Emerging complexity of the HuD/ELAVl4 gene; implications for neuronal development, function, and dysfunction

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ABSTRACT

Precise control of messenger RNA (mRNA) processing and abundance are increasingly being recognized as critical for proper spatiotemporal gene expression, particularly in neurons. These regulatory events are governed by a large number of *trans*-acting factors found in neurons, most notably RNA-binding proteins (RBPs) and micro-RNAs (miRs), which bind to specific *cis*-acting elements or structures within mRNAs. Through this binding mechanism, *trans*-acting factors, particularly RBPs, control all aspects of mRNA metabolism, ranging from altering the transcription rate to mediating mRNA degradation. In this context the best-characterized neuronal RBP, the Hu/ELAVl family member HuD, is emerging as a key component in multiple regulatory processes—including pre-mRNA processing, mRNA stability, and translation—governing the fate of a substantial amount of neuronal mRNAs. Through its ability to regulate mRNA metabolism of diverse groups of functionally similar genes, HuD plays important roles in neuronal development and function. Furthermore, compelling evidence indicates supplementary roles for HuD in neuronal plasticity, in particular, recovery from axonal injury, learning and memory, and multiple neurological diseases. The purpose of this review is to provide a detailed overview of the current knowledge surrounding the expression and roles of HuD in the nervous system. Additionally, we outline the present understanding of the molecular mechanisms presiding over the localization, abundance, and function of HuD in neurons.

Keywords: HuD/ELAVl4; RNA-binding protein; mRNA metabolism; post-transcriptional regulation

INTRODUCTION

The flow of genetic information from DNA to protein requires numerous stages of intricate control, with a myriad of regulatory events occurring between transcription and translation. These regulatory events are orchestrated by *trans*-acting factors, a group of regulatory molecules comprising RNA-binding proteins (RBPs) and multiple classes of noncoding RNAs (ncRNAs), that aggregate onto mRNAs and form messenger ribonucleoprotein (mRNP) complexes (Lukong et al. 2008; Carninci 2010). The past two decades have seen tremendous advances in the discovery of novel types of *trans*-acting factors and provided a greater understanding of their diverse and vital roles in mRNA metabolism. By binding to *cis*-acting elements and/or structures in mRNAs, *trans*-acting factors control a broad spectrum of processes that range from alternative splicing and polyadenylation in the nucleus to localization, stabilization, and translation in the cytoplasm (Carmody and Wente 2009; Licatalosi and Darnell 2010; Vazquez-Pianzola and Suter 2012; Wu and Brewer 2012). Some *trans*-acting factors, in particular, RBPs, have been shown to transition mRNAs encoding proteins with similar functions through different post-transcriptional stages, thereby effectively coupling distinct regulatory events during mRNA metabolism (Dahan et al. 2011). The coordination of groups of mRNAs along successive processing and regulatory events formed the premise of the RNA operon theory, which helps to clarify the organization and dynamics of post-transcriptional networks (Keene 2007). These networks, collectively known as the ribonome, confer numerous benefits to cells including increasing protein diversity from a fixed number of genes, accurate spatial and temporal gene expression, and rapid modification of protein levels (Mansfield and Keene 2009).

The advantages convened by post-transcriptional regulation are particularly necessary in neurons since these cells contain complex cellular architecture due to extensive neurite branching. In addition to playing critical roles in neuronal development, maintenance, and function, the importance of these regulatory events is further highlighted by the growing number of diseases associated with mutations of certain mRNAs or dysregulation of RBPs or ncRNAs, notably small ncRNAs termed micro-RNAs (miRs), that associate with them (Lukong et al. 2008; Cooper et al. 2009; Esteller 2011; Im and Kenny 2012). For example, defects affecting the normal expression of mRNAs and/or function of RBPs are central in the etiology and pathogenesis of a broad spectrum of neuronal diseases, including the neurodegenerative disorders fragile X syndrome, fragile X tremor ataxia syndrome

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(Li and Jin 2012), spinal muscular atrophy (SMA) (Fallini et al. 2012b), Huntington's disease (Krzyzosiak et al. 2012), and amyotrophic lateral sclerosis (ALS) (Fiesel and Kahle 2011; Strong and Volkening 2011).

The nervous system encompasses an extensive amount of RBPs, many of which are critical for neuronal development. In support of this idea, one genome-wide screen found that 85% of the 380 RBPs examined were expressed in the embryonic (E13.5) and early postnatal (P0) mouse brain (McKee et al. 2005). Most notable are the well-described RBPs that regulate various stages of mRNA metabolism and are important for proper neuronal differentiation and/or function, such as butyrate-response factor 1 (BRF1) (Sanduja et al. 2011), neuro-oncologic ventral antigens 1/2 (NOVA 1/2) (Darnell 2006), A+U binding factor 1 (AUF1) (Gratacos and Brewer 2010), tristetraprolin (TTP) (Sanduja et al. 2011), K homology splicing regulatory protein (KSRP) (Briata et al. 2012), and Hu/embryonic lethal abnormal vision-like (ELAVl) (Table 1; Pascale et al. 2008). The majority of RBPs can be clustered according to their similar molecular functions, such as those belonging to the translation and turnover regulatory (TTR)-RBP group. Although not limited to these functions, TTR-RBPs are involved in stabilizing/destabilizing mRNAs and promoting/inhibiting translation, and comprise various members, including TTP, KSRP, AUF, and Hu/ELAVl proteins (Table 1; Pullmann et al. 2007). Among all the RBPs expressed in neurons, the best characterized is the predominantly neuron-specific Hu member HuD. This review will focus on the multifunctional roles and diverse regulatory events controlling the expression of HuD in neurons.

TABLE 1. Select AU-rich element targeting RNA-binding proteins in neurons and their effects on mRNA metabolism

RNA-binding protein	Effect on mRNA
HuB/ELAVl2, HuC/ELAVl3, HuD/ELAVl4	Local transcription rate enhancement (Zhou et al. 2011)
	Alternative splicing (Zhu et al. 2006, 2008)
	Alternative polyadenylation (Zhu et al. 2006)
	Nuclear export (Kasashima et al. 1999)
	Stabilization (Jain et al. 1997; Anderson et al. 2000; Mobarak et al. 2000)
	Cytoplasmic transportation (Aronov et al. 2002)
	Translation repression (Kullmann et al. 2002)
	Translation enhancement (Antic et al. 1999; Kullmann et al. 2002; Fukao et al. 2009)
HuR/ELAVl1	Local transcription rate enhancement (Zhou et al. 2011)
	Alternative splicing (Izquierdo 2008)
	Alternative polyadenylation (Zhu et al. 2006)
	Nuclear export (Fan and Steitz 1998)
	Stabilization (Fan and Steitz 1998; Levy et al. 1998; Peng et al. 1998)
	Destabilization (Kim et al. 2009)
	Translation repression (Kullmann et al. 2002)
	Translation enhancement (Mazan-Mamczarz et al. 2003; Meng et al. 2005)
AU-rich element binding factor 1 (AUF1; p37, p40, p42, and p45)/hnRNPD	Mostly destabilization (Brewer and Ross 1989; Brewer 1991; Zhang et al. 1993)
	Translation enhancement (Liao et al. 2007)
K homology-type splicing regulatory protein (KSRP)	Transcription enhancement (Davis-Smyth et al. 1996)
	Alternative splicing (Min et al. 1997)
	Destabilization (Chen et al. 2001; Gherzi et al. 2004)
	Cytoplasmic transport (Snee et al. 2002)
Tristetraprolin (TTP)	Transcription repression (Liang et al. 2009; Schichl et al. 2009)
	Destabilization (Carballo et al. 1998)
	Translation repression (Pfeiffer and Brooks 2012; Qi et al. 2012; Tiedje et al. 2012)
Butyrate-response factor-1 (BRF1)	3′ End processing inhibition (Desroches-Castan et al. 2011)
	Destabilization (Stoecklin et al. 2002)
TINO/Mex3D/RKHD1	Destabilization (Donnini et al. 2004)
Polyadenylate-binding protein-interacting protein 2 (PAIP2)	Stabilization (Onesto et al. 2004)
	Translation repression (Khaleghpour et al. 2001)
CUG-binding protein 1 (CUG-BP1)	Alternative splicing (Philips et al. 1998)
	Destabilization (Moraes et al. 2006)
	Translation enhancement (Timchenko et al. 1999)
CUG-BP2	Alternative splicing (Zhang et al. 2002)
	RNA editing (Anant et al. 2001)
	Stabilization (Mukhopadhyay et al. 2003)
	Translation repression (Mukhopadhyay et al. 2003)
Nucleolin	Transcription repression (Yang et al. 1994)
	Stabilization (Chen et al. 2000)
	Destabilization (Zaidi and Malter 1995)
	Translation repression (Takagi et al. 2005)
	Translation enhancement (Fahling et al. 2005)

THE HuD GENE, mRNA, AND PROTEIN

The name of Hu/ELAVl genes originated from the first initials (Hu) of the patient in which they were identified and the close relation of these genes to the *Drosophila embryonic lethal abnormal vision* (*elav*) gene (Graus et al. 1987; Okano and Darnell 1997). In addition, Hu genes are also closely related to the *Drosophila sex lethal* (*sxl*), *found in neurons (fne)*, *and RNA-binding protein 9 (rbp9)* genes. These four *Drosophila* genes encode RBPs that display similar structures and overlapping functions in neurons to HuD (Bell et al. 1988; Robinow et al. 1988; Kim and Baker 1993; Samson and Chalvet 2003; Colombrita et al. 2013). In mammals, Hu proteins were first discovered as antigens targeted by auto-antibodies in patients with the neurological syndrome paraneoplastic encephalo-

myelitis and sensory neuropathy (PEM/SN) (Dalmau et al. 1990, 1992). This syndrome arises in a small subset of patients when the ectopic expression of Hu proteins in certain types of cancers, typically small cell lung carcinomas (SCLCs), provokes an autoimmune attack on the nervous system.

Out of the four Hu members, HuD was the first to be cloned and characterized, followed by HuC/ELAVl3, HuB/ELAVl2, and HuR/ELAVl1 (Szabo et al. 1991; King et al. 1994; Sakai et al. 1994; Ma et al. 1996). The expression of three Hu members—HuB, HuC and HuD—is predominantly restricted to neurons (nELAVs), while HuR is found in most cell types (Szabo et al. 1991; King et al. 1994; Good 1995; Ma et al. 1996; Okano and Darnell 1997). In addition, nELAVs share a higher degree of amino acid sequence identity (>80%) with each other compared to HuR (72.5%–73.6%) (Okano and Darnell 1997). Despite the high degree of sequence identity between nELAVs, variation of amino acid composition in key regions, particularly the N-terminal and linker regions (see below), and spatiotemporal expression suggest that HuD carries out some unique roles and is subjected to partially distinct regulation (Okano and Darnell 1997; Clayton et al. 1998; Hambardzumyan et al. 2009).

Overall, the *HuD* gene is well conserved among higher vertebrates and is located on chromosome 1 in humans and chromosome 4 in mice (Fig. 1A). The human and mouse *HuD* gene spans ∼146 kb of DNA and is divided into seven coding exons (E2 to E8) that encompass ∼44 kb of DNA (Sekido et al. 1994; Inman et al. 1998). For several years, the 5′ region of *HuD* was thought to contain three presumably untranslated exon 1 variants (termed E1a, E1b, and E1c) that are alternatively spliced to the 5′ end of exon 2 (Inman et al. 1998). Recent findings in our laboratory, however, have identified the expression of five additional HuD E1 variants, resulting in a total of eight (Fig. 1A,B; Bronicki et al. 2012). We also found that most E1 variants house an in-frame methionine codon (with the exception of $E1a^4$, since it harbors two stop codons downstream from the AUG), suggesting that they may encode alternate HuD N termini.

In addition to the 5′ end, *HuD* pre-mRNA is subjected to alternative splicing of exons (E) 6 and 7, resulting in three additional transcript isoforms (*HuD*, HuD_{mex}, and HuD_{pro}) (Fig. 1B,C), two of which are predominantly expressed in the postnatal mouse brain (*HuD* and HuD_{pro}) (Inman et al. 1998). The combinatorial complexity of HuD alternative 5′ and 3′ exons may produce up to 24 different mRNA variants. The HuD protein, like all Hu members, has a molecular weight of ∼40 kDa and contains three RNA recognition motifs (RRMs) with a linker region separating the second and third RRM (Fig. 1C; Good 1995; Liu et al. 1995; Okano and Darnell 1997). The RRM is the most abundant (found in up to 1% of human genes) and well-characterized RNA-binding domain, composed of ∼90 amino acids (Clery et al. 2008). In the *HuD* gene locus, E2 and E3, E4 and E5, and E8 encode the three RRMs, respectively, whereas the alternatively spliced E6

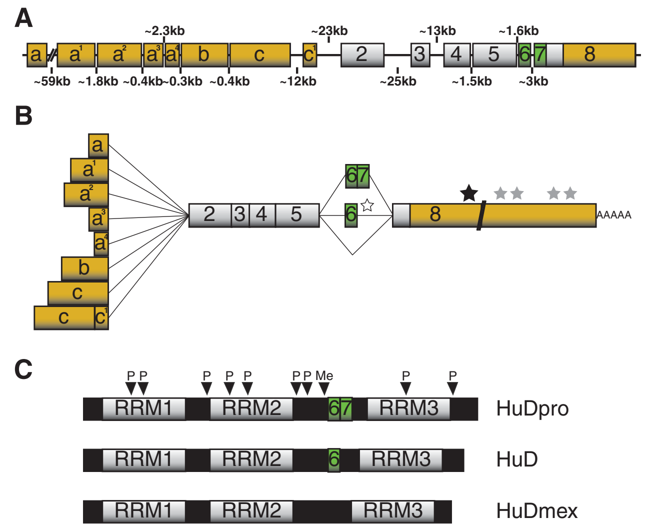

FIGURE 1. Organization of the mouse *HuD* gene, mRNA, and protein. (*A*) Mouse *HuD* gene organization with noncoding exons depicted as orange rectangles, coding exons as gray and green rectangles, and introns as black lines. (*B*) Alternative splicing/processing of *HuD* pre-mRNA produces different HuD isoforms. 5′/3′ UTRs and coding exons are represented by orange and gray rectangles, respectively. Green rectangles denote alternatively spliced exons near the 3′ end of the gene. Also shown are the approximate locations of the Hu/ELAVl (white star), miR-375 (black star), and putative ARE (gray stars) binding sites. (*C*) The three major HuD protein variants found in neurons with position of RRMs (gray) and amino acid extensions in the linker region (green) shown. "P" and "Me" indicate approximate locations of serine/threonine and arginine residues that are subjected to phosphorylation by PKC and methylation by CARM1, respectively.

and E7 encode the linker region (Inman et al. 1998). The HuD linker region contains multiple residues that are post-translationally modified and houses the nuclear export (NES) and putative nuclear localization (NLS) signals (Kasashima et al. 1999).

HuD utilizes its RRMs to recognize and bind specific target mRNAs. Deletion and mutational analysis of the HuD protein revealed that the first two RRMs bind to the *cis*-acting elements, typically in the 3′ UTR, while the third RRM interacts with the poly(A) tail of transcripts and stabilizes the RBP-mRNA complex (Chung et al. 1996, 1997; Ma et al. 1997; Ross et al. 1997; Anderson et al. 2000; Park et al. 2000; Wang and Tanaka Hall 2001; Beckel-Mitchener et al. 2002). Binding to the poly(A) tail seems to depend on the tail length, since results from in vitro binding assays demonstrated that the longer the poly(A) tail, the higher the affinity of Hu protein binding to its target mRNA (Beckel-Mitchener et al. 2002). Another function of the third RRM of HuD involves interacting with other proteins, including homo- and hetero-multimerizing with Hu members (Kasashima et al. 2002). Both the *Drosophila* ELAV and other mammalian Hu proteins have also been shown to form homo-multimers on target mRNAs, suggesting that complex formation is an evolutionarily conserved mechanism necessary for efficient function of Hu members (Levine et al. 1993; Gao and Keene 1996; Kasashima et al.

2002; Devaux et al. 2006; Fialcowitz-White et al. 2007; Toba and White 2008).

TEMPORAL AND SPATIAL EXPRESSION OF HuD

Converging findings suggest that HuD is one of the earliest markers of the neuronal phenotype (Good 1995; Okano and Darnell 1997; Wakamatsu and Weston 1997; Hambardzumyan et al. 2009). Multiple laboratories have demonstrated via Northern blots and quantitative RT-PCR that *HuD* mRNA levels are detectable by day 10 of mouse and rat embryonic brain development, peak at E16, and slowly decline until birth (Clayton et al. 1998; Hambardzumyan et al. 2009; Abdelmohsen et al. 2010). This temporal pattern of mRNA expression is similar to that of HuB and HuC, although there is some evidence that the relative abundance of each nELAV member varies at different time points of development (Good 1995; Okano and Darnell 1997; Hambardzumyan et al. 2009).

In situ hybridization analyses of *nELAV* expression in the mouse brain at day 14 of embryogenesis (E14) have revealed that the spatial expression of *HuD* mRNA is also partially unique compared to *HuB* and *HuC* (Okano and Darnell 1997), thus further supporting the notion that HuD is not completely functionally redundant. In the developing mouse and rat cerebral cortex, *HuD* mRNA is present in proliferating neuronal stem/progenitor cells in the ventricular zone, neuroblasts migrating in the intermediate zones, and terminally differentiating neurons in the cortical plate (Okano and Darnell 1997; Clayton et al. 1998). *HuD* transcripts are also present in several regions of the developing hippocampal formation, olfactory bulb, retina and spinal cord, such as sensory and motor neurons (Okano and Darnell 1997; Clayton et al. 1998; Bronicki et al. 2012). In the adult mouse and rodent nervous system, expression of *HuD* mRNA is mostly restricted to specific neuronal populations, including large pyramidal-like neurons in layer V of the neocortex and cerebellar cortex (Purkinje cells), the four *Cornu Amonis* (CA1-4) regions of the hippocampus, dorsal root ganglia, and motor neurons in the spinal cord, mitral cells in the olfactory bulb, ganglion and internal plexiform layers in the retina, and enteric nervous system neurons (Okano and Darnell 1997; Clayton et al. 1998; D'Autreaux et al. 2011).

At the subcellular level, HuD is predominantly expressed in the cytoplasm, with minor levels detected in the nucleus (Kasashima et al. 1999). In developing neurons, HuD is found in growth cones of extending neurites, while in mature neurons it is present in axons and dendrites, including at the pre- and post-neurite terminals, respectively (Aranda-Abreu et al. 1999; Aronov et al. 2002). Closer inspection of intraneuronal compartments revealed that HuD is localized to granules, along with other proteins, such as kinesin-like protein (KIF3A) and survival of motoneuron (SMN), within the cytoplasm and neurites (Aronov et al. 2002; Smith et al. 2004; Tiruchinapalli et al. 2008a,b; Hubers et al. 2010; Akten et al. 2011; Fallini et al. 2012b). In addition to being expressed in various neuronal subpopulations in the CNS and PNS, quantitative RT-PCR and Western blot analysis revealed relatively minor, although significant, HuD mRNA and protein levels in several nonneuronal tissues including lung, testes, liver, pituitary gland, pancreatic β cells, and fiber cells of the lens (Abdelmohsen et al. 2010; Bitel et al. 2010; Lee et al. 2012). These recent findings indicate that, in parallel to its key functions in neurons, HuD likely plays important roles in several other nonneuronal cell types.

Cis-ELEMENT AND TARGET mRNAs OF HuD

The most extensively studied HuD-targeted *cis*-acting elements are adenosine/uridine (A/U)-rich elements (AREs) commonly found in the 3′ UTR of almost 10% of cellular mRNAs (Halees et al. 2008). AREs usually range from 50 to 150 nt in size and, as their name indicates, consist of A- and U-rich stretches. These motifs vary considerably in sequence, and for simplification, they are categorized into three major classes; class I contains one to three AUUUA pentamers in a U-rich context, class II includes overlapping UUAUUUAUU nonamers, and class III consists of U-rich sequences without AUUUA pentamers (Bakheet et al. 2003, 2006). ARE motifs are typically found in short-lived transcripts encoding proteins with diverse functions such as cellular proliferation, differentiation, transcription, RNA metabolism, inflammation, and stress-response (Bakheet et al. 2006; Khabar 2010). These elements affect the stability of mRNAs by serving as target sites for ARE-binding RBP(s), also known as AU-binding proteins (AUBPs). Out of the roughly 20 currently known AUBPs, the majority are expressed in neurons and promote mRNA decay including, for example, AUF1, TTP, and KSRP (Table 1; Wu and Brewer 2012). Nevertheless, there are a few known AUBPs that enhance mRNA stability, with Hu proteins being the most notable (Brennan and Steitz 2001; Hinman and Lou 2008; Pascale et al. 2008).

The crystal structure of HuD bound to *c-fos* or tumor necrosis factor α (*TNF*α) mRNAs illustrates that specific residues within its first two RRMs preferentially bind the pyrimidine-rich X-U/C-U-X-X-U/C?-U-U/C consensus sequence where the "?" denotes ambiguity whether a cytosine in this position is tolerated (Wang and Tanaka Hall 2001). This report also showed that the amino acid residues interacting with RNA nucleotides are well conserved among all Hu proteins and the *Drosophila* ELAV protein. Several studies have utilized in vitro and in vivo approaches such as RNA electrophoretic mobility shift assays (REMSAs) and RNA-immunoprecipitation (IP), respectively, to demonstrate that HuD binds to ARE sequences in mRNAs (Liu et al. 1995; Chung et al. 1996, 1997; Deschenes-Furry et al. 2003; Ratti et al. 2008). However, it is important to point out that the presence of an AUUUA sequence is not always sufficient for HuD binding, indicating that HuD displays preference for specific ARE motifs (Toba et al. 2002).

Interestingly, a microarray screen of HuD-bound mRNAs found that less than half of its targets contain an ARE (Bolognani et al. 2010). Instead, the investigators identified three novel consensus motifs that are present in ∼80% of HuD-targeted mRNAs expressed in the mouse forebrain. The first motif consists of C- and U-rich stretches, with a prevalence of the former, and is located mostly in the 5′ and 3′ UTRs of mRNAs. The second and third motifs are U-rich with interspersed guanines (G) or adenosines (A), respectively. These latter two motifs are predominantly found in the 3′ UTR and occasionally in the 5′ UTR and coding regions of mRNAs. Recent high-throughput analyses, using variations of cross-linking and immunoprecipitation (CLIP) such as Photoactivatable-Ribonucleoside-Enhanced CLIP (PAR-CLIP) and individual-nucleotide resolution CLIP (iCLIP), of HuR and nELAV target mRNAs corroborate these findings by demonstrating that all four Hu proteins preferentially bind U-rich sequences interspersed with Gs or As (Lebedeva et al. 2011; Mukherjee et al. 2011; Uren et al. 2011; Ince-Dunn et al. 2012). These studies also found that Hu binding sites were mostly located in 3′ UTRs of mRNAs, with a significant portion in introns and relatively few in 5′ UTRs. In terms of HuD, the various types of *cis*-acting motifs that it targets are found in mRNAs encoding proteins with diverse functions in neurons ranging from cell cycle arrest to synapse formation (Bolognani et al. 2010).

Evidence that Hu proteins bind AREs originated from a study demonstrating preferential binding of HuB to A/GUUU A/G/U sequences located within the 3′ UTRs of mRNAs (Levine et al. 1993). This fundamental finding enabled the subsequent identification of the first target mRNA of HuD, *c-fos*, a member of the immediate early (IE) gene family of transcription factors (TFs) that encode proteins involved in cell proliferation and differentiation (Liu et al. 1995). Using REMSAs, the authors showed that HuD binds specifically to an ARE sequence in the *c-fos* 3′ UTR. Numerous other ARE-harboring transcripts, such as *N-myc* and *c-myc*, were later identified as targets of HuD using similar in vitro approaches, indicating that HuD binds short-lived mRNAs whose protein product functions in cell proliferation (Liu et al. 1995; Ross et al. 1997). In addition to these transcripts, HuD has been shown to bind and regulate several other mRNAs in vitro and in vivo, many of which encode proteins with important functions in neuronal differentiation such as cell cycle arrest ($p21^{cip1/waf1}$) (Joseph et al. 1998), neuroblast proliferation (MSI-1) (Ratti et al. 2006), neuron migration (MARCKS) (Wein et al. 2003), neurite extension ([GAP-43] [Chung et al. 1997], Tau [Aranda-Abreu et al. 1999], and AChE [Cuadrado et al. 2003; Deschenes-Furry et al. 2006; Bronicki and Jasmin 2012]), synapse formation (NOVA-1) (Ratti et al. 2008), and neuronal growth and survival (NGF, BDNF, and NT-3) (Table 2; Lim and Alkon 2012).

In addition to these HuD-targeted mRNAs, three independent high-throughput studies have unravelled functionally diverse classes of genes regulated by HuD. One of these studies purified HuD-containing mRNP complexes from HuD overexpressing (HuD-Tg) adult mouse forebrains and subsequently pulled down target mRNAs with GST-HuD for microarray examination. Gene ontology analysis identified several mRNAs whose proteins are involved in regulating RNA processing, nuclear export, translation, cell-to-cell signalling, and vesicle trafficking (Bolognani et al. 2010). Of particular interest, this study also found that HuD binds mRNAs of several RBPs, including its own, *HuB*, *HuR*, *Cugbp2*, *Musashi 2*, *and Staufen 2*, indicating a complex regulatory network between HuD and other components of the ribo-

TABLE 2. Established function(s) of HuD on neuronal target mRNAs

Gene	Effect of HuD on mRNA
Growth associated protein 43 (GAP-43)	Stabilization (Liu et al. 1995; Chung et al. 1997; Tsai et al. 1997; Anderson et al. 2000; Mobarak et al. 2000)
	Transportation into neurites (Smith et al. 2004)
$p21^{cip1/waf1}$	Stabilization (Joseph et al. 1998; Fujiwara et al. 2006)
Tau	Stabilization (Aronov et al. 2002)
	Transportation into neurites (Aranda-Abreu et al. 1999)
	Translation enhancement (Aronov et al. 2002)
$p27^{KIP}$	Translation repression (Kullmann et al. 2002)
Neuroserpin	Stabilization (Cuadrado et al. 2002)
N-myc	Stabilization (Manohar et al. 2002)
Myristoylated alanine-rich C kinase substrate (MARCKS)	Stabilization (Wein et al. 2003)
Acetylcholinesterase (AChE)	Stabilization (Deschenes-Furry et al. 2003)
Musashi 1 (Msi1)	Stabilization (Ratti et al. 2006)
Calcitonin/CGRP	Alternative splicing (exon exclusion) (Zhu et al. 2006)
	Alternative polyadenylation (Zhu et al. 2006)
Ikaros	Alternative splicing (Bellavia et al. 2007)
Neurofibromatosis (NF-1)	Alternative splicing (exon exclusion) (Zhu et al. 2008)
	Local transcription rate enhancement (Zhou et al. 2011)
Neuro-oncological ventral antigen 1 (NOVA1)	Stabilization (Ratti et al. 2008)
	Translation enhancement (Ratti et al. 2008)
LIM domain transcription factor (LMO4)	Stabilization (Chen et al. 2007)
HuD	Alternative splicing (exon inclusion) (Wang et al. 2010)
HuR	Alternative polyadenylation (Mansfield and Keene 2011)
Glutaminase (Gls1/Gls)	Alternative splicing (Ince-Dunn et al. 2012)
	Stabilization (Ince-Dunn et al. 2012)
	Translation enhancement (Ince-Dunn et al. 2012)
Nerve growth factor (NGF)	Stabilization (Lim and Alkon 2012)
Neurotrophin 3 (NT-3)	Stabilization (Lim and Alkon 2012)
Brain-derived growth factor (BDNF)	Stabilization (Lim and Alkon 2012)

nome (Pullmann et al. 2007; Mansfield and Keene 2009; Bolognani et al. 2010). A second study also employed a microarray approach to determine HuD-targeted mRNAs in dentate granule cells of HuD-Tg mice (Perrone-Bizzozero et al. 2011). The authors found that the data set is mostly enriched in mRNAs encoding proteins involved in neuronal development and axogenesis, such as Notch3 and Neurog2 (Perrone-Bizzozero et al. 2011). Last, a recent study used CLIP and microarray technologies to reveal that nELAVs regulate multiple transcripts, some of which encode components of the amino acid glutamate biosynthetic pathway, such as glutaminase 1, which indicates that an important function of nELAVs is to control neuronal activity (Ince-Dunn et al. 2012).

MULTILEVEL CONTROL OF mRNA METABOLISM BY HuD

Alternative splicing

Following binding to a target mRNA, HuD has been shown to control one or multiple regulatory events during the transcript's metabolism, with alternative splicing being one of the earliest. The first clues that HuD plays a role in alternative splicing were based on its significant homology to the *Drosophila* splicing factor *sxl* (Szabo et al. 1991) and experiments demonstrating that the *Drosophila* ELAV protein promotes neuron-specific alternative splicing (Koushika et al. 1996). Following these seminal findings, a more direct indication that HuD and the other three Hu proteins regulate alternative splicing in mammals emerged from a study that showed HuD binding to both exonic and intronic regions of *N-myc* pre-mRNA (Lazarova et al. 1999). A few years later, a series of papers predominantly from the Hua Lou laboratory corroborated that all Hu proteins function as auxiliary splicing factors (Hinman and Lou 2008). Using various complementary methods, such as REMSA and UV cross-linking/IP assays, one report demonstrated that nELAVs compete with TIA-1/TIAR RBPs for a U-rich intronic sequence in the calcitonin/calcitonin gene-related peptide (CGRP) pre-mRNA to block inclusion of exon 4. Through this mechanism, nELAVs promote expression of the neuron-specific CGRP isoform in neurons (Zhu et al. 2006). nELAVs were also shown to interact with intronic AREs surrounding exon 23a of the neurofibromatosis type 1 (*NF1*) pre-mRNA to block its inclusion by inhibiting U1/U6 snRNP and U2AF auxiliary splicing factor binding to the 5′ and 3′ splice sites (SSs), respectively (Zhu et al. 2008). Furthermore, overexpression and down-regulation of HuD in T-cell lines demonstrated that it mediates alternative splicing of the *Ikaros* gene, possibly by suppressing exon 4 inclusion (Bellavia et al. 2007; Hinman and Lou 2008). In addition to binding intronic sequences, co-IP assays revealed that Hu proteins interact with RNA polymerase II and histone deacetylase 2 (HDAC2) to regulate alternative splicing (Zhou et al. 2011). In vitro transcription elongation assays showed that, by blocking HDAC2 activity, histones surrounding specific exons remained hyperacetylated, which resulted in an increased RNAPII transcription rate and decreased exon inclusion at these genomic loci. Experiments with HuB and HuC deletion mutants indicated that the linker region and RRM3 are necessary for nELAV regulation of alternative splicing (Zhu et al. 2008; Zhou et al. 2011).

In contrast to this function, evidence that HuD and the two other nELAVs enhance exon inclusion also exists. For instance, all four Hu members were shown to promote inclusion of HuD exon 6 by binding to two ARE sequences in the downstream intron (Wang et al. 2010). In addition, a recent report identified intronic nELAV binding sites flanking an alternative 3′ SS in the *Gls1/Gls* gene and demonstrated that nELAVs generate a longer *Gls* mRNA variant, indicating that they may enhance inclusion of a *Gls* 3′ exon(s) (Ince-Dunn et al. 2012). The same report also used genome wide analysis to map the binding sites in nELAV targeted pre-mRNAs and revealed that these proteins preferentially bind introns surrounding cassette exons, especially near exon/intron splice junctions (Ince-Dunn et al. 2012). In line with previous studies, nELAVs were found to enhance exon inclusion when bound to 5′ SSs in the downstream intron and inhibit exon inclusion when bound to adjacent 3′ and 5′ SSs of alternative exons. Altogether, these studies clearly demonstrate that HuD, along with the other nELAV proteins, can promote or suppress exon inclusion by interacting with (or antagonizing) splicing, transcription, and chromatin components (Fig. 2A).

Alternative polyadenylation

In addition to alternative splicing, both the *Drosophila* ELAV and the mammalian Hu proteins were demonstrated to control alternative polyadenylation specifically in neurons (Soller and White 2003). Truncation of the mouse HuB protein revealed that all three RRMs are necessary for Hu proteins to perform this function (Zhu et al. 2006). Employing mainly REMSAs and in vitro cleavage and polyadenylation assays, one study determined that Hu proteins bind U-rich sequences downstream from the exon 4 poly(A) signal in calcitonin/CGRP to block binding of cleavage stimulation factor 64 (CstF64) and cleavage-polyadenylation specificity factor (CPSF), essential components of the cleavage and polyadenylation machinery. Through this mechanism Hu proteins prevent cleavage and polyadenylation at the nonneuronal exon 4 poly(A) signal, thereby promoting the neuron-specific CGRP pathway (Zhu et al. 2006). Moreover, all four Hu proteins were recently demonstrated to differentially regulate alternative polyadenylation of HuR in neurons (Mansfield and Keene 2011; also see Dai et al. 2012). Mansfield and Keene showed that there are three different HuR mRNA polyadenylation variants (1.5, 2.4, and 6.0 kb), which differ in 3′ UTR length. All four Hu members were found to bind and block U-rich sequences near the 2.4-kb polyadenylation site and thereby promote expression of the 6.0-kb transcript. Since the 6.0-kb variant is less stable and not as efficiently

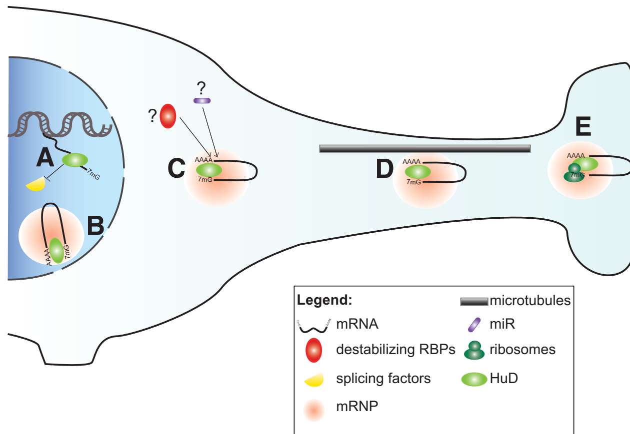

FIGURE 2. Model depicting the intracellular localizations and multiple post-transcriptional functions of HuD. (*A*) In the nucleus, HuD regulates alternative splicing and polyadenylation by competing for specific pre-mRNA binding sites with other *trans*-acting factors. (*B*) HuD forms part of an mRNP complex, and it likely facilitates mRNA export into the cytoplasm. (*C*) In the cytoplasm, HuD and destabilizing RBPs bind competitively or cooperatively to mRNAs. HuD might also prevent binding of miRs to the mRNA or antagonize their function. Through these mechanisms, HuD increases the half-lives of mRNAs. (*D*) HuD transports mRNAs to different compartments of neurons, most notably neurites, along microtubules. (*E*) At the synaptic terminal, HuD may promote or repress translation of transcripts.

translated as the 2.4-kb transcript, the authors postulated that the reduction of HuR expression prevents HuR's ability to promote proliferation and thereby permits neurons to terminally differentiate (Mansfield and Keene 2011). The role of Hu proteins in controlling alternative polyadenylation is likely extended to several of their target mRNAs, given that the ELAV protein in *Drosophila* was found to coordinate 3′ UTR extension of multiple mRNAs in neuronal cells (Hilgers et al. 2012).

Nucleocytoplasmic shuttling

Another chief function of HuD involves shuttling of target mRNAs into the cytoplasm. As previously mentioned, HuD contains a NES and putative NLS located in the variable linker domain, between the second and third RRM, which permit nucleocytoplasmic shuttling of HuD (Fig. 2B; Kasashima et al. 1999). Overexpression of wild-type and deletion mutant HuD proteins revealed that cytoplasmic shuttling of HuD requires the NES region (Kasashima et al. 1999). Moreover, in vitro binding assays showed that the first two RRMs of HuD associate with the primary general mRNA export adaptor complex (TAP-p15; also known as NXF1/NXT1) in a RNA-independent manner and that this interaction may be necessary for shuttling of HuD (Saito et al. 2004). Further studies are necessary to establish whether TAP-p45 and/or another export pathway, such as the karyopherin CRM1, is used by HuD for nuclear export of mRNAs (Carmody and Wente 2009).

mRNA stability

The initial demonstration that Hu proteins regulate mRNA stability was based on the observation that HuB enhances *GLUT1* mRNA half-life and, incidentally, translation (Jain et al. 1997). Following this landmark study, other reports described similar roles for HuR (Fan and Steitz 1998; Levy et al. 1998; Peng et al. 1998) and HuC/D (Anderson et al. 2000; Mobarak et al. 2000) in regulating mRNA stability. Since these initial findings, HuD has emerged as the best-described stabilizing RBP in neurons, as evidenced by the numerous studies outlining its role in increasing the half-lives of various neuronal transcripts (Fig. 2C). Among all of the HuD-regulated mRNAs, the most prominent is the *GAP-43* mRNA, which houses a class III ARE in its 3′ UTR (Chung et al. 1997; Tsai et al. 1997). HuD-dependent control of GAP-43 expression is particularly interesting because GAP-43 plays multiple roles in neurons, including functioning as a critical component in neural development (Maier et al. 1999; Mani et al. 2001; Shen et al. 2008) and synaptic remodeling during learning and memory (Routtenberg et al. 2000). Multiple studies have shown that overexpression or down-regulation of HuD results in increased or decreased, respectively, *GAP-43* mRNA levels and/or stability in cultured cells and the mouse nervous system (Anderson et al. 2000, 2001; Mobarak et al. 2000; Bolognani et al. 2006, 2007a). Interestingly, HuD is more effective at stabilizing *GAP-43* mRNAs that contain a longer poly(A) tail, indicating that HuD binds to both the ARE and poly(A) tail to increase *GAP-43* mRNA half-life (Beckel-Mitchener et al. 2002). In addition to *GAP-43* transcripts, HuD has also been shown to promote the stability of several other ARE-containing mRNAs in neurons, such as *AChE* (Deschenes-Furry et al. 2003) and *Nova1* (Ratti et al. 2008), both in vitro and in vivo (Table 2; Perrone-Bizzozero and Bird 2013). Despite mRNA stabilization being one of the better-known roles of HuD, the molecular events behind this function are not well understood.

One likely mechanism through which HuD and other Hu members stabilize mRNAs involves antagonizing destabilizing RBPs such as AUF1 from binding to *cis*-acting elements (Barreau et al. 2006). Once bound to a target mRNA, destabilizing RBPs may promote deadenylation through a variety of manners such as blocking poly(A) binding protein (PABP) binding and/or recruitment of decapping, deadenylation, and exosome components (Wu and Brewer 2012). Thus, it is feasible that, by binding to AREs or other motifs, HuD blocks access to the binding site and consequently prolongs mRNA half-life (Fig. 3). However, there is evidence indicating that this mechanism is more complex since both stabilizing and destabilizing RBPs can bind the same transcript simultaneously. Fluorescence resonance energy transfer (FRET) and biochemical techniques revealed that HuR interacts with AUF1, KSRP, and TIA-1 in the nucleus and cytoplasmic stress granules (David et al. 2007; David Gerecht et al. 2010). Moreover, these interactions require the presence

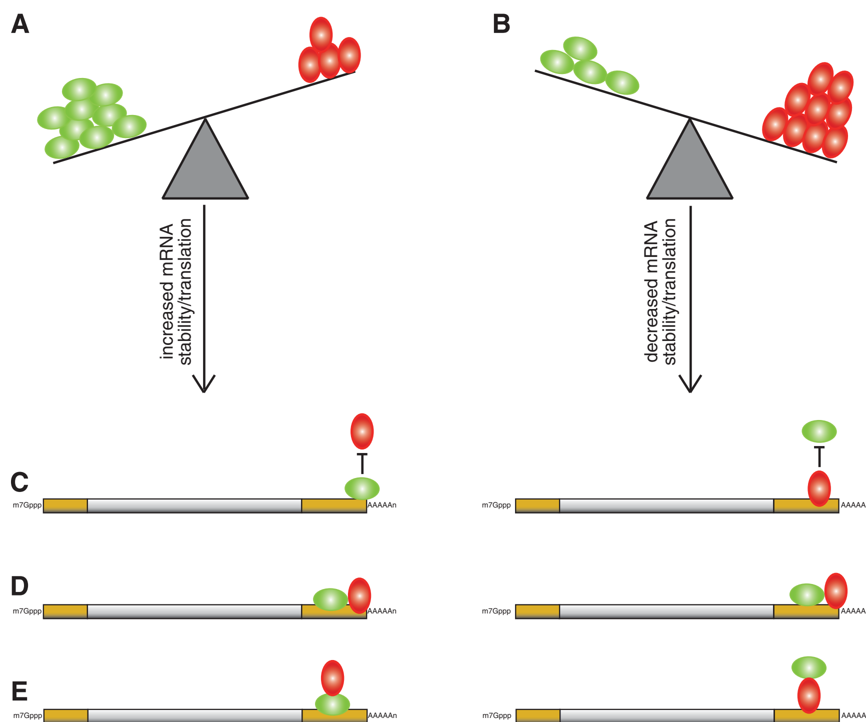

FIGURE 3. Relative levels of RBPs and their cooperative and competitive binding to mRNAs controls gene expression. The relative expression of RBPs that (*A*,*B*) promote (e.g., Hu/ELAVl proteins; green) or inhibit (e.g., AUF1; red) mRNA stability/translation influences gene expression. Functionally antagonistic RBPs can also bind (*C*) competitively or (*D*,*E*) cooperatively to regulate mRNA fate. Cooperative binding could potentially allow for a quicker response to extracellular cues in determining mRNA fate.

of RNA, since HuR and AUF1 were shown to simultaneously or competitively bind onto exclusive or common, respectively, *cis*-acting elements in the 3′ UTRs of *p21*$^{cip1/waf1}$, *Cyclin D*, *TNF*α, and β_2*-adrenergic receptor* mRNAs (Lal et al. 2004; David et al. 2007). Together, these findings suggest that regulation of mRNA stability, and likely other mRNA metabolism events, by Hu proteins is at least partially dependent on a balance between stabilizing and destabilizing RBPs (Fig. 3).

mRNA localization and translation

Along with promoting mRNA stability, compelling evidence supports a role for HuD in regulating both localization and translation of transcripts in the cytoplasm and possibly in neurites (Fig. 2D,E). For example, two target mRNAs of HuD involved in axonal outgrowth, namely *GAP-43* and *Tau*, were found to colocalize with HuD protein and polysomes in growth cones during neuronal differentiation (Smith et al. 2004; Atlas et al. 2007). Co-IP and immunocytochemistry experiments revealed that HuD interacts with a host of mRNP components involved in transport and translation in the cytoplasm and neurites such as the motor protein KIF3A (Aronov et al. 2002), IGF-II mRNA binding protein 1 (IMP-1) (Atlas et al. 2004, 2007), survival of motor neuron (SMN) (Hubers et al. 2010; Akten et al. 2011; Fallini et al. 2011), poly(A) binding protein (PABP), translation initiation factor 4E (eEF4E) (Tiruchinapalli et al. 2008b) and the microtubule-associated component MAP1B (Fujiwara et al. 2011b). Various conditions, including KCl depolarization of primary hippocampal neurons, cocaine, or seizure treatment of mice and phorbol ester or bryostatin-1 application to human neuroblastoma (NB) cells, result in an augmented abundance of HuD, its redistribution to neurites, and its increased association with mRNAs and mRNP components (Pascale et al. 2005; Tiruchinapalli et al. 2008a,b). Complementary to its role in mRNA localization, evidence for the direct involvement of HuD in regulating translation was unveiled when Fukao and colleagues showed that HuD binds to eIF4A and the poly(A) tail of transcripts, via its linker region and third RRM, to enhance cap-dependent protein synthesis (Fukao et al. 2009). Moreover, this mechanism was determined to be important for HuD-dependent neurite extension in PC12 cells.

In addition to enhancing translation, HuD is capable of repressing protein synthesis, as shown for *p27* and *preproinsulin* (*Ins2*) mRNAs (Kullmann et al. 2002; Lee et al. 2012). Even though the mechanism of translational silencing is unclear, it was demonstrated to require HuD binding to an internal ribosome entry site (IRES) in the 5′ UTR of *p27* mRNA (Kullmann et al. 2002) and a short ∼22-nt sequence in the 5′ UTR of *Ins2* mRNA (Lee et al. 2012). The report by Kullman et al. suggests that inhibition of p27 translation by Hu proteins is an event that sustains cells in a proliferative state (Kullmann et al. 2002). On the other hand, the study by Lee et al. showed that the repression of *Ins2* translation by HuD is likely required to maintain insulin homeostasis (Lee et al. 2012). Taking into account these findings, it is plausible that HuD inhibits translation of select target mRNAs in neuronal stem/precursor cells or in response to a neuronal stress such as hypoxia. Altogether, these studies indicate that HuD recruits mRNAs to microtubules and may transport them to neurites to control their local translation in response to extracellular cues, particularly during neuronal development and function.

MOLECULAR MECHANISMS REGULATING HuD

The key roles of HuD in neuronal development and plasticity and its implications in neuronal diseases raise important questions regarding the molecular mechanisms that regulate its expression. To date, only a few studies have started to describe

the post-translational, post-transcriptional, and transcriptional mechanisms that control the expression and function of HuD (Fig. 4). However, given the key functions of HuD in neurons, more systematic investigations of the molecular events that regulate its expression are necessary.

Post-translational control of HuD expression and function

Although some conditions, such as application of growth factors (Aranda-Abreu et al. 1999; Abdelmohsen et al. 2010), pharmacological agents (Pascale et al. 2005; Tiruchinapalli et al. 2008a,b), and cellular stress (Burry and Smith 2006), have been documented to alter the expression or function of HuD, the molecular pathways and downstream regulators involved are only beginning to emerge. Currently, there are only three known pathways that regulate the abundance or function of HuD: protein kinase C (PKC) (Mobarak et al. 2000; Pascale et al. 2005; Lim and Alkon 2012); coactivator-associated arginine methyltransferase 1 (CARM1) (Fujiwara et al. 2006; Hubers et al. 2010); and protein kinase B (PKB/AKT) (Fujiwara et al. 2011a).

The ubiquitously expressed serine/threonine PKC family consists of at least 10 isoforms with diverse functions ranging from controlling cell proliferation to synaptic remodeling (Amadio et al. 2006). The classical PKCα isoform was shown to increase nELAV levels and provoke their nuclear export following treatment of SH-SY5Y NB cells with the PKC-activating compounds phorbol esters and bryostatin-1 (Pascale et al. 2005). Moreover, stimulation of PKCα promotes its interaction with nELAV proteins resulting in their phosphorylation at threonine residues, redistribution to the neuronal cytoskeletal and membrane fractions, and stabilization of *GAP-43* mRNA. In addition, a recent study demonstrated that PKCε also interacts with HuD, and activation of both PKC isoforms by bryostatin-1 results in HuD phosphorylation on nine residues (Lim and Alkon 2012). Importantly, HuD is a focal downstream target of this neurite-extending pathway since nerve growth factor (NGF) or phorbol ester treatment of PC12 cells following HuD knockdown prevents neurite outgrowth (Mobarak et al. 2000).

Another pathway that has been documented to regulate HuD function involves the methyltransferase CARM1 (also known as protein arginine methyltransferase 4 [PRMT4]), a member of the PRMT family that performs a variety of cellular functions such as regulating transcription and RNA processing by methylating arginine residues in substrate proteins (Bedford and Clarke 2009). Using a variety of complementary techniques, including an in vitro protein methylation assay, CARM1 was demonstrated to interact with and methylate an amino acid located in the hinge region of rat (Arg^{236}) (Fujiwara et al. 2006) and mouse (Arg^{248}) (Hubers et al. 2010) HuD (Fig. 1C). Moreover, methylation of HuD decreased *p21^cip1/waf1^* mRNA stability and maintained neuronal precursor cells in a proliferative state. On the other hand, inhibition of HuD methylation or knockdown of CARM1 resulted in increased $p21^{cip1/waf1}$ mRNA levels, cell-cycle withdrawal, and neuronal differentiation. Surprisingly, although reduced CARM1 levels also increased *GAP-43* mRNA expression, they had no effect on other known HuD-targeted mRNAs, including *Tau* and *p27*, suggesting that stabilization of these other target mRNAs requires additional post-translational modifications of HuD and/or other *trans*-acting factors (Fujiwara et al. 2006; Hubers et al. 2010). These findings provide evidence that methylation of HuD by CARM1 may be a universal switch in neuronal precursors that regulates the transition from proliferation to neuronal differentiation.

A recent report found a direct link between the antagonistic effects of PKC and CARM1 on HuD function

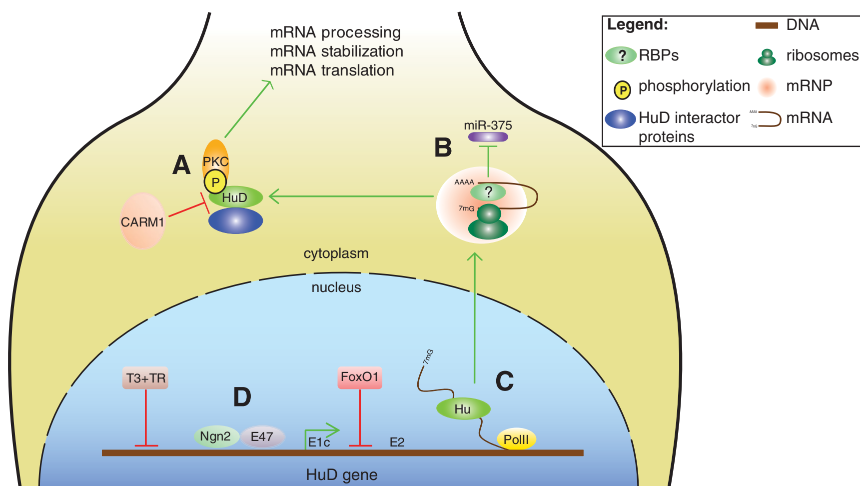

FIGURE 4. Simplified model of the molecular events governing HuD expression, processing, and function in neurons. (*A*) CARM1 methylation of HuD prevents its binding to specific mRNA targets and maintains neuronal precursors in an undifferentiated state. On the other hand, an increase in unmethylated HuD protein levels, its post-translational modification (such as phosphorylation by PKC isoforms), and interaction with other proteins (e.g., AKT1) enable HuD to regulate mRNA metabolism of proneural genes and promote neuronal differentiation/neurite extension. (*B*) The stability and translation of mature *HuD* mRNA is negatively controlled by miR-375 and potentially other *trans*-acting factors. (*C*) *HuD* pre-mRNA undergoes alternative splicing, including inclusion of E6 mediated by Hu proteins. (*D*) During neuronal development transcription of the *HuD* gene is positively regulated by the Ngn2-E47 heterodimer binding to E-boxes upstream of E1c. Synthesis of *HuD* mRNA is negatively regulated by thyroid hormone (presumably bound to thyroid hormone receptor) in neurons and FoxO1 in pancreatic β cells. Green and red lines represent pathways that promote and inhibit neuronal differentiation, respectively.

and cell fate (Lim and Alkon 2012). In hippocampal neurons, the study demonstrated using pathway-specific inhibitors that activated PKC isoforms interact and phosphorylate CARM1, which leads to decreased CARM1 methyltransferase activity. Furthermore, activating the classical PKC isoforms and simultaneously blocking CARM1 activity potentiates HuD binding to target mRNAs and hippocampal neurite extension. This study clearly illustrates that PKC activation negatively controls CARM1-mediated methylation of HuD to promote neuronal differentiation.

The phosphatidylinositol 3-kinase (PI3K)/AKT1 pathway was also found to regulate HuD function, specifically at the translational level (Fujiwara et al. 2011a). This finding is in agreement with PI3K/AKT1 being one of the prominent signalling pathways regulating translation (Roux and Topisirovic 2012). In the study by Fujiwara et al., HuD mutants were used to demonstrate direct binding of active (phosphorylated) AKT1 to a sequence in the linker region of HuD and that this interaction is required for HuD-dependent neurite outgrowth (Fujiwara et al. 2011a). In addition to binding HuD, chronic (7-day) activation of AKT1 enhances HuD protein levels, suggesting that AKT1 also positively controls HuD expression (Tiruchinapalli et al. 2008a). These results indicate that AKT1 increases the abundance of HuD protein and interacts with the HuD-mRNA complex to promote neurite elongation, possibly through regulation of HuD-mediated translation. Based on these findings, an emerging model suggests that, through post-translational control of HuD expression and function, activation of different signalling pathways can maintain neuronal precursor/stem cells in a proliferative state (CARM1) or promote neuronal differentiation (PKC and AKT1) (Fig. 4A).

Post-transcriptional control of HuD expression

Recent studies have started to decipher the molecular mechanisms controlling *HuD* mRNA (Fig. 4B) and pre-mRNA (Fig. 4C) expression. The mammalian *HuD* mRNA contains at least three alternate 5′ UTRs and a long 3′ UTR (∼2.6 kb), characteristics that are a strong indication of predisposition to post-transcriptional regulation. As mentioned above, previous studies, along with data from our laboratory, revealed eight HuD E1 variants, indicating the existence of at least eight different 5′ UTRs (Fig. 1A,B), with most containing an in-frame translation start site (Abe et al. 1994; Inman et al. 1998; Bronicki et al. 2012). RT-PCR analysis revealed that all eight exons are expressed in embryonic and adult mouse brain and are induced during neuronal differentiation. Moreover, our data suggest that E1c is the predominantly expressed E1 variant (Bronicki et al. 2012). Although the function of E1c and the rest of the E1 variants is unknown, it is tempting to speculate that they encode HuD 5′ UTRs and/or N-terminal peptides that have important functions such as in translational control or intracellular localization (Davuluri et al. 2008).

Analysis of the *Xenopus HuD* homolog *elrD* sequence illustrated that the 3′ UTR contains an ∼100-nt stretch of nucleotides with 90% identity to the human *HuD* 3′ UTR, implying that this fragment encompasses an important evolutionarily conserved regulatory structure and/or *cis*-element(s) (Good 1995). Indeed, analysis of HuD-targeted mRNAs in the mouse forebrain showed that HuD binds to its own transcript, although the role of this interaction remains unknown (Bolognani et al. 2010). One functional consequence of this auto-regulatory binding is presumably in controlling alternative splicing of its own pre-mRNA (see above) (Wang et al. 2010). Another possible significance of this interaction may be similar to the one found for the *Drosophila* ELAV homolog, which was demonstrated to negatively regulate its own transcript (Samson 1998; Borgeson and Samson 2005). This auto-regulatory mechanism is thought to occur when ELAV levels pass a certain threshold, resulting in increased ELAV binding to an AU-rich site in a noncoding 3′-terminal exon and production of a noncoding transcript (Borgeson and Samson 2005). Given the multifunctional roles of HuD on mRNA metabolism and the numerous putative ARE signatures in its 3′ UTR (L Bronicki and BJ Jasmin, unpubl.), it is conceivable that HuD also controls other regulatory events such as polyadenylation, stability, localization, and translation of its own mRNA.

Further support of the notion that *trans*-acting factors regulate HuD mRNA metabolism surfaced when miR-375 was demonstrated to target the 3′ UTR of HuD and decrease its abundance by reducing both its mRNA stability and translation (Abdelmohsen et al. 2010). Knockdown of miR-375 in cultured cells and mouse brain revealed that this microRNA prevents neurite outgrowth through negative control of HuD expression. Altogether, these studies stress that HuD transcripts are likely subjected to intricate post-transcriptional control involving RBPs and miRs.

Transcriptional control of HuD expression

Despite a few studies demonstrating direct or indirect regulation of HuD at post-translational, translational, and post-transcriptional levels, little was known regarding the molecular mechanisms that control transcription of this gene in neurons. As discussed above, a previous study suggested that at least three HuD E1 variants exist, pointing to the potential presence of multiple promoters (Inman et al. 1998). Work in our laboratory has shown that the mammalian HuD gene actually contains eight E1 variants that are well conserved among higher vertebrates, adding to the speculation that multiple promoters control HuD expression (Bronicki et al. 2012). In line with this possibility, the 5′ genomic region of *elrD* was demonstrated to contain two distinct promoters that drive neuron-specific expression of two E1 variants, E1 and E′1 (Nassar and Wegnez 2001). These variants encode transcripts exhibiting unique temporal expression during *Xenopus* nervous system development (Nassar 2011). Remarkably, E1 and E′1 partially align

with mouse E1b and E1c, respectively (L Bronicki and BJ Jasmin, unpubl.), implying that the developmental regulation and function of these two mammalian exons is evolutionarily conserved.

In addition to shedding light on the 5′ genomic region of HuD, some studies, including from our laboratory, have contributed to the understanding of the *trans*-acting factors and *cis*-acting elements that control transcription of *HuD*. Prior to our study, only two transcriptional mechanisms were known to regulate expression of the *HuD* gene—thyroid hormone (T3; presumably via activation of the thyroid hormone receptor) and TF forkhead box O1 (FoxO1)—and both of these factors were shown to negatively impact the abundance of HuD. T3 was demonstrated to decrease *HuD* mRNA synthesis in neuronal N2a cells, and T3 levels were inversely correlated with *HuD* mRNA levels in the rat brain (Cuadrado et al. 2003), whereas FoxO1 was recently found to negatively regulate transcription of *HuD* under low glucose conditions in pancreatic β cells (Lee et al. 2012). These findings raise the possibility that one or both of these transcriptional events maintain low/inhibit HuD expression in nonneuronal and neuronal precursor/stem cells.

Research in our laboratory has revealed that the induction of HuD during early neuronal differentiation of P19 cells, a multipotent embryonal carcinoma cell line, is under neuron-specific transcriptional control (Bronicki et al. 2012). Additionally, we demonstrated that a highly conserved 400-bp region upstream of the predominantly expressed E1 variant, E1c, houses several E-boxes, two of which are targeted by the basic helix-loop-helix transcription factor Neurogenin 2 (Ngn2). Importantly, using a combination of promoter-reporter assays, chromatin IPs, and electrophoretic mobility shift assays, we showed that Ngn2 binds these two E-boxes to promote expression of HuD only in cells differentiating into neurons. Given the established function of Ngn2 in neurogenesis (Guillemot 2007) and its emerging roles in learning and memory (Galichet et al. 2008) and neuronal diseases (Rostomily et al. 1997; Andersson et al. 2006; Kele et al. 2006; Uhrig et al. 2009), our findings suggest that Ngn2-dependent control of HuD transcription is central to neuronal development, function, and dysfunction (Fig. 4D).

FUNCTIONAL ROLES OF HuD IN THE NERVOUS SYSTEM

The multiple post-transcriptional effects exerted by HuD on mRNAs whose proteins have key roles in neurons (Table 2) and its expansive spatiotemporal expression within the nervous system strongly suggest that HuD acts as a "master regulator" of various neuronal processes (Deschenes-Furry et al. 2006; Perrone-Bizzozero and Bird 2013). Although the most-established role of HuD is in neurogenesis, studies are surfacing depicting its importance in neuronal function and survival, as well as plasticity during learning and memory and following neuronal injury. Additionally, there is accumulating support implicating misregulation and mutation of HuD in neuronal pathologies and neuroendocrine cancers, further stressing the significance of this protein in the nervous system.

Neuronal development

Multiple studies on primary and cultured neuronal cells have demonstrated that HuD controls almost all stages of a neuron's existence, including neuronal commitment, differentiation, and activity (Deschenes-Furry et al. 2006; Pascale and Govoni 2012). For example, neurosphere assays using neuronal stem/progenitor cells from the cerebral cortex of HuD knockout (−/−) mice revealed that HuD decreases self-renewal capacity and promotes cell cycle exit and differentiation of these cells. Adult HuD−/− mice were also found to contain an increased number of self-renewing cells in the subventricular zone (SVZ), illustrating in vivo the importance of HuD for inhibition of neuronal stem/progenitor cell self-renewal (Akamatsu et al. 2005). The ability of HuD to control these early differentiation processes requires post-transcriptional regulation of specific target transcripts such as mRNAs encoding cyclin-dependent kinase $p21^{cip1/waf1}$ (Fujiwara et al. 2006). Moreover, HuD-dependent neurogenesis requires commitment of cells to the neuronal lineage since ectopic expression of HuD in nonneuronal cells does not result in neuronal differentiation (Wakamatsu and Weston 1997).

Studies in which HuD levels were down-regulated or overexpressed in cultured cells have revealed an essential role for HuD in terminal differentiation, namely dendritic and axonal outgrowth (Wakamatsu and Weston 1997; Aranda-Abreu et al. 1999; Mobarak et al. 2000; Anderson et al. 2001; Hubers et al. 2010). In support of these findings, transgenic mice ectopically expressing HuD (HuD-Tg) in forebrain neurons under the control of the CAMKIIα promoter (Bolognani et al. 2007b) were shown to contain increased mossy fiber length (Perrone-Bizzozero et al. 2011). Conversely, detailed analysis of HuD−/− mice revealed a transient impairment in neurite extension of several cranial nerves around day 10.5 of embryonic development (Akamatsu et al. 2005). These studies provide a clear demonstration that HuD positively regulates neurite outgrowth. This function of HuD requires its shuttling to the cytoplasm and involves stabilization of various target mRNAs whose protein products promote microtubule assembly and growth cone development including GAP-43, Tau, and AChE (Chung et al. 1997; Aranda-Abreu et al. 1999; Kasashima et al. 1999; Deschenes-Furry et al. 2003).

Neuronal plasticity

Increasing evidence suggests that HuD also plays a role in different types of neuronal plasticity. In adult *HuD*−/− mice, an abnormal clasping reflex and poor performance on the rotarod test were observed, suggesting a sensory/motor defect

in these animals and a role for HuD at the axon terminal (Akamatsu et al. 2005). Additionally, HuD has been implicated in axonal regeneration in two models of nerve injury. A study in our laboratory demonstrated that, between 1 and 4 d following axotomy of superior cervical ganglion (SCG) neurons, HuD protein and mRNA levels, its interaction with *AChE* mRNA, and *AChE* transcript levels decreased (Deschenes-Furry et al. 2007). Importantly, the reduction in *AChE* mRNA abundance could be rescued by localized overexpression of HuD delivered via a viral vector. Although HuD expression was shown to decrease immediately following nerve injury, another study performed nerve crush of dorsal root ganglion (DRG) neurons which resulted in an increase of HuD protein and *GAP-43* mRNA expression within 7 d, lasting up to 21 d post-injury (Anderson et al. 2003). These two studies provide a strong indication that the eventual up-regulation of endogenous HuD levels post-nerve injury may be involved in axonal recovery following nerve damage. Thus increases in HuD levels after nerve injury may facilitate regeneration.

In parallel with this potential role, studies have demonstrated that HuD protein levels, as well as a cohort of its target mRNAs such as *GAP-43*, *CAMKII*α and *Homer*, increase in several brain regions including the dentate gyrus (DG) following neurotoxin-induced seizure (Bolognani et al. 2007a; Tiruchinapalli et al. 2008a; Winden et al. 2011). Moreover, glutamate receptor activation following cocaine treatment was shown to increase HuD protein and target mRNA abundance (e.g., *GAP-43*) and promote HuD localization to dendritic spines (Tiruchinapalli et al. 2008a). Along with the finding that several HuD-targeted mRNAs encode synaptic proteins (Bolognani et al. 2010), these studies indicate that HuD is important for various types of neuronal plasticity, potentially by directly acting at the synapse. However, further studies are necessary to elucidate the precise role of HuD in these forms of neuronal plasticity.

The function of HuD in neurogenesis and neuronal plasticity raised the possibility that it is also involved in hippocampus-dependent learning and memory. The first studies to link HuD to learning and memory demonstrated that expression of nELAV proteins and *GAP-43* mRNA increased in the cytoskeletal and membrane fractions of murine hippocampal neurons following spatial discrimination tasks (Quattrone et al. 2001; Pascale et al. 2004). Closer inspection of HuD expression revealed increased HuD protein levels in somatic and dendritic compartments of hilar and CA3 hippocampal neurons following a different learning paradigm, contextual fear conditioning (Bolognani et al. 2004). To further study the role of HuD in learning and memory, HuD-Tg mice were employed since these mice express HuD throughout the forebrain, including the hippocampus and cerebral cortex (Bolognani et al. 2006). Detailed analysis of these mice showed enhanced sprouting of GAP-43-containing mossy fibers to the CA3 region, ectopic expression of GAP-43 protein in the DG, and increased GAP-43 and calmodulin binding, possibly causing altered calcium signalling (Tanner et al. 2008). Furthermore, as mentioned above, microarray analysis of HuD-targeted mRNAs in DGCs demonstrated that HuD-Tg mice contained increased expression of transcripts whose products regulate neuronal development and axogenesis (Perrone-Bizzozero et al. 2011). Although these mice displayed physiological and molecular alterations including ectopic HuD protein expression throughout the hippocampus, they exhibited aberrant acquisition and retention of memories possibly due to the lack of strict temporal and spatial regulation of HuD protein (Bolognani et al. 2007b). Based on these findings, it was concluded that the precise spatiotemporal control of HuD levels is necessary for proper learning and memory.

Neurological disorders

Given its multiple important roles in neurons, it is not surprising that misregulation or misexpression of HuD is implicated in several neural pathologies and tumors (Pascale and Govoni 2012; Perrone-Bizzozero and Bird 2013). In accordance with its direct or indirect function in learning and memory, nELAVs have recently been linked to Alzheimer's disease (AD), a condition characterized by neuronal death, memory loss and eventual dementia. One hypothesis for the pathogenesis of AD revolves around cleavage of the amyloid precursor proteins (APPs) into β-amyloid (Aβ) fragments, a process that is promoted and prevented by β and α-secretase enzymes, respectively. Under nondisease conditions, α-secretases such as A disintegrin and metalloproteinase 10 (ADAM10) cleave APP to generate a growth promoting and neuroprotective fragment, soluble APPα, whereas in AD, several small Aβ fragments are produced by β and γ secretases (Claeysen et al. 2012). One of these Aβ fragments, the self-aggregating $A\beta_{1-42}$, is increasingly generated and accumulates in the cerebral plaques of AD patients (Jarrett et al. 1993; Gandy 2005). In the hippocampus of AD patients, nELAV protein abundance was found to decrease in parallel with clinical dementia progression, and their expression was inversely correlated with Aβ peptide levels both in vitro and in vivo (Amadio et al. 2009). The authors also determined that the interaction between nELAV proteins and *ADAM10* mRNA was disrupted following exposure to $A\beta_{1-42}$ leading to reduced ADAM10 protein levels (Amadio et al. 2009). These studies suggest that the decrease in nELAV protein levels leads to reduced ADAM10 expression which may be an important event in the development of AD.

In addition to its potential role in AD, three separate cohort genetic studies have linked single nucleotide polymorphisms (SNPs) within the *HuD* locus to the development of Parkinson's disease (PD), a degenerative disorder of the CNS. The first of these studies genotyped nine SNPs in the *HuD* gene locus from a Caucasian population and found that two of them, one in the first intron (rs967582) and one in the coding region (rs2494876), were associated

with age at onset (AAO) of PD (Noureddine et al. 2005). A second study analyzed five SNPs in the HuD locus from Norwegian, United States, and Irish PD and control samples and found that two SNPs (rs967582 and rs3902720) are associated with AAO of PD but only in Irish patient samples, possibly due to a Celtic founder effect (Haugarvoll et al. 2007). Last, a subsequent study employed samples from a Caucasian population to confirm that the rs967582 SNP is associated with increased risk of PD (DeStefano et al. 2008).

Also of particular relevance is the possible role of HuD in spinal muscular atrophy (SMA), a disease that results in degeneration of spinal cord motor neurons due to deletion or loss-of-function mutations in the survival of motor neuron 1 (SMN1) gene, resulting in inadequate expression of functional SMN protein (Burghes and Beattie 2009). A series of cell imaging and biochemical experiments in three independent studies demonstrated that HuD interacts with SMN protein in axonal compartments of neurons (Hubers et al. 2010; Akten et al. 2011; Fallini et al. 2011). Importantly, overexpression of HuD or of one of its target mRNAs (*cpg-15*) was found to partially rescue SMA-like defects related to cell morphology and recruitment of specific mRNAs into RNA granules (Hubers et al. 2010; Akten et al. 2011).

In line with its potential role in SMA, HuD has recently been associated with amyotropic lateral sclerosis (ALS), a neurodegenerative disorder resulting in progressive and fatal deterioration of upper and lower motor neurons (Pratt et al. 2012). Degenerating motor neurons in ALS patients commonly display cytoplasmic aggregates containing the DNA/RNA-binding protein TDP-43 (Neumann et al. 2006). Biochemical and cell imaging techniques were used to demonstrate that TDP-43 associates with HuD and that TDP-43-containing cytoplasmic aggregates sequester HuD, suggesting that misregulation of mRNA metabolism in the cytoplasm of motor neurons could be involved in the development of ALS (Fallini et al. 2012a).

HuD has also been linked to schizophrenia, a psychiatric disorder in which patients often experience hallucinations, dementia, and impaired cognition. One study employed a DNA microarray on samples from patients with chronic schizophrenia and determined that HuD transcript levels were increased in the dorsolateral prefrontal cortex (Hakak et al. 2001). The authors also found that there was augmented abundance of several mRNAs including those encoding proteins involved in neuronal development, synaptic plasticity, and neurotransmission. Interestingly, some of these mRNAs, such as *GAP-43*, are targets of HuD, indicating that increased levels of HuD in brains of schizophrenic patients may lead to altered post-transcriptional control and, consequently, an abundance of various HuD-targeted mRNA products (Perrone-Bizzozero and Bird 2013).

Converging studies have also implicated HuD in epilepsy, a neurological syndrome hallmarked by seizures. Using kainic acid to induce seizures in adult male rats, one study found that this treatment produced increased *HuD* and *GAP-43* mRNA levels in the DG (Bolognani et al. 2007a). Another report used immunocytochemistry and Western blot techniques to demonstrate increased dendritic localization of HuD in hippocampal neurons following pilocarpine-induced seizure (Tiruchinapalli et al. 2008a). Furthermore, this study illustrated via RNA IP and quantitative RT-PCR that several HuD-targeted mRNAs such as *GAP-43* and *Neuritin* also increased after seizures. A third study assessed gene expression using microarray platforms to show that *HuD* and several of its target mRNAs increased following kainic acid-induced seizures in rats (Winden et al. 2011). Based on these studies, it is tempting to speculate that the increase in HuD and its target mRNAs following seizure is a protective mechanism to minimize neurodegeneration.

Evidence is also mounting that HuD plays a role in certain types of cancers. Aberrant expression or function of HuD has been detected in several cancers of the central and peripheral nervous system, most prominently NBs and SCLCs (Szabo et al. 1991; Dalmau et al. 1992; Chagnovich and Cohn 1996; Ball and King 1997; Schramm et al. 1999; Stawski et al. 2012). For instance, in ∼40% of NB tumors, there is a partial or complete deletion of the short arm (p) in one chromosome 1 homolog, which encompasses the *HuD* gene locus (Muresu et al. 1994; Maris et al. 1995). In these cells, haploinsufficiency of HuD is thought to promote amplification of the *N-myc* gene and result in poor patient outcome (Grandinetti et al. 2006). Conversely, high levels of HuD and other nELAVs in NBs have been associated with a clinically favorable prognosis (Ball and King 1997). In line with this, a recent study screened various neuroendocrine lung tumors and found 11 mutations of unidentified consequence(s) and two mutations that prevent full-length protein expression in the *HuD* gene (D'Alessandro et al. 2010). Together, these findings imply that deletions or mutations of the *HuD* gene are associated with the etiology or progression of certain neuroendocrine cancers.

CONCLUSION AND PERSPECTIVE

The Hu member HuD is one of the earliest markers of the neuronal phenotype and is broadly expressed in the mature nervous system. HuD targets specific mRNAs to regulate multiple levels of their metabolism, including nuclear processing, mRNA stability, and translation. Through post-transcriptional regulation of various neuronal mRNAs, HuD has key roles in neurons, among which neuronal differentiation is the best described. In addition to this function, increasing evidence implicates HuD in neuronal plasticity, nerve injury, learning and memory, and multiple neuronal diseases. In light of the important roles attributed to HuD in developing and mature neurons, a few studies have started to elucidate the cellular and molecular events governing its expression and function. An emerging picture reveals elaborate regulation of HuD expression, notably at the transcriptional and post-transcriptional levels, since the *HuD* gene may contain

multiple promoters and harbors eight exon 1 variants and an extensive 3′ UTR. Accordingly, it is tempting to speculate that the alternative promoters and encoded 5′ UTRs (and possibly N termini) are essential for proper spatiotemporal control of HuD expression and function in neurons. For instance, aberrant abundance of the predominant HuD exon 1 variant, E1c, may occur in pathologies such as AD, leading to improper localization of HuD or post-transcriptional regulation of certain HuD-targeted mRNAs. Despite the recent progress, there is still a lack of knowledge of the molecular mechanisms presiding over the "master regulator" HuD, and thus, it becomes evident that a complete elucidation of these events is critical for improving our fundamental understanding of neuronal development, function, and diseases. Furthermore and equally important, a better comprehension of these events could ultimately lead to the development of therapeutic approaches for the treatment of neuronal injuries and disorders.

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