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ARTICLE

RNA binding protein HuD contributes to β-cell dysfunction by impairing mitochondria dynamics

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Abstract

Imbalanced mitochondrial dynamics in pancreatic β-cells contributes to β-cell dysfunction in diabetes; however, the molecular mechanisms underlying mitochondrial dynamics in the pathology of diabetes are not fully elucidated. We previously reported the reduction of RNA binding protein HuD in pancreatic β-cells of diabetes. Herein, we demonstrate that HuD plays a novel role in the regulation of mitochondrial dynamics by promoting mitochondrial fusion. We show enhanced mitochondrial fragmentation in the pancreas of *db/db* mice and HuD KO mice. Downregulation of HuD increases the number of cells with fragmented mitochondria and reduces the mitochondrial activity determined by mitochondrial membrane potential and ATP production in mouse insulinoma βTC6 cells. HuD binds to 3′-untraslated region of *mitofusin 2* (*Mfn2*) mRNA and positively regulates its expression. Ectopic expression of Mfn2 in βTC6 cells stably expressing short hairpin RNA against HuD (shHuD) restores HuD-mediated mitochondrial dysfunction. Taken together, our results suggest that HuD regulates mitochondrial dynamics by regulating Mfn2 level and its reduced expression leads to mitochondrial dysfunction in pancreatic β-cells.

Introduction

Mitochondria play crucial roles in cellular energy homeostasis and apoptosis by regulating cellular energy production, reactive oxygen species generation, and signal transduction [1–3]. Mitochondria continuously change their morphology through fusion and fission in response to the physiological demands of cells, which is critical to the organelle's function [4–6]. Abnormal regulation of mitochondrial dynamics has been implicated in the pathogenesis of several human diseases, in particular, metabolic disorders [5, 7–9]. Enhanced fission and reduced fusion were previously reported in obesity and diabetes [10, 11]. The differential expression of mitochondrial dynamics regulating proteins, such as dynamin related protein 1 (Drp1), optic atrophy protein 1 (Opa1), mitofusin 1/2 (Mfn1/2), and mitochondria fission factor (Mff), is responsible for the imbalance between fusion and fission, and contributes to mitochondrial dysfunction [12, 13]. Emerging evidence suggests that the tight regulation of mitochondrial dynamics plays an important role in the maintenance of mitochondrial homeostasis and in the pathogenesis of diseases; however, the underlying mechanisms need to be further elucidated.

Mitofusin 2 (Mfn2), a 757 aa protein containing several well-conserved domains, mediates oligomerization of two adjacent mitochondria via heptad-repeat domains, and regulates mitochondrial distribution, shape, and quality control [14]. Mfn2 has been shown to play a key role in metabolism: Mfn2 depletion leads to reduced mitochondrial membrane potential,

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cellular oxygen consumption, and mitochondrial proton leak, whereas Mfn2 overexpression promotes oxidative phosphorylation, glucose oxidation, and insulin sensitivity [12, 15–19]. Mfn2 also mediates the interaction between mitochondria and endoplasmic reticulum (ER) and regulates ER stress in response to metabolic stress [20]. Downregulation of Mfn2 expression has been reported in obesity and diabetes, and it suggests the implication of Mfn2 in the pathophysiology of metabolic diseases [12, 14, 15].

Mfn2 expression is regulated at the levels of transcription, posttranscription, and posttranslation. Estrogen-related receptor-α, peroxisome proliferator-activated receptor gamma coactivator 1-beta, and lysocardiolipin acyltransferase 1 regulate *Mfn2* transcription [21–23]. microRNAs (miRNAs) including miR-17, −31, −106b, −125a, −195, −214, –497, and −761, suppress, while a long noncoding RNA *Plscr4* increases, Mfn2 expression [24–36]. Phosphorylation-dependent ubiquitination by JNK and Wuwe1 also regulates the level of Mfn2 [37]. In addition, Mfn2 activity is affected by protein–protein interactions such as the Smad2-RIN complex [38]. Although several studies have identified the factors regulating the expression or activity of Mfn2, the underlying mechanisms in physiological or pathological conditions need to be further determined.

HuD, one of members of human antigen Hu/ELAVL family, plays an essential role in posttranscriptional regulation of gene expression by controlling mRNA turnover or translation in certain types of cells including neurons and pancreatic β-cells [39–45]. Herein, we identified HuD as a novel factor regulating mitochondrial dynamics and function by promoting Mfn2 expression. Mitochondrial fragmentation is enhanced in pancreatic islets of diabetic *db/db* mice and HuD knockout (KO) mice. Mitochondrial morphology and activity is regulated in an HuD-dependent manner in the mouse pancreatic β-cell line, βTC6. HuD increases Mfn2 expression by associating with 3′-untranslated region (3′UTR) of *Mfn2* mRNA, thereby promoting mitochondrial fusion. Ectopic expression of Mfn2 restores HuD-mediated mitochondrial dysfunction. Our data reveal that HuD plays an essential role in the regulation of mitochondrial homeostasis and its reduction may contribute to impaired mitochondrial function resulting β-cell dysfunction.

Results

Mitochondrial fragmentation is enhanced in diabetic islets

Mitochondria dysfunction has been reported in a diverse diabetes models and abnormal mitochondrial morphology is known to be associated with mitochondrial dysfunction in the pathogenesis of diabetes [11, 46–48]. We investigated the mitochondrial morphology in the pancreas of leptin receptor-deficient mice (*db/db* mice), one of the diabetes models, compared with normal C57BL/6J mice using an electron microscope. The results show that mitochondria in pancreatic islets of *db/db* mice were smaller than those of the control mice (Fig. 1a). Our previous study demonstrated that lower HuD expression in pancreatic islets contributed to β-cell dysfunction [44]. To elucidate the relationship between HuD and mitochondrial dynamics, we also analyzed the mitochondrial morphology of pancreatic islets in HuD KO mice. We found that mitochondria in pancreatic islets of KO mice were rounded and smaller than those derived from wildtype mice (Fig. 1b). These observations suggest that mitochondria fragmentation is enhanced in diabetic islets and HuD plays a role in the regulation of mitochondrial dynamics.

HuD functions as an essential regulator in the maintenance of mitochondrial morphology and function

Our previous studies shows HuD plays pivotal roles in pancreatic β-cells and its expression is reduced in the pancreas of *db/db* mice [42–44]. Figure 1b shows abnormal mitochondrial morphology in the pancreatic islets of HuD KO mice. To determine whether HuD has a potential to regulate mitochondrial dynamics, we analyzed mitochondrial morphology in βTC6 cells transfected with HuD siRNA or in a stable cell line expressing a short hairpin RNA against HuD (shHuD) [44] by staining with MitoTracker® or electron microscopy. Downregulation of HuD resulted in fragmentation of mitochondria in βTC6 cells as shown in Fig. 2a, c. To test the effect of HuD overexpression on mitochondrial dynamics, we transduced βTC6 cells with a retrovirus containing HA-tagged HuD (pHuD) and analyzed mitochondrial morphology. HuD overexpression increased mitochondrial fusion in βTC6 cells (Fig. 2b, d). These results suggest that HuD has a role in the regulation of mitochondrial dynamics by promoting mitochondrial elongation.

It has been suggested that dynamic changes in mitochondrial morphology are essential for their functions, including cellular respiration and ATP generation. An imbalance of mitochondria dynamics is usually linked to mitochondrial dysfunction and leads to pathogenesis of several diseases such as neurodegenerative diseases and diabetes [2–4, 46]. To address whether HuD affects to the function of mitochondria, we assessed mitochondrial activity by measuring mitochondrial membrane potential after HuD overexpression or knockdown in pancreatic β-cells. HuD overexpression increased, while HuD knockdown decreased mitochondrial membrane potential based on JC-1 staining (Fig. 3a). In addition, the level of mitochondrial ATP was positively regulated by HuD (Fig. 3b). These observations indicate that HuD expression is linked to mitochondrial function. Consistent with these results, a reduction in mitochondrial activity

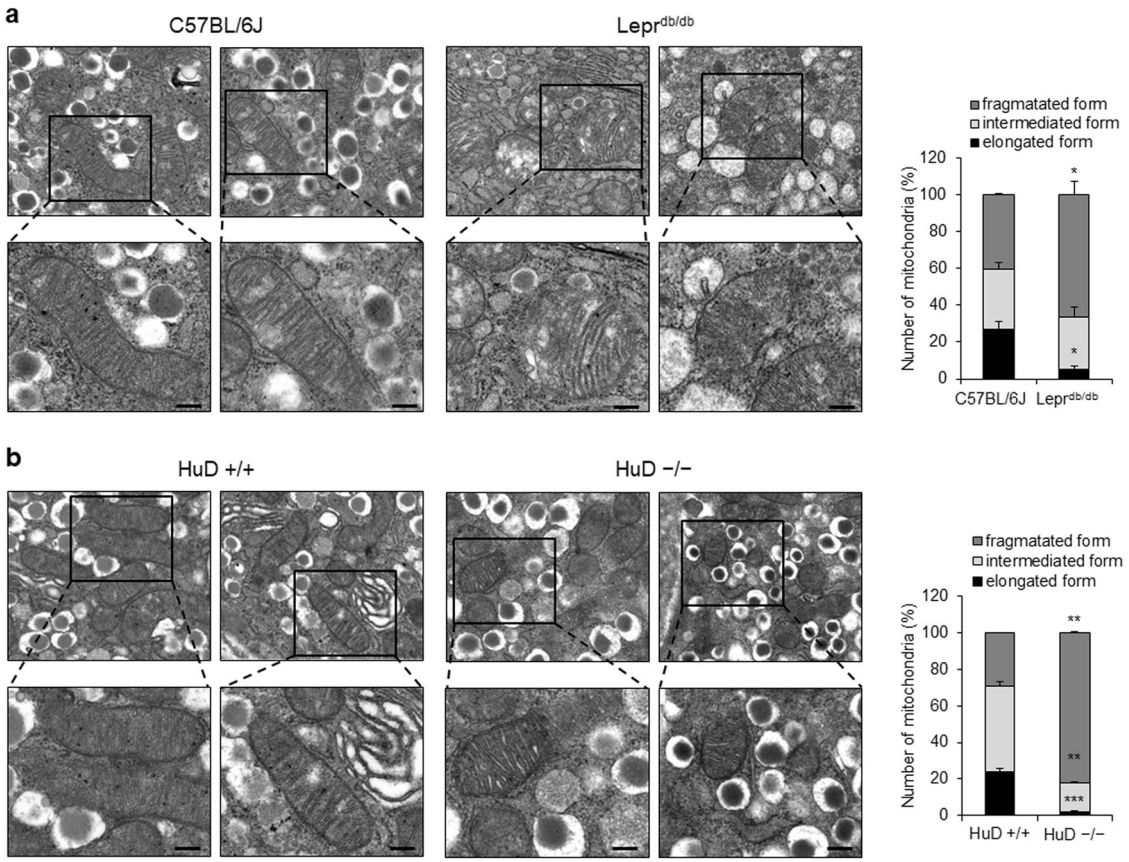

Fig. 1 Mitochondrial morphology in the pancreas of diabetic mice. Mitochondrial morphology was analyzed in the pancreas of normal *C57BL/6J* and diabetic (Lepr$^{db/db}$) mice (**a**) and the pancreas of HuD knockout mice (**b**) using a transmission electron microscope (TEM). Bar, 0.2 µm. Fifty mitochondria from each group were counted according to their relative length. Images are representative and the values indicate the mean ± SEM obtained from three independent analyses. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

was confirmed in shHuD cells, which indicated that the loss of HuD impaired mitochondrial function (Fig. 3c). In addition, we investigated the impact of HuD on cellular respiration by assessing oxygen consumption rate (OCR) between shHuD cells and shCtrl cells using the Seahorse FX24 Extracellular Flux Analyzer. Basal OCR and maximal OCR assessed by FCCP treatment in shHuD cells were lower than those in shCtrl cell (Fig. 3d), indicating that HuD down-regulation impaired basal and maximal rates of mitochondrial respiration. However, no significant change on electron flow through the ETC or nonmitochondrial respiration of both cells was observed. Taken together, these results suggest that HuD plays an essential role in the regulation of mitochondrial morphology and function in pancreatic β-cells.

HuD binds to *Mfn2* mRNA and regulates its expression

To determine the target mRNAs of HuD involved in the regulation of mitochondrial morphology and function, HuD-associated mRNAs were isolated and identified by HuD-RIP assay followed by microarray. The mRNAs enriched more than fivefold in HuD IP shown in Supplementary Table 1 were further screened by gene ontology-based analysis of genes related to mitochondrial function. This survey identified seven mRNAs including *apoptosis inducing factor mitochondria associated 2* (*AIFM2*), *diablo IAP-binding mitochondrial protein* (*DIABLO*), *frataxin* (*Fxn*), *heat shock 70 KDa Protein 9* (*HSPA9*, *mortalin*), *mitochondrial tumor suppressor gene 1* (*MTUS1*), *Mfn2*, and *mitochondrial transcription factor A* (*TFAM*) as putative targets of HuD (Fig. 4a). The association between HuD and these mRNAs was validated by RIP analysis followed by RT-qPCR, and the results showed that *Mfn2* mRNA was significantly enriched in HuD-IP compared with normal IgG (Fig. 4b). It suggests that HuD binds to *Mfn2* mRNA. HuD is known to regulate mRNA turnover and translation by binding to the 3′UTR of target mRNAs [42, 45, 49, 50]. Therefore, we further assessed the interaction between HuD and *Mfn2* mRNA via in vitro pull-down assay using biotin-labeled transcripts corresponding to the 5′UTR (Mfn2 5U) and 3′UTR of *Mfn2* mRNA (Mfn2 3U-1 and 3U-2) and

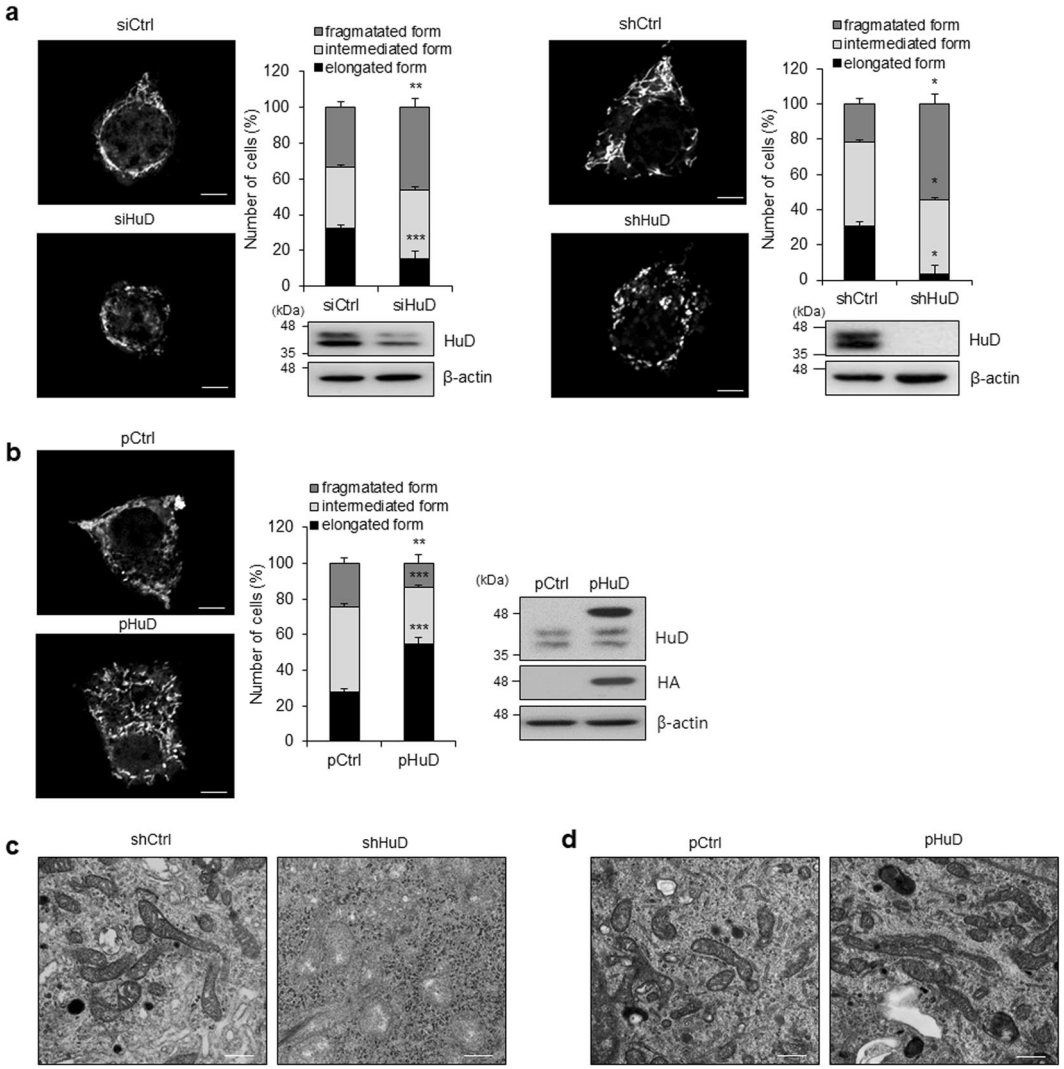

Fig. 2 Regulation of mitochondrial morphology by HuD. **a**, **b** HuD knockdowned βTC6 cells using either siRNA (left) or small hairpin RNA (right) (**a**) and HuD overexpressing βTC6 cells using a retrovirus carrying HA-HuD (pHuD) (**b**) were incubated with MitoTracker® and the mitochondrial morphology was analyzed using a fluorescence microscope. Bar, 4 μm. The number of mitochondria was assessed by counting at least 300 cells from three independent experiments. Western blot analysis shows relative level of HuD and β-actin. **c**, **d** Mitochondrial morphology of βTC6-shHuD cells and βTC6 cells after HuD overexpression (pHuD) was analyzed with a TEM. Bar, 0.5 μm. Images are representative and the values indicate the mean ± SEM from three independent analyses. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

streptavidin beads. As shown in Fig. 4c, HuD bound to both transcripts of Mfn2 3U-1 and 3U-2. These results indicate that *Mfn2* mRNA is the target of HuD.

HuD is downregulated in the pancreas of diabetic models [44]. We demonstrate that it binds to *Mfn2* mRNA (Fig. 4). To determine whether HuD affects Mfn2 expression, we assessed Mfn2 level using RT-qPCR and western blot analysis after HuD downregulation. Knockdown of HuD using HuD siRNA in βTC6 cells decreased both *Mfn2* mRNA and protein level (Fig. 5a), which was further confirmed in shHuD cells (Fig. 5b). In addition, we investigated the HuD-mediated regulation of Mfn2 expression by assessing the EGFP reporter level. Based on the interaction between HuD and the 3′UTR of *Mfn2* mRNA (Fig. 4c), we generated EGFP reporters containing the 3′UTR of *Mfn2* mRNA (EGFP-Mfn2-3U1 and 3U2) (Fig. 5c) and analyzed EGFP expression after transfection of HuD siRNA. HuD knockdown decreased the EGFP expressions of both Mfn2-3U1 and 3U2 reporters (Fig. 5d), indicating that HuD downregulation decreases Mfn2 expression. Taken together, these results suggest that HuD regulates Mfn2 expression by associating its 3′UTR.

The HuD/Mfn2 axis plays an essential role in the regulation of mitochondrial morphology

Mfn2 regulates mitochondrial function by controlling mitochondrial elongation [16, 51, 52]. Our observations

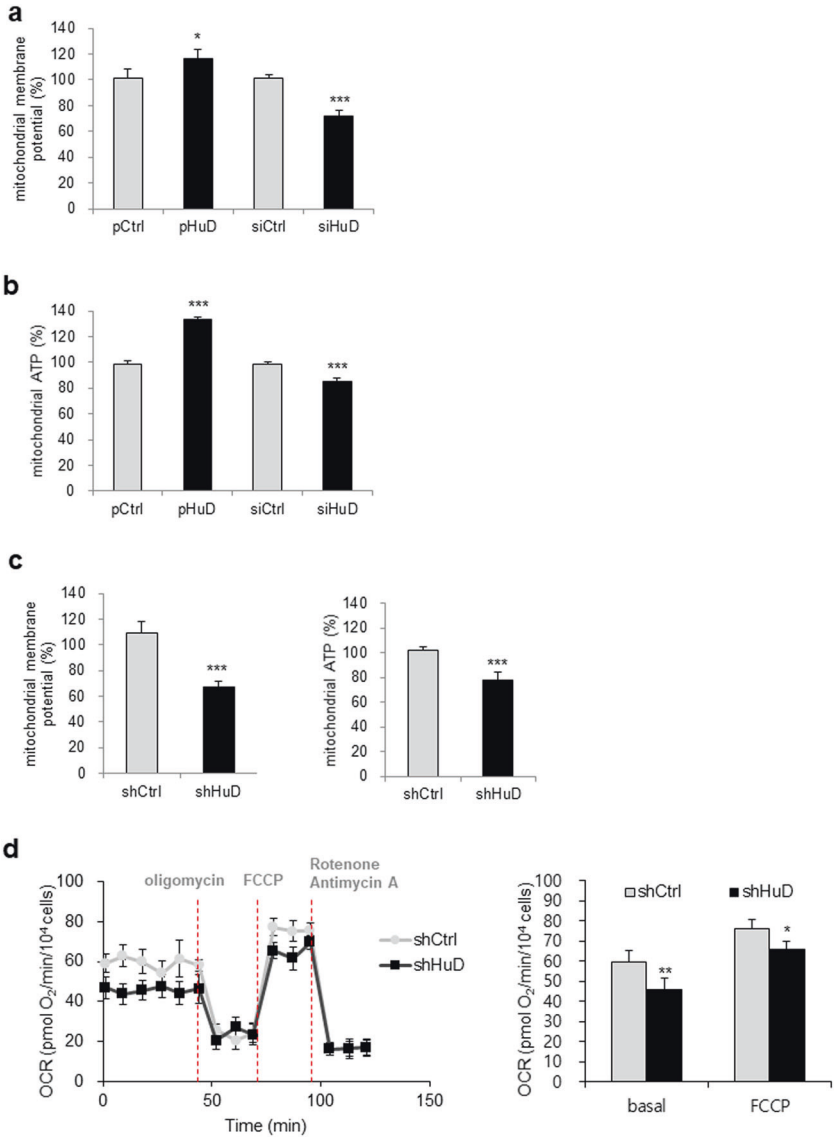

Fig. 3 Regulation of mitochondrial activity by HuD. **a**, **b** After transfection of βTC6 cells with HuD siRNA or pHuD with appropriate controls, mitochondrial membrane potential (**a**) and mitochondrial ATP levels (**b**) were analyzed. Cells were stained with tetraethyl benzimidazoly carbocyanine iodide (JC-1) and the fluorescence was measured at 530 nm (excitation)/590 nm (emission). Cells pretreated with galactose-containing media were incubated with the ATP detection reagent and the luminescence was measured using a microplate reader. **c** Mitochondrial membrane potential and ATP level were determined in βTC6-shHuD and βTC6-shCtrl cells. **d** Mitochondrial respiration in βTC6-shCtrl and βTC6-shHuD cells was determined by assessing the oxygen consumption rate. Cells were sequentially incubated with oligomycin, FCCP, rotenone, and antimycin A and OCR was measured using the Seahorse FX24 Extracellular Flux Analyzer. Data represent the mean ± SEM derived from three independent experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$

suggest that HuD acts as a novel factor regulating Mfn2 expression at the posttranscriptional level (Figs. 4 and 5). To evaluate whether the HuD/Mfn2 axis play a role in the regulation of mitochondrial morphology and function, we performed a complementation experiment by overexpressing myc-tagged Mfn2 in shHuD cells (Fig. 6a). As shown in Fig. 6b, ectopic expression of Mfn2 increased the portion of cells with elongated mitochondria in shHuD cells (Fig. 6b). In addition, mitochondrial membrane potential and mitochondrial ATP levels were moderately restored by Mfn2 overexpression (Fig. 6c). These results suggest that the HuD/Mfn2 axis regulates mitochondrial morphology and function, and aberrant expression of HuD in pancreatic β-cells results in mitochondrial dysfunction in diabetes.

Discussion

Tight regulation of mitochondrial structure and activity is critical for cellular homeostasis. Mitochondrial dysfunction is characterized by a reduction in ATP generation, mitochondrial membrane potential, and mitochondrial numbers, and is implicated in the pathology of several human diseases, such as diabetes, neurodegenerative diseases, and cancers. Dynamic regulation of mitochondrial morphology is essential for the normal function of mitochondria and is mediated by several key regulatory proteins including Drp1, Opa1, Fis1, Mff, and Mfn1/2. Herein, we demonstrate that the RNA binding protein HuD plays a novel role in mitochondrial dynamics via posttranscriptional regulation of Mfn2, resulting in mitochondrial dysfunction in pancreatic β-cells. We showed that HuD binds to 3′UTR of *Mfn2*

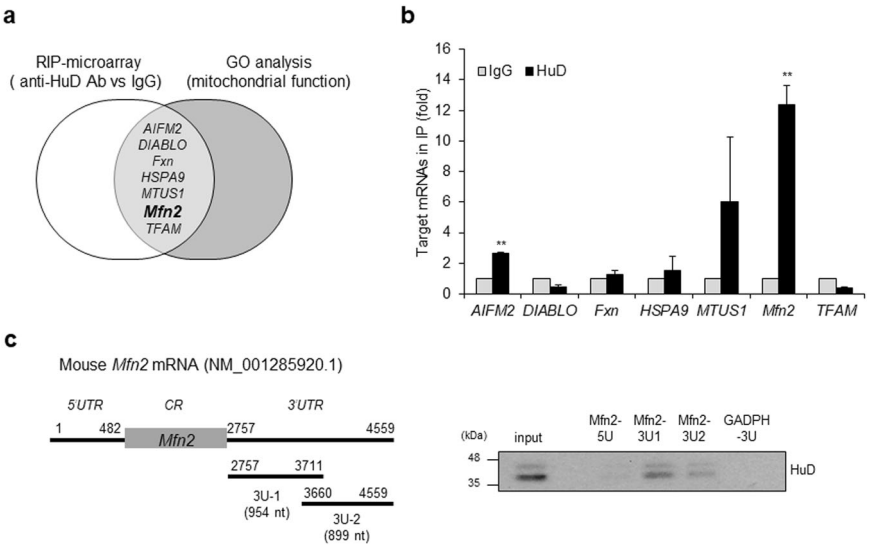

Fig. 4 Identification of target mRNAs of HuD. **a** Putative targets of HuD identified by both HuD-RIP analysis and GO term analysis (mitochondrial function). **b** The interaction between HuD and target mRNAs was confirmed by RIP-qPCR using anti-HuD and control IgG antibodies. *Gapdh* mRNA was used for normalization. **c** *Left:* A schematic of mouse *Mfn2* mRNA (NM_001285920). The 5′UTR (5U) and 3′UTR of *Mfn2* mRNAs (3U-1 and 3U-2) were transcribed in vitro and biotinylated for use in biotin pull-down analysis. *Right*: The biotinylated transcripts obtained from *Mfn2* mRNA were incubated with βTC6 cell lysates. The interaction between the transcripts and HuD was shown by western blot. Biotinylated *Gapdh* 3′UTR transcript was used as a negative control. Data represent the mean ± SEM derived from three independent experiments. $^{**}p < 0.01$

mRNA and promotes Mfn2 expression, thereby enhancing mitochondrial elongation. We also demonstrated mitochondrial fragmentation in pancreatic β-cells of diabetic mice, with a lower expression of HuD. Our data reveal that HuD regulates mitochondrial dynamics via posttranscriptional control of Mfn2 expression.

It is still disputed whether mitochondrial dysfunction is a cause or a consequence of diabetes; however, several studies suggest that abnormal mitochondrial function in pancreatic β-cells plays a decisive role in metabolic dysregulation and hyperglycemia [47, 48, 53, 54]. Alteration of mitochondrial morphology in pancreatic β-cells, in particular, fragmentation of mitochondria has been reported in diabetic pancreas [11, 46, 55–57]. Herein, we also report enhanced mitochondrial fission, matrix swelling, and disruption of cristae in pancreatic β-cells of diabetic mice (Fig. 1a). A few key proteins regulating mitochondrial fission and fusion, including Fis1 and Opa1 have been linked to β-cell dysfunction by affecting mitochondrial dynamics [9, 58, 59]. We propose that HuD-mediated Mfn2 regulation also mediates β-cell dysfunction. Because of the complex and possibly synergistic processes involved, elucidation of the molecular relationship between β-cell dysfunction and mitochondrial dynamics is needed.

Our previous report shows that the HuD level is downregulated in the pancreas of diabetic mice [44]. HuD is an RNA binding protein belonging to Hu antigen family and is expressed in certain types of tissues including neurons and pancreatic β-cells [42]. Various target mRNAs of HuD have been identified, while the regulatory mechanism is poorly understood. Posttranslational modifications including phosphorylation by protein kinase C and methylation by coactivator-associated arginine methyltransferase 1 regulate HuD activity [60, 61]. In addition, miRNA-375, one of the enriched miRNAs in the pancreas, acts as a negative regulator of HuD [41]. The miR-375 KO mice show impaired glucose homeostasis by regulating β-cell mass [62]. Therefore, it is worth exploring whether mitochondrial dysfunction occurred in miR-375 KO mice. Further studies exploring the regulation of HuD expression in diabetes may provide the molecular link underlying HuD-mediated β-cell dysfunction.

RIP analysis and in vitro pull-down assay using biotin-labeled transcripts reveal the interaction between HuD and 3′UTR of *Mfn2* mRNA (Fig. 4). Tracing the expression of reporters containing 3′UTRs of *Mfn2* mRNA may shed additional light on the interactions (Fig. 5d). Both 3U1 and 3U2 regions of 3′UTRs of *Mfn2* mRNA are regulated by HuD. The binding of 3U1 region to HuD is relatively strong, while that of 3U2 is moderate, but consistent. Although we could not determine the exact HuD-binding motifs on 3′UTRs of *Mfn2* mRNA, 3U1 appears to be subject to dominant regulation by HuD. Crosslinking immunoprecipitation and RNA sequencing analysis in pancreatic β-cells may provide additional information about the sequence responsible for HuD binding.

In conclusion, we propose that HuD is a novel regulatory protein affecting mitochondrial dynamics by enhancing

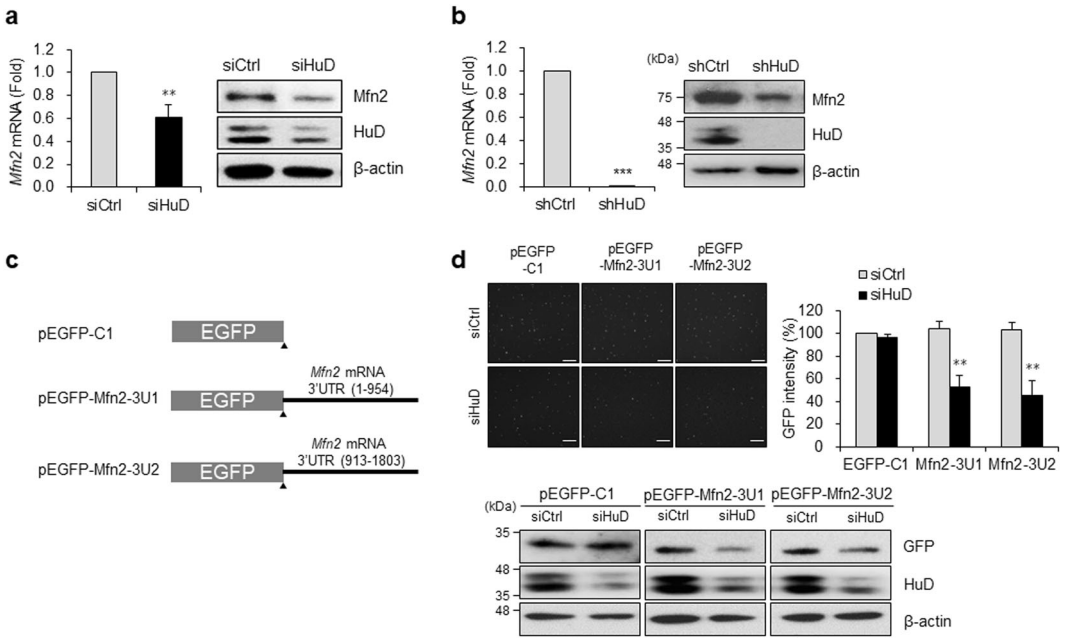

Fig. 5 Regulation of Mfn2 expression by HuD. *Mfn2* mRNA and protein levels were analyzed in βTC6 cells transfected with siHuD (**a**) or βTC6-shHuD (**b**) cells by RT-qPCR and western blot analysis, respectively. **c** Schematic representation of EGFP reporter constructs containing *Mfn2* mRNA 3′UTRs (pEGFP-Mfn2-3U1 and -3U2). **d** Following sequential transfection with a reporter plasmid after HuD knockdown in βTC6 cells, the relative EGFP expression was determined by measuring the fluorescence of EGFP and western blot analysis. Data represent the mean ± SEM from three independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$

Mfn2 expression. Our results suggest that downregulation of HuD in diabetes may contribute to impaired mitochondrial shape and function. Further studies investigating the differential expression of HuD in pancreatic β-cells and the underlying molecular mechanisms may provide additional insight into the role of HuD-mediated mitochondrial dynamics in the pathogenesis of diabetes.

Materials and methods

Cell culture, transfection of plasmids, and small interfering RNAs

Mouse pancreatic β-cell line, βTC6 cells were cultured in Dulbecco's Modified Eagle's Medium (Capricorn scientific, Ebsdorfergrund, Germany) supplemented with 10% fetal bovine serum and 1% antibiotics. βTC6 cell clones stably expressing shHuD plasmid (βTC6-shHuD) was established and maintained with puromycin (Invitrogen, Carlsbad, CA, USA). HuD overexpression plasmid (pHuD) was prepared by cloning CDS of mouse *HuD* mRNA into a retroviral vector pPGS-HA and viral particles were prepared using HEK293 cells. Enhanced green fluorescent protein (EGFP) reporters were cloned by inserting the 3′UTR of *Mfn2* mRNA (2757–4559, 1803 nt) into pEGFP-C1 vector (BD Bioscience, Franklin lake, NJ, USA). Plasmids (pEGFP, pHuD, pMfn2 (Addgene, #23213)) and small interfering RNAs (siRNAs; control siRNA (siCtrl) and HuD siRNA (siHuD)); Genolution Pharmaceuticals, Seoul, Korea) were transiently transfected using Lipofectamine^{TM} 2000 (Invitrogen).

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) containing a 1× protease inhibitor cocktail (Roche, Basel, Switzerland), separated by SDS polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies against HuD (Santa Cruz Biotechnology, Dallas, TX, USA), Mfn2 (Abcam, Cambridge, USA), EGFP (Santa Cruz Biotechnology), HA (Biolegend, San Diego, CA, USA), and β-actin (Abcam), and further incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology). Protein bands were detected using enhanced chemiluminescence (Bio-Rad, Hercules, CA, USA).

RNA analysis

Total RNA was isolated from whole cells using RNAiso plus Reagent (Takara Bio Inc, Shiga, Japan). After reverse transcription (RT) using random hexamers and reverse transcriptase (Toyobo, Osaka, Japan), the mRNA levels

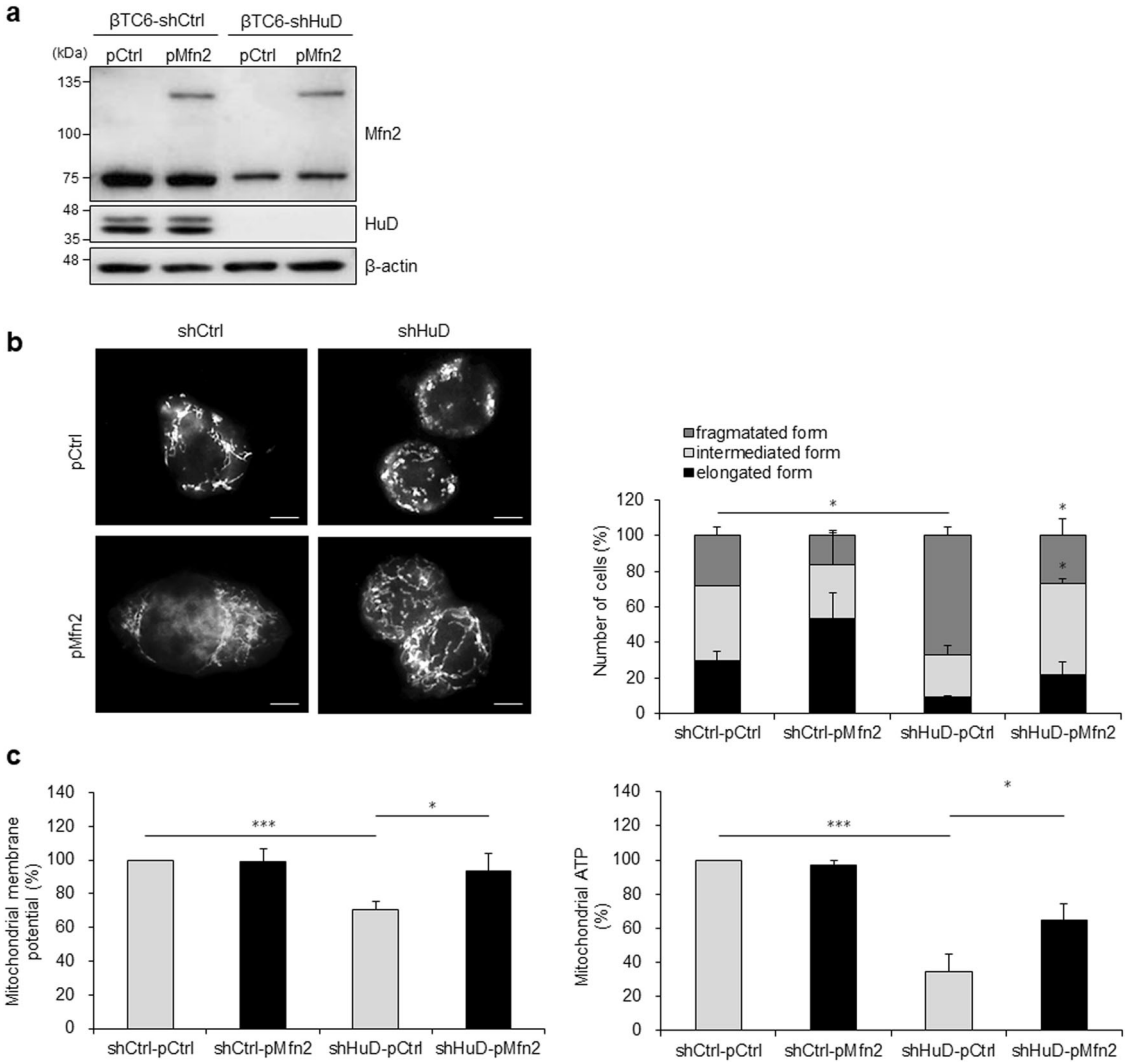

Fig. 6 Regulation of mitochondrial dynamics along the HuD/Mfn2 axis. βTC6-shCtrl and βTC6-shHuD cells were transfected with pMfn2 and pCtrl. **a** Mfn2 and HuD protein levels were analyzed by western blot analysis. **b** After transfection with pMfn2, cells were incubated with MitoTracker® and mitochondrial morphology was analyzed using a fluorescence microscope. Bar, 5 μm. **c** After transfection with pMfn2, mitochondrial membrane potential and ATP level were determined by JC-1 staining and mitochondrial ToxGlo™ assay. Data represent the mean ± SEM derived from three independent experiments. $*p < 0.05$; $***p < 0.001$

were quantified by real time quantitative PCR analysis using SensiFAST™ SYBR® Hi-ROX Kit (Bioline, London, UK) and gene-specific primer sets (Supplementary Table 2). RT-qPCR analysis was performed using StepOnePlus™ (Applied Biosystems, Foster City, CA, USA).

Ribonucleoprotein complex-immunoprecipitation and qPCR (RIP-qPCR) analysis

RNP complexes were immunoprecipitated using Protein A bead (Invitrogen) coated with anti-HuD or control IgG antibodies (Santa Cruz Biotechnology) at 4 °C overnight [43]. The immunoprecipitated RNP complexes were incubated with DNase I and proteinase K. RNAs were isolated from the complex and further analyzed by RT-qPCR using the primers listed (Supplementary Table 2).

Biotin pull down assay

To synthesize biotinylated transcripts, PCR fragments were prepared using forward primers, including T7 RNA polymerase promoter sequence (T7, 5′-CCAAGCTTCTAATA CGACTCACTATAGG-GAGA-3′). Primers used to prepare biotinylated transcripts *Mfn2* mRNA (NM_001285920.1) are listed in Supplementary Table 2. After purification of the PCR products, biotinylated transcripts were synthesized using the MaxiScript T7 kit (Ambion, Waltham, MA, USA) and biotin-CTP (Enzo Life Sciences, Farmingdale, NY, USA).

Whole-cell lysates (300 μg per sample) were incubated with 1 μg of purified biotinylated transcripts for 30 min at room temperature, and the complexes were isolated using streptavidin-coupled Dynabeads (Invitrogen). Proteins were isolated from the complex and further studied by western blot analysis [43].

Morphological analysis of mitochondria

βTC6 cells were incubated with 100 nM Mitotracker Red CMXRos (Invitrogen) for 30 min at 37 °C. Mitochondria morphology of either βTC6 cells treated with Mitotracker or mt-YFP expressing βTC6 cells was observed using a fluorescence microscope. The fluorescent images were acquired using an Axiovertcam mRM camera attached to an Axiovert 200M (Carl Zeiss, Jena, Germany).

For electron microscopy, tissues or cells were fixed with 1% glutaraldehyde and embedded using Epon 812. Ultra-thin sections were observed with a transmission electron microscope JEM 1010 (JEOL, Tokyo, Japan).

Measurement of the mitochondrial membrane potential and mitochondrial ATP level

Mitochondrial membrane potential was measured using the tetraethyl benzimidazoly carbocyanine iodide (JC-1) staining solution (Abcam) according to the manufacturer's protocol. Cells were incubated with the reagent for 15 min and the fluorescence was measured at 535 nm (excitation)/590 nm (emission) using the Synergy™ H1 multimode microplate reader (BioTek, VT, USA). Mitochondrial ATP level was also measured using the Mitochondrial Toxglo™ assay kit (Promega, Madison, WI, USA). After incubating cells with galactose-containing media, cells were incubated with the ATP detection reagent at 37 °C for 90 min and the luminescence was assessed with the Synergy™ H1 hybrid microplate reader (BioTek, VT, USA).

Analysis of oxygen consumption rate

OCR between βTC6-shCtrl and βTC6-shHuD cells was determined using the Seahorse FX24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA, USA) with an XF Cell Mito Stress Test Kit (Seahorse Bioscience) according to the manufacturer's instruction. An ATP synthase blocker, oligomycin (3 μM) and the mitochondrial uncoupler, FCCP (carbonyl cyanide 4-[trifluoromethoxy] phenylhydrazone, 1 μM) were used to measure the proton leakage and the maximal OCR, respectively. An inhibitor of complex I, rotenone (1 μM) and a complex III blocker, antimycin A (1 μM) were used to determine the non-mitochondrial respiration. The results were analyzed using a Seahorse XF Prep Station [63].

Statistical analysis

Data were expressed as mean ± SEM of three independent experiments. The statistical significances of the data were analyzed by Student's t test; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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