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HuD Regulates Coding and Noncoding RNA to Induce APP→Aβ Processing

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SUMMARY

The primarily neuronal RNA-binding protein HuD is implicated in learning and memory. Here, we report the identification of several HuD target transcripts linked to Alzheimer's disease (AD) pathogenesis. HuD interacted with the 3′ UTRs of *APP* mRNA (encoding amyloid precursor protein) and *BACE1* mRNA (encoding β-site APP-cleaving enzyme 1) and increased the half-lives of these mRNAs. HuD also associated with and stabilized the long noncoding (lnc)RNA *BACE1AS*, which partly complements *BACE1* mRNA and enhances BACE1 expression. Consistent with HuD promoting production of APP and APP-cleaving enzyme, the levels of APP, BACE1, *BACE1AS*, and Aβ were higher in the brain of HuD-overexpressing mice. Importantly, cortex (superior temporal gyrus) from patients with AD displayed significantly higher levels of HuD and, accordingly, elevated APP, BACE1, *BACE1AS*, and Aβ than did cortical tissue from healthy age-matched individuals. We propose that HuD jointly promotes the production of APP and the cleavage of its amyloidogenic fragment, Aβ.

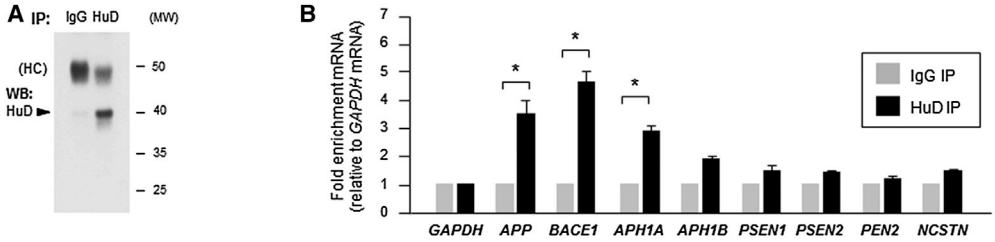
INTRODUCTION

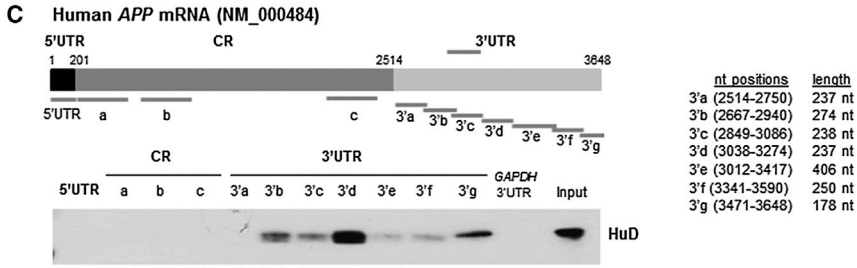
The posttranscriptional regulation of gene expression underlies many aspects of mammalian physiology and pathology. The two main groups of posttranscriptional regulators, RNA-binding proteins (RBPs) and noncoding RNAs (Moore, 2005; Morris et al., 2010), have been implicated in all steps controlling gene expression after transcription: pre-mRNA splicing and maturation, and mRNA transport, editing, stability, storage, and translation.

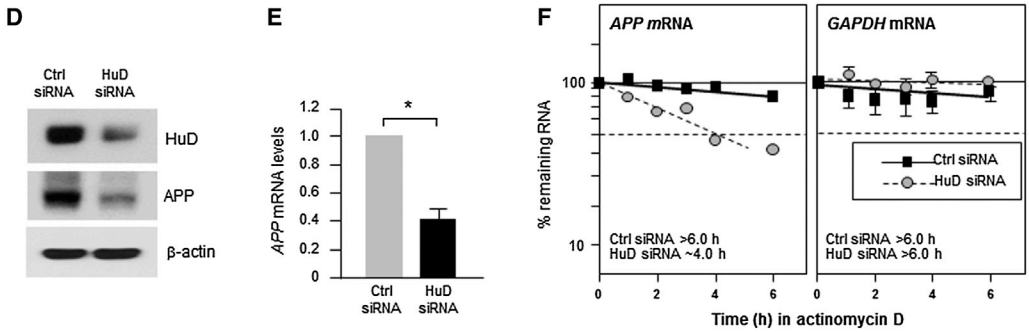
Among the noncoding RNAs that control gene expression post-transcriptionally, the best-characterized molecules are microRNAs (miRNAs; 22 nt in length), which typically associate with the 3′ UTR of target mRNAs and repress their translation and/or stability (Fabian et al., 2010). Long noncoding RNAs (lncRNAs) are also gaining recognition as posttranscriptional regulators of gene expression. Through complementary base pairing, lncRNAs can modulate the turnover and translation rate of target mRNAs; in the absence of complementarity, lncRNAs can suppress precursor mRNA splicing and translation by acting as decoys or competitors of RBPs and miRNAs (Yoon et al., 2013).

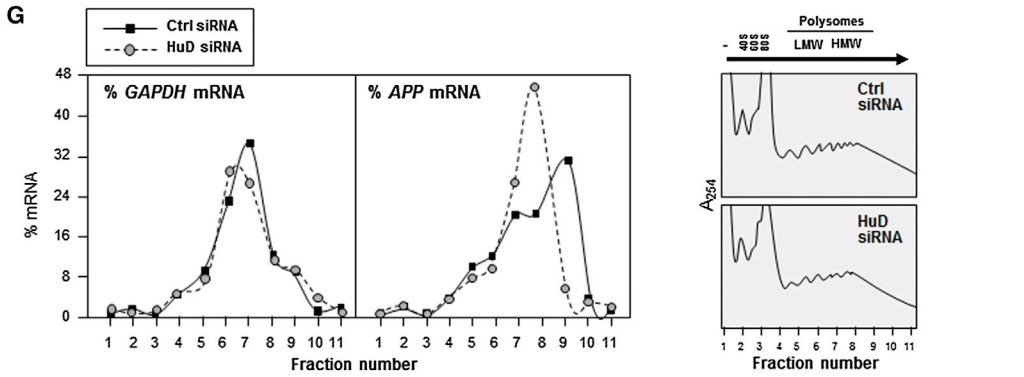
RBPs associate with a wide range of coding and noncoding RNAs and thus modulate many critical functions of the cell, including proliferation, survival, differentiation, motility, senescence, and apoptosis (Glisovic et al., 2008). Among them, the *elav* (embryonic lethal abnormal vision)/*Hu* (human antigen) group of proteins has been implicated primarily in controlling the stability and translation of target mRNAs. The Hu family comprises a ubiquitous member (HuR) and three predominantly neuronal members (HuB, HuC, and HuD). Elav/Hu proteins generally bind to U- and AU-rich RNA elements in target transcripts with which they associate via three highly conserved RNA recognition motifs (RRMs 1–3) (Hinman and Lou, 2008; Pascale et al., 2008).

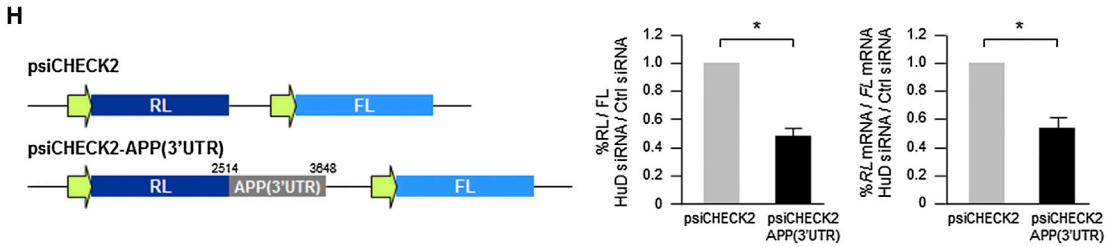
Unlike HuR, which is primarily nuclear, HuD is abundantly present in the cytoplasm. HuD expression is restricted to a few tissues, mainly neurons, gonads, and pancreatic β cells (Good, 1995, Lee et al., 2012). Several lines of evidence indicate that in cultured neurons, HuD promotes neurite outgrowth (Kasashima et al., 1999; Abdelmohsen et al., 2010), but the physiological role of HuD in animals appears to be complex. Although adult HuD-knockout (KO) mice do not exhibit morphological defects, HuD-KO embryos display transient impairment in cranial nerve development, and neurospheres derived from these mice generate fewer neurons compared to wild-type (WT) mice (Akamatsu et al., 2005). At the same time, expression of HuB, HuC, and HuD specifically increases in areas of mouse and rat brain











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associated with spatial learning, implicating these Hu proteins in learning and memory. In these tissues, elevated HuD is associated with enhanced production of GAP-43 (growth-associated protein-43), encoded by a HuD target mRNA (Anderson et al., 2001; Pascale et al., 2004). The roles of HuD in neuronal development and memory have been reviewed (Deschênes-Furry et al., 2006; Pascale et al., 2008; Perrone-Bizzozero et al., 2011).

HuD targets include many mRNAs that encode proteins preferentially expressed in neurons (e.g., GAP-43, acetylcholinesterase, tau, PSD-95, neuroserpin, musashi-1, and HuD itself), as well as proteins expressed in other tissues (e.g., c-Myc, N-myc, RhoA, c-Fos, VEGF, p21, p27, Bcl-2, NCAM1, and MARCKS) (Deschênes-Furry et al., 2006; Pascale et al., 2008; Abdelmohsen et al., 2010; Bolognani et al., 2010). With the exception of p27 and insulin, whose translation is repressed by HuD (Kullmann et al., 2002; Lee et al., 2010), HuD generally promotes the expression of target mRNAs. A recent survey of HuD target transcripts in human neuroblastoma cells (Abdelmohsen et al., 2010) revealed a number of HuD-interacting mRNAs implicated in the synthesis and processing of amyloid precursor protein (APP) into its amyloidogenic fragment, Aβ. HuD binds *APP* mRNA and *β-site APP-cleaving enzyme 1* (*BACE1*) mRNA, the latter encoding the β-secretase that cleaves APP in the critical first proteolytic-processing step that leads to the generation of Aβ. HuD also bound to and increased the abundance of *BACE1AS*, a lncRNA that stabilizes *BACE1* mRNA and promotes BACE1 expression (Faghihi et al., 2008). Our findings indicate that HuD may coordinate the production and cleavage of APP and further suggest that this regulatory paradigm contributes to Alzheimer's disease (AD) pathogenesis, characterized by the accumulation of toxic aggregates of Aβ peptide.

RESULTS

HuD Associates with mRNAs Involved in APP Processing

RNAs associated with HuD were identified by immunoprecipitation (IP) of ribonucleoprotein (RNP) complexes (RIP analysis) with anti-HuD in parallel with control immunoglobulin G (IgG) IP. The interaction of HuD in the IP material (Figure 1A) with bound RNAs was assayed by reverse transcription (RT) and subsequent real-time, quantitative PCR (qPCR) amplification. An earlier survey in the human neuroblastoma BE(2)-M17 cells (Abdelmohsen et al., 2010) revealed that *APP* mRNA was a potential target of HuD. Experiments to investigate this possibility directly revealed that *APP* mRNA was significantly enriched in HuD IP samples compared with IgG IP samples and additionally showed that several HuD-bound mRNAs encoded proteases that cleave APP to generate Aβ peptide. Among them, the *BACE1* mRNA was also significantly enriched, as previously observed by Bolognani et al. (2010), whereas HuD associated less prominently with mRNAs encoding components of the γ-secretase complex (*PSEN1* and *PSEN2* mRNAs, encoding presenilins, and *APH1A* and *APH1B* mRNAs, encoding presenilin-stabilization factors) (Figure 1B). The *PEN2* mRNA (encoding presenilin enhancer 2) and the *NCSTN* mRNA (encoding nicastrin, a component of the γ-secretase protein complex) showed no significant enrichment in HuD IP (Figure 1B). We thus focused on analyzing the interaction of HuD with *APP* and *BACE1* mRNAs in human neuroblastoma SK-N-F1 cells.

To identify areas of interaction of HuD with *APP* mRNA, biotinylated segments spanning the 5′ UTR, coding region (CR) and 3′ UTR of the *APP* mRNA were synthesized in vitro and incubated with cytoplasmic lysates of SK-N-F1 cells. After pull-down using streptavidin-coated beads, HuD association with the biotinylated transcripts was assessed by western blot (WB) analysis. As shown in Figure 1C, several *APP* 3′ UTR segments associated with HuD in vitro, but segments of the *APP* CR, the APP 5′ UTR, or a negative control RNA (*glyceraldehyde 3-phosphate dehydrogenase* [*GAPDH*] 3′ UTR) did not. Although HuD bound multiple *APP* 3′ UTR segments, the most robust affinity and regulation (Figure S1) were mapped to segment 3′d. The consequences of the interaction of HuD with the *APP* mRNA were assessed by silencing HuD; 48 hr after transfecting SK-N-F1 cells with a small interfering RNA (siRNA) directed to

Figure 1. HuD Binds to *APP* 3′ UTR and Enhances *APP* mRNA and Stability and Translation

(A) Analysis of HuD in SK-N-F1 cells following IP with IgG and anti-HuD and WB analysis. HuD (arrowhead), immunoglobulin heavy chain (HC), and molecular weight (MW) markers are indicated.

(B) Limited analysis (using RIP) to survey HuD-interacting mRNAs encoding proteins with roles in APP production or processing. Following IP as in (A), mRNA levels in the IP materials were measured by RT-qPCR analysis, normalized to *GAPDH* mRNA levels in each IP reaction, and represented as "Fold enrichment" relative to IgG IP.

(C) Top, schematic of *APP* mRNA, depicting the 5′ UTR, CR, and 3′ UTR, and the different biotinylated RNA segments (gray lines) tested for binding to HuD after pull-down using streptavidin beads; HuD was detected by WB analysis. Biotinylated *GAPDH* 3′ UTR was included as negative control "Input," 20 μg of whole-cell lysate.

(D and E) Forty-eight hours after transfecting Ctrl or HuD siRNAs, the levels of proteins APP, HuD, and loading control β-actin were assessed by WB analysis (D), and the reduction in *APP* mRNA levels by RT-qPCR analysis (normalized to *GAPDH* mRNA levels) (E).

(F) Cells transfected as in (D) were treated with actinomycin D, and the levels of *APP* mRNA and *GAPDH* mRNA (encoding a housekeeping protein) were assessed by RT-qPCR; the $t_{1/2}$ of *APP* and *GAPDH* mRNA were quantified by measuring the time required for transcript levels to decline to 50% of their original abundance relative to time 0 hr.

(G) Polysomes were prepared from cells transfected as in (D) by fractionating cytoplasmic extracts through sucrose gradients. The arrow indicates the direction of sedimentation. The minus sign ("−") indicates no ribosomal components. Small (40S) and large (60S) ribosomal subunits and monosomes (80S) in fractions 2–4, and progressively larger polysomes, ranging from low to high molecular weight (LMW and HMW, respectively) in fractions 6–11 are shown in the right panel. The distribution of *APP* and *GAPDH* mRNAs was studied by RT-qPCR analysis of RNA in gradient fractions and is represented as percentage (%) of total RNA in the gradient (left).

(H) Left, schematic of reporters prepared to analyze the influence of *APP* 3′ UTR on gene expression. In the right panels, 12 hr after transfecting SK-N-F1 cells with Ctrl or HuD siRNAs, cells were further transfected with psiCHECK2 or psiCHECK-APP(3′ UTR), and 24 hr later, luciferase activity was measured (RL/FL) for each transfected plasmid and normalized to luciferase activity (RL/FL) in Ctrl siRNA (left). *RL* mRNA and *FL* mRNA levels in each transfection group, normalized to *GAPDH* mRNA, were quantified by RT-qPCR analysis and plotted relative to the changes in the psiCHECK2 group (right).

Graphs in (B), (E), (F), and (H) display the mean and SD from three independent experiments. *$p < 0.05$.

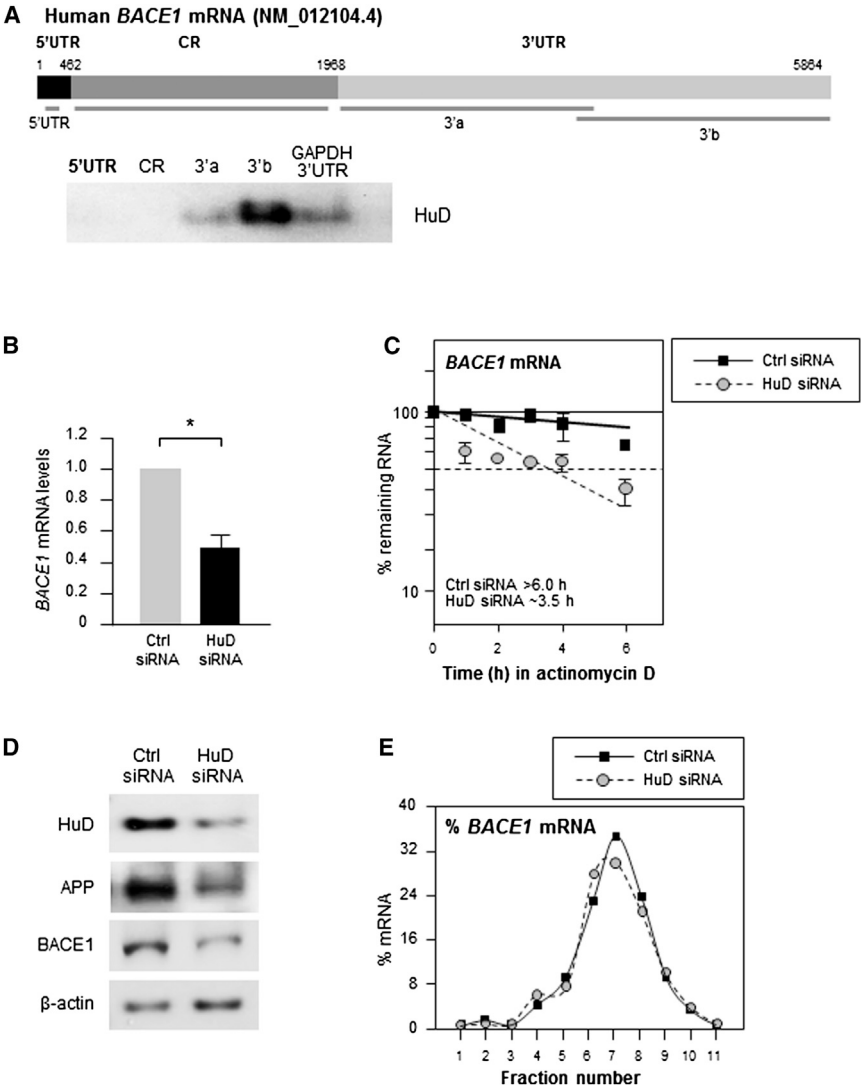


Figure 2. HuD Associates with *BACE1* mRNA

(A) Top, schematic of *BACE1* mRNA, indicating the different biotinylated RNA segments (gray underlines) that were prepared and tested for binding to HuD after pull-down using streptavidin beads; HuD was detected by WB analysis as explained in Figure 1C.

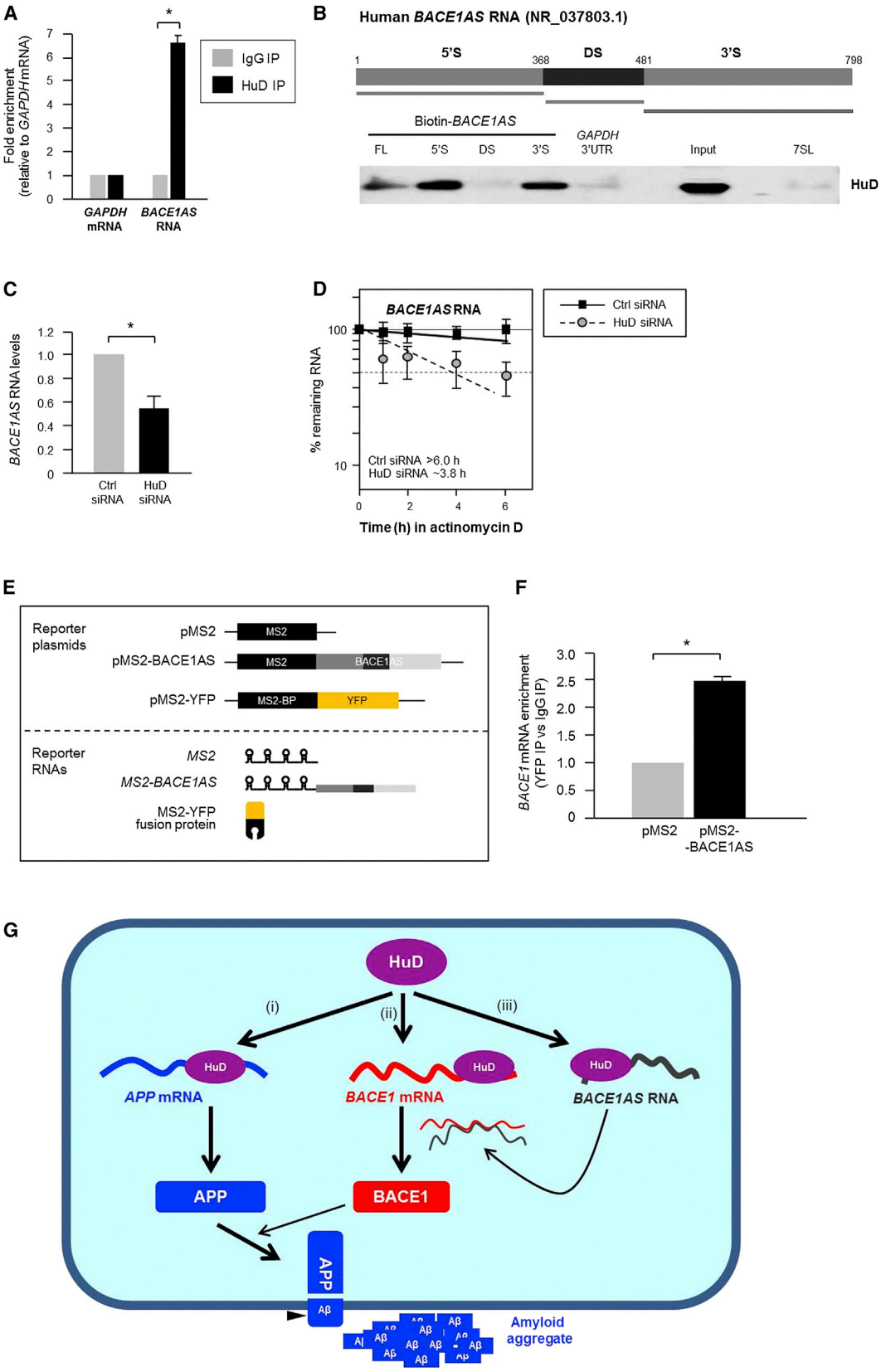
(B–E) Forty-eight hours after transfecting SK-N-F1 cells with the siRNAs indicated, *BACE1* mRNA levels were measured by RT-qPCR analysis (*$p < 0.05$) (B), and the $t_{1/2}$ of *BACE1* mRNA (C) was quantified as explained in Figure 1F. The levels of BACE1 protein were assessed by WB analysis (D), and the relative distribution of *BACE1* mRNA on polysomes (E) was studied as explained in Figure 1G.

HuD, the decline in HuD levels caused a marked decrease in the levels of APP and *APP* mRNA, as assessed by WB and RT-qPCR analyses, respectively (Figures 1D and 1E).

HuD Enhances the Stability and Translation of *APP* mRNA

Because HuD was shown to stabilize a number of target mRNAs, we investigated if the loss in *APP* mRNA after silencing HuD was due at least in part to changes in *APP* mRNA stability. After silencing HuD expression in SK-N-F1 cells, actinomycin D was used to inhibit de novo transcription; the time needed for *APP* mRNA to be reduced to 50% of its initial abundance (its half-life [$t_{1/2}$]) was then calculated by measuring *APP* mRNA levels using RT-qPCR and normalizing to *18S* rRNA levels. As shown in Figure 1F, *APP* mRNA $t_{1/2}$ in control cells (Ctrl siRNA; >6.0 hr) was much longer than that measured in HuD siRNA cells (~4.0 hr). The $t_{1/2}$ of *GAPDH* mRNA, a stable mRNA that encodes a housekeeping protein, was not shortened by HuD silencing (Figure 1F). HuD was also shown to promote mRNA translation (Fukao et al., 2009); to investigate if HuD also affected APP translation, we silenced HuD in SK-N-F1 cells and quantified the fraction of *APP* mRNA associated with the translation machinery in each transfection group. Cytoplasmic extracts from the Ctrl siRNA and HuD siRNA groups were fractionated on sucrose gradients, and the relative abundance of *APP* mRNA in each fraction indicated the association of *APP* mRNA with the cellular polysomes and hence its translation status. The polysome-fractionation pattern was identical in both groups, suggesting that silencing HuD does not affect translation globally. In Ctrl siRNA cells, *APP* mRNA levels were very low in nontranslating and low-translating fractions of the gradient (fractions 1–4, where free RNA and 40S and 60S subunits, as well as 80S monosomes, are found), but they were abundant in the actively translating fractions of the gradient (fractions 5–10, spanning low and high molecular weight polysomes) (Figure 1G) and peaking at fraction 9. However, in HuD siRNA-transfected cells, *APP* mRNA showed a leftward shift on the gradient, peaking at fraction 8, indicating that *APP* mRNA formed on average smaller polysomes after silencing HuD. These results agree with a role for HuD both elevating *APP* mRNA abundance and enhancing the translation of *APP* mRNA.

These effects were further examined using heterologous luciferase reporter vectors that expressed Renilla luciferase (RL) lacking or containing the *APP* 3′ UTR (psiCHECK2 or psiCHECK2-APP[3′ UTR], respectively). The ratio of RL to firefly luciferase (FL) (encoded by an internal control reporter transcript within the same plasmid) was set as one ("1") for the parent vector (psiCHECK2). The lower RL/FL ratios for HuD siRNA-transfected cells (~48%) relative to those for Ctrl



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siRNA-transfected cells indicated that the presence of *APP* 3′ UTR reduced luciferase production when HuD was silenced (Figure 1H, left). As shown in Figure 1H, right, there was a parallel reduction in *RL* mRNA levels compared with *FL* mRNA levels in HuD-silenced cells (~52%), indicating that accelerated decay of the *RL-APP(3′UTR)* chimeric mRNA contributed to the decrease in RL expression. In sum, HuD enhanced both the stability and translation of *APP* mRNA.

HuD Stabilizes *BACE1* mRNA and *BACE1AS* RNA

Next, we examined the interaction of HuD with *BACE1* mRNA. HuD associated in vitro with partial biotinylated segments of the 3′ UTR but not the CR or 5′ UTR of *BACE1* mRNA, showing preferential binding to a distal *BACE1* 3′ UTR segment (3′b) (Figures 2A and S2). The levels of *BACE1* mRNA were lower in the HuD siRNA group, and this reduction was achieved at least in part through a decline in *BACE1* mRNA stability (Figures 2B and 2C), just as seen for *APP* mRNA (Figure 1F); nontargets of HuD, including mRNAs encoding γ-secretases, did not show this trend (Figure S2). BACE1 protein levels were correspondingly lower, but the translation of *BACE1* mRNA did not appear to be affected by HuD silencing (Figures 2D and 2E).

The stability of *BACE1* mRNA was not previously reported to be affected by other RBPs, but it was shown to be stabilized via interaction with the lncRNA *BACE1AS* (Faghihi et al., 2008). Thus, we asked if the present regulatory paradigm involved *BACE1AS*. RIP analysis revealed that *BACE1AS* was robustly present in HuD RNP complexes (Figure 3A) and that HuD associated in vitro with partial biotinylated segments of *BACE1AS* RNA; negative controls *GAPDH* 3′ UTR and the lncRNA *7SL* were included (Figure 3B). This interaction likely stabilized *BACE1AS* because silencing HuD lowered the steady-state levels (Figure 3C) and the $t_{1/2}$ of *BACE1AS* (Figure 3D). Further evidence that *BACE1* mRNA and *BACE1AS* interacted in our study system was obtained by engineering a plasmid that expressed a chimeric *BACE1AS* tagged with MS2 RNA hairpins (pMS2-BACE1AS). By 36 hr after transfecting SK-N-F1 cells with pMS2 or pMS2-BACE1AS along with plasmid pMS2-YFP, which expressed a fluorescent fusion protein, MS2-YFP (Lee et al., 2010; Figure 3E), RIP analysis was carried out with anti-YFP to bring down the YFP-MS2 protein bound to MS2-bearing RNAs. As shown in Figure 3F, *BACE1* mRNA, as measured by RT-qPCR analysis in the IP samples, was significantly more abundant in *MS2-BACE1AS* IP than in control IP (pMS2 transfection group), indicating that *BACE1* mRNA and *BACE1AS* associated physically in SK-N-F1 cells.

Collectively, these results suggest a model whereby HuD jointly controls three transcripts on the route to generating amyloidogenic Aβ: (1) HuD binds to *APP* mRNA and enhances APP levels, (2) HuD binds to *BACE1* mRNA and promotes BACE1 production, and (3) HuD binds to *BACE1AS* RNA and increases its levels, further augmenting BACE1 production. By acting on functionally related RNAs, HuD serves as a master coordinator of Aβ production (Figure 3G).

HuD Influence on APP→Aβ Production in the Brain

Based on the model proposed presented in Figure 3G, we hypothesized that HuD may affect Aβ production in the brain. We tested this possibility using two different models. In transgenic mice overexpressing HuD (HuD-Tg mice) as a tagged protein (Myc-HuD; Figure 4A), the levels of Aβ40, as measured by ELISA (Experimental Procedures), were significantly higher than in WT mice (Figure 4B), and the levels of *Elavl4* mRNA (encoding HuD), *App* mRNA, *Bace1* mRNA, and *Bace1as* RNA were higher in hippocampus, cortex, and cerebellum, relative to the levels in age-matched WT mice (Figure 4C).

We also studied the levels of HuD in the brains of patients with AD. Brain sections from the superior temporal gyrus (STG) from ten persons with AD and ten age-matched healthy individuals were used for RNA and protein analysis. RNA prepared from these samples was analyzed by RT-qPCR; as shown in Figure 4D, *ELAVL4* mRNA and *BACE1AS* RNA were markedly higher in AD brains relative to healthy brains, whereas *APP* mRNA and *BACE1* mRNA were moderately higher in patients with AD. The relative levels of protein followed these general differences (Figure 4E; Faghihi et al., 2008), with greater than 2-fold higher levels of HuD and BACE1 in AD brains. In keeping with the higher levels of BACE1, Aβ peptide (Aβ40 and Aβ42) was also more abundant in AD brains (Figure 4F). In summary, in the brains of HuD-overexpressing mice, the levels of APP, BACE1, and Aβ40 correlated with the heightened abundance of HuD, whereas in human AD brains, HuD levels were significantly elevated and correlated with higher levels of BACE1, *BACE1AS*, and Aβ.

Figure 3. lncRNA *BACE1AS* Is the Target of HuD and *BACE1* mRNA

(A) RIP analysis of the interaction of *BACE1AS* RNA with HuD.

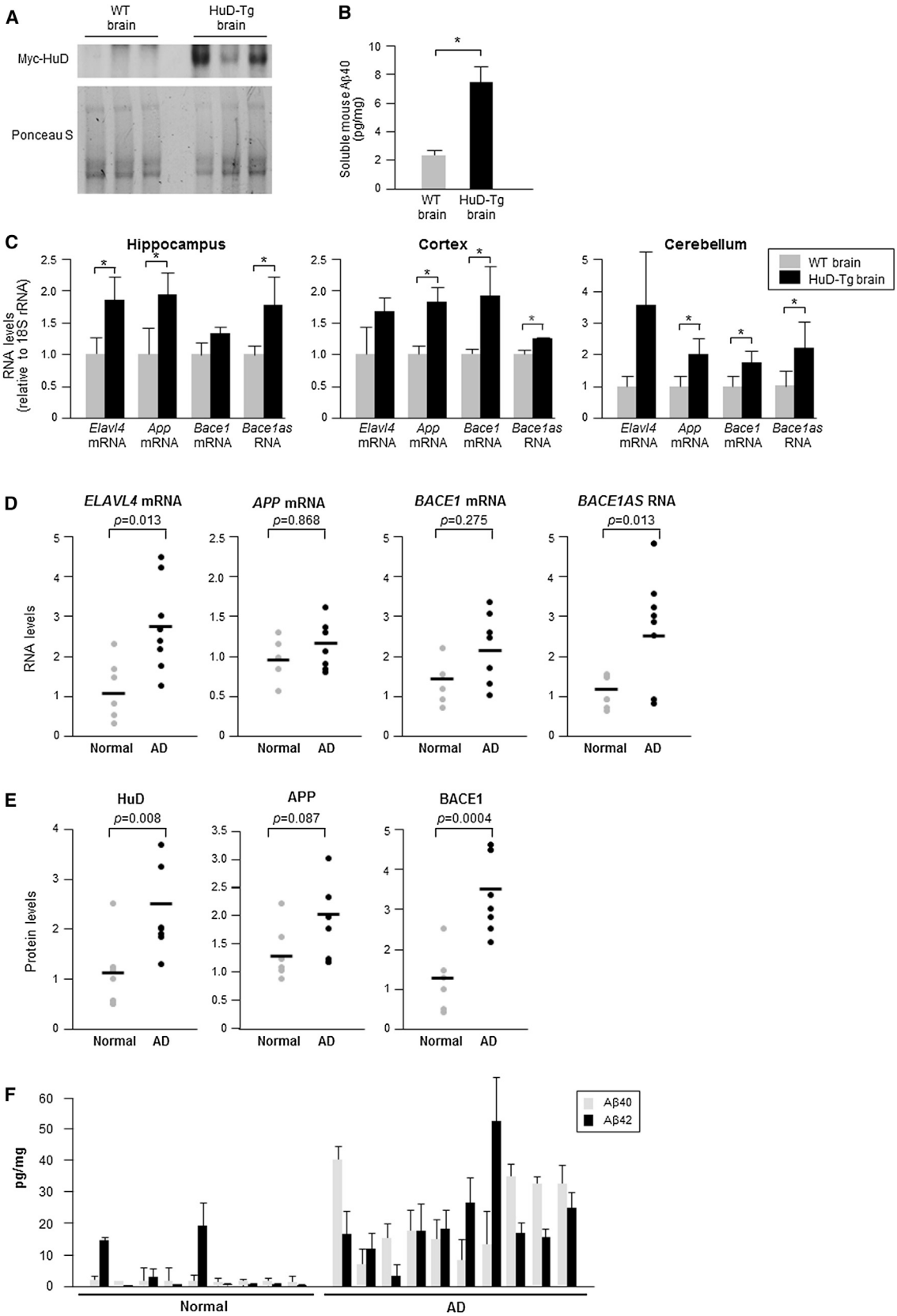
(B) Schematic of human *BACE1AS* RNA, depicting the 5′ and 3′ segments (5′S and 3′S) as well as the double-stranded (DS) segment. The interaction of biotinylated *BACE1AS* RNA with HuD was assayed by biotin pull-down (biotinylated segments assayed are shown; gray underlines). FL, full-length *BACE1AS*. *GAPDH* 3′ UTR and *7SL* were included as negative controls.

(C and D) Forty-eight hours after transfecting SK-N-F1 cells with the siRNAs indicated, *BACE1AS* RNA levels were measured by RT-qPCR analysis (C), and the $t_{1/2}$ of *BACE1AS* was quantified as in Figure 1F (D).

(E and F) SK-N-F1 cells were transfected with the plasmids shown (schematic). Plasmid pMS2, which expressed 24 repeats of MS2 hairpins, or plasmid pMS2-BACE1AS (expressing full-length *BACE1AS*) was cotransfected along with plasmid pMS2-YFP (expressing a fusion protein [MS2-YFP] capable of detecting MS2-tagged RNA) (E). By 24 hr after transfection of the plasmids in (E), lysates were subjected to RIP analysis using an antibody against YFP. The presence of *BACE1* mRNA in IP samples from each transfection group was assessed by RT-qPCR analysis (F).

(G) Schematic of proposed influence of HuD upon the expression and β-cleavage of APP. (1) HuD binds to *APP* mRNA and promotes *APP* mRNA stabilization and translation. (2) HuD binds to *BACE1* mRNA and stabilizes it. (3) HuD binds to *BACE1AS* and stabilizes it, in turn increasing *BACE1* mRNA stability and BACE1 production. Arrowhead points to subsequent cleavage by γ-secretase that releases Aβ peptide (blue).

In (A), (C), (D), and (F), the graphs reflect the mean and SD from three independent experiments. *$p < 0.05$.



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DISCUSSION

Our results indicate that HuD controls the expression of three RNAs affecting APP production and processing into Aβ. The three-pronged actions of HuD include binding to *APP* mRNA, causing it to be more stable and translated; binding to *BACE1* mRNA, increasing its stability and hence BACE1 production; and binding to and stabilizing *BACE1AS*, which further contributes to the production of BACE1. In keeping with this regulatory paradigm, HuD levels were positively linked to Aβ levels in three systems: in neuroblastoma SK-N-F1 cells expressing normal versus silenced HuD levels, in the brains of WT and HuD-overexpressing mice, and in the brains from AD and normal subjects.

The discovery that HuD influences the production of the key pathogenic peptide Aβ raises the immediate question of what determines the levels of HuD in neuronal tissues. Among the possible transcriptional regulators of HuD expression is FoxO1; the repression of *ELAVL4* gene transcription in pancreatic β cells was relieved by exposure to high glucose or insulin (Lee et al., 2012). Whether FoxO1 also suppresses *ELAVL4* gene transcription in neuronal tissues and whether this regulation is dependent on glucose/insulin await further study. Posttranscriptional regulators of HuD production include the miRNA miR-375, which binds to *ELAVL4* mRNA, lowers its stability, and represses HuD translation (Abdelmohsen et al., 2010; Supplemental Discussion).

The notion that a single RBP, HuD, can jointly control the expression of three transcripts (*APP* mRNA, *BACE1* mRNA, and *BACE1AS* RNA) that are functionally related supports the "posttranscriptional operon/regulon" model put forth by Keene and Tenenbaum (2002). According to this model, a single RBP can associate with and coordinate the expression of multiple mRNAs that share a given RNA sequence with affinity for the RBP and whose encoded gene products are implicated in a specific cellular function. To establish a simple analogy with a "transcriptional" operon/regulon, the RBP functions as the equivalent of a transcription factor that binds to a shared DNA sequence present in the promoter of genes encoding proteins with related functions (as in eukaryotes) or synthesizes a polycistronic RNA whose individual RNA components encode proteins functionally linked. In light of our findings, the traditional posttranscriptional operon/regulon model would help to explain HuD function; we proposed a slightly revised version of the model that includes noncoding RNA.

Our studies did not identify a specific RNA element present in the three transcripts. However, most RBPs do not have strict sequence requirements and instead associate with loosely variable RNA elements, often within the context of a given secondary structure. Approaches such as photoactivatable-ribonucleoside-enhanced crosslinking and IP identification of HuD-bound RNAs can give us a more precise view of the subset of transcripts with which HuD interacts. A more complete understanding of the collection of HuD-interacting coding and noncoding RNAs will be particularly informative as we strive to understand the underlying causes of Aβ processing in AD.

EXPERIMENTAL PROCEDURES

Cell Culture, siRNA, and Plasmids

Human neuroblastoma SK-N-F1 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum and antibiotics. The plasmids and siRNAs used are described in Supplemental Experimental Procedures.

Protein Analysis

WB analysis and fractionation of polyribosomes are explained in Supplemental Experimental Procedures. Aβ levels (Aβ40 and Aβ42) in SK-N-F1 cells were measured in conditioned media, and Aβ in human brain samples was assayed directly from protein lysates. Human Aβ was measured by ELISA (Invitrogen; KHB3481 and KHB3441), and the manufacturer's protocol included incubation with guanidine; [methyl(phenyl)-λ3-oxidanyl]formic acid solution. Aβ levels in mouse brain lysates were analyzed by ELISA (Invitrogen).

RNA Analysis

RNA-binding assays RIP (RNP IP) and biotin pull-down, as well as mRNA $t_{1/2}$ measurements, are explained in Supplemental Experimental Procedures. TRIzol (Invitrogen) was used to extract total RNA, and acidic phenol (Ambion) was used to extract RNA for RIP analysis (Lee et al., 2010). RT was performed using random hexamers and Maxima Reverse Transcriptase (Thermo Scientific), and real-time qPCR was done using gene-specific primers (Supplemental Experimental Procedures). RT-qPCR was performed using SYBR Green Master Mix (Kapa Biosystems) in an Applied Biosystems 7300 instrument.

Human Subjects

The sample consists of 20 participants (19 females and 1 male) from the Baltimore Longitudinal Study of Aging (BLSA) (National Institute on Aging [NIA], National Institutes of Health); cognitive status was determined based on standardized consensus diagnostic procedures for the BLSA (Driscoll et al., 2012) and eventually came to autopsy. Participants were between 55 and 98 (AD) and 56 and 95 (normal) years of age. All studies were approved by the local institutional review boards, and all participants gave written informed consent prior to each assessment. In addition, next of kin or legally designated power of attorney provided consent for autopsy. STG regions of the brain were used for protein and RNA analysis.

Figure 4. In Mice Overexpressing HuD, the Levels of *App* mRNA, *Bace1* mRNA, *Bace1as* RNA, and Aβ Are Elevated; in AD Brains, HuD, HuD Target Transcripts and Encoded Proteins, and Aβ Are Elevated

(A) The levels of tagged HuD in whole-brain lysate from mice (three WT and three overexpressing the Myc-HuD transgene [9-month-old females]) were assessed by WB analysis. Ponceau S staining was used to assess sample loading.

(B) ELISA was used to measure soluble Aβ in WT and HuD-Tg (whole-brain lysates; three mice per genotype).

(C) The levels of *Elavl4* mRNA (encoding HuD), *App* mRNA, *Bace1* mRNA, and *Bace1as* in each mouse brain group (n = 3 mice per genotype) were measured by RT-qPCR; data were normalized to *18S* rRNA.

(D and E) Lysates prepared from STG from normal subjects (n = 10) and from AD subjects (n = 10) were assayed for levels of *ELAVL4* mRNA, *APP* mRNA, *BACE1* mRNA, and *BACE1AS* RNA using RT-qPCR (D) and for the encoded proteins (six normal, seven AD) by WB analysis followed by densitometric analysis (E). RNA levels were normalized to *GAPDH* mRNA levels, protein levels to β-actin levels.

(F) The levels of Aβ40 and Aβ42 were assayed in ten normal and ten AD brains (STG) by using ELISA.

In (B)–(F), the graphs reflect the mean and SD from three independent experiments. p values are indicated in (D) and (E). *$p < 0.05$.

Animals

HuD-Tg (Bolognani et al., 2010; a kind gift from Dr. N.I. Perrone-Bizzozero) and WT mice in the C57BL/6J background were obtained from in-house breeding colonies of the NIA (Baltimore) (details are provided in Supplemental Experimental Procedures).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Results, Supplemental Discussion, Supplemental Experimental Procedures, and two figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.04.050.

AUTHOR CONTRIBUTIONS

M.-J.K., K.A., E.R.H., S.J.M., I.G., R.G., J.H.N., and J.L.M. performed experiments. M.-J.K., K.A., E.R.H., I.G., R.G., J.H.N., O.P., and S.M.R. analyzed the data. K.A., S.J.M., X.Y., E.K.L., M.A.F., C.W., J.C.T., O.P., N.P.-B., R.d.C., M.P.M., and M.G. contributed reagents and expertise. M.-J.K., S.M.R., R.d.C., M.P.M., and M.G. wrote the paper.

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REFERENCES

Abdelmohsen, K., Hutchison, E.R., Lee, E.K., Kuwano, Y., Kim, M.M., Masuda, K., Srikantan, S., Subaran, S.S., Marasa, B.S., Mattson, M.P., and Gorospe, M. (2010). miR-375 inhibits differentiation of neurites by lowering HuD levels. Mol. Cell. Biol. *30*, 4197–4210.

Akamatsu, W., Fujihara, H., Mitsuhashi, T., Yano, M., Shibata, S., Hayakawa, Y., Okano, H.J., Sakakibara, S., Takano, H., Takano, T., et al. (2005). The RNA-binding protein HuD regulates neuronal cell identity and maturation. Proc. Natl. Acad. Sci. USA *102*, 4625–4630.

Anderson, K.D., Sengupta, J., Morin, M., Neve, R.L., Valenzuela, C.F., and Perrone-Bizzozero, N.I. (2001). 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*, 250–258.

Bolognani, F., Contente-Cuomo, T., and Perrone-Bizzozero, N.I. (2010). Novel recognition motifs and biological functions of the RNA-binding protein HuD revealed by genome-wide identification of its targets. Nucleic Acids Res. *38*, 117–130.

Deschênes-Furry, J., Perrone-Bizzozero, N.I., and Jasmin, B.J. (2006). The RNA-binding protein HuD: a regulator of neuronal differentiation, maintenance and plasticity. Bioessays *28*, 822–833.

Driscoll, I., Troncoso, J.C., Rudow, G., Sojkova, J., Pletnikova, O., Zhou, Y., Kraut, M.A., Ferrucci, L., Mathis, C.A., Klunk, W.E., et al. (2012). Correspondence between in vivo (11)C-PiB-PET amyloid imaging and postmortem, region-matched assessment of plaques. Acta Neuropathol. *124*, 823–831.

Fabian, M.R., Sonenberg, N., and Filipowicz, W. (2010). Regulation of mRNA translation and stability by microRNAs. Annu. Rev. Biochem. *79*, 351–379.

Faghihi, M.A., Modarresi, F., Khalil, A.M., Wood, D.E., Sahagan, B.G., Morgan, T.E., Finch, C.E., St Laurent, G., 3rd, Kenny, P.J., and Wahlestedt, C. (2008). Expression of a noncoding RNA is elevated in Alzheimer's disease and drives rapid feed-forward regulation of beta-secretase. Nat. Med. *14*, 723–730.

Fukao, A., Sasano, Y., Imataka, H., Inoue, K., Sakamoto, H., Sonenberg, N., Thoma, C., and Fujiwara, T. (2009). The ELAV protein HuD stimulates cap-dependent translation in a Poly(A)- and eIF4A-dependent manner. Mol. Cell *36*, 1007–1017.

Glisovic, T., Bachorik, J.L., Yong, J., and Dreyfuss, G. (2008). RNA-binding proteins and post-transcriptional gene regulation. FEBS Lett. *582*, 1977–1986.

Good, P.J. (1995). A conserved family of elav-like genes in vertebrates. Proc. Natl. Acad. Sci. USA *92*, 4557–4561.

Hinman, M.N., and Lou, H. (2008). Diverse molecular functions of Hu proteins. Cell. Mol. Life Sci. *65*, 3168–3181.

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

Keene, J.D., and Tenenbaum, S.A. (2002). Eukaryotic mRNPs may represent posttranscriptional operons. Mol. Cell *9*, 1161–1167.

Kullmann, M., Göpfert, U., Siewe, B., and Hengst, L. (2002). ELAV/Hu proteins inhibit p27 translation via an IRES element in the p27 5'UTR. Genes Dev. *16*, 3087–3099.

Lee, E.K., Kim, H.H., Kuwano, Y., Abdelmohsen, K., Srikantan, S., Subaran, S.S., Gleichmann, M., Mughal, M.R., Martindale, J.L., Yang, X., et al. (2010). hnRNP C promotes APP translation by competing with FMRP for APP mRNA recruitment to P bodies. Nat. Struct. Mol. Biol. *17*, 732–739.

Lee, E.K., Kim, W., Tominaga, K., Martindale, J.L., Yang, X., Subaran, S.S., Carlson, O.D., Mercken, E.M., Kulkarni, R.N., Akamatsu, W., et al. (2012). RNA-binding protein HuD controls insulin translation. Mol. Cell *45*, 826–835.

Moore, M.J. (2005). From birth to death: the complex lives of eukaryotic mRNAs. Science *309*, 1514–1518.

Morris, A.R., Mukherjee, N., and Keene, J.D. (2010). Systematic analysis of posttranscriptional gene expression. Wiley Interdiscip. Rev. Syst. Biol. Med. *2*, 162–180.

Pascale, A., Gusev, P.A., Amadio, M., Dottorini, T., Govoni, S., Alkon, D.L., and Quattrone, A. (2004). Increase of the RNA-binding protein HuD and posttranscriptional up-regulation of the GAP-43 gene during spatial memory. Proc. Natl. Acad. Sci. USA *101*, 1217–1222.

Pascale, A., Amadio, M., and Quattrone, A. (2008). Defining a neuron: neuronal ELAV proteins. Cell. Mol. Life Sci. *65*, 128–140.

Perrone-Bizzozero, N.I., Tanner, D.C., Mounce, J., and Bolognani, F. (2011). Increased expression of axogenesis-related genes and mossy fibre length in dentate granule cells from adult HuD overexpressor mice. ASN Neuro *3*, 259–270.

Yoon, J.H., Abdelmohsen, K., and Gorospe, M. (2013). Posttranscriptional gene regulation by long noncoding RNA. J. Mol. Biol. *425*, 3723–3730.