysed with RIPA buffer; after incubating the lysates with at 37 °C for 5 min, absorbance was read at 540 nm by spectrometry (Victor3, Perkin Elmer, Inc., Waltham, MA, USA).

2.6. Subcellular fractionation

Nuclear fractions were prepared by centrifugation after incubation with digitonin [30]. In brief, cells were suspended in hypotonic buffer (20 mM Tris–HCl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 0.5% NP-40) and incubated with 40 μg/ml digitonin for 10 min on ice. After centrifugation at 2100 ×*g*, 4 °C for 10 min, the supernatant was collected (cytosolic fraction). Insoluble fractions were washed, and incubated in RIPA buffer; the nuclear fractions were collected after centrifugation at 14,000 ×*g* for 10 min at 4 °C.

2.7. Polysome analysis

Forty-eight hours after transfection of siHuD and pHuD with appropriate controls, βTC6 cells were incubated with 100 μg/ml cycloheximide for 15 min and lysed with polysome extraction buffer [20 mM Tris–HCl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 0.5% (*v*/v) Nonidet P-40, 1× protease inhibitor mixture, and RNase inhibitor]. Polysome extracts were collected by centrifugation at 10,000 ×*g* for 10 min, and then the lysates were further fractionated by ultracentrifugation through linear sucrose gradients [31]. RNAs from each fraction were isolated and cDNA was synthesized as described above. The relative levels of *Insig1* and *Gapdh* mRNAs were analyzed by RT-qPCR using specific primer set (Table 1).

2.8. De novo protein synthesis analysis

Nascent translation of EGFP was examined by incubating βTC6 cells with 1 mCi L-[^{35}S]methionine and L-[^{35}S]cysteine (PerkinElmer Life Sciences, Shelton, CT, USA) per 35-mm plate for 15 min. After lysis with RIPA buffer, IP reactions were carried out using mouse IgG or anti-GFP (Santa Cruz Biotechnology), and the immunoprecipitated material was separated by SDS-PAGE and transferred to PVDF membranes [27–28]. Images were obtained by Pharose FX Plus system (Bio-Rad) and analyzed using Quantity One software (Bio-Rad).

2.9. Luciferase assay

Cells were lysed with 200 μl of 1× reporter lysis buffer, and 20 μl of which was used for measurement of luciferase activities using the Dual-Luciferase® reporter assay system (Promega, Madison, WI, USA). Luminescence was measured using a luminometer (Berthold Technologies, Bad Wildbad, Germany). The amount of luciferase activity in each transfection group was normalized to the concentration of protein measured using Bradford assay.

2.10. Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde, immersed in 20% sucrose before freezing, and then sectioned at a thickness of 5 μm. After antigen unmasking, slides were incubated with anti-INSIG1 (Abcam, ab70784) antibody and then with secondary antibody. Microscopy was performed with a LSM-710 microscope (Carl Zeiss MicroImaging).

2.11. Statistical analysis

All experiments were conducted at least three times and all samples were analyzed in triplicate. Results are presented as the mean ± SEM. The statistical significance was calculated by unpaired Student's *t*-test.

3. Results

3.1. Downregulation of HuD promotes accumulation of triglycerides in pancreatic β cells

Intracellular lipid accumulation in the pancreas has been linked to pancreatic βcell dysfunction; however, the mechanisms that regulate lipid accumulation in β cells is not fully elucidated [14,17]. To assess the role of the RNA-binding protein HuD in this process,

we generated stable cell lines derived from mouse pancreatic insulinoma βTC6 cells in which HuD was silenced through stable expression of shHuD (Fig. 1a). We analyzed the relative lipid accumulation in βTC6 cells stably expressing shHuD (βTC6-shHuD) compared to control cells (βTC6-shCtrl). As shown in Fig. 1b, the relative lipid levels in both cell lines were assessed by Oil Red O staining, and enhanced lipid accumulation was seen in βTC6-shHuD cells. Since cellular lipids are stored as triglycerides (TGs), we further measured TG content using a triglyceride determination kit. TG content increased in both βTC6-shHuD and βTC6-shCtrl cells during culture; however, downregulation of HuD significantly increased TG levels (Fig. 1c). These results indicate that HuD has a novel function in the regulation of cellular lipid level in pancreatic β cells.

Sterol regulatory element binding protein (SREBP) function as key regulators of lipid metabolism by regulating gene expression involved in lipid synthesis; SREBP1c in particular is known to be related to pancreatic βcell dysfunction [13,32]. Thus, we assessed SREBP1c expression levels in both βTC6-shCtrl and βTC6-shHuD cells by Western blot analysis and found that the levels of SREBP1c remained constant. However, in βTC6-shHuD cells we observed a specific decrease in INSIG1, a negative regulator of SREBP-mediated regulation of lipid synthesis (Fig. 1d). Taken together, these results indicate that downregulation of HuD might result in a decrease of INSIG1 expression linked to an increase in TG accumulation.

3.2. HuD regulates INSIG1 expression by interacting with its 3′UTR

HuD belongs to the Hu family of RBPs that regulates various aspects of RNA metabolism; HuD in particular modulates the translational efficiency and turnover of mRNA in brain and pancreatic β cells [22,23, 26–28]. Given that INSIG1 levels were lower in βTC6-shHuD cells (Fig. 1d), we examined whether HuD regulates INSIG1 expression at the RNA level in pancreatic β cells. To test this possibility, we performed RIP analysis using an anti-HuD antibody followed by RNA extraction and RT-qPCR analysis. Our results revealed an enrichment in *Insig1* mRNA in the RNP complexes containing HuD (Fig. 2a). We then sought to identify the regions of interaction by testing biotinylated fragments of *Insig1* mRNA (Fig. 2b) spanning the 5′UTR (5U), coding region (CR), and 3′UTRs (3U-1, 3U-2, and 3U-3) after incubation with cell lysates, the interaction between HuD and biotinylated RNA fragments was assessed by Western blotting using anti-HuD antibody. As shown in Fig. 2b, HuD was capable of associating selectively with the 3′UTR (segment 3U-3) of *Insig1* mRNA. To further evaluate if fragment 3U-3 was essential for INSIG1 regulation by HuD, we generated EGFP reporter constructs containing the 3U-3 sequence of *Insig1* mRNA after the EGFP coding sequence (pEGFP + *Insig1* 3U-3; Fig. 2c). The constructs were transfected into βTC6 cells expressing normal (siCtrl) or reduced HuD (siHuD) as well as into cells overexpressing HuD (pHuD) or a control plasmid (pcDNA); the relative EGFP expression was assessed by Western blotting (Fig. 2d). HuD silencing selectively decreased EGFP expression in the pEGFP + *Insig1* 3U-3 group, but not in the control reporter group (pEGFP). Conversely, HuD overexpression upregulated slightly EGFP expression in the pEGFP + *Insig1* 3U-3 group. Taken together, these results indicate that the 3′UTR of *Insig1* mRNA (3U-3) participates in the regulation of INSIG1 expression by HuD.

3.3. HuD promotes translation of Insig1 mRNA

Next, we tested whether HuD regulates endogenous INSIG1 expression in βTC6 cells by transiently overexpressing HuD using a plasmid vector or silencing HuD using siRNAs directed at HuD, then measuring *Insig1* mRNA and INSIG1 protein levels by RT-qPCR and Western blotting analyses, respectively. Neither HuD silencing nor overexpression significantly changed *Insig1* mRNA levels (Fig. 3a). However, silencing HuD decreased INSIG1 levels and overexpressing HuD increased it (Fig. 3b), suggesting that HuD may regulate INSIG1 expression by promoting its translation. To test this hypothesis further, we examined the distribution of *Insig1* mRNA on polysomes of βTC6 cells transfected with HuD siRNA or plasmids (along with controls) [28]. Polyribosomes (polysomes) were fractionated by centrifugation through sucrose density gradients and 12 fractions were collected. As shown in Fig. 3c, polysomes were observed in fractions 6–12, and the overall polysome profiles were not affected by HuD silencing (siCtrl compared with siHuD) or by HuD overexpression (pcDNA compared with pHuD); differences in the overall distribution of gradients may reflect differences in cell toxicity after transfection or differences in gradients (Fig. 3c, left). Consistently, however, *Insig1* mRNA distribution shifted substantially among the fractions after HuD silencing or overexpression: *Insig1* mRNA was found preferentially in lighter polysomes (fraction 6) of HuD siRNA-transfected cells, while it was found in heavier fractions (fractions 7 and 8) in HuD-overexpressing cells (Fig. 3c, right). The distribution of *Gapdh* mRNA remained unchanged among the different groups. These observations suggest that HuD silencing decreases the association of *Insig1* mRNA with polysomes, and HuD overexpression enhances it, in agreement with a role for HuD as enhancer of INSIG1 translation.

To test the translational regulation of *Insig1* mRNA by HuD using an alternative method, we sought to investigate the nascent translation of *Insig1* mRNA after HuD silencing or overexpression; unfortunately, the anti-INSIG1 antibody used in this study did not work for immunoprecipitation (data not shown). Instead, we analyzed *de novo* synthesis of [^{35}S]EGFP reporter protein from plasmid pEGFP + *Insig1*-3U-3 by immunoprecipitation using anti-EGFP antibody. As expected, nascent EGFP translation decreased as a result of HuD silencing and was enhanced by HuD overexpression (Fig. 3d). These results indicate that HuD promotes INSIG1 expression by enhancing translation of *Insig1* mRNA.

3.4. HuD silencing increases the transcriptional activity of SREBP1c in pancreatic β cells

As shown in Figs. 1 and 3, downregulation of HuD decreased the expression of INSIG1, a negative regulator of SREBP-mediated lipid biosynthesis, and increased TG content in βTC6 cells without a significant increase in SREBP1c. Because SREBP1c activates gene expression involved in the biosynthesis of FFA and TG [33,34], and its activity is regulated through proteolytic cleavage-dependent translocation from the cytoplasm to the nucleus [35], we sought to test whether downregulation of HuD increased the transcriptional activity of SREBP1c after stimulation with palmitate, an SREBP1c activator [32,33,36,37]. To this end, we first examined the translocation of SREBP1c in HuD-silenced cells after incubation with 0.4 mM palmitate. The relative level of nuclear SREBP1c was determined by fractionation followed by Western blot analysis. As shown in Fig. 4a, HuD silencing slightly increased nuclear SREBP1c levels, and palmitate treatment enhanced its accumulation in the

nucleus compared to the untreated group. To further test whether HuD silencing affected the transcriptional activity of SREBP1c, we investigated the relative expression of target genes involved in lipogenesis. HuD silencing resulted in an increase in the expression of two lipogenic genes, *Fasn* and *Gpat*, and the increase in expression of those genes was enhanced after palmitate treatment (Fig. 4b). These results suggest that downregulation of HuD increases the expression of lipogenic genes by promoting the nuclear localization of SREBP1c, thereby enhancing its transcriptional activity.

To further test if downregulation of HuD raises the transcriptional activity of SREBP1c, we generated a luciferase reporter construct (pGL3-SRE × 3-Luc) containing three copies of the sterol response element (SRE; ATCACCCCAC) as described in Ref. [8] (Fig. 4c). The transcriptional activity of SREBP1c was assessed by measuring luciferase activity in βTC6-shCtrl cells and βTC6-shHuD cells. The relative luciferase activity from SRE-containing vector was higher than that seen from the control luciferase reporter, indicating that endogenous SREBPs are functional (Fig. 4d). Importantly, βTC6-shHuD cells also displayed higher reporter activity, while palmitate treatment further enhanced the transcriptional activity of SREBPs. Besides transient transfection of siRNAs, we also investigated the relative nuclear SREBP1c levels in stable cell lines with or without palmitate treatment and observed an increase in nuclear translocation of SREBP1c in βTC6-shHuD cells after palmitate treatment (Supplementary Fig. 1a). Finally, the expression of SREBP target genes was increased in βTC6-shHuD cells and this regulation was enhanced after palmitate treatment (Supplementary Fig. 1b). Taken together, these results indicate that downregulation of HuD promotes the nuclear translocation of SREBP1c, thereby increasing the expression of lipogenic genes.

3.5. Differential expression of INSIG1 and HuD in the pancreas of HuD-null mice and db/db mice

After gaining evidence that HuD increased INSIG1 expression and enhanced the transcriptional actions of SREBP1c, we examined whether HuD might also regulate INSIG1 expression in pancreatic β cells in vivo. Immunohistochemical analysis of pancreases from HuD *null* mice (HuD−/−) using anti-INSIG1 antibody revealed that INSIG1 levels were slightly downregulated in HuD−/− mice compared with control mice (HuD+/+) (Fig. 5). This result supports the hypothesis that HuD functions as a regulator of lipid regulation through its effects on INSIG1 expression in pancreatic β cells. Since previous reports indicated that a rise in intracellular TG in the pancreas was responsible for the pathogenesis of metabolic disorder including T2DM [1,4,6], we hypothesized that HuD may be downregulated in a model system of diabetes. To test this possibility, we investigated the relative expression levels of HuD in the pancreas of *db/db* mice, an animal models to study T2DM. *HuD* mRNA, as assessed by RT-qPCR, and HuD protein, as assessed by Western blot analysis, were lower in *db/db* mice (Fig. 6a and b). In addition, the relative levels of HuD and INSIG1 in islets of the pancreas, as assessed by immunohistochemistry, decreased in *db/db* mice (Fig. 6c). Taken together, HuD regulates intracellular TG homeostasis in pancreatic β cells; decreasing HuD elevates TG production and contributes to βcell dysfunction.

4. Discussion

Energy surplus results in TG accumulation in adipose tissues as well as non-adipose tissues including myocytes, hepatocytes, and pancreatic β cells, which leads to internal organ steatosis and organ dysfunction [6, 38–41]. It was previously reported that patients with T2DM showed higher pancreatic TG levels compared to non-diabetic cohorts, and intracellular lipid accumulation in the pancreas appeared even before the onset of T2DM associated with obese or pre-diabetic status [6,41, 42]. Therefore, reduction in TG levels favorably contributes to preventing pancreatic steatosis and βcell dysfunction. However, the mechanisms responsible for fine-tuning intracellular TG level in pancreatic β cells in response to various stimuli are largely unknown. In this study, we present evidence that the RNA-binding protein HuD plays an essential role in the regulation of TG accumulation in pancreatic β-cells. We show that downregulation of HuD resulted in an increase in intracellular TG levels in βTC6 cells by post-transcriptionally reducing the levels of INSIG1, a negative regulator of cellular lipid biosynthesis, and that HuD downregulation promoted the nuclear localization of SREBP1c, thereby facilitating lipogenic gene expression (Fig. 6).

INSIGs, including INSIG1 and INSIG2, regulate lipid production by binding to SCAP and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase). INSIGs promote the retention of SCAP-SREBP complexes in the ER and ubiquitin-proteasomal degradation of HMG-CoA reductase, thereby influencing lipid metabolism, lipogenesis, and glucose homeostasis in diverse tissues [43,44]. When cellular lipid levels decline or higher levels of nuclear SREBPs are required, the interaction between the SCAP and INSIG decreases and the translocation of SCAP-SREBP complex into the Golgi apparatus increases. In the Golgi apparatus, two resident proteases sequentially process the SREBP precursor and release mature SREBP that is rapidly mobilized to the nucleus [44,45]. We observed that HuD silencing in pancreatic βTC6 cells downregulated INSIG1 expression and resulted in an increase in nuclear SREBP1c (Fig. 4), uncovering a novel function for HuD in cellular lipid maintenance. Although INSIG proteins also direct cholesterol synthesis by interacting with HMG-CoA reductase and promoting its proteasomal degradation, we did not investigate whether HuD influences cholesterol biosynthesis in pancreatic β cells in this study. It is possible that cellular cholesterol levels are also differentially regulated depending on the relative abundance of HuD, since HuD directly affects the levels of INSIG proteins, which are upstream regulators of cholesterol biosynthesis. Further study is required to elucidate the exhaustively the roles of HuD in lipid homeostasis.

The expression of INSIGs is regulated at the transcriptional, posttranscriptional, and post-translational levels. SREBPs transcriptionally activate *Insig1* genes in βTC6 cells in response to cellular lipid levels [46,47]. Ubiquitination-mediated degradation of INSIG1 by the proteasome is another important regulatory step for maintaining cellular lipid homeostasis [48,49]. Likewise, insulin, leptin, and several reagents including wortmannin, unsaturated fatty acids, and silibinin are also widely involved in the regulation of INSIG expression in various cell types [46,50–54]. Recently, it was also reported that one of the SREBP-responsive miRNAs, miR-96, functions as a negative regulator of INSIG2

expression [55]. Adding to this complex regulatory paradigm, our data indicate that the regulation of *Insig1* mRNA translation is an additional mechanism governing INSIG1 production, thus influencing SREBP-mediated TG accumulation in pancreatic β cells. We observed that HuD increased INSIG1 protein levels without changing *Insig1* mRNA levels significantly (Fig. 3B) and thus hypothesized that HuD regulated the translation rather than the stability of *Insig1* mRNA. When we investigated the alternative possibility that HuD might reduce INSIG1 protein stability, we observed no significant changes in INSIG1 protein levels after up to 6 h of cyclohexamide treatment (data not shown); additional studies will be needed to fully examine whether HuD influences INSIG1 protein stability.

HuD is a turnover- and translation-regulatory RNA-binding protein (TTR-RBP), expressed mainly in the brain, where it regulates neuronal outgrowth and brain development at the post-transcriptional level by affecting RNA metabolism including mRNA stability or translation [22,56,57]. Our previous studies showed that HuD is also found in pancreatic β cells, where it regulates insulin biosynthesis and autophagosome formation [27,28], establishing HuD as a pivotal post-transcriptional regulator of gene expression in pancreatic β cells. Insulin or glucose stimulation increases the production of *HuD* mRNA, while miR-375 lowers HuD levels, indicating that HuD expression is regulated by transcriptional and post-transcriptional mechanisms [27,58]. Additionally, HuD methylation by CARM1 and phosphorylation by protein kinase C or Akt affect the RNA-binding activity of HuD and determine the expression of HuD target genes [59–62]. However, the regulation of HuD expression in certain cell types during various physiological and pathological conditions has not been studied extensively. Whether HuD expression or activity is differentially regulated in pancreatic β cells or various stage of diabetes was not investigated in this study and will be addressed in a separate study. Such an expansion of this work will provide critical insight into the possible roles of HuD during the maintenance of βcell homeostasis and the pathogenesis of diabetes-related diseases.

Taken together, our data support a model whereby HuD has a pivotal role in the maintenance of lipid homeostasis by enhancing the expression of INSIG1 expression, thereby regulating intracellular TG accumulation (Fig. 6d). We propose that the HuD-INSIG1 regulatory axis plays an essential role in the maintenance of intracellular TG homeostasis and provides novel insight into the intracellular mechanisms that control TG levels. Further studies are warranted in order to examine whether HuD expression or activity is differentially regulated during the pathogenesis of metabolic diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

[1]. Paolisso G, Tataranni PA, Foley JE, Bogardus C, Howard BV, Ravussin E, A high concentration of fasting plasma non-esterified fatty acids is a risk factor for the development of NIDDM, Diabetologia 38 (1995) 1213–1217. [PubMed: 8690174]

[2]. Listenberger LL, Han X, Lewis SE, Cases S, Farese RV Jr., Ory DS, Schaffer JE, Triglyceride accumulation protects against fatty acid-induced lipotoxicity, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 3077–3082. [PubMed: 12629214]

[3]. Prentki M, Vischer S, Glennon MC, Regazzi R, Deeney JT, Corkey BE, Malonyl-CoA and long chain acyl-CoA esters as metabolic coupling factors in nutrient-induced insulin secretion, J. Biol. Chem 267 (1992) 5802–5810. [PubMed: 1556096]

[4]. Zhou YP, Grill V, Long term exposure to fatty acids and ketones inhibits B-cell functions in human pancreatic islets of Langerhans, J. Clin. Endocrinol. Metab 80 (1995) 1584–1590. [PubMed: 7745004]

[5]. Shimabukuro M, Zhou YT, Levi M, Unger RH, Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 2498–2502. [PubMed: 9482914]

[6]. Tushuizen ME, Bunck MC, Pouwels PJ, Bontemps S, van Waesberghe JH, Schindhelm RK, Mari A, Heine RJ, Diamant M, Pancreatic fat content and beta-cell function in men with and without type 2 diabetes, Diabetes Care 30 (2007) 2916–2921. [PubMed: 17666465]

[7]. Sakai J, Duncan EA, Rawson RB, Hua X, Brown MS, Goldstein JL, Sterol-regulated release of SREBP-2 from cell membranes requires two sequential cleavages, one within a transmembrane segment, Cell 85 (1996) 1037–1046. [PubMed: 8674110]

[8]. Briggs MR, Yokoyama C, Wang X, Brown MS, Goldstein JL, Nuclear protein that binds sterol regulatory element of low density lipoprotein receptor promoter. I. Identification of the protein and delineation of its target nucleotide sequence, J. Biol. Chem 268 (1993) 14490–14496. [PubMed: 8390995]

[9]. Wang X, Sato R, Brown MS, Hua X, Goldstein JL, SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis, Cell 77 (1994) 53–62. [PubMed: 8156598]

[10]. Kim JB, Sarraf P, Wright M, Yao KM, Mueller E, Solanes G, Lowell BB, Spiegelman BM, Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1, J. Clin. Invest 101 (1998) 1–9. [PubMed: 9421459]

[11]. Foretz M, Guichard C, Ferre P, Foufelle F, Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 12737–12742. [PubMed: 10535992]

[12]. Wang H, Maechler P, Antinozzi PA, Herrero L, Hagenfeldt-Johansson KA, Bjorklund A, Wollheim CB, The transcription factor SREBP-1c is instrumental in the development of beta-cell dysfunction, J. Biol. Chem 278 (2003) 16622–16629. [PubMed: 12600983]

[13]. Takahashi A, Motomura K, Kato T, Yoshikawa T, Nakagawa Y, Yahagi N, Sone H, Suzuki H, Toyoshima H, Yamada N, Shimano H, Transgenic mice overexpressing nuclear SREBP-1c in pancreatic beta-cells, Diabetes 54 (2005) 492–499. [PubMed: 15677507]

[14]. Li J, Liu X, Ran X, Chen J, Li X, Wu W, Huang H, Huang H, Long Y, Liang J, Cheng J, Tian H, Sterol regulatory element-binding protein-1c knockdown protected INS-1E cells from lipotoxicity, Diabetes Obes. Metab. 12 (2010) 35–46. [PubMed: 19758361]

[15]. Yang T, Espenshade PJ, Wright ME, Yabe D, Gong Y, Aebersold R, Goldstein JL, Brown MS, Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER, Cell 110 (2002) 489–500. [PubMed: 12202038]

[16]. Chen K, Jin P, He HH, Xie YH, Xie XY, Mo ZH, Overexpression of Insig-1 protects beta cell against glucolipotoxicity via SREBP-1c, J. Biomed. Sci 18 (2011) 57. [PubMed: 21843373]

[17]. Moore MJ, From birth to death: the complex lives of eukaryotic mRNAs, Science 309 (2005) 1514–1518. [PubMed: 16141059]

[18]. Morris AR, Mukherjee N, Keene JD, Systematic analysis of posttranscriptional gene expression, Wiley Interdiscip. Rev. Syst. Biol. Med 2 (2010) 162–180. [PubMed: 20836020]

[19]. Fabian MR, Sonenberg N, Filipowicz W, Regulation of mRNA translation and stability by microRNAs, Annu. Rev. Biochem. 79 (2010) 351–379. [PubMed: 20533884]

[20]. Lunde BM, Moore C, Varani G, RNA-binding proteins: modular design for efficient function, Nat. Rev. Mol. Cell Biol. 8 (2007) 479–490. [PubMed: 17473849]

[21]. Good PJ, A conserved family of elav-like genes in vertebrates, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 4557–4561. [PubMed: 7753842]

[22]. Akamatsu W, Fujihara H, Mitsuhashi T, Yano M, Shibata S, Hayakawa Y, Okano HJ, Sakakibara S, Takano H, Takano T, Takahashi T, Noda T, Okano H, The RNA-binding protein HuD regulates neuronal cell identity and maturation, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 4625–4630. [PubMed: 15764704]

[23]. Anderson KD, Sengupta J, Morin M, Neve RL, Valenzuela CF, Perrone-Bizzozero NI, Overexpression of HuD accelerates neurite outgrowth and increases GAP-43 mRNA expression in cortical neurons and retinoic acid-induced embryonic stem cells in vitro, Exp. Neurol. 168 (2001) 250–258. [PubMed: 11259113]

[24]. Kasashima K, Terashima K, Yamamoto K, Sakashita E, Sakamoto H, Cytoplasmic localization is required for the mammalian ELAV-like protein HuD to induce neuronal differentiation, Genes Cells 4 (1999) 667–683. [PubMed: 10620013]

[25]. Mobarak CD, Anderson KD, Morin M, Beckel-Mitchener A, Rogers SL, Furneaux H, King P, Perrone-Bizzozero NI, The RNA-binding protein HuD is required for GAP-43 mRNA stability, GAP-43 gene expression, and PKC-dependent neurite outgrowth in PC12 cells, Mol. Biol. Cell 11 (2000) 3191–3203. [PubMed: 10982410]

[26]. Perrone-Bizzozero N, Bolognani F, Role of HuD and other RNA-binding proteins in neural development and plasticity, J. Neurosci. Res 68 (2002) 121–126. [PubMed: 11948657]

[27]. Lee EK, Kim W, Tominaga K, Martindale JL, Yang X, Subaran SS, Carlson OD, Mercken EM, Kulkarni RN, Akamatsu W, Okano H, Perrone-Bizzozero NI, de Cabo R, Egan JM, Gorospe M, RNA-binding protein HuD controls insulin translation, Mol. Cell 45 (2012) 826–835. [PubMed: 22387028]

[28]. Kim C, Kim W, Lee H, Ji E, Choe YJ, Martindale JL, Akamatsu W, Okano H, Kim HS, Nam SW, Gorospe M, Lee EK, The RNA-binding protein HuD regulates autophagosome formation in pancreatic beta cells by promoting autophagy-related gene 5 expression, J. Biol. Chem. 289 (2014) 112–121. [PubMed: 24275661]

[29]. Schwartz EA, Zhang WY, Karnik SK, Borwege S, Anand VR, Laine PS, Su Y, Reaven PD, Nutrient modification of the innate immune response: a novel mechanism by which saturated fatty acids greatly amplify monocyte inflammation, Arterioscler. Thromb. Vasc. Biol. 30 (2010) 802–808. [PubMed: 20110572]

[30]. Lal A, Mazan-Mamczarz K, Kawai T, Yang X, Martindale JL, Gorospe M, Concurrent versus individual binding of HuR and AUF1 to common labile target mRNAs, EMBO J. 23 (2004) 3092–3102. [PubMed: 15257295]

[31]. Lee EK, Kim HH, Kuwano Y, Abdelmohsen K, Srikantan S, Subaran SS, Gleichmann M, Mughal MR, Martindale JL, Yang X, Worley PF, Mattson MP, Gorospe M, hnRNP C promotes APP translation by competing with FMRP for APP mRNA recruitment to P bodies, Nat. Struct. Mol. Biol. 17 (2010) 732–739. [PubMed: 20473314]

[32]. Diraison F, Ravier MA, Richards SK, Smith RM, Shimano H, Rutter GA, SREBP1 is required for the induction by glucose of pancreatic beta-cell genes involved in glucose sensing, J. Lipid Res. 49 (2008) 814–822. [PubMed: 18178930]

[33]. Amemiya-Kudo M, Shimano H, Hasty AH, Yahagi N, Yoshikawa T, Matsuzaka T, Okazaki H, Tamura Y, Iizuka Y, Ohashi K, Osuga J, Harada K, Gotoda T, Sato R, Kimura S, Ishibashi S, Yamada N, Transcriptional activities of nuclear SREBP-1a, −1c, and −2 to different target promoters of lipogenic and cholesterogenic genes, J. Lipid Res. 43 (2002) 1220–1235. [PubMed: 12177166]

[34]. Pai JT, Guryev O, Brown MS, Goldstein JL, Differential stimulation of cholesterol and unsaturated fatty acid biosynthesis in cells expressing individual nuclear sterol regulatory element-binding proteins, J. Biol. Chem 273 (1998) 26138–26148. [PubMed: 9748295]

[35]. Brown MS, Goldstein JL, A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 11041–11048. [PubMed: 10500120]

[36]. Tanabe K, Liu Y, Hasan SD, Martinez SC, Cras-Meneur C, Welling CM, Bernal-Mizrachi E, Tanizawa Y, Rhodes CJ, Zmuda E, Hai T, Abumrad NA, Permutt MA, Glucose and fatty acids synergize to promote B-cell apoptosis through activation of glycogen synthase kinase 3beta independent of JNK activation, PLoS One 6 (2011), e18146. [PubMed: 21541314]

[37]. Kato T, Shimano H, Yamamoto T, Ishikawa M, Kumadaki S, Matsuzaka T, Nakagawa Y, Yahagi N, Nakakuki M, Hasty AH, Takeuchi Y, Kobayashi K, Takahashi A, Yatoh S, Suzuki H, Sone H, Yamada N, Palmitate impairs and eicosapentaenoate restores insulin secretion through regulation of SREBP-1c in pancreatic islets, Diabetes 57 (2008) 2382–2392. [PubMed: 18458149]

[38]. Lee Y, Hirose H, Ohneda M, Johnson JH, McGarry JD, Unger RH, Beta-cell lipotoxicity in the pathogenesis of non-insulin-dependent diabetes mellitus of obese rats: impairment in adipocyte–beta-cell relationships, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 10878–10882. [PubMed: 7971976]

[39]. Lee Y, Wang MY, Kakuma T, Wang ZW, Babcock E, McCorkle K, Higa M, Zhou YT, Unger RH, Liporegulation in diet-induced obesity. The antisteatotic role of hyperleptinemia, J. Biol. Chem. 276 (2001) 5629–5635. [PubMed: 11096093]

[40]. Wang MY, Grayburn P, Chen S, Ravazzola M, Orci L, Unger RH, Adipogenic capacity and the susceptibility to type 2 diabetes and metabolic syndrome, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 6139–6144. [PubMed: 18413598]

[41]. Lingvay I, Esser V, Legendre JL, Price AL, Wertz KM, Adams-Huet B, Zhang S, Unger RH, Szczepaniak LS, Noninvasive quantification of pancreatic fat in humans, J. Clin. Endocrinol. Metab. 94 (2009) 4070–4076. [PubMed: 19773401]

[42]. Lee Y, Lingvay I, Szczepaniak LS, Ravazzola M, Orci L, Unger RH, Pancreatic steatosis: harbinger of type 2 diabetes in obese rodents, Int. J. Obes 34 (2010) 396–400.

[43]. Dong XY, Tang SQ, Insulin-induced gene: a new regulator in lipid metabolism, Peptides 31 (2010) 2145–2150. [PubMed: 20817058]

[44]. Sun LP, Seemann J, Goldstein JL, Brown MS, Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: Insig renders sorting signal in Scap inaccessible to COPII proteins, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 6519–6526. [PubMed: 17428919]

[45]. Jeon TI, Osborne TF, SREBPs: metabolic integrators in physiology and metabolism, Trends Endocrinol. Metab. 23 (2012) 65–72. [PubMed: 22154484]

[46]. Yabe D, Xia ZP, Adams CM, Rawson RB, Three mutations in sterol-sensing domain of SCAP block interaction with insig and render SREBP cleavage insensitive to sterols, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 16672–16677. [PubMed: 12482938]

[47]. Janowski BA, The hypocholesterolemic agent LY295427 upregulates INSIG-1, identifying the INSIG-1 protein as a mediator of cholesterol homeostasis through SREBP, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 12675–12680. [PubMed: 12242342]

[48]. Lee JN, Gong Y, Zhang X, Ye J, Proteasomal degradation of ubiquitinated Insig proteins is determined by serine residues flanking ubiquitinated lysines, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 4958–4963. [PubMed: 16549805]

[49]. Gong Y, Lee JN, Lee PC, Goldstein JL, Brown MS, Ye J, Sterol-regulated ubiquitination and degradation of Insig-1 creates a convergent mechanism for feedback control of cholesterol synthesis and uptake, Cell Metab. 3 (2006) 15–24. [PubMed: 16399501]

[50]. Keeton AB, Bortoff KD, Franklin JL, Messina JL, Blockade of rapid versus prolonged extracellularly regulated kinase 1/2 activation has differential effects on insulin-induced gene expression, Endocrinology 146 (2005) 2716–2725. [PubMed: 15731359]

[51]. Lee JN, Zhang X, Feramisco JD, Gong Y, Ye J, Unsaturated fatty acids inhibit proteasomal degradation of Insig-1 at a postubiquitination step, J. Biol. Chem. 283 (2008) 33772–33783. [PubMed: 18835813]

[52]. Ka SO, Kim KA, Kwon KB, Park JW, Park BH, Silibinin attenuates adipogenesis in 3T3-L1 preadipocytes through a potential upregulation of the insig pathway, Int. J. Mol. Med. 23 (2009) 633–637. [PubMed: 19360322]

[53]. Goldstein JL, DeBose-Boyd RA, Brown MS, Protein sensors for membrane sterols, Cell 124 (2006) 35–46. [PubMed: 16413480]

[54]. Nogalska A, Sucajtys-Szulc E, Swierczynski J, Leptin decreases lipogenic enzyme gene expression through modification of SREBP-1c gene expression in white adipose tissue of aging rats, Metab. Clin. Exp 54 (2005) 1041–1047. [PubMed: 16092054]

[55]. Jeon TI, Esquejo RM, Roqueta-Rivera M, Phelan PE, Moon YA, Govindarajan SS, Esau CC, Osborne TF, An SREBP-responsive microRNA operon contributes to a regulatory loop for intracellular lipid homeostasis, Cell Metab. 18 (2013) 51–61. [PubMed: 23823476]

[56]. Kim W, Kyung Lee E, Post-transcriptional regulation in metabolic diseases, RNA Biol. 9 (2012) 772–780. [PubMed: 22664919]

[57]. Hinman MN, Lou H, Diverse molecular functions of Hu proteins, Cell. Mol. Life Sci. 65 (2008) 3168–3181. [PubMed: 18581050]

[58]. Poy MN, Hausser J, Trajkovski M, Braun M, Collins S, Rorsman P, Zavolan M, Stoffel M, miR-375 maintains normal pancreatic alpha- and beta-cell mass, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 5813–5818. [PubMed: 19289822]

[59]. Fujiwara T, Mori Y, Chu DL, Koyama Y, Miyata S, Tanaka H, Yachi K, Kubo T, Yoshikawa H, Tohyama M, CARM1 regulates proliferation of PC12 cells by methylating HuD, Mol. Cell. Biol. 26 (2006) 2273–2285. [PubMed: 16508003]

[60]. Pascale A, Amadio M, Scapagnini G, Lanni C, Racchi M, Provenzani A, Govoni S, Alkon DL, Quattrone A, Neuronal ELAV proteins enhance mRNA stability by a PKCalpha-dependent pathway, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 12065–12070. [PubMed: 16099831]

[61]. Sanna MD, Quattrone A, Ghelardini C, Galeotti N, PKC-mediated HuD-GAP43 pathway activation in a mouse model of antiretroviral painful neuropathy, Pharmacol. Res. 81 (2014) 44–53. [PubMed: 24565699]

[62]. Lim CS, Alkon DL, PKCepsilon promotes HuD-mediated neprilysin mRNA stability and enhances neprilysin-induced Abeta degradation in brain neurons, PLoS One 9 (2014), e97756. [PubMed: 24848988]

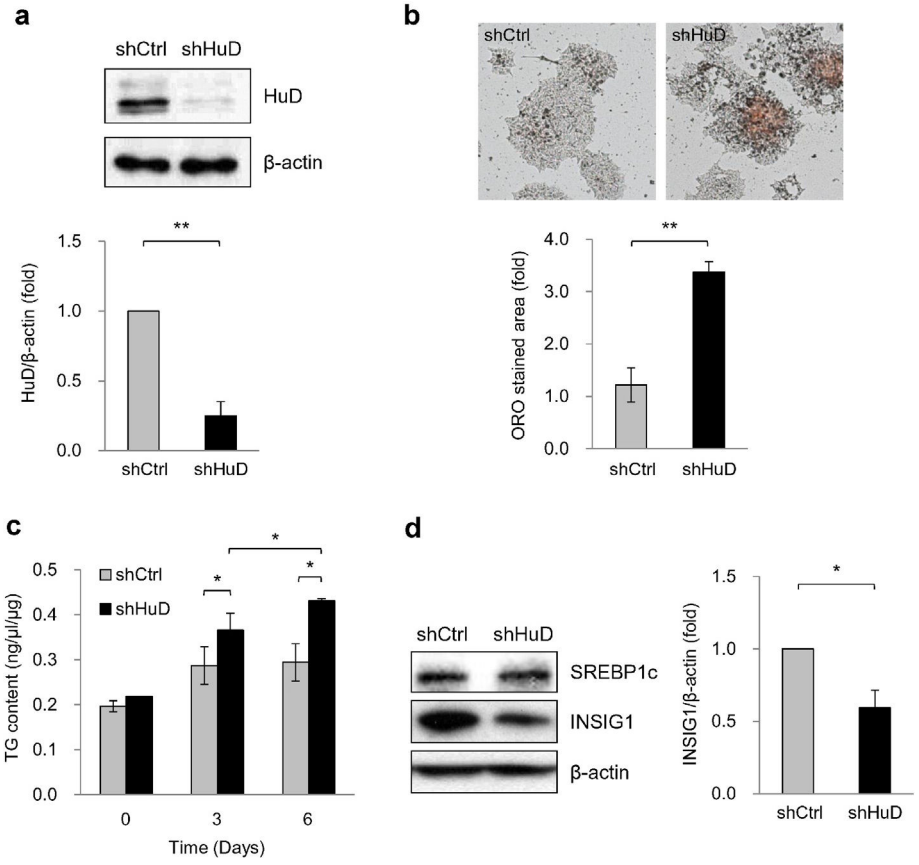

Fig. 1.

Downregulation of HuD accumulates triglyceride in pancreatic β cells. (a) Cell lysates from pancreatic βTC6 cells expressing pshHuD or control plasmid (βTC6-shHuD or βTC6-shCtrl) were prepared and the levels of HuD and housekeeping control protein β-actin levels were assessed by Western blot analysis. (b) Cells were fixed and incubated with 0.2% Oil-Red-O solution for 30 min and lipid accumulation was observed using a Zeiss Axioimager M1 microscope. Quantification of the Oil-Red-O-stained area of the lipid droplet in βTC6 cells was analyzed using ImageJ software. (c) The relative triglyceride content in cells was determined at 0, 3, and 6 days. (d) In cells processed as described in (a), the levels of SREBP1c, INSIG1, and β-actin were determined by Western blot analysis. Images in (a), (b), and (d) are representative from three independent experiments. Data in (c) represent the means ± SEM from three independent experiments; *, $p < 0.05$, **, $p < 0.01$.

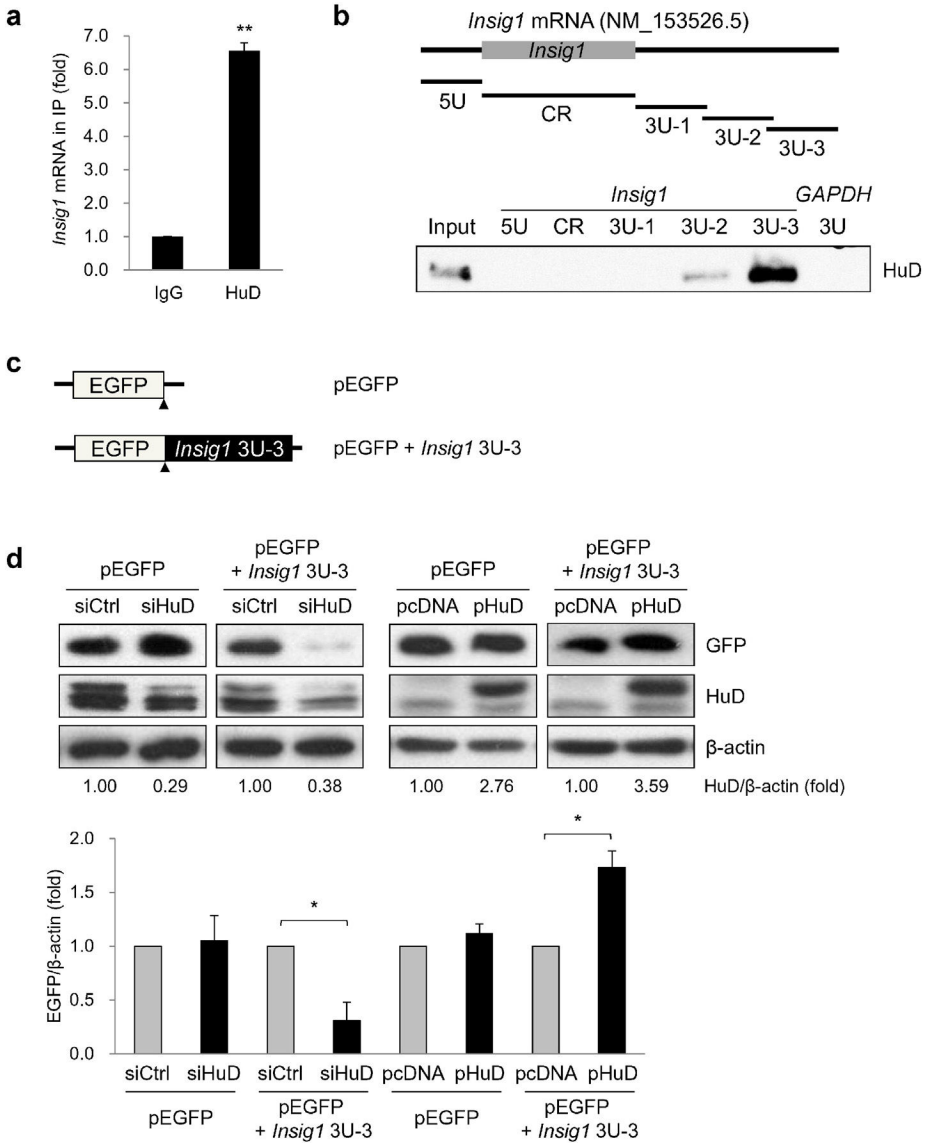

Fig. 2.

HuD regulates INSIG1 expression by interacting with the *Insig1* 3′UTR. (a) βTC6 cell lysates was subjected to RIP followed by RT-qPCR analysis to measure the enriched *Insig1* mRNA in anti-HuD IP compared control IgG IP. *Gapdh* mRNA was used for normalization. (b) Top, schematic of the *Insig1* mRNA depicting the 5′UTR, coding region, and 3′UTR as well as the biotinylated transcripts (5U, CR, 3U-1, 3U-2, and 3U-3) that were synthesized for biotin pulldown analysis. Bottom, after incubation of each biotinylated transcript with βTC6 cell lysate, the interactions between the biotinylated transcripts and HuD were analyzed by Western blot analysis using anti-HuD antibody. The numbers indicate the mean ± SEM from three independent experiments. (c) Schematic of reporter plasmids pEGFP (control) and pEGFP + *Insig1* 3U-3. (d) Forty-eight hours after transfection of HuD-direct siRNA (siHuD) or overexpression of HuD using a plasmid (pHuD) along with appropriate controls (siCtrl and pcDNA, respectively), together with each reporter plasmid, the levels of GFP, HuD, and loading control β-actin were assessed by Western blot analysis. Data in

(a) represent mean ± SEM from three independent experiments; *, $p < 0.05$, **, $p < 0.01$. Images in (b) and (d) are representative from three independent experiments.

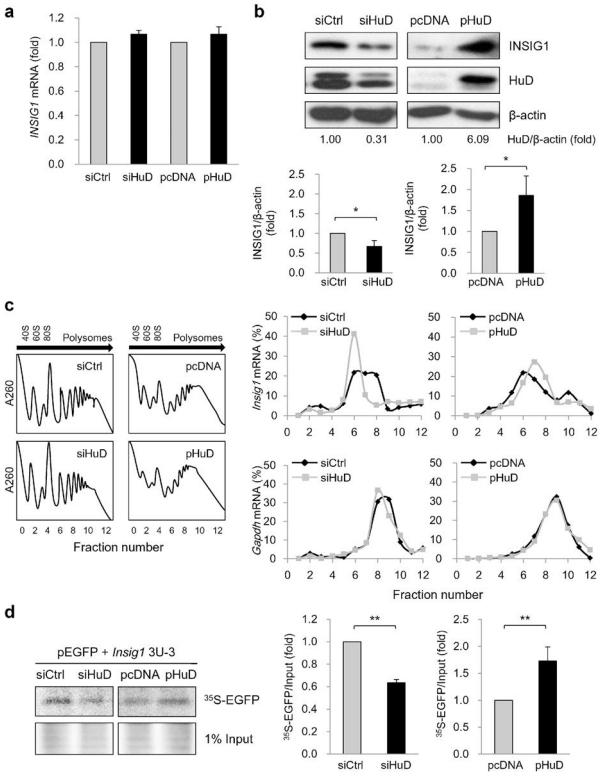

Fig. 3.

HuD promotes translation of *INSIG1* mRNA. (a) Forty-eight hours after transfection of βTC6 cells with either of HuD-direct siRNA (siHuD) or overexpression plasmid (pHuD) along with appropriate controls (siCtrl and pcDNA, respectively), the levels of *Insig1* mRNA were measured by RT-qPCR and normalized to *Gapdh* mRNA. (b) Lysates prepared from βTC6 cells transfected as described in (a) were used to assess the level of INSIG1, HuD, and loading control β-actin by Western blot analysis. (c) Lysates prepared from βTC6 cells transfected as described in (a) were fractionated through sucrose gradients (left), and the relative distribution of *Insig1* mRNA and *Gapdh* mRNA in each of 12 fractions was studied by RT-qPCR analysis (right). 40S, small ribosome subunits; 60S, large ribosome subunits; 80S, monosome; polysomes, polyribosomes. Polysome profiles are representative of two different fractionation analyses. (d) Nascent EGFP production was assessed by a brief (20-min-long) incubation with L-[^{35}S]methionine and L-[^{35}S]cysteine after transfection of HuD siRNA or HuD plasmid along with appropriate controls. Lysates were subjected to IP using anti-EGFP or anti-GAPDH antibodies, and the incorporation of radiolabeled amino acids into newly synthesized EGFP proteins was assessed by SDS-PAGE and visualized using a PharoseFX Plus. The data are representative of three independent experiments and the numbers indicate fold changes in EGFP levels. Numbers indicate the mean ± SEM from three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

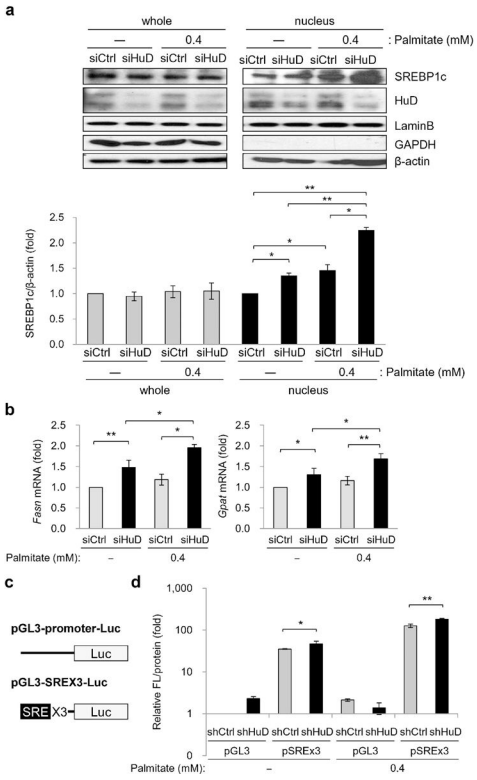

Fig. 4.

Downregulation of HuD enhances the transcriptional activity of SREBP1c in pancreatic β cells. (a) Forty-eight hours after transfection with siCtrl or siHuD, βTC6 cells were incubated with or without 0.4 mM of palmitate for 72 h, and the nuclear fractions were prepared using digitonin. The levels of nSREBP1c, HuD, lamin B, GAPDH, and loading control β-actin were assessed by Western blot analysis. (b) RNAs were prepared from cells as described in (a) and the relative expression of *Fasn* and *Gpat* mRNAs was determined by RT-qPCR analysis and normalized to the levels of *Gapdh* mRNA. (c) Schematic of reporter plasmids; control vector (pGL3-promoter-Luc) and luciferase vector containing triplicate SRE binding domains (pGL3-SREx3-Luc). (d) 100 ng of pGL3-SREx3-Luc or control plasmid was transfected to βTC6-shHuD or βTC6-shCtrl cells and both cell populations were incubated with or without 0.4 mM palmitate for 15 h. The relative luciferase activity was determined by measuring luminescent signal and normalized to each concentration of proteins. Data represent the means ± SEM from three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

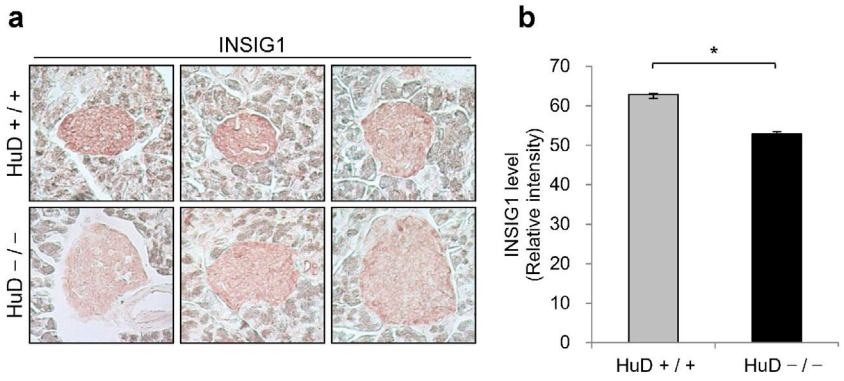

Fig. 5.

INSIG1 level was downregulated in HuD-null mice. (a and b) Pancreatic sections from WT (HuD+/+) and HuD-null (HuD−/−) mice were stained with anti-INSIG1 antibody. The intensity of INSIG1-positive areas in twenty islets from three pairs of mice was quantified using ImageJ. Data represent the means ± SEM. *, $p < 0.05$.

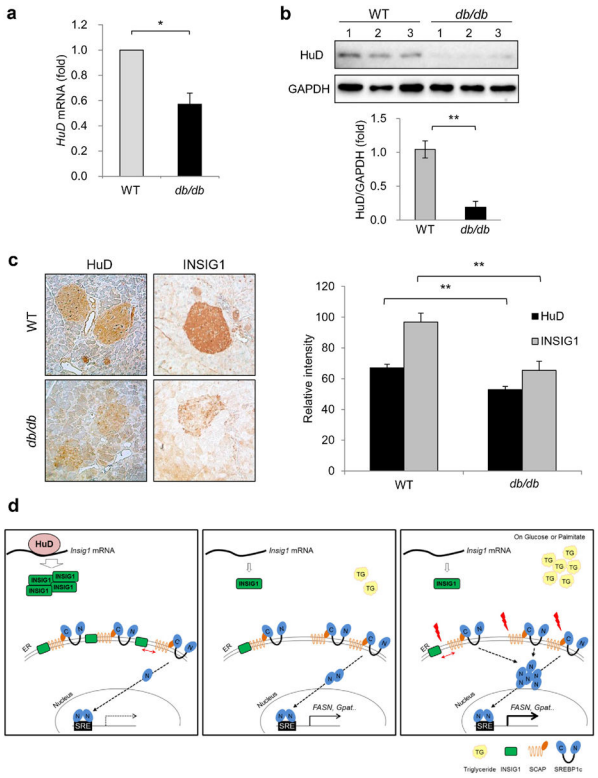

Fig. 6.

HuD expression was downregulated in the pancreas of db/db mice. (a and b) The relative levels of *HuD* mRNA and HuD protein in the pancreases of WT and db/db mice was analyzed by RT-qPCR and Western blot analysis. (b) Relative HuD and INSIG1 protein levels in the islet of WT or *db/db* mice (n = 3) was analyzed by immunohistochemistry using HuD and INSIG1 antibody. The intensity of HuD and INSIG1 was quantified using ImageJ. Data represent the means ± SEM. *, $p < 0.05$; **, $p < 0.01$. (d) Schematic diagram of INSIG1 regulation by HuD in pancreatic β-cells. HuD positively regulates INSIG1 expression at the post-transcription level by binding to the 3′UTR of *Insig1* mRNA and enhancing its translation, thereby suppressing transcriptional activity of nSREBP1 (left). Downregulation of HuD results in the transcriptional activation of lipid biosynthetic genes such as *Fasn* and *Gpat* by nSREBP1; glucotoxic and lipotoxic conditions can promote the action of nSREBP1 by suppressing HuD function and thus lowering INSIG1 levels.

Table 1

Primer sequences used in this study.

Primers for BPD	Sequences		
Mouse GAPDH 3U-F	5′-CCAAGCTTCTAATACGACTCACTATAGGGAGACCTCAACGACCACTTTGTCA-3′		
Mouse GAPDH 3U-R	5′-GGTTGAGCACAGGGTACTTTAT-3′		
Mouse INSIG1-5U-F	5′-CCAAGCTTCTAATACGACTCACTATAGGGAGAACCTTCCATGCTGTGCCTGGACGCCG-3′		
Mouse INSIG1-5U-R	5′-CGGCGTCCAGGCACAGCATGGAAGGTTCTCCCTATAGTGAGTCGTATTAGAAGCTTGG-3′		
Mouse INSIG1-CR-F	5′-CCAAGCTTCTAATACGACTCACTATAGGGAGAATGCCCAGGCTGCACGACCAC-3′		
Mouse INSIG1-CR-R	5′-TCAGTCACTGTGAGGCTTTTCCG-3′		
Mouse INSIG1-3U-1-F	5′-CCAGCTTCTAATACGACTCACTATAGGGAGAGACTGAGCTTCAGCACATGGT-3′		
Mouse INSIG1-3U-1-R	5′-TTTTGGTACCGTGCAGAACCCTGAC-3′		
Mouse INSIG1-3U-2-F	5′-CCAAGCTTCTAATACGACTCACTATAGGGAGAGGTTCTGCACTGCGATG-3′		
Mouse INSIG1-3U-2-R	5′-TTTTGGTACCCCAGGTGTCCACAGGT-3′		
Mouse INSIG1-3U-3-F	5′-CCAAGCTTCTAATACGACTCACTATAGGGAGAGGACACCTGGTTTGG-3′		
Mouse INSIG1-3U-3-R	5′-CACAATGCTGCAAACTTAGCACG-3′		
Primers for EGFP-reporter	Sequences		
mouse INSIG1-3U-3-F	5′-AAAAAGATCTTAAGGACACCTGGGTT-3′		
mouse INSIG1-3U-3-R	5′-AAAAGGTACCCACAATGCTGC-3′		
Primers for PCR	Sequences	Product size	Primer efficiency[a]
Mouse Insig1-F	5′-CACGACCACGTCTGGAACTAT-3′	213 bp	96.5%
Mouse Insig1-R	5′-TGAGAAGAGCACTAGGCTCCG-3′		
Mouse Fasn-F	5′-GGAGGTGGTGATAGCCGGTAT-3′	140 bp	101.7%
Mouse Fasn-R	5′-TGGGTAATCCATAGAGCCCAG-3′		
Mouse Gpat-F	5′-CAACACCATCCCCGACATC-3′	148 bp	107.4%
Mouse Gpat-R	5′-CCGCAGCATTCTGATAACGC-3′		
Mouse Gapdh-F	5′-AGGTCGGTGTGAACGGATTTG-3′	123 bp	103.8%
Mouse Gapdh-R	5′-TGTAGACCATGTAGTTGAGGTCA-3′		
Primers for cloning of pGL3 reporter	Sequences		
pGL3-SREX3-Luc F	5′-CATCACCCCACATCACCCCACATCACCCCACA-3′		

pGL3-SREX3-Luc R	5′-GATCT GTGGGGTGATGTGGGGTGATGTGGGGTGATGGTAC-3′

[a]Primer efficiency for RT-qPCR was calculated from the slope of the calibration curve.