The RNA-binding Protein HuD Regulates Autophagosome Formation in Pancreatic β Cells by Promoting Autophagy-related Gene 5 Expression*

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Background: Autophagy-related gene 5 (ATG5) has a pivotal role in the formation of autophagosomes. ATG5 expression was increased by the RNA-binding protein HuD at the mRNA level.

Results: ATG5 expression was increased by the RNA-binding protein HuD at the mRNA level.

Conclusion: HuD promotes autophagosome formation by elevating ATG5 expression in pancreatic β cells.

Significance: HuD is a novel regulator of autophagosome formation and autophagy flux in pancreatic β cells.

 Tight regulation of autophagy is critical for the fate of pancreatic β cells. The autophagy protein ATG5 is essential for the formation of autophagosomes by promoting the lipidation of microtubule-associated protein LC3 (light chain 3). However, little is known about the mechanisms that regulate ATG5 expression levels. In this study, we investigated the regulation of ATG5 expression by HuD. The association of HuD with ATG5 mRNA was analyzed by ribonucleoprotein complex immunoprecipitation and biotin pull-down assays. HuD expression levels in pancreatic β cells were knocked down via siRNA, elevated by overexpression of a HuD-adigm, HuD-null mice displayed lower ATG5 and LC3 levels in LC3-positive autophagosomes. In keeping with this regulatory paradigm, HuD contributed to the lipidation of LC3 and the formation of autophagosomes. However, little is known about the mechanisms that regulate ATG5 expression levels. In this study, we investigated the regulation of ATG5 expression by HuD, and conversely, HuD overexpression did not alter ATG5 mRNA translation. Through its effect on ATG5, HuD contributed to the lipidation of LC3 and the formation of LC3-positive autophagosomes. In keeping with this regulatory paradigm, HuD-null mice displayed lower ATG5 and LC3 levels in pancreatic β cells. Our results reveal HuD to be an inducer of ATG5 expression and hence a critical regulator of autophagosome formation in pancreatic β cells.

Autophagy is a catabolic process that leads to the degradation and recycling of unnecessary or dysfunctional cellular components (1). It is primarily activated in response to stress conditions induced by a multitude of physiological and pathological situations, including starvation, growth factors, oxidative stress, and endoplasmic reticulum stress (2, 3). Autophagy also occurs at basal levels in most tissues for the selective elimination of misfolded or aggregated proteins, damaged organelles, and intracellular pathogens, thereby protecting against various diseases. It is a highly regulated process that has key roles in cell survival, development, and homeostasis, helping to maintain a balance between synthesis and degradation in cells undergoing intense metabolic activity and is deregulated in various pathological conditions, including cancer, neurodegenerative diseases, and type 2 diabetes (4, 5).

The hallmark of autophagy is the formation of a double-membrane vesicle with cytosolic contents that is called an autophagosome (6). The formation of the autophagosome and their delivery to lysosomes are controlled by conserved key regulators known as autophagy-related (ATG) proteins including ATG5, ATG7, ATG8 (or LC3, for microtubule-associated protein light chain 3), and ATG12 (7). They mediate the conjugation of LC3I to phosphatidylethanolamine to form LC3II, which specifically associates with autophagosomes, a critical step in autophagy (8, 9). LC3 conjugation, which can be monitored by a shift in electrophoretic mobility from the LC3I to LC3II by Western blot analysis, and LC3-positive puncta are used as well accepted markers for autophagosome formation and autophagy (10). Accumulating data indicate that ATG5 plays critical roles in autophagosome formation under most circumstances by promoting LC3I lipidation to LC3II (11, 12). The activity of ATG5 during the autophagic process is regulated by several post-translational modifications including acetylation (13) and phosphorylation (14). Accordingly, deletion of ATG5 suppresses the lipidation of LC3I to LC3II, inhibits autophagy (15, 16), and was sufficient to cause pathological conditions including neurodegeneration and neurolog-
Table 1: Primer list used in this study

| Primers Sequences |
|-------------------|
| **For BPD** |
| Mouse GADPH 3’-F | 5’-GGTGAAAGCAAGTCTTTTCTTTA-3’ |
| Mouse GADPH 5’-R | 5’-GCAGCTGCTAGATATGGCTG-3’ |
| Mouse ATG5 5’-F | 5’-TCCTGACGTGGAATGGTTGACA-3’ |
| Mouse ATG5 5’-R | 5’-TCCTGACGTGGAATGGTTGACA-3’ |
| Mouse EGFP 5’-F | 5’-GGAGTCTGTTGAGAGGTTTCC-3’ |
| Mouse EGFP 3’-R | 5’-GGAGTCTGTTGAGAGGTTTCC-3’ |
| **For cloning of HuD fusion plasmid** |
| Mouse HuD 5’-F | 5’-AAAAGATCTTACTGGAGAGGTTGACA-3’ |
| Mouse HuD 3’-R | 5’-AAAAGATCTTACTGGAGAGGTTGACA-3’ |
| **For RT-qPCR** |
| Mouse ATG5 5’-F | 5’-GTCTCCCTCAGAATGTTGACAT-3’ |
| Mouse ATG5 3’-R | 5’-GTCTCCCTCAGAATGTTGACAT-3’ |
| Mouse GAPDH 5’-F | 5’-GTCTCCCTCAGAATGTTGACAT-3’ |
| Mouse GAPDH 3’-R | 5’-GTCTCCCTCAGAATGTTGACAT-3’ |
| EGFP 5’-F | 5’-CACAGGAGGGCGAGAGACA-3’ |
| EGFP 3’-R | 5’-CGATCTGCTCTCCTCTCCT-3’ |

HuD Enhances Autophagosome Formation

Recent studies have identified microRNAs that post-transcriptionally regulate ATG transcripts including ATG5 mRNA (18–20). Here, we describe the identification of HuD/human antigen D/embryonic lethal abnormal vision-like 4 (ELAVL4) (18–20). Here, we describe the identification of HuD/human antigen D/embryonic lethal abnormal vision-like 4 (ELAVL4) as a new autophagy regulatory RNA-binding protein that associates with ATG5 mRNA and controls its translation in pancreatic β cells. Like other Hu/ELAV family members (HuR, HuB, and HuC), HuD has three RNA recognition motifs that mediate its association with the UTR of mRNAs bearing specific sequences that are often AU- and U-rich (21). Through these associations, HuD can modulate the stability or/and the translation of target mRNAs, often enhancing it, but other times repressing it (22–24). The roles of HuD in the regulation of neuronal development and plasticity were characterized using animal models (25, 26). We previously found that HuD is also expressed in pancreatic β cells in addition to neurons (27). In this study, we showed that the binding of HuD to the 3’-UTR of ATG5 mRNA enhances ATG5 mRNA translation, thereby promoting the conversion of LC3I to LC3II and leading to an increase of LC3-positive autophagosomes. Because of this regulatory process, HuD-null (HuD−/−) mice expressed lower levels of ATG5 and LC3 in β cells.

Materials and Methods

**Cell Culture, Transfection, Plasmid, and Small Interfering RNAs**—βTC6 cells were cultured in DMEM (Invitrogen), supplemented with 10% fetal bovine serum and antibiotics. U2OS cells stably expressing GFP-LC3 was established and maintained as described in Ref. 28. The pHuD plasmid was constructed by inserting the mouse coding HuD sequence into the pRFP-C1 plasmid. Enhanced green fluorescent protein (EGFP)3 reporters were cloned by inserting 3’-UTR fragments from the ATG5 mRNA into pEGFP-C1 (BD Bioscience). siRNAs (control siRNA (siCtrl; Qiagen), HuD siRNA (Santa Cruz Biotechnology)), ATG5 siRNA (Damarcon), and miR-181 precursor (Bioneer, South Korea), and the Myc-tagged HuD (pHuD) and EGFP reporter plasmids were transfected using Lipofectamine RNAiMAX or Lipofectamine 2000 (Invitrogen).

**Western Blot Analysis**—Whole cell lysates were prepared using radioimmune precipitation assay buffer (10 mm Tris-HCl, pH 7.4, 150 mm NaCl, 1% Nonidet P-40, 1 mm EDTA, and 0.1% SDS), separated by electrophoresis in SDS-containing polyacrylamide gels, and transferred onto PVDF membranes (Millipore). Incubation with primary antibodies against ATG5 (Epitomics), LC3 (Abcam), HuD (Santa Cruz Biotechnology), EGFP (Santa Cruz Biotechnology), or β-actin (Abcam) was followed by incubations with the appropriate secondary antibodies conjugated with HRP (Santa Cruz Biotechnology) and detection using enhanced luminescence (GE Healthcare).

**RNA Analysis and Ribonucleoprotein Complex Immunoprecipitation**—Total RNA was prepared from whole cells using TRIzol (Invitrogen). After RT using random hexamers and reverse transcriptase (Toyobo), the abundance of transcripts was assessed by quantitative PCR (qPCR) analysis using the SYBR green PCR master mix (Kapa Biosystems) and gene-specific primer sets (Table 1). RT-qPCR analysis was performed on Applied Biosystems model 7300 instruments.

3 The abbreviations used are: EGFP, enhanced green fluorescent protein; qPCR, quantitative PCR; IP, immunoprecipitation; 3U1, first 3’-UTR; 3U2, second 3’-UTR; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide.
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Ribonucleoprotein complex immunoprecipitation analysis was performed using anti-HuD primary antibodies or control IgG (Santa Cruz Biotechnology) (27). In brief, RNP complexes were immunoprecipitated using HuD or control IgG antibodies and incubated with DNase I and protease K; RNA in the immunoprecipitation (IP) samples was isolated and further analyzed by RT-qPCR using the primers listed (Table 1).

**Biotin Pulldown Analysis**—To synthesize biotinylated transcripts, PCR fragments were prepared using forward primers that contained the T7 RNA polymerase promoter sequence (T7, CCAAGCTTCTAACTAGCAGCTAATAGGGGA). The primers used to prepare biotinylated transcripts spanning the ATG5 mRNA (NM_053069.5) are listed in Table 1. After purification of the PCR products, biotinylated transcripts were synthesized using the MaxiScript T7 kit (Ambion) and biotinylated-mRNA (Enzo Life Sciences). Whole cell lysates (200 μg/sample) were incubated with 1 μg of purified biotinylated transcripts for 30 min at room temperature, and then complexes were isolated using streptavidin-coupled Dynabeads (Invitrogen). Proteins present in the pulldown material were studied by Western blot analysis as described in Ref. 27.

**Polysome Analysis**—48 h after transfection of siHuD and pHuD with appropriate controls, βTC6 cells were preincubated with cycloheximide (100 μg/ml, 15 min) and lysed with polysome extraction buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM MgCl2, 0.5% (v/v) Nonidet P-40, 1× protease inhibitor mixture, and RNase inhibitor, followed by centrifugation at 10,000 × g for 10 min. Lysates were further fractionated by ultracentrifugation through linear sucrose gradients as described in Ref. 29. RNAs from each fraction were isolated, and cDNA was synthesized as described above. The relative levels of ATG5 and GAPDH mRNAs were analyzed by RT-qPCR using specific primer set.

**Immunostaining**—HuD−/− mice and wild-type littersmates (HuD+/+) have been described previously (25) and were used at 14 weeks of age. Pancreases from fasted HuD+/+ and HuD−/− mice were fixed in 4% paraformaldehyde, immersed in 20% sucrose before freezing, and then sectioned at a thickness of 7 μm. After antigen unmasking, the slides were incubated with primary antibodies (anti-insulin (Sigma) or anti-ATG5 and anti-LC3 (Abcam)), followed by secondary antibodies (Invitrogen). Slides were viewed using a LSM-710 confocal microscope (Carl Zeiss). For quantification, digital images of multiple sections from three mice per group, separated by at least 200 μm from each section, at a magnification of 40× were obtained, and signal intensity was determined using LSM Image Browser software (Carl Zeiss).

**Nascent Translation Assay**—Nascent translation of EGFP was studied by incubating βTC6 cells with 1 μCi [35S]methionine and l-[35S]cysteine (PerkinElmer Life Sciences) per 35-mm plate for 15 min. After lysis, IP reactions were carried out using mouse IgG or anti-GFP (Santa Cruz Biotechnology), and reactions were resolved by SDS-PAGE, transferred to PVDF membranes, and visualized using a PharoseFX Plus system (Bio-Rad) analyzed using Quantity One software (Bio-Rad).

**Electron Microscopy**—For transmission electron microscopic observation, βTC6 cells were fixed with 1% osmium tetroxide and embedded using Epon 812. Ultrathin sections were observed with a transmission electron microscope (JEM 1010).

**RESULTS**

**HuD Binds to the 3′-UTR of ATG5 mRNA**—During experiments that identified HuD as an RNA-binding protein that binds to insulin mRNA and controls its translation (27), we also identified ATG5 mRNA as another target of HuD in pancreatic β cells. We confirmed the interaction between HuD and ATG5 mRNA by ribonucleoprotein complex immunoprecipitation with an anti-HuD antibody. ATG5 mRNA associated with HuD in the RNP complexes was further analyzed by RT followed by qPCR analysis in mouse insulinoma βTC6 cells. Ins2 mRNA was used as a positive control for the HuD IP (27). As seen in Fig. 1A, ATG5 mRNA is significantly enriched in the anti-HuD IP relative to the control IgG IP (Fig. 1A), supporting the idea that ATG5 mRNA is a part of HuD RNP complexes. We further identified a potential HuD binding region in ATG5 mRNA using biotin-labeled RNA probes (27). Biotinylated segments of the ATG5 mRNA spanning the 5′–3′UTR, coding region, and 3′-UTR (3U1 and 3U2) were synthesized (Fig. 1B, top), and the RNP complexes containing HuD and biotinylated RNAs were detected using streptavidin-coated beads. As shown in Fig. 1B, HuD has specific affinity for the 3U2 fragment of the ATG5 mRNA, but not for the ATG5 5′-UTR, coding region, 3U1, or a negative control RNA, the 3′-UTR of GAPDH mRNA. Experiments to examine the association of other RNA-binding proteins and ATG5 mRNA reveal that ATG5 mRNA associates with HuR but not with AU1, hnrNP L, TIAR, and NF90 (Fig. 1B). Further assays using fragments of the ATG5 3U2 reveal that segment B, spanning positions 819–1038, predominantly interacts with HuD (Fig. 1C). In sum, HuD associates with the ATG5 mRNA through its 3′-UTR. We then set out to study the functional link between HuD and ATG5 mRNA.

**HuD Promotes the Translation of ATG5 mRNA by Interacting with the ATG5 3′-UTR**—Next, we tested whether the 3U2 region of ATG5 mRNA is responsible for the regulation of ATG5 expression by HuD in βTC6 cells. We generated EGFP reporter constructs harboring the 3U1 or 3U2 segment of ATG5 mRNA downstream of the coding region of EGFP mRNA (pATG5 3U1 or pATG5 3U2; Fig. 2A) and transfected the reporter constructs into siRNA-transfected (siCtrl or siHuD) or plasmid-transfected (pcDNA or pHuD) βTC6 cells. As observed in Fig. 2B, EGFP mRNA remains unchanged after both silencing and overexpression of HuD, as measured using RT-qPCR (Fig. 2B). However, HuD silencing leads to selectively decreased expression of EGFP reporter in the pATG5 3U2 group, but not in the control reporter group (pEGFP), as assessed by Western blot analysis. Conversely, HuD overexpression up-regulates the expression of EGFP reporter in the pATG5 3U2 group, but not in the control group (Fig. 2C). However, EGFP expression levels are not changed by HuD silencing or overexpression in the groups transfected with pATG5 3U1 (Fig. 2D), the region that shows no direct interaction with HuD (Fig. 1B).

Previously, miR-181 was shown to be a negative regulator of ATG5 expression by targeting 3′-UTR (20). To determine
whether miR-181 competes with HuD the regulation of ATG5 expression, we examined the level of EGFP in miR-181- and/or HuD-transfected cells. Whereas the level of EGFP expressed by the control (pEGFP) is not affected by miR-181 or HuD expression, expression of EGFP by pATG5 3U2 is down-regulated by miR-181 transfection and restored by HuD overexpression (Fig. 2E).

Likewise, endogenous ATG5 mRNA levels in βTC6 cells remained unchanged after both silencing and overexpression of HuD (Fig. 3A). However, the levels of ATG5 protein were significantly reduced by silencing HuD via siRNA and, conversely, increased by ectopic expression of HuD (pHuD) (Fig. 3B). Collectively, these results indicate that HuD can associate with the 3′-UTR in ATG5 mRNA and that HuD increases ATG5 abundance, likely by enhancing its translation.

To investigate whether HuD regulates translation of ATG5 mRNA, we tested the relative association of ATG5 mRNA with polyribosomes by polysome fractionation in βTC6 cells after HuD silencing or HuD overexpression as described previously (29). Cytoplasmic lysates were fractionated through sucrose gradients, with the lightest components sedimenting at the top (fractions 1 and 2), small (40 S) and large (60 S) ribosomal subunits and monosomes (80 S) appearing in fractions 3–5, and progressively larger polyribosome appearing in fractions 6–12 (Fig. 3C). Polysome profiles are not affected by HuD silencing or HuD overexpression (Fig. 3C). Whereas in siCtrl cells, the distribution of polysome-associated ATG5 mRNA levels peaks in fraction 7, HuD silencing reduces the average peak size of ATG5 mRNA polysomes such that most of the ATG5 mRNA is found in the lighter molecular weight polysomes in fraction 6 (Fig. 3D, left top). In HuD-overexpressing cells, polysome-associated ATG5 mRNA is found among the larger polysomes in fractions 7 and 8 (Fig. 3D, right top). The distribution of the housekeeping GAPDH mRNA associating with polysomes largely overlaps between two groups (Fig. 3D, bottom). The relative distribution of ATG5 mRNA is significantly enriched in the polysome fraction (fractions 7–11) compared with the free subunits/monosome fraction (fractions 1–6; Fig. 3E). To provide additional evidence for the translational regulation of ATG5 mRNA by HuD, the distribution of EGFP mRNA in the polysome fractions is analyzed in Fig. 3F. Polysome-associated EGFP mRNA containing ATG5 mRNA 3′-UTR is found in the heavier polysome fractions in HuD-overexpressing cells, whereas is found in the lighter molecular weight polysomes in HuD-silenced cells (Fig. 3F). Together, these findings suggest that HuD increases the association of the ATG5 mRNA with high molecular weight, actively translating polysome fractions, and therefore that HuD promotes ATG5 expression by enhancing ATG5 mRNA translation.

To further confirm the translational regulation of ATG5 mRNA by HuD, we also studied the nascent translation of ATG5; however, the ATG5 antibody used in this study did not work for IP (data not shown). Instead, we measured the de novo synthesis of the reporter protein expressed from pATG5 3U2 using an EGFP antibody. Briefly, following transfections, cells were incubated for a short time period (20 min) with [35S]Met and [35S]Cys, whereupon lysates were prepared, and the abundance of newly synthesized [35S]EGFP was measured by IP. As shown Fig. 3G, nascent EGFP translation is enhanced by HuD overexpression but down-regulated by HuD silencing, which suggests that HuD enhances the translation of ATG5 mRNA.

HuD Increases Autophagosome Formation—ATG5 is responsible for LC3I conjugation to phosphatidylethanolamine to form LC3II, as well as for the membrane localization of autophagic machinery and the formation of autophagosomes (7). Because LC3II is localized in autophagosome-associated puncta, assessing LC3 lipidation is a useful way to monitor autophagy in mammals (10). By monitoring the change in LC3II levels in βTC6 cells expressing different levels of HuD, we investigated whether HuD-mediated modulation of ATG5
expression is involved in the formation of autophagosomes. As shown in Fig. 4A, ectopic expression of HuD increases the levels of LC3II, which, conversely, is reduced by HuD silencing. Because autophagy induction is mediated by AMPK activation, we next treated U2OS cells stably expressing GFP-LC3 with AICAR, which is an AMPK activator, or colchicine, which is an autophagic-lysosomal delivery inhibitor, for 16 h and examined whether HuD affects GFP-LC3II conversion, as well as the formation of GFP-positive puncta, using Western blot analysis and fluorescence microscopy, respectively (Fig. 4, B and C). AICAR or colchicine treatment leads to elevated levels of GFP-LC3II in control cells (transfected with pcDNA), whereas HuD-overexpressing cells (pHuD) have higher levels of GFP-LC3II, as assessed by Western blot analysis (Fig. 4B). Consistent with these results, HuD overexpression facilitates the formation of GFP-positive puncta in both untreated cells and AICAR/colchicine-treated cells (Fig. 4, C and D).

The ratio of the number of cells having GFP-LC3 puncta relative to the number of GFP-positive cells is shown in Fig. 4D. AICAR or colchicine treatment increases GFP-LC3II accumulation in both control and HuD-overexpressing cells (Fig. 4D), indicating that HuD-mediated conversion of LC3I to LC3II contributes to the formation of autophagosome. We also examined the effect of HuD in autophagosome formation by transmission electron microscopic analysis (Fig. 4E). As expected, the number of autophagosomes is reduced in HuD silencing cells. The essential role of ATG5 in the regulation of autophagy has previously been demonstrated by showing impaired LC3 conversion and autophagosome formation in ATG5-deficient mice (11). To test whether HuD-mediated modulation of LC3 con-
version is dependent on ATG5, HuD (pHuD) was ectopically expressed in U2OS/GFP-LC3 cells (28) with either siCtrl or ATG5 siRNA (siATG5), and then the cells were incubated with AICAR or colchicine (Fig. 5). As anticipated, siATG5 transfection diminishes GFP-LC3 conversion after AICAR treatment (third lane versus fourth lane). In agreement with results in Fig. 4B, overexpression of HuD enhances GFP-LC3 conversion in both the vehicle (first lane versus fifth lane) and AICAR/colchi-
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**FIGURE 4.** HuD enhances autophagosome formation. 

**A.** 48 h after transfection of siRNA (siCtrl or siHuD) or 24 h after transfection of plasmids (pcDNA or pHuD) into βTC6 cells, the levels of HuD, LC3, and loading control β-actin were assessed by Western blot analysis. Relative density for the indicated proteins is shown on the bottom. The data represent the means ± S.E. from three independent experiments. 

**B.** U2OS cells stably expressing GFP-LC3 were incubated with 200 μM AICAR or 0.4 μg/ml colchicine for 16 h after transfection with pcDNA or pHuD. The levels of HuD, GFP-LC3, and loading control β-actin were analyzed by Western blot analysis. Relative density for the indicated proteins is shown on the bottom. The data represent the means ± S.E. from three independent experiments. 

**C.** 24 h after transfection of pcDNA or pHuD, U2OS/GFP-LC3 cells were incubated with 200 μM AICAR or 0.4 μg/ml colchicine for 16 h and examined under fluorescence microscope. 

**D.** The ratio of GFP-LC3 punctated cells to total GFP-positive cells was analyzed under a fluorescence microscope. The data represent the means ± S.E. of three independent experiments. 

**E.** 48 h after transfection of siCtrl or siHuD, βTC6 cells were fixed overnight and subjected to transmission electron microscopic analysis. The arrows indicate autophagosomes.

**FIGURE 5.** Regulation of autophagosome formation by HuD is ATG5 dependent. 

48 h after transfection of siRNAs (siCtrl or siATG5), U2OS/GFP-LC3 cells were transfected with pcDNA or pHuD for 24 h and then treated with 200 μM AICAR (A) or 0.4 μg/ml colchicine (B) for an additional 16 h. The levels of ATG5, HuD, GFP-LC3, and loading control β-actin were assessed by Western blot analysis. Relative density for the indicated proteins is shown on the bottom. The data are representative of three independent experiments.
cine-treated groups (third lane versus seventh lane), respectively. However, the HuD-mediated increase of GFP-LC3 conversion is largely impaired by ATG5 silencing (sixth lane and eighth lane) as assessed by Western blot analysis (Fig. 5). This observation was recapitulated in /H9252 TC6 cells (data not shown).

Together, these data suggest that HuD contributes to the lipidation of LC3II and to autophagosome formation by directly regulating ATG5 expression.

Decreased ATG5 and LC3 Levels in Pancreatic /H9252 Cells of HuD /H11002 /H11002 Mice

/HuD /H11002 /H11002 mice are known to show impaired neuronal differentiation and decreased proliferation of neural stem/progenitor cells (25). In our previous report (27), we found increased insulin levels in pancreatic /H9252 cells from HuD /H11002 /H11002 mice, compared with HuD /H11001 /H11001 mice. Because HuD increased ATG5 levels and affected LC3 conversion in cultured cells, we tested the possibility that HuD also elicits this effect in pancreatic β cells in vivo. Analysis of pancreases from HuD /H11002 /H11002 mice reveals lower expression of ATG5 in the pancreatic β cells from HuD /H11002 /H11002 mice compared with HuD /H11001 /H11001 mice, as assessed by immunofluorescence (Fig. 6A). Furthermore, the perinuclear accumulation of LC3, which is apparent mainly during longer starvation periods (30) and is considered a reliable marker of autophagy (31), is reduced in pancreatic β cells from HuD /H11002 /H11002 mice, compared with those from HuD /H11001 /H11001 mice (Fig. 6B). Thus, our data suggest that HuD plays an important role in autophagosome formation by regulating ATG5 translation in pancreatic β cells in vivo.

DISCUSSION

In type 2 diabetes, it is well known that an increased number of autophagic vacuoles and increased cell death is linked to pancreatic β cell dysfunction. ATG5 is a critical factor for autophagosome formation under most circumstances by facilitating the conversion of LC3I to LC3II. In this study, we identified HuD as a novel regulator of autophagosome formation, a role that is linked to the binding of HuD to the ATG5 mRNA 3’-UTR, which leads to enhanced ATG5 expression. In pancreatic β cells, HuD overexpression elevated the level of ATG5 and enhanced the conversion of LC3I to LC3II, whereas HuD silencing lowered ATG5 levels and suppressed LC3II conversion. Moreover, ectopic expression of HuD in U2OS cells, which do not express endogenous HuD, enhanced the conversion of GFP-LC3I to GFP-LC3II and the formation of GFP-LC3 puncta after AICAR or colchicine treatment. Taken together, these data support the hypothesis that HuD is one of the pivotal regulators of autophagosome formation in pancreatic β cells.

/HuD has been extensively studied in neuronal system (25, 32–37). It has been reported to associate with AU-rich RNA elements in 3’-UTRs from several mRNAs, and this association often results in mRNA stabilization (29, 38, 39). However, our previous studies found that HuD was also expressed in pancreatic β cells and repressed insulin translation through binding to the insulin mRNA 5’-UTR (27), in turn lowering insulin production. A similar function for HuD as a translational repressor...
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has also been identified previously, with HuD being shown to repress the translation of p27 target mRNA and other RNAs (38–40). In contrast, HuD binding to the Nova1 mRNA enhanced its stability and translation (41). The highly conserved protein HuR, a ubiquitous member of the Hu family, also associates with the ATG5 mRNA 3′-UTR (Fig. 1B). The possibility that HuR may also be involved in the regulation of ATG5 expression awaits further investigation.

The results presented in this study show that the binding of HuD to ATG5 mRNA facilitates ATG5 translation by enhancing the assembly of large polysomes to increase ATG5 levels, which promotes the conversion of LC3I to LC3II and the formation of autophagosomes. A recent study by Tekirdag et al. (20) showed that miR-181a affects starvation- and rapamycin-induced autophagy by down-regulating ATG5 levels in human MCF, Huh7, and K562 cells. Overexpression of miR-181a resulted in decreased autophagy activity during starvation or rapamycin treatment by targeting the ATG5 mRNA 3′-UTR. Another study reported that mimics and inhibitors of miR-181a modulated ATG5 expression levels as well as those of a luciferase reporter containing the ATG 3′-UTR (42). HuD and miR-181 have different binding sites in 3′-UTR of ATG5 mRNA (Fig. 1C) and regulate ATG5 expression in opposite ways; HuD enhances ATG5 expression, whereas miR-181 inhibits it, which indicates that they might competitively regulate ATG5 expression (Fig. 2E). Further studies will be needed to elucidate the molecular mechanisms underlying the regulation of ATG5 expression through various post-transcriptional regulators.

Our results add to a small but emerging body of evidence that ATG5 expression is regulated at the post-transcriptional level in pancreatic β cells. The fact that HuD−/− mice express lower levels of ATG5 and reduced perinuclear accumulation of LC3 in pancreatic β cells compared with HuD+/+ mice (Fig. 6) leads us to hypothesize that HuD−/− mice may show altered autophagy activity. Further studies are underway to monitor autophagy influx in HuD−/− animals after stress responses with heavy involvement of β cell activity, including endoplasmic reticulum stress and changes in circulating glucose. This expansion of the work will provide critical insight into the possible pathological roles of HuD during stress-induced autophagy leading to β cell dysfunction.

Tight regulation of autophagy influx is critical for maintaining normal pancreatic β cell homeostasis and preserves pancreatic function, structure, and mass in response to stresses such as diabetes-induced oxidative stress, which causes the abnormal cytoplasmic accumulation of proteins (43). It has been shown that the loss of autophagy (ATG7−/−) in β cells increases the accumulation of ubiquitinated proteins and damaged organelles in the cytoplasm (44), and ATG7-deficient pancreatic β cells showed reduced β cell mass caused by increased apoptosis and decreased cell proliferation. Moreover, ATG7−/−/RIP-Cre mice developed diabetes with obvious β cell loss when fed a high fat diet (45). Our studies add to an expanding body of evidence that underscores the complex function of HuD in preserving glucose homeostasis in pancreatic β cells.

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