CHARACTERIZATION OF MULTIPLE EXON 1 VARIANTS AND NEURON-SPECIFIC TRANSCRIPTIONAL CONTROL OF MAMMALIAN *HUD*

by

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ABSTRACT

The RNA-binding protein (RBP) and Hu/ELAV family member HuD regulates mRNA metabolism of genes that encode proteins involved in neuronal differentiation, learning and memory, and certain neurological diseases. Given the important functions of HuD in a variety of processes, we set out to characterize the 5' genomic region of the mammalian *HuD* gene and determine the mechanisms that regulate its mRNA expression in neurons using P19 cells and mouse brain as models.

Bioinformatic and 5'RACE (rapid amplification of cDNA ends) analyses of the *HuD* 5' genomic flanking region identified eight conserved leader exons (E1s), two of which are novel. Expression of all E1 variants was established in differentiating P19 cells, mouse embryonic (E14.5) and adult brains. Through several complementary approaches, we determined that the abundance of *HuD* mRNA is predominantly under transcriptional control in differentiating neurons. Sequential deletion of the 5' regulatory region upstream of the predominantly expressed E1c variant revealed a well-conserved 400 bp DNA region that contains five E-boxes and is capable of directing expression of HuD specifically in neurons. Using electrophoretic mobility shift assays (EMSAs), chromatin immunoprecipitations (ChIPs), and E1c 5' regulatory region (RR) deletion and mutation analysis, we found that two of these E-

boxes are targeted by neurogenin 2 (NGN2/NEUROG2) and that this mechanism is important for induction of HuD mRNA in neurons. Additional deletion and mutation of the E1c 5' RR revealed that putative *cis*-acting elements for Kruppel-like factors (KLFs) and nuclear DEAF-1-related (NuDR) transcription factors also positively regulate transcription of HuD.

Together, our findings reveal that the intricate transcriptional regulation of mammalian HuD involves eight leader exons and potentially alternate promoters. We further demonstrate that transcription of HuD requires neuron-specific control by NGN2 and possibly KLF and NuDR transcription factors. To our knowledge, this is the first study to identify transcriptional events that positively regulate expression of HuD.

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GLOSSARY

3'/5'UTR	3'/5' untranslated region
5'RACE	5' rapid amplification of cDNA ends
AChE	acetylcholinesterase
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
ARE	AU-rich element
AUBP	AU-binding protein
AUF1	AU-rich element RNA-binding protein 1
BDNF	brain derived neurotrophic factor
bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
BRF1	butyrate-regulated factor 1
C/EBP	CCAAT/enhancer binding protein
CA1-3	cornu ammonis 1-3
CamKII	Ca^{2+}/calmodulin-dependent protein kinases II
CARM1	coactivator-associated arginine methyltransferase 1
CBC	cap-binding complex
cDNA	complimentary DNA
ChIP	chromatin immunoprecipitation
CGRP	calcitonin gene related peptide
CLIP	crosslinking and immunoprecipitation
CNS	central nervous system
CP	cortical plate
CPSF	cleavage and polyadenylation specificity factor
CRM1	chromosome region maintenance 1
Cstf	cleavage stimulation factor
CUG-BP	CUG-binding protein 1
DEPC	Diethylpyrocarbonate
DG	dentate gyrus
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DRG	dorsal root ganglia
EGF	epidermal growth factor
eIF4	eukaryotic initiation factor 4
ELAV	embryonic lethal abnormal vision
EMSA	electrophoretic mobility shift assay
ESC	embryonic stem cell
EST	expressed sequence tag
FRET	foster resonance energy transfer

FXS	Fragile X syndrome
FXTAS	Fragile X-associated tremor/ataxia syndrome
GAP-43	growth associated protein-43
HDAC	histone deacetylase
IE gene	immediate early gene
IP	immunoprecipitation
IRES	internal ribosome entry site
ISH	*in situ* hybridization
ITAF	IRES *trans*-acting factor
IZ	intermediate zone
KLF	krueppel-like factor
KSRP	K-homology splicing regulator protein
LMO4	LIM domain transcription factor
lncRNA	long non-coding ribonucleic acid
MARCKS	myristoylated alanine-rich C kinase substrate
MAP1B	microtubule associated protein 1B
miR	micro-RNA
mRNA	messenger ribonucleic acid
mRNP	messenger ribonucleoprotein
MyT1	myelin transcription factor 1
Msi	musashi
ncRNA	non-coding ribonucleic acid
NES	nuclear export signal
NFX1	nuclear transcription factor, X box-binding protein 1
NGF	nerve growth factor
Ngn	neurogenin
NLS	nuclear localization signal
NOVA	neuro-oncological ventral antigen
NSC	neural stem cell
NuDR	nuclear deformed epidermal autoregulatory factor
OB	olfactory bulb
ORF	open reading frame
PABP	poly(A)-binding protein
PAIP2	polyadenylate-binding protein-interacting protein 2
PAP	poly(A) polymerase
PARN	poly(A)-specific ribonuclease
PD	Parkinson’s disease
PEM	paraneoplastic encephalomyelitis
PKC	protein kinase c
PNS	peripheral nervous system
RA	retinoic acid
RBP	RNA-binding protein
REMSA	RNA electrophoretic mobility shift assay
RIP	RNA immunoprecipitation
5’RR	5’ regulatory region
RNAPII	RNA polymerase II
RRM	RNA recognition motif

Rta	retina
SCG	superior cervical ganglia
SCLC	small cell lung cancer
SGZ	subgranular zone
SMA	spinal muscular atrophy
SMN	survival of motor neuron
SN	sensory neuropathy
SNP	single-nucleotide polymorphism
snRNP	small nuclear ribonucleoprotein
Str	striatum
SVZ	subventricular zone
Sxl	sex-lethal
TF	transcription factor
TIA-1	T-cell intracellular antigen 1
TIAR	TIA-1-related protein
tiRNA	transcription initiation RNA
TK	thymidine kinase
TSS	transcription start site
TTP	tristetraprolin
VEGF	vascular endothelial growth factor
VZ	ventricular zone

Chapter 1

1: INTRODUCTION

1.1 Molecular mechanisms controlling mRNA metabolism

In eukaryotic cells, proper spatiotemporal gene expression necessitates intricate control at multiple levels, including transcriptional, post-transcriptional, translational and post-translational. Among these, the post-transcriptional level encompasses the most diverse molecular mechanisms, affecting all aspects of messenger ribonucleic acid (mRNA) metabolism and playing a central role in various cellular and biological processes (Moore, 2005; Lukong et al., 2008; Kishore et al., 2010; Bian and Sun, 2011). Post-transcriptional control of mRNAs largely depends on *cis*-acting elements inherent to mRNAs and *trans*-acting factors, such as RNA-binding proteins (RBPs) and microRNAs (miRs), that bind these sequences (Barreau et al., 2006; Krol et al., 2010; Lee and Gorospe, 2011; Matoulkova et al., 2012). Over the past decade, tremendous technological advancements have aided in unravelling the molecular events governing mRNA expression and function. For instance, complete sequencing of animal genomes and many expressed RNAs revealed that mammals, especially humans, contain numerous other types of non- protein-coding RNAs (ncRNA). These ncRNAs range from tiny transcription initiation RNAs (tiRNAs) to long non-coding RNAs (lncRNAs) and contribute substantially to the complexity of post-transcriptional events controlling mRNA expression (Carninci, 2010; Esteller, 2011). Despite our improved understanding of the instrumental roles RBPs and ncRNAs play in

gene expression, the molecular mechanisms that regulate these factors are not well-defined and merit thorough investigation.

1.2 Post-transcriptional regulation in neurons

In general, post-transcriptional control of gene expression produces increased protein diversity from a fixed number of genes, accurate spatiotemporal regulation of gene expression, subcellular localization of mRNA and quick modification of protein expression. This regulation is achieved through a broad spectrum of processes, ranging from alternative splicing and polyadenylation in the nucleus to localization, stabilization and translation in the cytoplasm (Hollams et al., 2002; Carmody and Wente, 2009; Licatalosi and Darnell, 2010; Wu and Brewer, 2012; Vazquez-Pianzola and Suter, 2012). These mechanisms are especially important in neurons, which often contain complex cellular architecture due to extensive neurite branching.

Moreover, increasing evidence indicates that the *trans*-acting factors that govern these events are essential at all stages of a neuron's life cycle, including neuronal development, maintenance and function (Bramham and Wells, 2007; Swanger and Bassell, 2011; Jung et al., 2012; Qureshi and Mehler, 2012). The importance of these regulatory events is further highlighted by the growing number of diseases associated with mutations of specific mRNAs or dysregulation of RBPs or miRs that associate with them (Lukong et al., 2008; Lau and de Strooper, 2010; Im and Kenny, 2012). Defects affecting normal post-transcriptional control of mRNAs are involved in the pathogenesis of a broad spectrum of neuronal diseases, such as the neurodegenerative disorders fragile

X syndrome (FXS), fragile X tremor ataxia syndrome (FXTAS; Li and Jin, 2012), spinal muscular atrophy (SMA; Fallini et al., 2012), Huntington's disease (Krzyzosiak et al., 2012) and amyotrophic lateral sclerosis (ALS; Fiesel and Kahle, 2011; Strong and Volkening, 2011).

1.3 The mammalian nervous system contains various RNA-binding proteins

RBPs regulate mRNA metabolism by binding to specific sequence motifs, known as *cis*-acting elements or structures (Barreau et al., 2006). Throughout their existence, mRNAs stably or dynamically interact with a myriad of RBPs, resulting in the formation of a heterogeneous mRNA ribonuclear protein (mRNP) complex. mRNP components control mRNA expression and function by coordinating multiple post-transcriptional mechanisms, such as coupling transcription to mRNA processing, localization, stability and translation (Dahan et al., 2011). Regulatory coupling of mRNA metabolism events formed the premise of the RNA operon theory, which states that transcripts encoding proteins with similar functions are often synchronously controlled at multiple post-transcriptional levels by one or more mRNP component(s) (Keene, 2007).

The central and peripheral nervous systems (CNS and PNS) contain a vast array of RBPs that are either neuron-specific or ubiquitously expressed. For example, a genome-wide screen found that 85% of the 380 mouse RBPs examined were expressed in the brain (McKee et al., 2005). Of particular interest are the well-described RBPs that regulate mRNA metabolism and are important for proper cellular development 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) and K homology splicing regulatory protein (KSRP; Briata et al., 2012; Table 1.1). Amongst all of the RBPs expressed in the brain, the best characterized are the neuron-specific Hu/ELAV family members.

1.4 The Hu/ELAV family of RBPs

Hu/ELAV proteins are orthologues of the *Drosophila sex lethal* (*sxl*) and *embryonic lethal abnormal vision* (*elav*) genes that also encode RBPs (Robinow et al., 1988; Bell et al., 1988; Szabo et al., 1991). Evidently, these proteins were given their names based on their close relation to the *Drosophila elav* gene and the name of the patient in which the Hu antibody was identified (Graus et al., 1986; Musunuru and Darnell, 2001). HuD was discovered as an antigen for autoantibodies in patients with the neurologic syndrome paraneoplastic encephalomyelitis and sensory neuropathy (PEM/SN; Szabo et al., 1991). This inflammatory disorder occurs in a small percentage of patients with small cell lung carcinomas (SCLCs) that abnormally express specific Hu/ELAV members. The ectopic expression of these proteins leads to an autoimmune attack on the nervous system (Dalmau et al., 1990; Dalmau et al., 1992). In addition to HuD, three other Hu/ELAV proteins were discovered that are also associated with the disorder; the ubiquitously expressed HuR/ELAVl1, the neuron and gonad-restricted HuB/ELAVl2 and the neuron-specific HuC/ELAVl3 (Hinman and Lou, 2008; Pascale et al., 2008). The three predominantly neuron-restricted

Table 1.1 Neuronal AU-rich element RNA-binding proteins and their effects on mRNA stability

RNA-binding protein	Effect on mRNA stability	References:
HuB/ELAVl2	stabilization	(Jain et al., 1997)
HuC/ELAVl3	stabilization	(Abe et al., 1996)
HuD/ELAVl4	stabilization	(Anderson et al., 2000; Mobarak et al., 2000)
HuR/ELAVl1	stabilization	(Peng et al., 1998; Fan and Steitz, 1998; Levy et al., 1998)
AUF1 (p37, p40, p42 and p45)/hnRNPD	mostly destabilization	(Brewer and Ross, 1989; Brewer, 1991; Zhang et al., 1993)
K homology-type splicing regulatory protein (KSRP)	destabilization	(Chen et al., 2001; Gherzi et al., 2004)
tristetraprolin (TTP)	destabilization	(Carballo et al., 1998)
butyrate-response factor-1 (BRF1)	destabilization	(Stoecklin et al., 2002)
TIA-1-related protein (TIAR)	no effect	(Piecyk et al., 2000)
T-cell intracellular antigen 1 (TIA-1)	no effect	(Piecyk et al., 2000)
TINO	destabilization	(Donnini et al., 2004)
polyadenylate-binding protein-interacting protein 2 **(**PAIP2)	stabilization	(Onesto et al., 2004)
CUG-binding protein 1 (CUG-BP1)	destabilization	(Moraes et al., 2006)
CUG-BP2	stabilization	(Mukhopadhyay et al., 2003)
nucleolin	stabilization	(Sengupta et al., 2004)

Hu/ELAV members (nELAVs; HuB/C/D) share a higher degree of amino acid sequence identity (>80%) with each other than with HuR (72.5-73.6%; Okano and Darnell, 1997; Figure 1.1). Despite their high degree of sequence identity between nELAVs, slight variation of amino acid composition in key regions (see below) and spatiotemporal expression suggest that HuD carries out some unique functions (Okano and Darnell, 1997; Clayton et al., 1998; Hambardzumyan et al., 2009).

1.5 The HuD gene, mRNA and protein

The *HuD* gene is well conserved among higher vertebrates and is located on chromosome 4 in mice and chromosome 1 in humans. The mouse *HuD* gene spans approximately 146kb of DNA and is divided into seven coding exons (E2 to E8) that cover around 44kb of DNA (Sekido et al., 1994; Inman et al., 1998; Figure 1.2A). Based on alignment of available published *HuD* cDNA sequences, the 5' region of *HuD* contains at least 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). Moreover, the *HuD* pre-mRNA is subjected to alternative splicing of micro-exons 6 and 7 resulting in three additional transcript isoforms (*HuD*, HuD_{mex} and HuD_{pro}; Figure 1.2), two of which are predominantly expressed in the postnatal mouse brain (*HuD* and HuD_{pro}; Inman et al., 1998; Steller et al., 1996; Tora et al., 2000). The HuD protein, like all Hu/ELAV members, contains three RNA-Recognition Motifs (RRMs) with a linker region

Figure 1.1 Alignment of mouse Hu/ELAV amino acid sequences

The amino acid sequences of mouse HuD, HuB, HuC and HuR proteins were compared using Basic Local Alignment Search Tool (BLAST). Residues conservation among all, three or two Hu/ELAVs are dented by asterisks, colons or periods, respectively. Amino acid coverage of the three RRMs, predicted NLS and NES are represented by green, orange and blue lines, respectively. The methylatable arginine (R) of HuD is coloured red. Adapted from (Okano and Darnell, 1997).

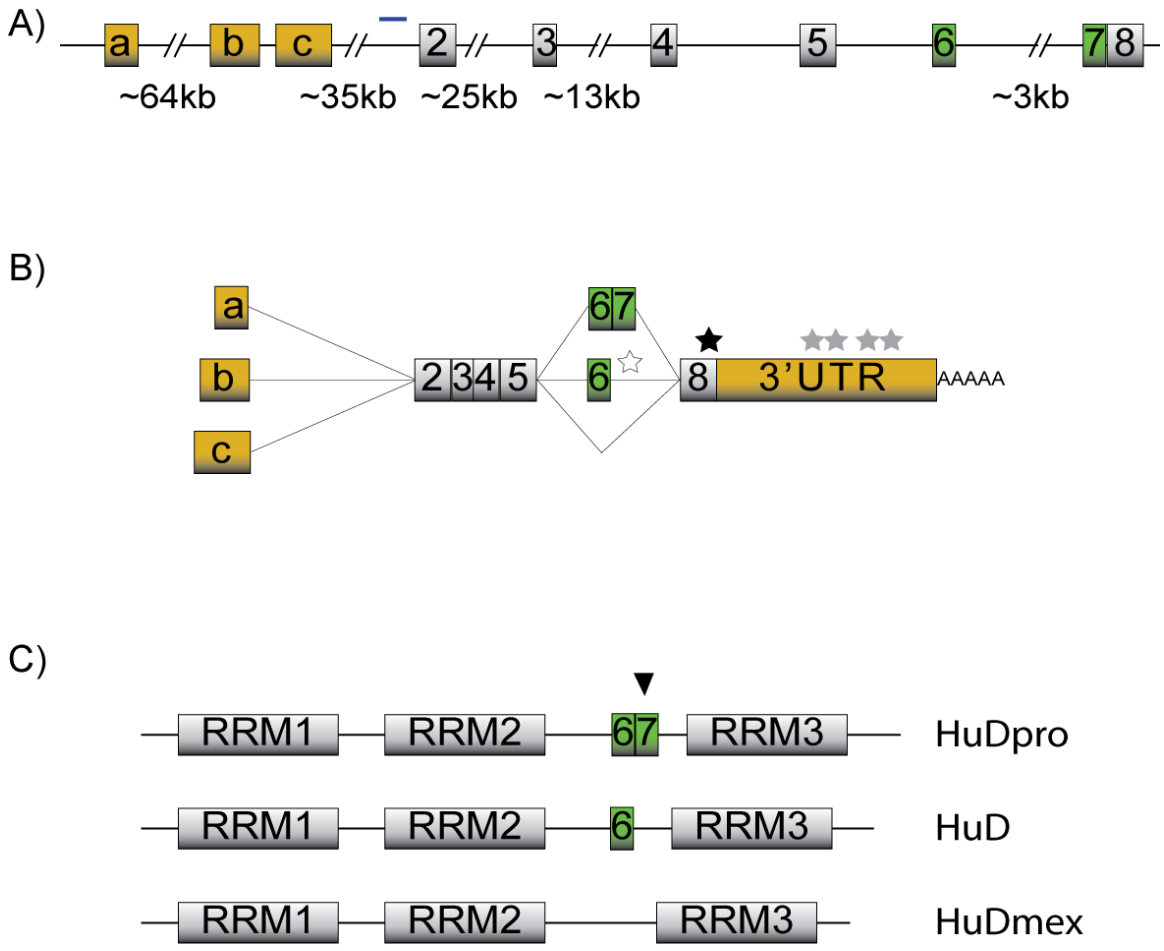


Figure 1.2 Organization of the mouse *HuD* gene, mRNA and protein

A) Mouse *HuD* gene locus with leader exons in orange (a, b and c), coding exons in grey and green (2-8) and introns in black are depicted. The FoxO1 binding site is represented by a blue rectangle whereas the location of the T3-TR binding site is unknown. **B)** Alternative splicing at the 5' and near the 3' end of *HuD* pre-mRNA produces different HuD isoforms. Non-coding, constitutive and alternatively spliced 3' exons are represented by orange, grey and green boxes, respectively. Also shown is the approximate location of the HuD (white star), miR-375 (black star) and putative ARE (grey stars) binding sites. **C)** The three major HuD protein variants with the RRMs shown in grey and linker region in green. Black triangle identifies the location of the arginine residue that is subjected to methylation by CARM1. Adapted from (Deschenes-Furry et al., 2006).

separating the second and third RRM (Okano and Darnell, 1997; Liu et al., 1995). The three RRMs are encoded by E2 and E3 (RRM1), E4 and E5 (RRM2) and E8 (RRM3), whereas the alternatively spliced E6 and E7 encode the linker region (Inman et al., 1998). The linker region contains multiple sites for post-translational modification and houses the nuclear export (NES) and putative nuclear localization (NLS) signals (Kasashima et al., 1999; Figures 1.1 and 1.2).

1.6 Temporal and spatial expression of HuD

Converging findings indicate that HuD is an early marker of the neuronal phenotype (Hambardzumyan et al., 2009; Wakamatsu and Weston, 1997). Quantitative RT-PCR assays have shown that *HuD* mRNA levels are detected by day 10 of murine embryonic brain development, peak at E16 and slowly decline until birth (Hambardzumyan et al., 2009; Abdelmohsen et al., 2010). This pattern of mRNA expression is similar to that of the other two neuronal *nELAVs*; however, *HuD* mRNA levels appear to be more prominent at E10 compared to *HuB* and *HuC* (Hambardzumyan et al., 2009), which strongly supports the notion that HuD is not completely functionally redundant.

In situ hybridization analysis of *nELAV* expression in the developing mouse brain has revealed that spatial expression of *HuD* mRNA is also partially unique compared to *HuB* and *HuC* (Okano and Darnell, 1997; Clayton et al., 1998). In the developing cerebral cortex, *HuD* mRNA is localized to proliferating neuronal progenitors in the ventricular zone, neuroblasts migrating in the intermediate zones and terminally differentiating neurons in the cortical plate.

HuD transcripts are also present in several sub-regions of the hippocampal formation, olfactory bulb, retina and spinal cord, such as sensory and motor neurons, during development. In the adult rodent, expression of *HuD* mRNA is mostly restricted to specific neuronal populations, including large pyramidal-like neurons in layer V of the neocortex, the four CA regions of the hippocampus, Purkinje cells in the cerebellum, 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; Wakamatsu and Weston, 1997; D'Autreaux et al., 2011).

At the sub-cellular level, immunocytochemistry detected minor presence of HuD protein in the nucleus and predominant expression in the cytoplasm (Kasashima et al., 1999). In developing neurons HuD is found in growth cones of extending neurites, while in mature neurons it is detected in axons and dendrites, including at the pre- and post-neurite terminals, respectively (Aranda-Abreu et al., 1999; Aronov et al., 2002; Tiruchinapalli et al., 2008a). Closer inspection of intraneuronal compartments revealed that HuD is localized to granules, along with other proteins, such as survival of motoneuron (SMN), within the cytoplasm and neurites (Smith et al., 2004; Hubers et al., 2010; Fallini et al., 2011; Figure 1.3).

In addition to being expressed in various neuronal subpopulations in the CNS and PNS, quantitative PCR and Western blot assays detected relatively minor HuD mRNA and protein levels in several non-neuronal tissues,

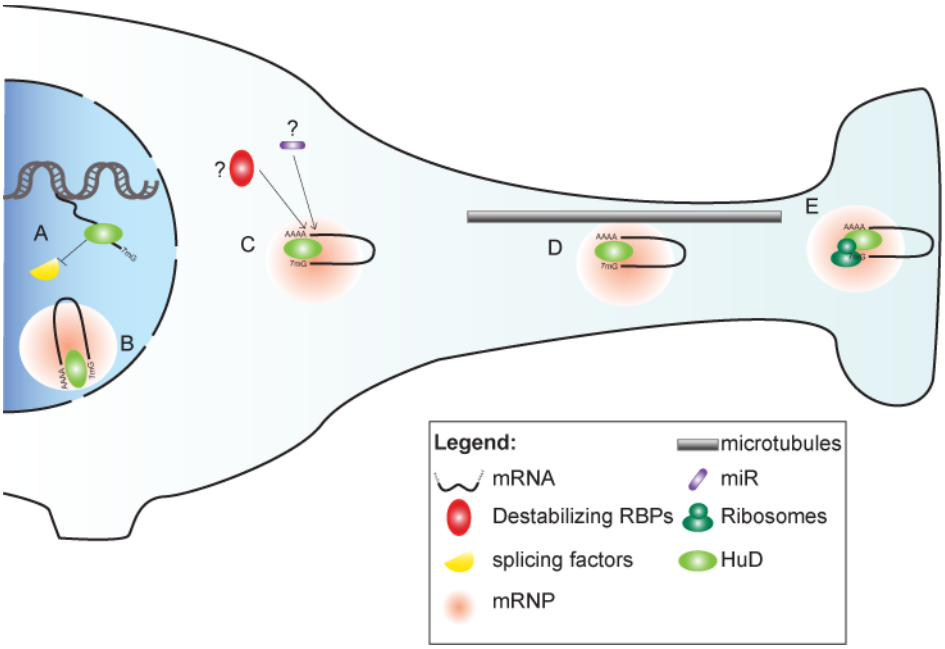


Figure 1.3 Model illustrating the multiple post-transcriptional functions performed by HuD

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 competes or binds cooperatively with destabilizing RBPs onto mRNAs. HuD might also prevent binding of miRs to the mRNA. Through these two mechanisms, HuD increases the half-life of mRNAs. **D)** HuD transports mRNAs to different regions of the neuron along microtubules, including neurites. **E)** At the synaptic terminal, HuD either promotes or represses translation of transcripts. Adapted from (Deschenes-Furry et al., 2006; Bronicki and Jasmin, 2012).

including lung, kidney, heart, liver and pancreatic β cells (Abdelmohsen et al., 2010; Lee et al., 2012).

1.7 Structural analysis of the HuD-RNA interaction

HuD is a *trans*-acting factor that recognizes and binds to specific mRNA targets with its three RRMs (Liu et al., 1995; Chung et al., 1996; Park et al., 2000b; Wang and Tanaka Hall, 2001). The RRM is the most abundant (found in up to 1% of human genes) and well-characterized RNA-binding domain (RBD; Clery et al., 2008). Structurally, the RRM is composed of ~90 amino acids that typically form four-stranded β-sheets and two α-helices, resulting in a $\beta_1\alpha_1\beta_2\beta_3\alpha_2\beta_4$ topology (Maris et al., 2005). Interaction between RRMs and single stranded RNA is usually accomplished through the β-sheets, whereas RRM-protein interactions occur through residues in the α-helices. N- and C- terminal extensions, post-translational modification and interactions with other proteins alter the RNA binding specificity of the RRMs (Clery et al., 2008).

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 binds the poly(A)-tail of transcripts and stabilizes the RBP-mRNA complex (Anderson et al., 2000; Chung et al., 1996; Park et al., 2000b; Chung et al., 1997; Ross et al., 1997; Ma et al., 1997; Beckel-Mitchener et al., 2002). This mechanism seems to depend on the length of the poly(A) tail, since *in vitro* binding assays have found that the longer the poly(A) tail, the higher the affinity of Hu/ELAV binding to the target mRNA (Beckel-Mitchener et al., 2002). Another function of the third HuD RRM involves interacting with other proteins, including

multimerization with other Hu/ELAV members (Kasashima et al., 2002). Both the Drosophila ELAV and mammalian Hu/ELAV proteins have also been shown to form multimers in an RNA dependent manner (Kasashima et al., 2002; Gao and Keene, 1996; Fialcowitz-White et al., 2007; Toba and White, 2008).

The most extensively studied HuD targeted *cis*-acting element is the adenosine/uridine (A/U)-rich element (ARE) found in the 3'UTR of ~10% of cellular mRNAs (Halees et al., 2008). AREs usually range from 50-150 nucleotides in size and, as their name suggests, consist of A- and U-rich regions. AREs vary considerably in sequence and for simplification they are categorized into three major classes; class I contain one to three AUUUA pentamers in a U-rich context, class II include overlapping UUAUUUAUU nonamers and class III consist of U-rich sequences without any AUUUA pentamers (Bakheet et al., 2003; Bakheet et al., 2006). Completion of the crystal structure of HuD bound to *c-fos* or tumor necrosis factor α (*TNFα*) mRNAs illustrated 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 X is any nucleotide, U is uridine, C is cytosine and ? denotes uncertainty whether the C is tolerated in this position. This study also showed that the amino acid residues interacting with RNA bases are well conserved amongst all Hu/ELAV members (Wang and Tanaka Hall, 2001). Over the years, through several *in vitro* and *in vivo* techniques, such as REMSA and RNA-IP, respectively, studies have demonstrated that HuD binds to ARE sequences in mRNAs (Liu et al., 1995; Chung et al., 1996; Chung et al., 1997; Deschenes-Furry et al., 2003; Ratti et al., 2008). However, the presence of

an ARE is not always sufficient for HuD binding, indicating that HuD displays preference for specific ARE motifs (Toba et al., 2002).

Interestingly, one study performed a microarray screen of HuD mRNA targets, following immunoprecipitation with myc-HuD and pull-down with GST-HuD, and found that less than half of these targets contain an ARE (Bolognani et al., 2010). Additionally, this study identified three novel consensus motifs that are present in ~80% of HuD-targeted mRNAs expressed in the mouse forebrain. The first motif consisted of C and U-rich stretches, with a prevalence of the former, and was 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 were predominantly found in the 3'UTR and occasionally in the 5'UTR and coding regions of mRNAs. Recent high throughput analysis, namely cross-linking and immunoprecipitation (CLIP), of HuR and nELAV mRNA targets further supported the findings that Hu/ELAVs preferentially bind U-rich sequences interspersed with Gs (Lebedeva et al., 2011; Mukherjee et al., 2011; Uren et al., 2011; Ince-Dunn et al., 2012). These studies also found that Hu/ELAV binding sites were mostly located in 3'UTRs of mRNAs and less often in introns and 5'UTRs, suggesting that subtle sequence differences might distinguish some mRNA targets of HuD from those of its family members. These different types of HuD-targeted *cis*-acting motifs are found in mRNAs encoding proteins with diverse functions in neurons, ranging from proliferation to synapse formation (Bolognani et al., 2010).

1.8 mRNA targets of HuD

The first mRNA target of HuD identified was *c-fos*, a member of the immediate early (IE) gene family of transcription factors (TFs) that play a role in cell proliferation and differentiation (Liu et al., 1995). Using RNA electrophoretic mobility shift assays (REMSAs), the authors showed that HuD binds specifically to an ARE sequence in the *c-fos* 3'UTR. Numerous other ARE-harbouring transcripts, such as *N-myc* and *c-myc*, were subsequently identified as targets of HuD using similar *in vitro* approaches, indicating that HuD binds short-lived mRNAs whose proteins function in cell proliferation (Liu et al., 1995; Ross et al., 1997). In addition to these mRNAs, HuD was demonstrated to bind transcripts that encode proteins with important functions in neuronal differentiation, including neuronal commitment ($p21^{waf}$; 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; Deschenes-Furry et al., 2003), synapse formation (NOVA-1; Ratti et al., 2008; and CPG-15; Nedivi et al., 1998; Javaherian and Cline, 2005) and neuronal survival (NGF, BDNF and NT-2; Lim and Alkon, 2012; Table 1.2). Moreover, a recent study used HITS-CLIP and microarray technologies to reveal that nELAVs regulate components of the glutamate biosynthetic amino acid pathway (Ince-Dunn et al., 2012). Gene ontology analysis of microarray-derived mRNA targets found that HuD also binds several mRNAs whose proteins are involved in regulation of RNA processing, nuclear export, translation, cell-to-cell

Table 1.2 Confirmed HuD mRNA targets in neurons and their prominent functions

Gene	Prominent functions	Reference
growth associated protein 43 (GAP-43)	cytoplasmic adaptor protein neuritogenesis	(Liu et al., 1995; Chung et al., 1997; Tsai et al., 1997)
c-Myc	transcription factor cell proliferation	(Liu et al., 1995)
c-fos	transcription factor cell proliferation	(Liu et al., 1995)
$p21^{waf}$	cyclin-dependent kinase inhibitor differentiation	(Joseph et al., 1998)
tau	microtubule stabilizer neuritogenesis	(Aranda-Abreu et al., 1999)
vascular endothelial growth factor (VEGF)	growth factor vasculogenesis and angiogenesis	(King, 2000)
$p27^{KIP}$	cyclin-dependent kinase inhibitor cell proliferation	(Kullmann et al., 2002)
myristoylated alanine-rich C kinase substrate (MARCKS)	membrane bound enzyme cell motility, secretion and cell cycle	(Wein et al., 2003)
acetylcholinesterase (AChE)	synaptic enzyme hydrolysis of acetylcholine	(Deschenes-Furry et al., 2003)
musashi 1 (Msi1)	RNA-binding protein cell proliferation	(Ratti et al., 2006)
neuro-oncological ventral antigen 1 (NOVA1)	RNA-binding protein survival and maintenance of motoneurons	(Ratti et al., 2008)
Ca^{2+}/calmodulin-dependent protein kinases II (CamKII)	protein kinase neuronal plasticity	(Tiruchinapalli et al., 2008a)
neuritin	membrane bound protein neuritogenesis	(Tiruchinapalli et al., 2008a)
neuroligin	anchoring protein Synaptogenesis	(Tiruchinapalli et al., 2008a)
LIM domain transcription factor (LMO4)	transcription co-factor synaptic plasticity	(Chen et al., 2007a)
nerve growth factor (NGF)	neurotrophin neuronal growth and survival	(Lim and Alkon, 2012)
brain-derived growth factor (BDNF)	neurotrophin neuronal growth and survival	(Lim and Alkon, 2012)

signalling and vesicle trafficking (Bolognani et al., 2010). Of particular interest, this study also found that HuD binds to its own mRNA and those of *HuB* and *HuR*, indicating a complex regulatory feedback network amongst Hu/ELAV members. HuD was also shown to bind mRNAs of other RBPs pointing to a complex regulatory network amongst RBPs, as previously described for the translation and turnover RBPs such as HuR, AUF1 and TIA-1 (Pullmann et al., 2007).

1.9 Multilevel control of mRNA metabolism by HuD

Following binding to a target RNA motif, HuD controls one or many aspects of mRNA metabolism, including alternative splicing, polyadenylation, nuclear export, stability, localization and translation. As discussed in the sections below, the post-transcriptional events governed by HuD partially depend on the location of its binding site within the mRNA (5'UTR, exon, intron, or 3'UTR).

1.9.1 Promoting and inhibiting splicing of alternative exons

Pre-mRNA contains intermittent non-coding (introns) and protein coding (exons) regions that are subject to constitutive, and occasionally alternative, splicing. Constitutive splicing involves sequential excision of introns and ligation of the remaining exons to form a mature transcript. Conversely, alternative splicing mediates ligation of specific exons in different combinations, thereby producing multiple mature mRNA isoforms from one precursor mRNA (pre-mRNA). By regulating inclusion and exclusion of RNA fragments, alternative splicing produces additional regulation of gene expression and diversifies the

proteome. Recent high throughput sequencing revealed that more than 90% of human genes undergo alternative splicing (Kalsotra and Cooper, 2011).

Splicing, whether constitutive or alternative, can occur during transcription (co-transcriptionally) or following release of the transcript from the transcription machinery (post-transcriptionally; Han et al., 2011). In both co- and post-transcriptional splicing, the mechanism involves components of a large ribonucleoprotein complex, termed the spliceosome, interacting with specific sites in the pre-mRNA. The spliceosome is composed of over 150 core proteins and several small nuclear ribonuclearprotein particles (snRNPs); U1, U2, U4/U6 and U5. Each intron contains specific sequence elements, namely 5' splice site (SS), 3'SS, branch point (BP) and polypyrimidine-rich tract (PPT), which constitute the core splicing signals. Simplistically, spliceosome assembly involves U1 binding to the 5'SS followed by U2 binding to the BP and 3'SS and subsequently recruitment of the U4/U6/U5 subcomplex to complete spliceosome maturation (Smith et al., 2008). In addition to the core splicing machinery, auxiliary splicing factors can modulate inclusion or exclusion of certain exons and introns by binding to splicing enhancers or silencers (Han et al., 2011; Matlin et al., 2005; Wang and Burge, 2008). For example, the alternative splicing regulators NOVA 1/2 promote splicing of the Agrin pre-mRNA to enhance inclusion of two exons, which changes the function of the Agrin protein at the neuromuscular junction (Ruggiu et al., 2009).

Previous experiments with the *Drosophila* ELAV protein have demonstrated that it controls alternative splicing of certain genes (Koushika et al.,

1996; Lisbin et al., 2001; Soller and White, 2003), suggesting that the mammalian Hu/ELAV proteins perform a similar function. A major indication that Hu/ELAVs regulate alternative splicing surfaced from a study that showed HuD binding to both exonic and intronic regions of *N-myc* pre-mRNA (Lazarova et al., 1999). Subsequently, a series of papers, predominantly from the Hua Lou laboratory, corroborated that all Hu/ELAVs function as auxiliary splicing factors (Hinman and Lou, 2008). Using various methods, such as REMSA and UV cross-linking/ immunoprecipitation (IP) assays, the authors revealed that all four Hu/ELAVs promote inclusion of HuD E6 by binding to two surrounding ARE sequences (Wang et al., 2010b).

In contrast to this function, evidence that nELAVs suppress exon inclusion also exists. For instance, nELAV proteins 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 also bind to 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'SSs, respectively (Zhu et al., 2008). Furthermore, over-expression 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 (Hinman and Lou, 2008; Bellavia et al., 2007). Experiments with the truncated forms of HuB and HuC, containing only RRMs 1 and 2, indicated that the linker region and/or

RRM3 are necessary for nELAV regulation of alternative splicing (Zhu et al., 2008). In addition to binding intronic sequences, co-IP assays revealed that Hu/ELAVs interact with RNA polymerase II and histone deacetylase 2 (HDAC2) to regulate alternative splicing. *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. Both the hinge region and RRM3 were found to be important for these interactions and the ability of nELAVs to elicit alternative splicing (Zhou et al., 2011). A recent genome wide analysis has mapped the binding sites in nELAV targeted pre-mRNAs revealing that these proteins preferentially bind introns surrounding alternative exons, especially at exon/intron splice junctions (Ince-Dunn et al., 2012). Altogether, these studies demonstrate that HuD, along with the other nELAV proteins, promotes or suppresses exon inclusion by interacting with splicing, transcription and chromatin components (Figure 1.3A).

1.9.2 Regulating alternative polyadenylation

Most mammalian mRNAs undergo polyadenylation, a nuclear process that results in template-independent addition of ~250 As to their 3' end. The polyadenylation event is biphasic, entailing consensus motif recognition, for example the polyadenylation sequence AAUAAA, and cleavage at 10-35 nucleotides downstream of this sequence followed by addition of a poly(A) tail. The cleavage and polyadenylation machinery consists of multiple factors, including cleavage-polyadenylation specificity factor (CPSF), cleavage

stimulation factor (CstF) and poly(A) polymerase (PAP; Mandel et al., 2008). Similar to alternative splicing, polyadenylation can occur during transcription (co-transcriptional) or following transcription termination (post-transcriptional) and affects various intracellular processes. For example, longer and shorter poly(A) tails generally promote mRNA stability and 3'-5' degradation by the exosome, respectively. Also, through a mechanism involving mRNA circularization, longer poly(A) tails enhance translation compared to shorter poly(A) tails (Eckmann et al., 2011).

Over 50% of mRNAs contain more than one polyadenylation signal and are therefore susceptible to alternative polyadenylation (Tian et al., 2005). A subset of these mRNAs harbours U-rich sites up- or down-stream of the cleavage site that are targeted by *trans*-acting factors (Licatalosi and Darnell, 2010; Zhang et al., 2010). However, the *trans*-acting factors that bind these sites and regulate alternative polyadenylation, especially in a cell-specific manner, are only starting to emerge.

Both the *Drosophila* ELAV and the mammalian Hu/ELAVs 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/ELAV to produce this function (Zhu et al., 2006). Through REMSAs and *in vitro* polyadenylation assays, one study showed that Hu/ELAVs bind U-rich sequences downstream of the exon 4 poly(A) signal in calcitonin/CGRP to prevent binding of CstF64 and CPSF (Zhu et al., 2006). The study illustrated that through this mechanism Hu/ELAV proteins blocked

polyadenylation of the non-neuronal exon 4 to promote the neuron-specific pathway. More recently, Hu/ELAV proteins were demonstrated to regulate alternative polyadenylation of HuR in neurons (Mansfield and Keene, 2011). There are three different HuR mRNA polyadenylation variants (1.5, 2.4 and 6.0kb), which differ in 3'UTR length. All four Hu/ELAV members were found to bind and block U-rich sequences near the 2.4kb polyadenylation site and thereby promote expression of the 6.0kb transcript. Since the 6.0kb variant is less stable and not as efficiently translated as the 2.4kb transcript, the authors postulated that the reduction of HuR expression permits neurons to terminally differentiate since HuR protein plays a major role in proliferation (Mansfield and Keene, 2011). Together, these studies reveal that Hu/ELAVs play significant roles in pre-mRNA alternative splicing and polyadenylation (Figure 1.3A).

1.9.3 Exporting mRNAs from the nucleus

Once mRNAs are processed in the nucleus, they typically undergo export to the cytoplasm through nuclear pore complexes (NPCs), where they can eventually be translated. Given the sequence and length variability of mRNAs, the coupling of different transcripts to a nuclear export pathway is linked to their processing and assembly into mRNPs. In mammals, the primary general mRNA export pathway requires the TAP-p15 (also known as NXF1/NXT1) complex (Carmody and Wente, 2009). Another general mRNA export pathway, via CRM1, is less often used and usually exports short-lived transcripts, such as those that contain AREs in their 3'UTRs (Fornerod et al., 1997; Brennan et al., 2000; Hutten and Kehlenbach, 2007). These nuclear receptor adaptor proteins directly or

indirectly recognize and interact with specific peptide sequences on RBPs, such as NES. On the other hand, protein cargo is usually transported via the karyopherin family, which consists of nuclear export (exportins) and import (importins) receptors. The former receptors also recognize the NES, whereas the latter receptors recognize the NLS on cargo proteins (Kohler and Hurt, 2007).

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 (Kasashima et al., 1999; Figure 1.3B). Overexpression of wild type and 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 TAP-p15 nuclear receptor, in a RNA independent manner, and this interaction may be necessary for shuttling of HuD (Saito et al., 2004). Further studies are necessary to establish whether TAP-p45 or another export pathway is used by HuD for nuclear export of mRNAs.

1.9.4 Increasing half-life of mRNAs

Stability of mRNAs is one of the major post-transcriptional events influencing mRNA abundance and consequently protein expression. Stability of transcripts is largely dependent on *trans*-acting factors, such as RBPs and micro-RNAs, and mRNA *cis*-acting elements found mostly in the 3'UTR but also occasionally in the 5'UTR and coding region (Wu and Brewer, 2012). Although the use of novel ribonomic techniques, including microarray and deep sequencing of transcripts obtained from RNA immunoprecipitation (RIP-chip) and

CLIP (CLIP-seq/HITS-CLIP), has expanded our understanding of *cis*-acting motifs, identifying these stability determinants remains elusive (Schoenberg and Maquat, 2012). Nevertheless, the few *cis*-acting elements that have been discovered thus far function to promote degradation of the transcript by recruiting decay machinery (Chen et al., 1998; Grosset et al., 2000; Marquis et al., 2006; Vlasova and Bohjanen, 2008). Degradation of most transcripts commences with 3' end deadenylation followed by decapping of transcript and 5' to 3' or 3' to 5' exoribonucleolytic decay of the mRNA. On the other hand, some transcripts are first cleaved at the body by endoribonucleases prior to exoribonucleolytic degradation at both ends (Schoenberg and Maquat, 2012; Figure 1.4).

One of these destabilizing *cis*-acting motifs, the ARE, is typically found in transcripts encoding proteins with diverse functions, such as cellular proliferation, differentiation, transcription, RNA metabolism, inflammation and stress-response (Bakheet et al., 2006; Khabar, 2010). This element affects the stability of mRNAs by serving as a target site for ARE-binding RBP(s), also known as AU-binding proteins (AUBPs). Out of the roughly 20 currently known AUBPs, the majority promote mRNA decay including, for example AUF1, TTP and KSRP (Wu and Brewer, 2012). However, there are few known AUBPs that enhance mRNA stability (Table 1.1).

The best-described stabilizing AUBPs are the Hu/ELAV members, partly due to the established role of HuD in increasing the mRNA half-life of specific neuronal transcripts (Hinman and Lou, 2008; Pascale et al., 2008; Deschenes-Furry et al., 2006; Figure 1.3C). One of the prominent targets of HuD is the

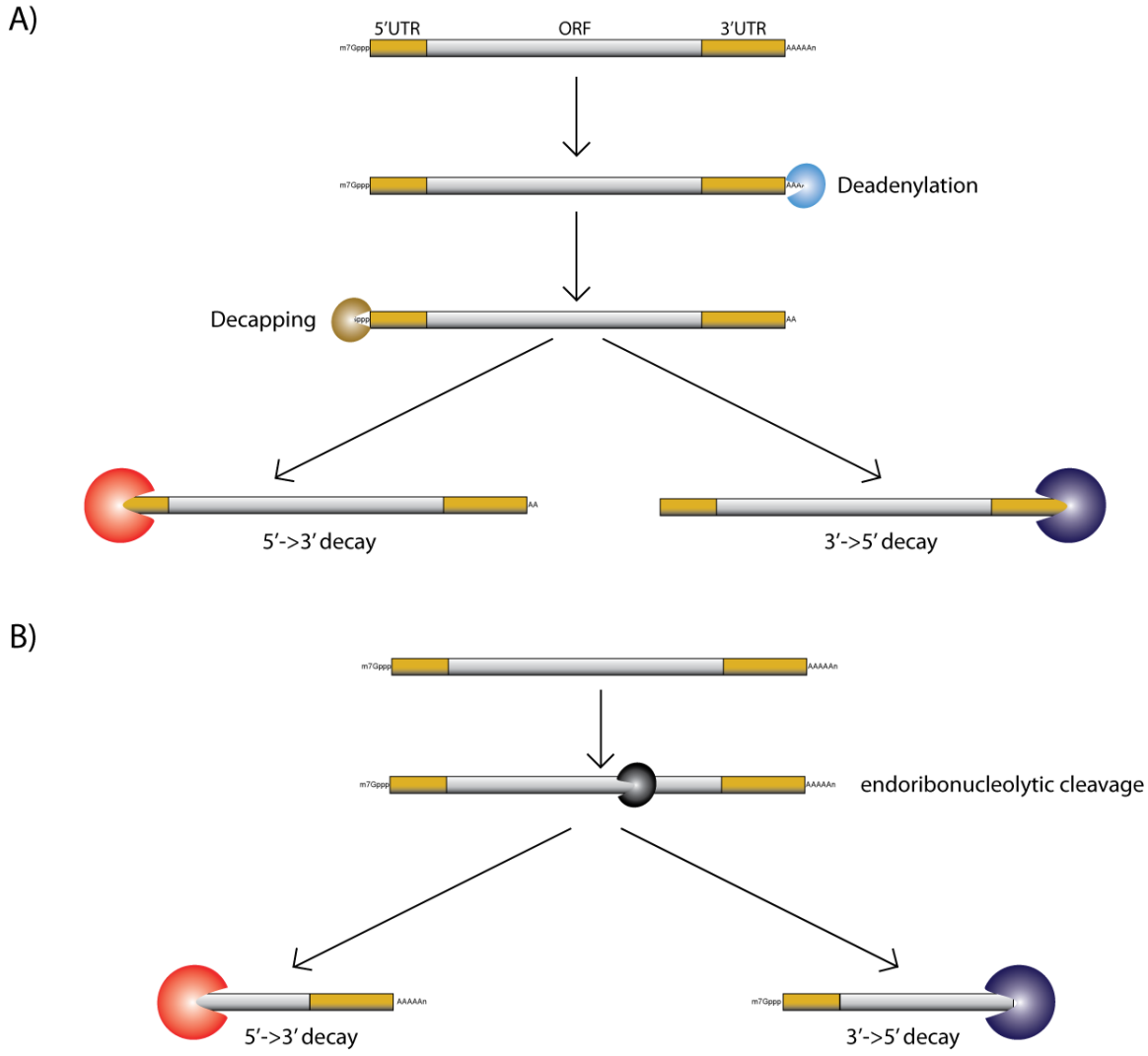


Figure 1.4 mRNA decay pathways in mammalian cells

Within mammalian cells, mRNAs are degraded through either the **A)** exoribonuclease or **B)** endoribonuclease decay pathways. These processes involve the function of decapping, deadenylating, 5'-3' exonucleolytic, 3'-5' exonucleolytic and endonucleolytic enzymes and the exosome. UTR; untranslated region and ORF; open reading frame. Adapted from (Schoenberg and Maquat, 2012).

GAP-43 transcript, which houses a class III ARE in its 3'UTR (Chung et al., 1997; Tsai et al., 1997). Interest in the control of GAP-43 expression stemmed from convincing evidence that it is essential for neural development (Maier et al., 1999; Mani et al., 2001; Shen et al., 2008) and involved in synaptic remodelling during learning and memory (Routtenberg et al., 2000). Multiple studies have shown that over-expression or down-regulation of HuD protein results in increased or decreased *GAP-43* mRNA levels and/or stability, respectively, in cultured cells and mouse brain (Anderson et al., 2000; Mobarak et al., 2000; Anderson et al., 2001; Bolognani et al., 2006; Bolognani et al., 2007b). Interestingly, HuD is more effective at stabilizing *GAP-43* mRNA that contains a longer poly(A) tail, indicating that HuD binds to both the ARE and poly(A) tail to increase the *GAP-43* mRNA half-life (Beckel-Mitchener et al., 2002). In addition to *GAP-43* transcripts, HuD has also been shown to promote stability of several other ARE-containing mRNAs, such as *AChE* (Deschenes-Furry et al., 2003) and *Nova1* (Ratti et al., 2008), both *in vitro* and *in vivo*. Despite mRNA stabilization being one of the better-known roles of HuD, the molecular events behind this function are not well described.

A possible mechanism through which HuD and other Hu/ELAV 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 methods, such as blocking PABP binding (Sagliocco et al., 2006) and/or recruitment of the decapping enzyme (Lykke-Andersen and Wagner, 2005),

deadenylase poly(A) ribonuclease (PARN; Lai and Blackshear, 2001) or exosome (Chen et al., 2001; Torrisani et al., 2007). Thus, it is feasible that by binding to AREs or other motifs, HuD blocks access to the motif and consequently prolongs the mRNA half-life (Figure 1.3C). However, there is evidence indicating that this mechanism is more complex since both stabilizing and destabilizing RBPs can bind the same transcript simultaneously. Foster resonance energy transfer (FRET) and immunocytochemistry assays revealed that HuR interacts with AUF1, KSRP and TTP in the nucleus and cytoplasm, including in P-bodies and stress granules (David et al., 2007; David Gerecht et al., 2010). Moreover, these interactions are thought to require the presence of mRNA, 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* and *Cyclin D* mRNAs (Lal et al., 2004). Together, these findings suggest that Hu/ELAV regulation of mRNA stability, and likely metabolism, is at least partially dependent on a balance between stabilizing and destabilizing RBPs (Figure 1.5).

1.9.5 Trafficking and translation of mRNAs

Another stage of post-transcriptional regulation occurs during mRNA transport, a process that ultimately also affects protein production. Transportation of mRNAs is necessary particularly in neurons given the large cytoplasmic area created by intricate neurite branching. In recent years, our understanding of the molecular components involved in mRNA transport has greatly improved. Active transport of mRNAs requires mRNPs composed of *trans*-acting factors that bind

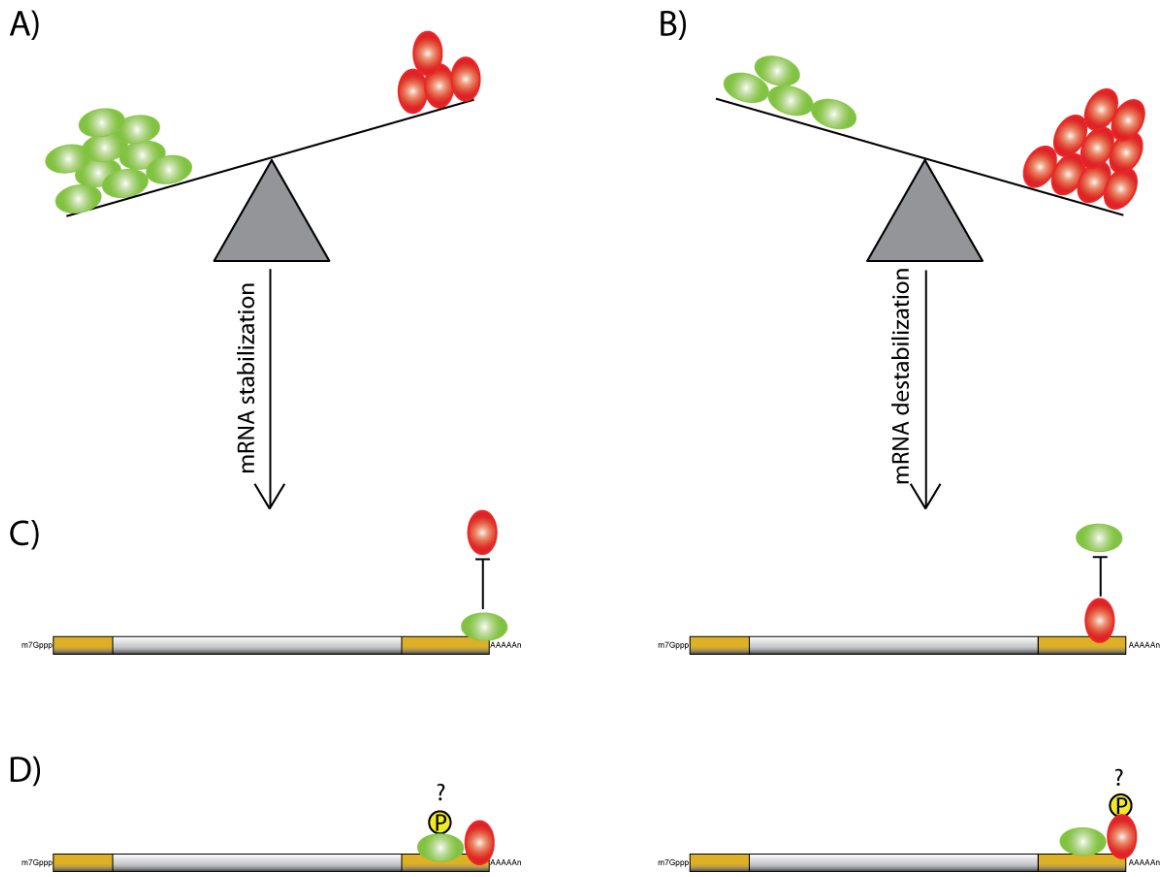


Figure 1.5 The balance in intracellular expression and activity between stabilizing and destabilizing RBPs controls mRNA half-life

High relative levels of **A)** stabilizing (e.g. Hu/ELAVs; green) or **B)** destabilizing (e.g. AUF1; red) RBPs promotes mRNA stability or decay, respectively. Moreover, stabilizing and destabilizing RBPs can bind **C)** competitively or **D)** cooperatively to influence mRNA fate. Cooperative binding presumably allows for rapid determination of mRNA fate in response to extracellular cues. Question mark denotes possible post-translational modification (e.g. phosphorylation) of mutually bound RBPs to control mRNA fate.

to specialized secondary or tertiary RNA structures, called localization elements (LE) or zipcodes, typically found in the 3'UTR (Jambhekar and Derisi, 2007). Specific components of mRNPs play individual roles, including transporting the transcript along microtubules (e.g. kinesins and dyneins) or actin filaments (e.g. myosins) and repressing translation (e.g. Pumilio 2; Marchand et al., 2012). Compelling evidence now exists demonstrating that specific mRNP components can direct ribosome-dependent local mRNA translation at the neurite terminal (Jung et al., 2012; Wang et al., 2010a; Liu-Yesucevitz et al., 2011).

The process of translation is comprised of three main phases- initiation, elongation and termination (Sonenberg and Hinnebusch, 2009). Since the rate-limiting step of protein synthesis is translation initiation, a large amount of research has been conducted to delineate the molecular machinery that performs and regulates this phase. In most cellular contexts, translation initiation occurs through a cap-dependent mechanism, which involves binding of the cap-binding complex (CBC), also known as eukaryotic initiation factor-4F (eIF4F), to the m^7G cap structure on the mRNA 5' end. eIF4F is composed of a cap-binding protein (eIF4E), RNA helicase (eIF4A) and scaffolding protein that indirectly binds the 43S preinitiation complex (eIF4G). Following assembly at the 5'end, the 43S complex scans the mRNA 5'UTR in the 5' to 3' direction for an initiation codon (AUG). Once identified, recruitment of the large ribosome (80S) onto the mRNA occurs and protein synthesis proceeds until a trinucleotide termination signal is detected and translation is terminated (Jackson et al., 2010). The second type of protein synthesis initiation mechanism is cap-independent, also known as

internal ribosome entry site (IRES)-mediated, translation. This process occurs independently of CBC formation and ribosomes are directly recruited by poorly defined IRES *trans*-acting factors (ITAFs) to the IRES in the mRNA 5'UTR. IRES-mediated translation occurs in 3-5% of mRNAs, usually under conditions of cellular stress (Komar and Hatzoglou, 2011).

Compelling evidence supports a role for HuD in regulating both localization and translation of transcripts in the cytoplasm and possibly in neurites (Figure 1.3C and D). For instance, two HuD target mRNAs that are 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 in the cytoplasm and neurites, such as the motor protein KIF3A (Aronov et al., 2002), translation regulator IMP-1, survival of motor neuron (SMN; Hubers et al., 2010; Fallini et al., 2011; Akten et al., 2011) and the microtubule-associated component MAP1B (Fujiwara et al., 2011b). Moreover, *in vitro* experiments showed that HuD binds to eIF4A and the poly(A)-tail of transcripts, functions that require both the linker region and third RRM of HuD, to enhance cap-dependent protein synthesis and PC12 neurite extension (Fukao et al., 2009). Together, these studies indicate that HuD recruits mRNAs to microtubules and may transport them to neurites to promote their local translation.

In addition to enhancing translation, both HuD and HuR are capable of repressing protein synthesis under certain contexts, such as cellular stress, as

shown for p27 and IGF-IR, respectively (Kullmann et al., 2002; Meng et al., 2005). Although the translational silencing mechanisms are unclear, it requires Hu/ELAV binding to an IRES in the mRNA 5'UTR (Kullmann et al., 2002; Meng et al., 2005). Since several HuD mRNAs targets encode proteins involved in axonal outgrowth, synapse formation and vesicle trafficking, these studies suggest that HuD regulates neuritogenesis and synaptogenesis partly through control of mRNA transport and local translation.

1.10 Diverse roles of HuD in the nervous system

The multiple post-transcriptional effects of HuD on mRNAs whose proteins have various key roles in neurons and its expression in several neuronal subtypes strongly points to central function(s) mediated by HuD in the nervous system. Studies have demonstrated the importance of HuD in neurogenesis and neuroplasticity during learning and memory and following neuronal injury. Additionally, there is increasing evidence implicating misregulation and mutation of HuD in neuropathies and cancers, further stressing the significance of HuD in the nervous system.

1.10.1 Neuronal development

As previously mentioned, HuD regulates multiple aspects of mRNA metabolism of genes that encode proteins with diverse roles (Table 1.2), ranging from neural precursor proliferation to synaptic remodeling (Figure 1.3). These prominent molecular functions of HuD strongly suggest that it acts as a "master" regulator of neuronal development. Indeed, studies on primary and cultured

neuronal cells have demonstrated that HuD controls almost all stages of a neuron's existence (Deschenes-Furry et al., 2006; Pascale and Govoni, 2012). For example, neurosphere assays using cerebral cortex stem/progenitors from HuD knockout (-/-) mice revealed that HuD decreases self-renewal capacity, promotes cell cycle exit and differentiation of neuronal stem/progenitor cells. Adult HuD-/- mice were also found to contain an increased number of self-renewing cells in the subventricular zone (SVZ), further emphasizing the importance of HuD for the 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 $p21^{waf}$ (Fujiwara et al., 2006). Moreover, HuD-dependent neurogenesis requires commitment of cells to the neuronal lineage since ectopic expression of HuD in non-neuronal cells does not result in neuronal differentiation (Wakamatsu and Weston, 1997; Anderson et al., 2001).

Studies in which HuD levels were down-regulated or over-expressed in cultured cells have revealed the essential role of HuD in terminal differentiation, namely dendritic and axonal outgrowth (Mobarak et al., 2000; Wakamatsu and Weston, 1997; Aranda-Abreu et al., 1999; Hubers et al., 2010; Anderson et al., 2001). In support of these findings, ectopic expression of HuD in mouse forebrain neurons (HuD-Tg) was shown to increase 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 clearly demonstrate that HuD positively regulates neurite outgrowth. This function of HuD requires it to shuttle to the cytoplasm and involves stabilization of various mRNA targets whose protein products promote microtubule assembly and growth cone development, including GAP-43, Tau and AChE (Kasashima et al., 1999; Aranda-Abreu et al., 1999; Chung et al., 1997; Deschenes-Furry et al., 2003).

1.10.2 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 detected, 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 for four days following axotomy of superior cervical ganglion (SCG) neurons, *HuD* mRNA levels, its interaction with *AChE* mRNA and *AChE* transcript levels decreased. Importantly, the reduction in *AChE* mRNA abundance could be rescued by localized overexpression of HuD delivered via a viral vector (Deschenes-Furry et al., 2007). Although HuD expression was shown to decrease immediately following nerve injury, another study performed nerve crush of dorsal root ganglion (DRG) neurons to demonstrate that HuD protein and *GAP-43* mRNA expression was increased by 7 days post injury and remained elevated up to 21 days (Anderson et al., 2003). These two studies raise the possibility that the

delayed up-regulation of endogenous HuD levels post nerve injury is involved in recovery following nerve damage.

In parallel with this potential role, studies have demonstrated that HuD protein levels, as well as a cohort of its mRNA targets, increased in several brain regions, including the dentate gyrus (DG), following neurotoxin-induced seizure (Bolognani et al., 2007b; Tiruchinapalli et al., 2008b; Winden et al., 2011). Moreover, glutamate receptor activation was shown to increase HuD protein and target mRNA abundance and promote HuD localization to dendritic spines (Tiruchinapalli et al., 2008a). Along with the finding that several *HuD* mRNA targets encode synaptic proteins (Bolognani et al., 2010), these studies indicate that HuD is important for multiple types of neuronal plasticity, potentially by directly acting at the synapse.

The function of HuD in neuronal plasticity has raised the possibility that it is involved in hippocampus-dependent learning and memory. The initial study to illustrate the involvement of HuD in 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). Subsequently, another study 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). These exciting findings led to the development of transgenic mice over-expressing HuD (HuD-Tg) in forebrain neurons (Bolognani et al., 2007a). Analysis of HuD-Tg

mice showed enhanced sprouting of GAP-43-containing mossy fibers to the CA3 region, ectopic expression of GAP-43 protein in DG and increased GAP-43 and calmodulin binding, possibly causing altered calcium signalling (Bolognani et al., 2006; Tanner et al., 2008). Moreover, laser capture microdissection of DG cells coupled with microarray analysis of *HuD* mRNA targets 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 changes, including ectopic HuD protein levels throughout the hippocampus, they demonstrated improper acquisition and retention of memories, possibly due to the lack of proper temporal and spatial regulation of HuD protein (Bolognani et al., 2006; Bolognani et al., 2007a). Based on these findings, it is clear that precise spatiotemporal control of HuD levels are necessary for proper learning and memory.

1.10.3 Neuronal diseases

Given its multiple important roles in neurons, it is not surprising that misregulation and/or misexpression of HuD are implicated in several neuropathies (Pascale and Govoni, 2012) and cancers (Dalmau et al., 1990; Sekido et al., 1994; Ball and King, 1997). In accordance with its direct or indirect function in learning and memory, HuD has recently been linked to the progression of Alzheimer's disease (AD), a neuropathy characterized by memory loss, neuronal death and eventual dementia (Amadio et al., 2009). The authors of this study found that nELAV protein expression was inversely correlated with

beta-amyloid (Aβ) peptide levels, whose abnormal accumulation is a hallmark of AD, both *in vitro* and *in vivo*. In addition to its potential role in AD, three separate cohort genetic studies have linked a single nucleotide polymorphism (SNP) located within intron 2 of the *HuD* locus to development of Parkinson's disease (PD), a degenerative disorder of the CNS, in Caucasian populations (Noureddine et al., 2005; Haugarvoll et al., 2007; 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 reduced survival of motor neuron (SMN) protein expression (Burghes and Beattie, 2009). Converging findings from three independent studies demonstrated that HuD interacts with SMN protein in axonal compartments of neurons (Hubers et al., 2010; Fallini et al., 2011; Akten et al., 2011). Importantly, overexpression of HuD, or its mRNA target *cpg-15*, was found to partially rescue SMA-like defects related to cell morphology or recruitment of specific mRNAs into RNA granules.

Evidence is also mounting that HuD may act as a tumor suppressor in some types of cancers. Aberrant expression of HuD has been detected in several cancers of the central and peripheral nervous system, most prominently in SCLCs (Szabo et al., 1991; Dalmau et al., 1992; Schramm et al., 1999; Stawski et al., 2012). Although the genetic composition of the *HuD* gene in most of these neoplasms is unknown, a recent study screened SCLCs and identified 11 mutations of unidentified consequence(s) and 2 mutations that prevent protein expression in the *HuD* gene (D'Alessandro et al., 2010). Interestingly, high levels of HuD and other nELAVs in neuroendocrine neoplasms have been associated

with clinically favourable outcomes (Ball and King, 1997; Graus et al., 1997). In line with this, vaccination of mice, containing HuD-expressing tumors, with HuD DNA resulted in decreased tumor growth (Carpentier et al., 1998; Ohwada et al., 1999). Together, these findings imply that genetic mutations in the *HuD* gene are associated with the etiology and/or progression of certain cancers and that vaccination with the wild-type *HuD* gene may produce a clinically favourable outcome for some cancer sufferers.

1.11 Molecular mechanisms regulating HuD

The important roles of HuD in neuronal development and plasticity and its implications in neuronal diseases raise questions regarding the molecular mechanisms that regulate its expression. To date, a few studies have commenced describing the post-translational, post-transcriptional and transcriptional mechanisms that control expression and function of HuD; however, given the key functions of HuD in neurons, more systematic investigations of the molecular events that regulate its expression are warranted.

1.11.1 Post-translational control of HuD expression and function

Although some conditions, such as application of growth factors (Mobarak et al., 2000; Aranda-Abreu et al., 1999), specific pharmaceuticals (Pascale et al., 2005) and cellular stress (Burry and Smith, 2006), have been documented to alter 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 expression and/or function of HuD; protein kinase

C (PKC; Mobarak et al., 2000; Lim and Alkon, 2012; Pascale et al., 2005), coactivator-associated arginine methyltransferase 1 (CARM1; Hubers et al., 2010; Fujiwara et al., 2006) 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 remodelling (Amadio et al., 2006). The classical PKCα isoform, was shown to increase nELAV levels and promote their nuclear export following treatment of SH-SY5Y neuroblastoma cells with the PKC-activating compounds phorbol esters and bryostatin-1 (Pascale et al., 2005). Moreover, activation of PKCα promoted 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 resulted in HuD phosphorylation on nine residues (Lim and Alkon, 2012). Importantly, HuD is a critical downstream target of this neurite-extending pathway since phorbol ester treatment of PC12 neuronal cells following HuD knockdown prevented 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 perform a variety of cellular functions such as regulating transcription and RNA processing by methylating arginine residues (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 mouse HuD at Arg^{248}, located in the hinge region (Figure 1.1), *in vitro* and *in vivo* (Hubers et al., 2010; Fujiwara et al., 2006). Moreover, methylation of HuD decreased $p21^{waf}$ 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 $p21^{WAF}$ 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 mRNA targets, including *tau* and *p27*, suggesting that stabilization of these other mRNA targets requires additional post-translational modification of HuD and/or *trans*-acting factors (Hubers et al., 2010; Fujiwara et al., 2006). These findings demonstrate that methylation of HuD by CARM1 may be a universal switch in neuronal precursors that regulates the transition from proliferation to differentiation.

Interestingly, a recent study found a direct link between the PKC and CARM1 effects on HuD (Lim and Alkon, 2012). In hippocampal neurons, the authors demonstrated using pathway-specific inhibitors that activated PKC isoforms interact and phosphorylate CARM1, which lead to decreased CARM1 methyltransferase activity. Furthermore, activating the classical PKC isoforms and simultaneously blocking CARM1 activity further increased 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 (Hers et al., 2011). In the HuD study, the authors used HuD mutants to demonstrate that active (phosphorylated) AKT1 binds directly to a sequence in the linker region of HuD, without phosphorylating it, and that this interaction is required for HuD-dependent neurite outgrowth. 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., 2008b). Together, these results indicate that AKT1 increases expression of HuD protein and interacts with the HuD-mRNA complex to promote neurite elongation, possibly through regulation of HuD-mediated translation.

1.11.2 Post-transcriptional control of HuD expression

Recent studies have started to decipher the molecular mechanisms regulating HuD mRNA. The mammalian *HuD* transcript contains at least three alternate 5'UTRs and a long 3'UTR (~2.6kb), features that are a strong indication of post-transcriptional regulation. Analysis of the *Xenopus HuD* homologue *ElrD* sequence illustrated that the 3'UTR contains a ~100nt stretch of nucleotides with 90% identity to the human *HuD* 3'UTR, suggesting that this fragment encompasses an important evolutionarily conserved regulatory structure and/or

cis-element (s) (Good, 1995). Indeed, analysis of HuD targets in the mouse forebrain showed that HuD binds to its own mRNA, although the location and function of this interaction was not described (Bolognani et al., 2010). One previously described functional consequence of this binding is regulating splicing of its own pre-mRNA (see above; Wang et al., 2010b). Another possible significance of this interaction may be similar to the one found for the *Drosophila* ELAV homologue, which has also been demonstrated to auto-regulate its own transcript (Samson, 1998; Borgeson and Samson, 2005). In this case, ELAV binding to its own mRNA was indirectly demonstrated to function as a negative regulatory loop that is activated when protein levels reached a certain threshold, thereby preventing further ELAV protein production. Given the multifunctional roles of HuD on mRNA metabolism, it is possible that HuD also regulates polyadenylation, stability, localization and/or translation of its own mRNA.

Also of particular interest to the post-transcriptional regulation of HuD, one study has found that miR-375 targets the 3'UTR of HuD and decreases its abundance by reducing both its mRNA stability and translation. Furthermore, knockdown of miR-375 in culture cells and in mouse brain revealed that through negative control of HuD expression, miR-375 prevents neurite outgrowth (Abdelmohsen et al., 2010). These studies stress that HuD transcripts are likely subject to intricate post-transcriptional control.

1.11.3 Transcriptional control of HuD expression

Despite some studies demonstrating regulation of HuD at post-translational and transcriptional levels, little is known regarding the molecular

mechanisms that control transcription of HuD in neurons. Nevertheless, detailed analysis of the mammalian *HuD* 5' regulatory region (RR) and a few studies on transcriptional events controlling HuD and its homologues have provided some insight. No consensus promoter region(s) for the mammalian *HuD* gene has been identified in mammals; however, the three *HuD* E1 variants point to the existence of multiple promoters (Inman et al., 1998). In agreement with this view, the *Xenopus elrD* 5' genomic region was found to contain two distinct E1 variants. Using promoter-reporter assays, the authors also demonstrated that the 5'RR of each variant was capable of driving neuron-specific expression of two different *elrD* mRNA isoforms (Nassar and Wegnez, 2001; Nassar, 2011).

Notwithstanding the lack of information on mammalian HuD promoter(s), a few *trans*-acting factors and *cis*-acting elements in the putative promoter regions of *HuD*, and those of its orthologues and paralogues, have been identified. For instance, one study showed through nuclear run-on assays that the thyroid hormone (T3) decreases transcription of *HuD* in cultured neuronal cells. The authors also demonstrated that *HuD* mRNA abundance was inversely correlated to T3 levels in the rat brain (Cuadrado et al., 2003). Given that HuD is expressed in several non-neuronal tissues (see above), a recent report examined the control of HuD expression in pancreatic beta cells. The authors found that the TF forkhead box O1 (FoxO1), under low glucose levels, negatively regulates transcription of *HuD* in these cells (Lee et al., 2012). In addition to binding motifs for T3 and FoxO1, the mammalian *HuD* 5'RR may also contain CCAAT boxes, E-boxes and MyT1 sites since they were identified in the 5'RR of other nELAV

members (King, 1996; Park et al., 2000a). Importantly, these studies indicate that along with the lack of information regarding mechanisms regulating expression of *HuD* mRNA, currently only factors that negatively regulate transcription of *HuD* are known.

1.12 Statement of problem

The predominantly neuron-specific HuD controls mRNAs, whose proteins are necessary for neuronal development, maintenance and function, at multiple levels. The implications of HuD in neuronal plasticity and several neuronal diseases emphasize the importance of this protein to the survival of vertebrate metazoans. To date, a few studies have started to delineate the nature of the molecular events presiding over expression of HuD in neurons; however, only mechanisms that negatively regulate its mRNA abundance were revealed (Abdelmohsen et al., 2010; Lee et al., 2012; Cuadrado et al., 2003). Thus, in light of these findings and central roles of HuD in neurons, it appears critical to characterize the 5' genomic region of mammalian *HuD* and investigate the *trans*-acting factors and *cis*-acting elements controlling its expression in neurons.

1.13 Hypothesis

Based on the current literature describing the pattern of expression of HuD and the control of HuD orthologues and paralogues, we hypothesize that neuron-specific transcriptional mechanisms are key regulatory events controlling the abundance and pattern of expression of HuD in developing neurons.

1.14 Objectives

1) Establish a neuronal differentiation model to study *HuD* regulation.
2) Identify whether *HuD* is transcriptionally and/or post-transcriptionally regulated during neurogenesis.
3) Characterize the leader exons in the 5' genomic region of *HuD*.
4) Determine the spatiotemporal expression and function of the predominant *HuD* E1 variant.
5) Define the *cis*-acting element(s) and *trans*-acting factor(s) that regulate neuron-specific expression of *HuD*.

Chapter 2

2: MATERIALS AND METHODS

2.1 Cell culture

Dr. Ilona Skerjanc (University of Ottawa, Ottawa, Canada) kindly provided the P19 cell line. Cells were cultured in growth media consisting of alpha Modified Eagle's Medium (αMEM), supplemented with 1 m*M* L-glutamine, penicillin (20 units/ml), streptomycin (20 mg/ml), 2.5% fetal bovine serum and 7.5% fetal calf serum (ATCC). N2a and C_2C_{12} cells were grown in Dulbecco's modified Eagle medium (DMEM), supplemented with 1 m*M* L-glutamine, penicillin (20 units/ml), streptomycin (20 mg/ml), and 10% fetal bovine serum (growth media). All cells were incubated at 37°C in a saturated humidity atmosphere containing 95% air and 5% CO_2.

To induce P19 differentiation into neuronal or muscle lineages, cells were resuspended at a density of $1x10^5$ cells/well in growth media containing 0.5μM *all-trans* RA (Sigma-Aldrich) or 0.8% (vol/vol) DMSO (Sigma-Aldrich), respectively, in ultra low-attachment six-well plates (Corning). All media were changed every two days.

2.2 Animals and neurospheres

Whole brain was extracted from adult (10–12 weeks) and embryonic (E10, 14 and E18) C57BL/6 (Jackson Laboratories) male and female mice. For the embryonic time points, days were counted from the first appearance of a vaginal

plug. Animals were housed two per cage, on a regular 12h day/night cycle and had *ad libitum* access to both standard food chow and water. The University of Ottawa Animal Care and Use Committee and Animal Care and Veterinary Service approved all procedures.

Neurosphere cultures were derived from the striatum of embryonic day 14 (E14) CD1 albino mouse embryos (Charles River) as previously described (Reynolds and Weiss, 1996; Fernando et al., 2005). Cells were subcultured only once per collection and grown in NeuroCult Proliferation Medium (StemCell Technologies) supplemented with 20ng/ml basic fibroblast growth factor (bFGF) in 75-cm^2 tissue culture flasks (Corning). To differentiate the cells, neurospheres were transferred to poly-L-ornithine-coated plates and maintained in bFGF-less NeuroCult differentiation medium. Media was changed every 24hrs for these experiments.

2.3 RNA extraction, semi-quantitative RT-PCR and real-time quantitative PCR

Total RNA was isolated from P19, N2a and C_2C_{12} cells and mouse brain with TRIzol as recommended by the manufacturer (Invitrogen) and as previously described (Boudreau-Lariviere et al., 2000). RNA was subjected to DNAse treatment (Promega) and subsequently stored at -80°C until use. RNA samples were quantified using the Gene Quant II spectrophotometer (Amersham Biosciences). Reverse Transcription (RT) reactions were performed as previously described (Clow and Jasmin, 2010). Briefly, RNA samples were diluted to 50ng/µl and subsequently used in an RT reaction mix containing 5mM

$MgCl_2$, 1x PCR buffer, 1mM dNTP, 1U/µl RNase inhibitor, 5U/µl MuLV reverse transcriptase, and 2.5µM random hexamers. RT mixes were placed in a thermocycler at 42°C for 1 h, followed by 5 min. at 99°C to deactivate the enzymes. Diethylpyrocarbonate (DEPC)-treated water was added to RT mix as a negative control. RT reactions with no MuLV reverse transcriptase were also performed to confirm no DNA contamination.

Semi-quantitative polymerase chain reaction (PCR) and quantification was performed as previously described (Deschenes-Furry et al., 2007). Briefly, PCR reaction mix contained a final concentration of 2mM $MgCl_2$, 1x PCR buffer, 2.5U/100 µl AmpliTaq DNA polymerase (PerkinElmer), 20µM primers and 100ng RT reaction mixture. All PCR reactions were preheated to 94°C for 2 min and the subsequent PCR parameters were: 94°C for 30-60s, 60°C for 30-60s, and 72°C for 30-60s. The optimum annealing temperature (50°C to 70°C) and cycle number within the linear range of amplification for each set of primers was determined empirically.

Real-time quantitative PCR was performed as previously described (Clow and Jasmin, 2010). The MX3005p real-time PCR system (Stratagene) and QuantiTect SYBR Green PCR kit (QIAGEN) were used for all qPCR reactions. Quantified values of gene expression levels were obtained by using the ΔΔCt method (values were normalized to *18S*). Specificity of amplified products was determined by using the same controls as in semi-quantitative PCR. Annealing temperatures were 60°C, unless otherwise specified.

2.4 5'RACE assay

5'-RACE was performed using the FirstChoice RLM-RACE kit (Ambion) according to the manufacturer's protocol. RNA (100ng) from 4-day RA-differentiated P19 cells with a specific inner reverse E2 primer (Table 2.1) was used for the assay. PCR products were resolved on a 1.5% agarose gel, purified using a Gel Extraction Kit (Qiagen), cloned into pCR2.1-TOPO plasmids (Invitrogen) and sequenced. All novel sequences are available on Genbank (accession numbers; DQ460221 and DQ437511).

2.5 Actinomycin D experiments

Actinomycin D experiments were performed as previously described (Gramolini and Jasmin, 1999). For these, undifferentiated and 4-day differentiated (with RA or DMSO) P19 cells were treated with 4µg/µl Actinomycin D (Sigma-Aldrich). Total RNA from cell lysates was harvested at different time points (0, 2, 4 and 8hrs) post-treatment using TRIzol and processed for qPCR analysis. The average ratios of RA treated versus DMSO treated samples from four independent experiments (with their standard errors) were converted to natural logarithms (y-axis) and then plotted against time (x-axis). Trend lines were then fit to the plotted data using exponential regression using Microsoft Excel software. The half-life was calculated as $t1/2 = (\ln(50/b)/m)$ where m is the slope of the trend line.

Table 2.1 Primer sequences

Name	Forward	Reverse
HuD	CAATACGGTCGCATCATCAC	CCTTTGATGGCTTCTTCTGC
HuD v2	ACGCATCCTGGTTGATCAAG	CTGATTCTGATGAGAGTGTCCTCT
GAP-43	GCTCAGCGGAGACAGAAAGTG	CACATCGGCTTGTTTAGGCTC
18S	CGCCGCTAGAGGTGAAATC	CCAGTCGGCATCGTTTATGG
GAPDH	GGGTGTGAACCACGAGAAAT	TTCCACAATGCCAAAGTT
Brachyury T	CTGGACTTCGTGACGGCTG	TGACTTTGCTGAAAGACACAGG
Ngn2	AAGGCACAGCCAGAAGAAAA	CGTCTTCTGACCAAACAGCA
NeuroD	CTTGAAGCCATGAATGCAGA	GGCTTTTGATCCTCCTCCTC
Mash1	AGATGAGCAAGGTGGAGACG	TGGAGTAGTTGGGGGAGATG
KLF6	GAGTTCCTCCGTCATTTCCA	GTCGCCATTACCCTTGTCAC
NuDR	AGAATGAGCTGCCCACAACT	TCAAAGGTCAGTGCTCCAGA
E1a	GGACCCAGTGAGAAGCGACT	GTTCTGGAGCCTCATCTTCG
E1a[1]	AGAACAGGAGGCAAGGTCTG	ATTAGGGCAGTTCCAGAGCA
E1a[2]	TGAAATCAGCAGGACGCTTA	CCTGAATTCCTCTTGGGTCA
E1a[3]	GGCTGCCTGATATGGGATTA	CATAAATGAGCAGTTTCTTGACTC
E1a[4]	GACCTGCAGTTGTGACAGGA	CCTTTTACCTTCTTCCAGCTCA
E1b	CCACCCCCTCCCAATAATAG	CAGCGCTTCGACTCTTCTCT
E1c	GAACGTTGAGATGGGCAGTT	ATACAAGCCGCCTCCTCTTT
E1c (entire exon)	GACTCGAGATCTCTCCATTTCTGTGCTG	GTAAGCTTCATCTTCAAGCCATTCCACT
E1c[1]	ACGCTCCTTTCTGCTTTTGA	GCATTTGGCATGATCTTTTG
E2	---	TGACCCAAGAGGAATTCAGG
pLUC3.4	GGCTGCCTGATATGGGATTA	CCATTCCACTCCATCTGTGA
pLUC1.0	GCTAGCCGACCTGCAGTTGTGACAGG	AGATCTCCAAGCCATTCCACTCCATC
pLUC0.6	CCACCCCCTCCCAATAATAG	CCATTCCACTCCATCTGTGA
pLUC1.0Me2	CCAATCAGAGTCTGGCTTCTGCACACAGATGGTCCCATAAATG	CATTTATGGGACCATCTGTGTGCAGAAGCCAGACTCTGATTGG
pLUC1.0Me3	AGAGTCTGGCTTCAGCTGACTGAAC	TTTACATCCATTTATGGGACGTTCAGTC

	GTCCCATAAATGGATGTAAA	AGCTGAAGCCAGACTCT
pLUC1.0Me2+3	CCCAGCCAATCAGAGTCTGGCTTCT GCACACTGAACGTCCCATAAATGGAT GTAAAAAGG	CCTTTTTACATCCATTTATGGGACGTTC AGTGTGCAGAAGCCAGACTCTGATTGG CTGGG
ChIP_E2+3	GCCGGGCTTTAATTACCAGA	TGAACAGAGCCTTGCTGAAA
ChIP_β RARE	CTGCTGGGAGTTTTTAAGC	GGCAAAGAATAGACCCTCC
hRAR ORF	TAGAATTCTTCGAGGGGAAAGATGTA CG	TTCTCGAGCGGGGGAGTGGGTGGCC
KLF ORF	GAATTCGCCATCCAGTTTGCATGA	GAATTCCCTCTGCTCCTTCAGAGGTG
3'UTR (L)	GCTCTAGAGACTTTATAAGCCCGCGT TG	GGTCTAGAACACACTTTCATTTATTGTC TGGA
3'UTR (E)	GGTCTAGACAACAAAGCCCACAAATC CT	GGTCTAGAACACACTTTCATTTATTGTC TGGA
CAAT Mut	ATGTAAAAAGGGGAAGCTTCGAGGC GGCTTCAGCAAGGCTCTGTTCAGTTG	CAACTGAACAGAGCCTTGCTGAAGCCG CCTCGAAGCTTCCCCTTTTTACAT
E5 Mut	CCAATTTCAGCAAGGCTCTGTTCTGT ACTTCTTATCTACATCCT	CGATTCTAGGATGTAGATAAGAAGTAC AGAACAGAGCCTTGCT
GATA Mut	CGAGCCAATTTCAGCAAGGCTCTGTA GTCTTGTTCTTATCTACATCCTAGAAT C	GATTCTAGGATGTAGATAAGAACAAGA CTACAGAGCCTTGCTGAAATTGGCTCG
KLF Mut	GCCAAGCTTCGAGCCAATTTCAGCTC ACCTCTGTTCAGTTGTTC	GTAGATAAGAACAACTGAACAGAGGTG AGCTGAAATTGGCTCG
NuDR Mut	GCTCTGTTCAGTTGTTCTTATCTACAT CCTAAGTACGGGGGTTTCAGCTCA	TGAGCTGAAACCCCCGTACTTAGGATG TAGATAAGAACAACTGAACAGAGC

2.6 Nuclear run-on assays

Nuclear run on assays were performed as previously described (Rolfe and Sewell, 1997). Nuclear extracts were harvested from undifferentiated or differentiated P19 cells using the ProteoJet Nuclear and Cytoplasmic Protein Extraction kit (Fermentas), according to manufacturer's protocol. Nuclei were split into two aliquots and incubated in a transcription reaction mix containing 20% glycerol, 30mM Tris-HCl, pH 8.0, 2.5mM MgCl2, 150mM KCl, 1mM DTT, 40U of RNAsin and 0.5 mM of rATP, rCTP, rGTP and rUTP (+) or DEPC-treated water (-; Promega). Reactions were incubated for 30min at 30°C, then RNA was TRIzol extracted and real time qPCR was performed using HuD and *18S* primers. Expression levels were quantified using the ΔCt method and values were normalized to *18S* rRNA.

2.7 Promoter-reporter plasmids, mutagenesis and Luciferase assays

The PC1G2-*Ngn2*, pcDNA3-*NeuroD*, pcDNA3-*Mash1*, pcDNA3-*E47* and pcDNA3 His-*Deaf-1* constructs were kindly provided by Drs. Carole Schuurmans (University of Calgary, Calgary, Canada), Valerie Wallace (OHRI, Ottawa, Canada), Marc Ekker (University of Ottawa, Ottawa, Canada) and Paul Albert (OHRI, Ottawa, Canada), respectively. pcDNA3- *KLF6* was generated by PCR amplification of the *KLF6* ORF (Table 2.1) from N2a cells. The bacterial artificial clone (BAC clone RP23-283L16.1; UK, Pubmed AL627425.15), encompassing the mouse HuD gene locus, was kindly provided by The Wellcome Trust Sanger Institute (BioScience LifeSciences, UK). All HuD promoter fragments were PCR

amplified or restriction enzyme digested, resolved on a 1.5% agarose gel, gel extracted (Qiagen) and ligated into the MCS of the PGL4.14 vector (Stratagene). To create pLUC3.4, E1a[3] forward and E1c reverse primers (Table 2.1) were used to PCR amplify a 3.820Kb fragment, from the RP23 clone, which was then subcloned into a TA TOPO4.0 vector. This construct was digested with Not1, blunted with Klenow fragments (Fermentas) and then digested with Spe1. The resulting 3.742Kb fragment was inserted between the Nhe1 and EcoRV sites in the PGL4.14 vector. The pLUC2.5 construct was produced by cutting out a ~900bp region from the 5' end of the insert in pLUC3.4 with EcoR1. pLUC1.3 was generated through partial digestion of pLUC2.5 with HindIII. A ~1.7kb fragment was then gel purified (Qiagen) and inserted into the HindIII site of an empty pGL4.14 vector. Primers, containing Nhe1 and BglII restriction enzyme sites (Table 2.1), were used to PCR amplify the ~1.3kbp fragment inside pLUC1.0. pLUC0.7 was produced by digesting pLUC1.0 with HindIII and inserting the fragment into the HindIII site in pGL4.14. The pLUC0.72 and pLUC0.29 constructs were produced by double digestion of pLUC1.0 with NheI/BglII and NheI/HindIII, respectively, and the DNA fragments were inserted into compatible sites in the PGL4.14 vector. pLUC0.6 was generated through PCR amplification of a ~940bp region (Table 2.1) from the pLUC3.4 plasmid and subcloned into pCR2.1-TOPO. A 0.933kb fragment was excised from this construct with EcoR1, blunted with Klenow fragments and inserted into the blunted HindIII site in PGL4.14. A ~2.3kb and ~2.7kb fragment of the HuD 3'UTR was PCR amplified (with primers attached to xba1 sites) and subcloned into the

corresponding site in the PGL4.14 plasmid, creating pLUC3'UTR(L) and pLUC3'UTR(E), respectively. Either the E1c 5'RR (-573/+365) or thymidine kinase (TK) was used as a promoter. The Expand Long Template PCR kit (Roche) was used to perform all these PCRs, according to the manufacturer's instructions.

To generate FLAG-tagged *Ngn2*, the *Ngn2* ORF was digested with EcoRI from PC1G2-*Ngn2*, and inserted into pcDNA3.1 FLAG (Invitrogen). pCDNA3.1/His-*Ngn2* was produced by excising the *Ngn2* ORF from PC1G2-*Ngn2* with EcoRI and ligating into the same restriction site in pCDNA3.1/His (Invitrogen). FLAG-tagged and pcDNA3-hRAR was produced by PCR amplifying the hRAR ORF (Openbiosystems) and subcloning the fragment into pcDNA3.1 FLAG. Nucleotide mutations for different DNA *cis*-elements were generated using specific primers (Table 2.1) and the QuikChange Lightning Site Directed Mutagenesis Kit (Stratagene), according to the manufacturer's instructions. All insertions and orientations were verified by sequencing.

Transfections were performed using the LipofectAMINE 2000 reagent kit (Promega), as per manufacture's recommendation. Undifferentiated P19 cells (70-80% confluent) were transfected with a total of 1-12µg of plasmid DNA (*Ngn2*, *NeuroD* or *Mash1* cDNA containing constructs, either promoter-reporter or parental vector and phRG-TK vector [Promega], for transfection efficiency) one day prior to harvesting the cells. For differentiation experiments, P19 cells were transfected and then differentiated with RA or DMSO for 4 days, prior to harvesting. To determine reporter gene activity, transfected P19 cells were

washed with cold 1X PBS, scraped, and lysed in 1X reporter lysis buffer followed by three freeze-thaw cycles. Extracts were then centrifuged (10,000 x *g* for 10 min at 4 °C), and the resulting supernatant was assayed using the Dual-Luciferase Reporter Assay kit (Promega) and a Lumat AT 9507 luminometer (Berthold Technologies). Firefly luciferase activity was normalized to renilla (phRGTK) luciferase activity and expressed as fold activity over parental control.

2.8 In situ hybridization

In situ hybridization was performing as previously described (Young and Chang, 1998) with minor modifications. A cDNA fragment encoding HuD E1c was PCR amplified from P19 cells and subcloned into pCR4.0 TOPO plasmids (Invitrogen). Subsequently, the constructs were linearized with Pst1 or Not1 and used as templates for T7 or T3 RNA polymerase-mediated *in vitro* transcription of radiolabeled antisense and sense probes, respectively. The *in vitro* transcription mix contained 1 µg/µl DNA template, 1x transcription buffer, 10 mM DTT, 0.6 mM ATP and GTP, 0.5U/µl of RNase inhibitor, 1.5 µCi/µl ^{35}S-UTP and ^{35}S-CTP (Perkin Elmer) and 0.075 U/µl of T3/T7 RNA polymerase. The transcription reactions were incubated at 37°C for 2 h, cDNA templates were digested with 0.05 U/µl RNase-free DNase I and mixtures were cleared from unincorporated nucleotides with NAP-5 Sephadex columns (Amersham Biosciences). Radioprobes were subsequently ethanol precipitated and resuspended in 10mM DTT and hybridization buffer; 50% Formaldehyde, 300mM NaCl, 20mM Tris-HCl,

5mM EDTA pH 8.0, 10% Dextran Sulphate, 1X Denhardt's Solution and 50 µg/ml tRNA. Purified probes were stored at –80°C until use.

Mouse E14.5 embryos were cleared from the extra-embryonic membranes and the heads were removed and fixed in RNAse-free 4% paraformaldehyde (diluted in PBS) at 4 °C overnight before freezing in OCT at -80°C. Fourteen micrometer sections were mounted onto Superfrost plus microscope slides (Fisher) and post-fixed in 2% and then 4% PFA. Sections were permeabilized with 20 µg/µl proteinase K in 50 mM Tris-HCl and 5 mM EDTA, acetylated with 2.5% (w/v) acetic anhydride in a solution containing 100 mM triethanolamine, pH 7.5 and dehydrated. For hybridization, ~200,000 cpm *HuD* E1c antisense and sense probes were placed on tissue sections and slides were subsequently developed 1-2 weeks later in Kodak Dektol developer, and viewed and photographed using an Axiophot microscope (Carl Zeiss).

2.9 Electrophoresis mobility shift assay

EMSAs were conducted as previously described (Hellman and Fried, 2007). Briefly, NGN2, E47, hRAR and LacZ recombinant proteins were produced using the TNT Quick Coupled Transcription/Translation Kit (Promega) from pCDNA3.1/*His-Ngn2*, pCDNA3-*E47*, pcDNA3-*hRAR* and pCDNA3.1/*myc-His/LacZ* (Invitrogen), respectively, according to manufacturer's protocol. For production of the DNA probes, a 0.29kb fragment was digested out of the pLUC1.0 and pLUC1.0M2+3 plasmids using Nhe1/HindIII to obtain the wild-type and mutant sequences, respectively. DNA fragments were end-labeled with ^{32}P γ-ATP (Perkin Elmer) and T4 polynucleotide kinase (Fermentas). EMSA

reactions were performed by incubating ~50 000cpm of labeled probe with 1.5µl of recombinant NGN2, E47 hRAR and/or LacZ, 0.02µg/µl salmon sperm, 1.25µg/µl BSA, 1mM DTT, 7.5mM MgCl2, 20mM HEPES buffer, 0.2mM EDTA, 0.1M KCl and a proteinase inhibitor (Roche). For competition assays, a non-radioactive 0.29kb DNA fragment was added in molar excess (5-10X) 10 min before adding radiolabeled DNA. The reactions were incubated for 1hr at 30°C, resolved on non-denaturing 4.5% polyacrylamide gels and exposed on BioMAX MR film (Eastman Kodak).

2.10 Chromatin Immunoprecipitation

ChIP experiments were performed as previously described (Ravel-Chapuis et al., 2007), using the EZ-ChIP protocol (Millipore). An empty plasmid (FLAG-Basic) or plasmids containing the *Ngn2*/*hRAR* ORFs (FLAG-*Ngn2* and FLAG-*hRAR*, respectively) were transfected into undifferentiated P19 cells. Twenty-four hours after transfection, cells were fixed for 1hr in 1% formaldehyde, washed and resuspended in SDS lysis buffer. The lysate was sonnicated, incubated with 1 µg of αIgG (Santa Cruz), αFLAG (Sigma-Aldrich) or αPOLII (a kind gift from Dr. J. Côté) antibody overnight, and immunoprecipiated with Protein A/G PLUS beads (Santa Cruz). Immunoprecipitates were washed 5X and reverse cross-linked at 65°C for 4-5hrs. Following phenol/chloroform extraction, qPCR was performed with specific primers (Table 2.1) to identify regions of interest.

2.11 Immunocytochemistry

Immunocytochemistry was performed as previously described (Ravel-Chapuis et al., 2007). P19 cells were fixed and permeabilized with 4% formaldehyde and 0.5% Triton X-100, respectively. The cells were then incubated for 1hr with primary anti-HuB/C/D (Invitrogen) antibody diluted in 1% BSA, rinsed with PBS, and incubated for 1 h with Alexa Fluor 594 secondary antibody (Invitrogen). Subsequently, cells were washed, mounted with DAPI (Vectashield) mounting medium and visualized on an Axio Imager.Z1 microscope (Carl Zeiss). Images were processed with AxioVision software (Carl Zeiss) and Photoshop (CS5; Adobe).

2.12 Co-immunoprecipitation

Co-immunoprecipitations were performed as previously described (Ravel-Chapuis et al., 2012). P19, N2a and C_2C_{12} cells were transfected with indicated constructs and whole-cell lysates were extracted 24 h later using NP-40 buffer (25mM Tris, pH 7.5, 200mM NaCl, 0.5% NP-40, 2mM EDTA, 2mM MgCl2, and protease inhibitors [Complete]). Cell extracts were then precleared with protein A/G PLUS beads (Santa Cruz) and 5% of lysate were kept for the input control. Protein A/G PLUS beads were coated with 0.5-1 µg of specified antibodies and then incubated with cell extract overnight at 4°C. Beads were washed four times with NP-40 buffer, resuspended in 2X loading buffer, and used for Western blot analysis.

2.13 Statistical Analysis

The data were analyzed using two-tailed Student t-tests and the level of significance was set at $p<0.05$. Mean $\pm$ S.E.M. are presented throughout.

Chapter 3

Please note that part of the following work has been recently published in the Journal of Neuroscience (Bronicki et al., 2012). See appendix A on page 179 for permission to use published material.

3: RESULTS

3.1 Neuronal development models

HuD is predominantly expressed in neurons but low levels have also been detected in early neuronal progenitors and some non-neuronal cells (e.g. skeletal muscle and pancreatic beta cells; Okano and Darnell, 1997; Abdelmohsen et al., 2010). These findings indicate that developmentally early and cell-specific mechanisms govern expression of HuD. Accordingly, for our first aim we set out to establish a neuronal differentiation model to study the expression and regulation of HuD. We sought a model that demonstrates increased abundance of HuD mRNA and protein during early neuronal development, as previously described *in vitro* (Steller et al., 1996; Mansfield and Keene, 2011) and *in vivo* (Okano and Darnell, 1997; Hambardzumyan et al., 2009). To achieve this, we assessed the abundance of HuD in three different neuronal differentiation models.

3.1.1 Neurospheres

Initially, we tested the neurosphere model, which involves extraction of regions of the embryonic mouse brain and culturing them. When plated in non-coated culture dishes, these cells form suspended spheres called neurospheres and proliferate in the presence of epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF/FGF2). Neurospheres can be differentiated into a heterogeneous population consisting of neurons, astrocytes and

oligodendrocytes following removal of growth factor and plating on poly-L-ornithine-coated culture plates (Reynolds and Weiss, 1996; Vescovi et al., 1993). For these experiments, we obtained cells from the striatum of CD-1 mice, cultured them in the presence of bFGF and differentiated them by removing FGF. We harvested genomic RNA from proliferating and 6 day differentiated neurospheres and performed semi-quantitative RT-PCR to assess *HuD*, *GAP-43* (mRNA target of HuD and a neuronal differentiation marker) and *GAPDH* mRNA levels. The HuD primers used for these experiments spanned exons 6 and 7 of the mouse transcript (Table 2.1; HuDv2) and detected the two most abundant isoforms, HuD_{pro} and HuD (Inman et al., 1998; Steller et al., 1996; Tora et al., 2000). Our results showed that *GAP-43* transcript levels slightly decreased and *HuD* mRNA levels did not significantly change between proliferating and 6 day differentiated neurospheres (Figure 3.1A-C). These findings suggest that under the conditions used, neurospheres were not an ideal model to study HuD expression and regulation during neuronal development.

3.1.2 Brain embryonic development

As a second approach, we profiled expression of *HuD* and *GAP-43* mRNAs during embryonic neuronal development *in vivo*. We obtained whole brain genomic RNA from three different time points of mouse embryonic brain development (E10, E14 and E18) and assayed expression of *HuD*, *GAP-43*, *tau* (also neuronal development markers) and *GAPDH* mRNAs via semi-quantitative RT- PCR. We detected similar *HuD* mRNA levels between E10 and E18, and

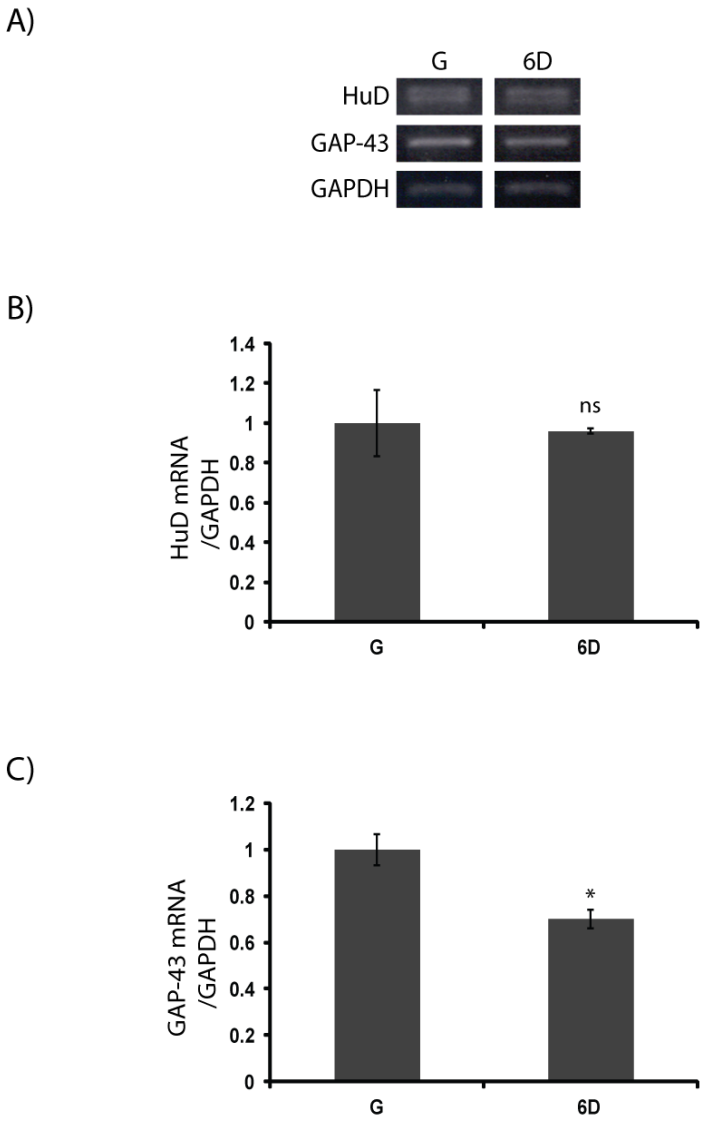


Figure 3.1 Expression of *HuD* and *GAP-43* mRNAs during neurosphere differentiation

Cells from E10.5 mouse forebrain were dissociated and plated in the presence of FGF to allow neurospheres formation (G). Neurospheres were subsequently differentiated for six days (6D) by removing growth factors. RNA from both time points was harvested and analysed via semi-quantitative RT-PCR. **(A)** Ethidium bromide-stained 1% agarose gels showing intensity of RT-PCR products. Graphs representing the quantification (Kodak Imager) of RT-PCR product intensity for **(B)** *HuD* and **(C)** *GAP-43*, normalized to *GAPDH*. Note that both major HuD variants, *HuD* and HuD_{pro}, were quantified together. Data are means ± SEM, n=3. *, $p<0.05$; ns, $p>0.05$ (Student's t-test).

observed a trend toward increased expression of *HuD* transcripts at E14 (Figure 3.2A and B). This trend is in line with previous findings that showed peak expression of *HuD* mRNA at E16 during mouse embryonic brain development (Hambardzumyan et al., 2009; Abdelmohsen et al., 2010). On the other hand, we observed a significant increase in *GAP-43* and *tau* mRNA levels by E18 of embryonic development (Figure 3.2A, C and D). These latter results are concomitant with previous studies outlining the important roles of GAP-43 in morphological differentiation of neurons (Maier et al., 1999; Routtenberg et al., 2000; Mani et al., 2001; Shen et al., 2008). Since we did not observe a significant augmentation in *HuD* mRNA levels *in vivo*, we set out to perform the majority of experiments in cell culture models and complement some of the findings using brain tissue.

3.1.3 The P19 cell line

Subsequently, we assessed the expression of HuD in the well-described pluripotent embryonal teratocarcinoma P19 cell line. These cells are conveniently differentiated into neuroectodermal or mesodermal lineages following a four-day treatment with all-trans retinoic acid (RA) or dimethyl sulfoxide (DMSO), respectively (McBurney et al., 1982). Equally important, these cells are easy to work with and amenable to various manipulations, such as DNA transfection (McBurney, 1993). Here, through immunocytochemistry experiments with a pan-neuronal anti-Hu antibody (16A11), we demonstrated that 4-day RA, but not DMSO, treated P19 cells express the principally neuron-restricted nELAV proteins (Figure 3.3A). We then set out to determine the levels of *HuD* mRNA at

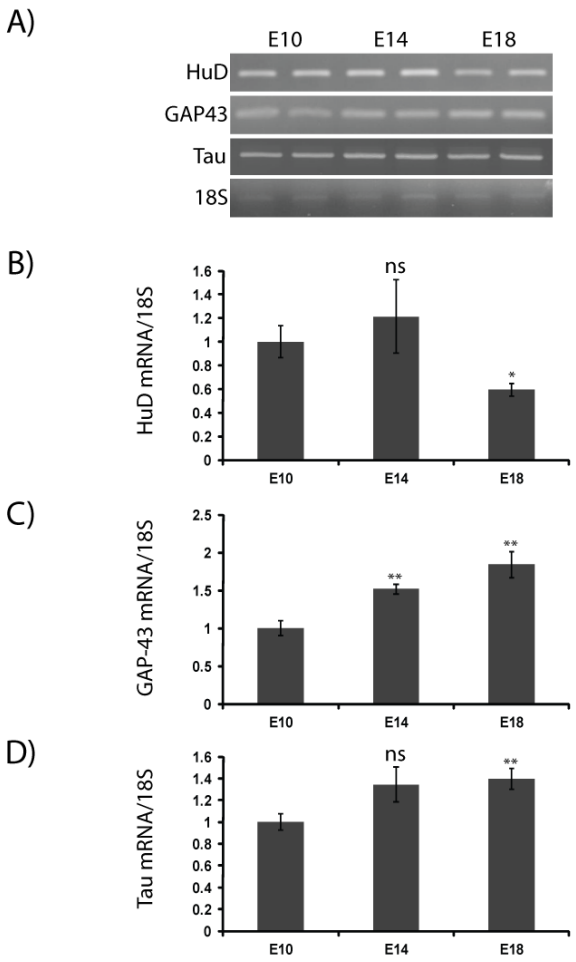


Figure 3.2 Expression of *HuD*, *GAP-43* and *tau* mRNAs during mouse embryonic brain development

Mouse brains were harvested at E10, E14 and E18 time points and processed for semi-quantitative RT-PCR analysis. **(A)** Ethidium bromide-stained 1% agarose gels showing intensity of RT-PCR products. Graphs representing the quantification (Kodak Imager) of RT-PCR product intensity for **(B)** *HuD*, **(C)** *GAP-43* and **(D)** *tau*, normalized to *18S*. Data are means ± SEM, n=4. *, $p<0.05$; **, $p<0.01$; ns, $p>0.05$ (Student's t-test).

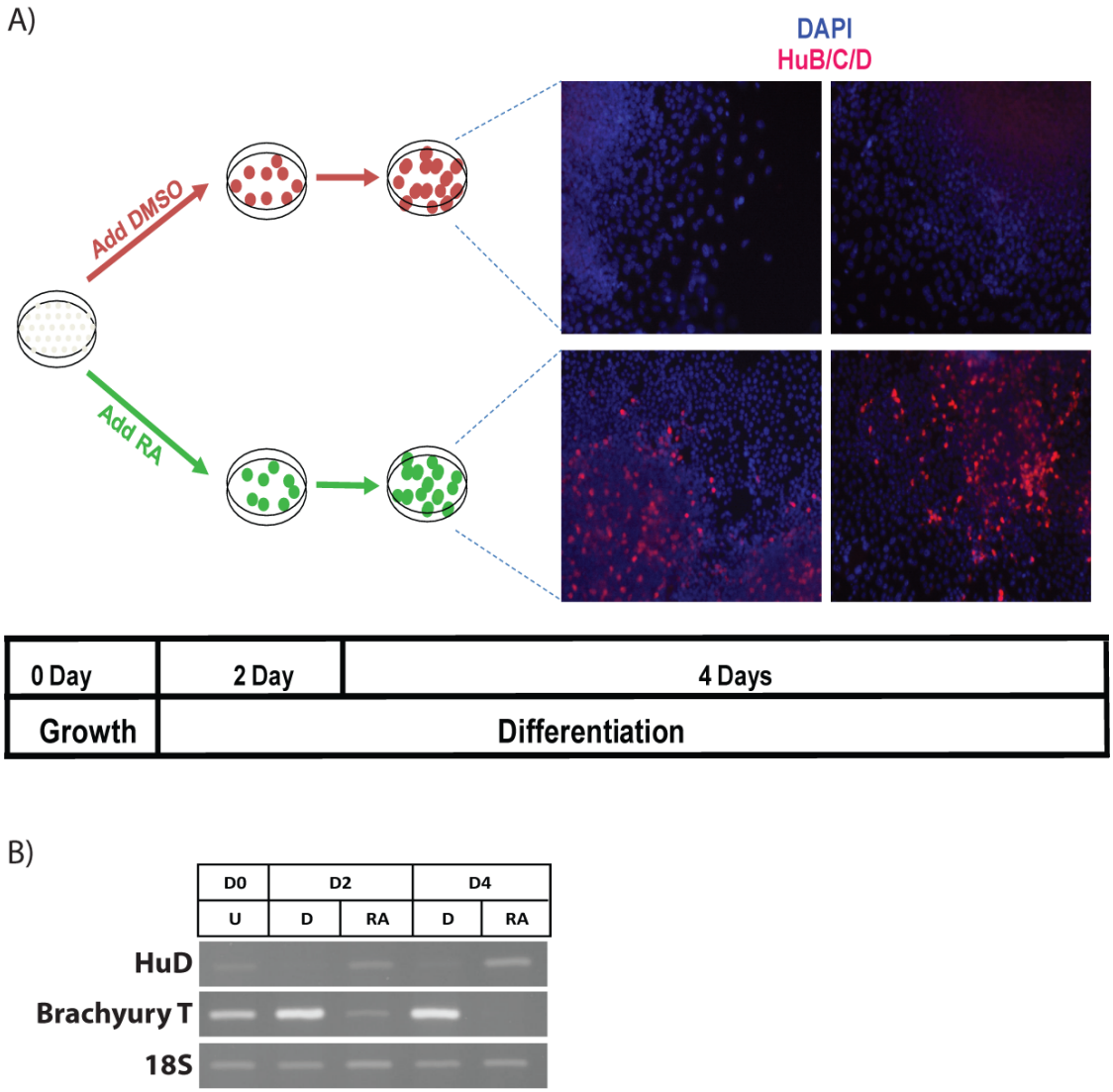


Figure 3.3 Differentiation of P19 cells into neuronal and mesodermal lineages

(A) Schematic diagram illustrating P19 cell differentiation into neurons or mesodermal cells following RA or DMSO treatment, respectively. *Right*; representative images of 4-day DMSO (top) or RA (bottom) -treated P19 cells immunostained with the anti-HuB/C/D antibody (16A11) showing the presence of nELAVs only in neuronal cells. n=2. Cell nuclei were counter stained with DAPI. **(B)** Ethidium bromide-stained 1% agarose gels showing RT-PCR product intensity of *HuD*, *Brachyury T* and *18S* transcripts during P19 cell differentiation. n=3.

different time-points of P19 differentiation into neuronal and mesodermal lineages. Semi-quantitative RT-PCR assays demonstrated that *HuD* transcripts were induced in differentiating neuronal cells by day 2, with the highest expression observed at day 4, compared to day 0 (Figure 3.3B). We also detected low *HuD* mRNA levels at different time points of mesodermal differentiation (Figure 3.3B), which is in agreement with recent studies showing minor *HuD* mRNA expression in skeletal muscle (Abdelmohsen et al., 2010). As a positive control for mesodermal differentiation, we also performed RT-PCR assays with *brachyury T*-specific primers, an mRNA encoding a TF important for proper mesodermal development (Showell et al., 2004), and found that it is highly expressed in mesoderm but barely detectable in neuronal P19 cells (Vidricaire et al., 1994). These experiments validated P19 cells as a useful model to investigate the mechanisms governing neuron-restrictive HuD expression during early development.

3.2 Neuron-specific expression of HuD is transcriptionally regulated

For our second objective, we wanted to determine whether expression of *HuD* mRNA is governed by neuron-specific transcriptional and/or post-transcriptional mechanisms. We first quantified *HuD* mRNA levels during early P19 differentiation into neurons and mesoderm to further assess its temporal neuron-specific expression. These experiments revealed a significant increase in the abundance of *HuD* transcripts after one day of neurogenesis ($p<0.05$), with the highest levels measured at 4 days ($p<0.05$), compared to undifferentiated

cells (Figure 3.4A). On the other hand, *HuD* mRNA levels remained unchanged during mesodermal differentiation. As a positive control for neuronal differentiation, we also quantified expression of *GAP-43* mRNA, a well-known *HuD* mRNA target. A robust (p<0.05) induction in *GAP-43* transcripts was detected after one day and its expression levels were increased only during neuronal differentiation (Figure 3.4B). The discordance between *HuD* and *GAP-43* mRNA levels during early P19 neurogenesis (Figure 3.4A and 3.4B) is likely a consequence of transcriptional induction of GAP-43 transcripts during early neurogenesis (Zhao et al., 2012). The implications of these findings are that early neuron-specific molecular events control expression of *HuD* mRNA during neurogenesis.

Based on these findings, we set out to determine if the neuron-specific induction of HuD is dependent on transcriptional and/or post-transcriptional events. To achieve this, we performed modified nuclear run-on assays using nuclei isolated from 4-day RA (neuronal lineage) and DMSO (mesodermal lineage) -treated P19 cells. Our results demonstrated increased levels of *HuD* mRNA in actively transcribing nuclei obtained from neuronal (p<0.05), but not mesoderm (p>0.05) cells (Figure 3.5). We also detected a small, although not significant (p>0.05), augmentation in *HuD* mRNA synthesis in mesodermal cells, which likely accounts for the low levels of *HuD* transcripts detected in these cells (Figure 3.5). These findings indicate that transcriptional mechanisms are, at least partially, responsible for the neuron-specific induction of HuD.

Recent studies have demonstrated that the abundance of *HuD* mRNA is

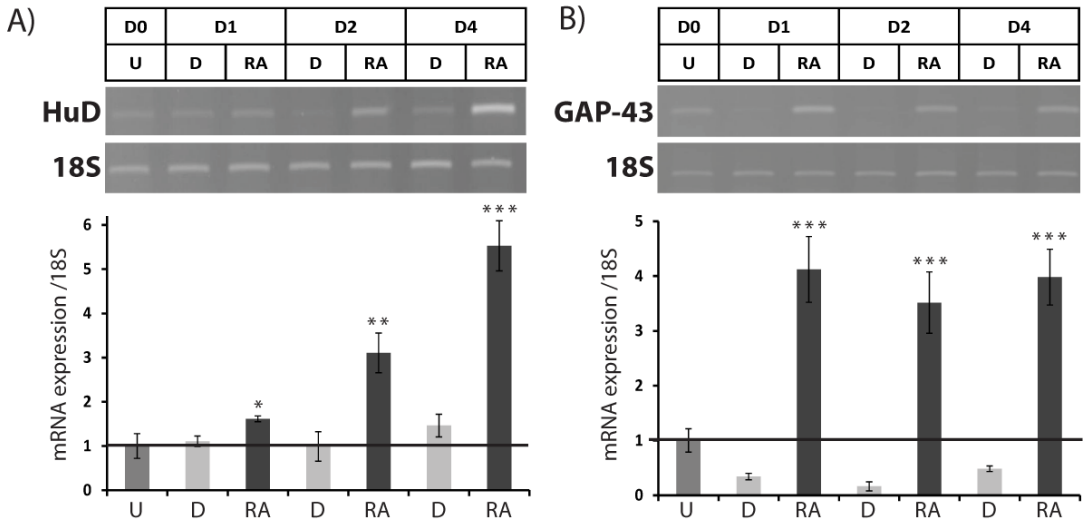


Figure 3.4 Neuron-specific expression of *HuD* mRNA in differentiating P19 cells

Representative ethidium bromide-stained 1% agarose gels (*top*) and quantification (*bottom*) showing increase in mRNA expression of **(A)** pan (all E1 isoforms) *HuD* and **(B)** *GAP-43* at different time points during P19 cell neuronal or mesodermal differentiation. Image J software (NIH) was used to quantify RT-PCR band intensities. Values were normalized over *18S*. Data are means ± SEM, n=3**.** *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ns, $p>0.05$ (Student's t-test).

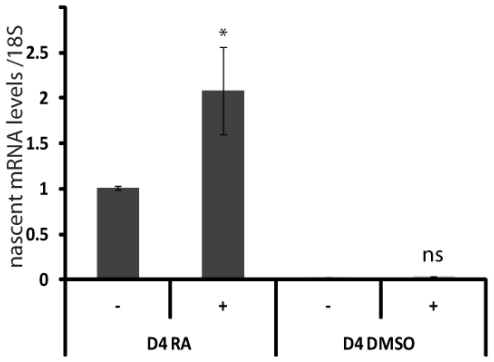


Figure 3.5 Transcription of *HuD* during early neuronal development

Nuclear run-on assay showing altered nascent *HuD* transcript synthesis in neuronal and mesoderm P19 cells. Nuclei from neuronal and mesoderm cells were harvested and incubated in a transcription reaction mixture (+) or vehicle (-). Endogenous *HuD* and *18S* mRNA levels were quantified using real-time quantitative RT-PCR. Values were normalized to *18S*. Data are means ± SEM, n=4. *, $p<0.05$; ns, $p>0.05$ (Student's t-test).

also subject to post-transcriptional control by RBPs and at least one miR (Abdelmohsen et al., 2010; Bolognani et al., 2010). *HuD* contains a long 3'UTR that is well conserved amongst all species (Figure 3.6A). In view of these findings, and since we (Figure 3.4A) and others have detected basal expression of HuD in non-neuronal cells (Abdelmohsen et al., 2010; Lee et al., 2012), we decided to complement our transcription assays by determining whether stability of *HuD* mRNA is also regulated in a cell-specific manner. To this end, we initially treated neuronal and mesodermal P19 cells with actinomycin D and measured endogenous *HuD* mRNA levels at different time-points thereafter. Using this approach, we obtained analogous endogenous *HuD* mRNA half-lives in both cell types (neurons; 3.81±0.67h and mesodermal cells; 4.00±1.00h; $p>0.05$), indicating that similar post-transcriptional mechanisms regulate expression of *HuD* mRNA in developing neuronal and mesoderm cells (Figure 3.6B). These results are comparable to the previously published half-life values of *HuD* transcripts obtained in embryonic stem cells (Sharova et al., 2009).

To further investigate this issue, we conducted a series of experiments to elucidate whether the *HuD* 3'UTR has an effect on *HuD* mRNA levels in developing neurons. Initially, we generated chimeric *HuD* 3'UTR (accession number NM_010488; nucleotides 1,613 to 3,900) reporter constructs, transfected them into P19 cells and measured luciferase activity following 4-days of neuronal or mesodermal differentiation. Our results revealed similar relative luciferase activity in both cell types, suggesting that the ~2.3kb fragment of the *HuD* 3'UTR

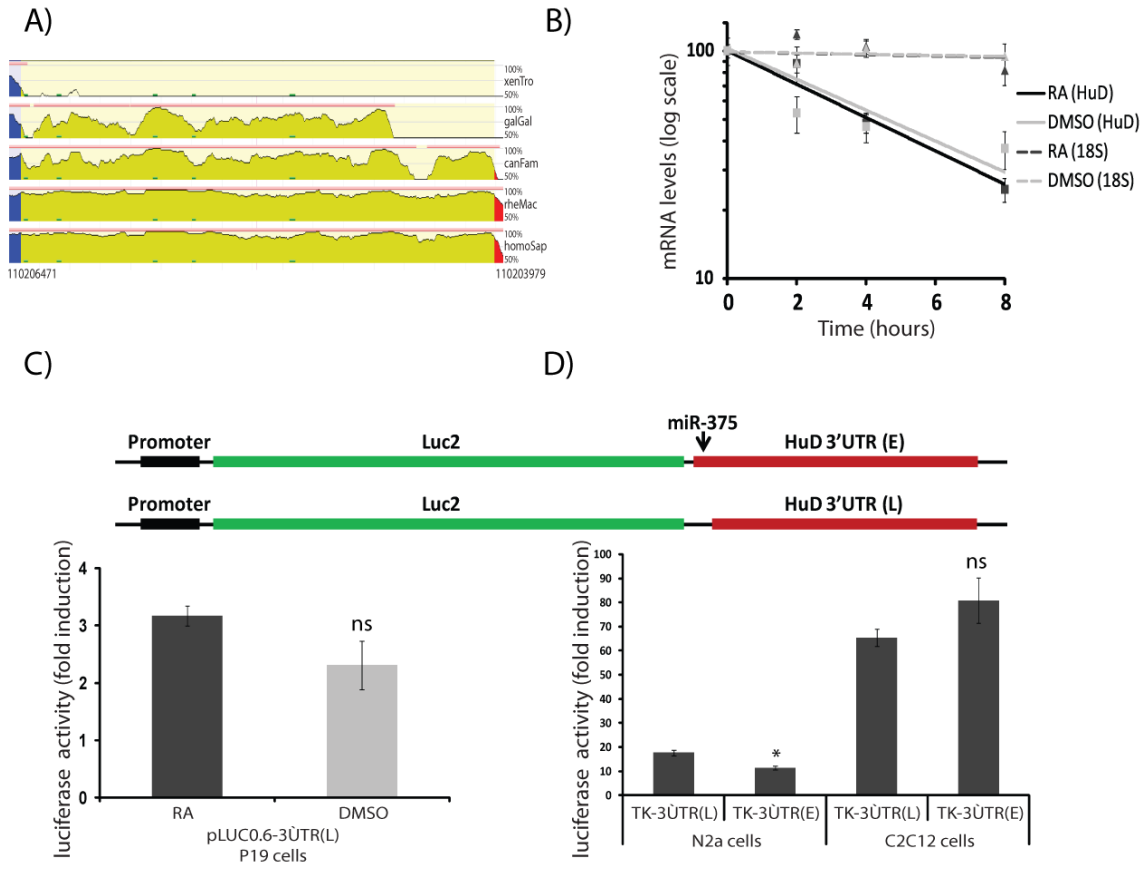


Figure 3.6 Similar post-transcriptional regulation of *HuD* mRNA between early neuronal and mesodermal cells

(A) Evolutionary Conserved Region (ECR) browser (Ovcharenko, M.A et al. 2006) was used to generate a schematic showing the DNA percent identity of the *HuD* 3'UTR sequence between *Mus musculus, Homo sapiens, Rhesus macaque, Canis farmiliaris, Gallus gallus and Xenopus tropicalis*. Pink horizontal lines are well-conserved regions (>70%). Red, yellow and blue indicate intergenic regions, UTRs and coding regions, respectively. **(B)** Endogenous mRNA stability of *HuD* and *18S* transcripts in neuronal and non-neuronal cells. Four-day neuron and mesoderm differentiated P19 cells were treated with actinomycin D (4µg/µl) for the indicated time points. Endogenous *HuD* mRNA expression was quantified through qPCR. n=4 **(C)** The chimeric luciferase–*HuD* 3'UTR constructs contained the 0.6kb (-573/+365) promoter, the firefly luciferase-coding region and majority of the mouse *HuD* 3'UTR (~2.1kb). Note that there's no significant difference in the expression of chimeric luciferase-HuD 3'UTR transcripts in the RA versus DMSO treated P19 cells. n=4. **(D)** Regulation of the full-length (~2.5kb) versus the shorter (~2.1kb) *HuD* 3'UTR in neuroblasts and myoblasts. Chimeric constructs were created where the full length (E) or shorter (L) *HuD* 3'UTR were cloned downstream of the luciferase ORF, driven by the thymidine kinase (TK) promoter. Both constructs were transfected into N2a and C_2C_{12} cells. All values were standardized to renilla luciferase promoter (phRG) activity. Data are means ± SEM, n=3. *, $p<0.05$; ns, $p>0.05$ (Student's t-test).

is not differentially regulated between 4-day neuronal and mesodermal P19 cells (Figure 3.6C).

Since we obtained these results, a study has emerged demonstrating that *HuD* is post-transcriptionally regulated by miR-375 through a binding site in its 3'UTR (Abdelmohsen et al., 2010). To test whether miR-375 affects abundance of *HuD* transcripts, we compared reporter activity between wild-type and miR-375 site deleted chimeric *HuD* 3'UTR constructs (Figure 3.6D). For these experiments, we used N2a neuroblastoma and C_2C_{12} myoblasts culture cell lines as surrogates for differentiated P19 neuronal and mesodermal lineages, respectively. Our results show that the wild-type construct (E) produced slightly less reporter activity compared to deletion construct (L) in N2a cells ($p<0.05$), while no significant difference in luciferase activity was detected between the two constructs in C_2C_{12} cells (Figure 3.6D). These results indicate that regulation of both *HuD* 3'UTR constructs is comparable in immature neuronal and muscle cells. Taken as a whole, these studies stress that expression of *HuD* mRNA is primarily transcriptionally regulated in developing P19 neurons.

3.3 The 5' genomic region of *HuD* contains eight E1 variants

Although the 5' end of the mammalian *HuD* gene is relatively uncharacterized, one previous study identified three non-coding exon 1 variants (E1a, E1b and E1c) upstream of E2 by aligning available mouse mRNA sequences (Inman et al., 1998). For our third objective, we wanted to gain further insight into these *HuD* E1 variants. To this end, we searched the NCBI RefSeq database (http://www.ncbi.nlm.nih.gov/RefSeq) and spliced expressed sequence

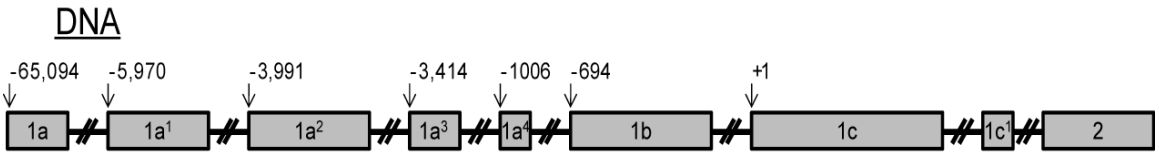
tags (ESTs) listed in the USCS genome browser (http://genome.ucsc.edu/) for related mouse and human sequences. Interestingly, alignment of existing *HuD* mRNA sequences (see Table 3.1 for accession numbers) with the *HuD* 5' genomic flanking region revealed six distinct 5' ends, providing additional evidence that multiple leader exons exist. To determine whether these E1 variants are expressed in developing mouse neurons, 5′-RACE experiments were conducted using RNA from 4-day differentiated P19 neurons. Using a *HuD* exon 2-specific reverse primer, we obtained a diverse range (50-400bp) of PCR products, which were subsequently subcloned and sequenced. Alignment of 5'RACE clones with the available leader exons revealed two novel *HuD* E1 splice variants (E1a^4 and E1c^1; Figures 3.7A and B and Table 3.1). These eight leader exons were mapped onto the *HuD* gene locus and named in accordance with the previously suggested nomenclature (Inman et al., 1998; E1a, E1a^1, E1a^2, E1a^3, E1a^4, E1b, E1c and E1c^1; Figure 3.7).

Examination of the exon/intron boundaries revealed the presence of canonical splice donor sites at the 3' end of each exon (Figure 3.7B). Moreover, sequencing of 5' RACE clones showed that each E1 variant was individually spliced to a common E2, except for E1c^1, which was found between E1c and E2. Interspecies nucleotide sequence comparison demonstrated that most E1 variants were highly conserved (>85%) amongst higher vertebrates except for E1a and E1c^1, which were conserved but to a lesser degree (Figures 3.7B and 3.8). Interestingly, almost all E1 isoforms contain an in-frame translation initiation codon (ATG), which raises the possibility that part of these exons encode

Table 3.1 Accession numbers for *HuD* E1 variants from Refseq, EST and Ensembl databases

	E1a	E1a[1]	E1a[2]	E1a[3]	E1a[4]	E1b	E1c	E1c[1]
RefSeq mouse mRNA	NM_001163397.1	n/a	n/a	n/a	n/a	NM_001038698.1	NM_001163399.1 NM_010488.4	n/a
Ensembl mouse transcript # and ID	Elavl4-001 (ENSMUST00000106603)	Elavl4-007 (ENSMUST00000106601)	Elavl4-008 (ENSMUST00000142722)	Elavl4-006 (ENSMUST00000106600)	n/a	Elavl4-002 (ENSMUST00000102723) Elavl4-003 (ENSMUST00000106598) Elavl4-011 (ENSMUST00000153906)	Elavl4-004 (ENSMUST00000106597) Elavl4-005 (ENSMUST00000102722) Elavl4-012 (ENSMUST00000138972)	Elavl4-010 (ENSMUSG00000028546)
Mouse ESTs	CO432423 CX217483 CF726962	n/a	BY095572 BY077759 BY277368 BY279276	BB868926	JX178292	BU701986 BG807610 BQ769443 CD776418 BU057510 BG807621 BI985239 BU920121 BB626228 BI991095 BU918620 BG804672 BG807880 CN663544 W18631 CA329034 BE653746 BY789145 BB872444 BB873655	CA527274 CJ056024 BQ770553 BB626275 BY274447 BB648788 BY290872 CJ133636 CD552588 BP769869 BB869084	DQ437511.1
RefSeq human mRNA	NM_001144777.1	NM_001144776.1	n/a	NM_001144775.1	n/a	NM_001144774.1 NM_021952.3	n/a	n/a
Ensembl human transcript # and ID	Elavl4-201 (ENST00000448907)	Elavl4-001 (ENST00000371827)	n/a	Elavl4-002 (ENST00000357083)	n/a	Elavl4-004 (ENST00000371823) Elavl4-003 (ENST00000371824)	Elavl4-005 (ENST00000371821) Elavl4-006 (ENST00000371819)	n/a
Human ESTs	DC351092 DA904958 AL525200 BX386941	n/a	n/a	BM509527 BQ267762	n/a	CK005107 DB472040 DC360842 DC361109 DC337942 DC352242 DC360175 BI604034 DB501125 AL530909 AU100542	DC367063 DC363892 BM693606 BI914284 DC366017 AA214645 DC338260 BP192961 DC365508 BX464139 AW965319 BX282543 DN993403 BM928123 BM928402 BP193370 BM711406 BM716630 DA608723 AA161265	n/a

A)



B)

Exon	Mouse vs Human (% nt identity)	Splice Donor (E1)	Splice Acceptor (E2)	Number of ESTs (mouse)	Number of ESTs (human)	E14.5	Adult
1a	63	AGgt	agGT	3	4		
1a^1	93	AGgt	agGT	0	0		
1a^2	98	AGgt	agGT	4	0		
1a^3	96	TGgt	agGT	1	2		
1a^4	85	AGgt	agGT	0	0		
1b	87	TGgt	agGT	20	11		
1c	95	TGgt	agGT	11	20		
1c^1	32	AGgt	agGT	1	0		

C)

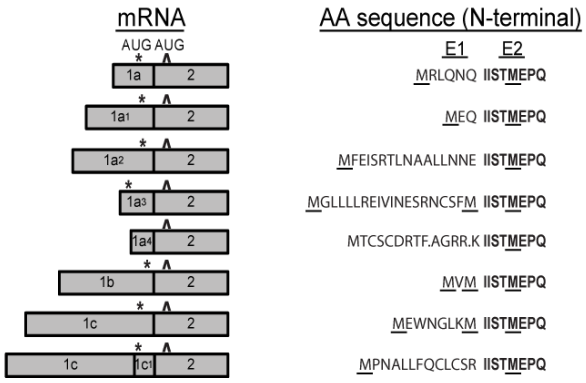


Figure 3.7 The *HuD* 5'RR contains eight alternative E1 variants

(A) Schematic diagram illustrating the genomic organization of *HuD* E1 splice variants and their transcription start sites (TSS) relative to the TSS of E1c. **(B)** Table showing percent identity between mouse and human sequences, the splice donor/acceptor sites and the known number of human and mouse ESTs for each leader exon. Also displayed are representative ethidium bromide-stained 1.5% agarose gels showing RT-PCR product of each *HuD* E1 variant in embryonic (E14.5) and adult (2 months) mouse brain. **(C)** Schematic demonstrating alternative splicing of each E1 variant to a common E2, the favoured (^) versus putative alternate (*) translation start site (AUG) and the possible alternate N-terminal amino acid (AA) sequences encoded by each E1 variant. Methionines (M) are underlined. Identification of E1 variants is based on 5'RACE assays and bioinfomatic analysis.

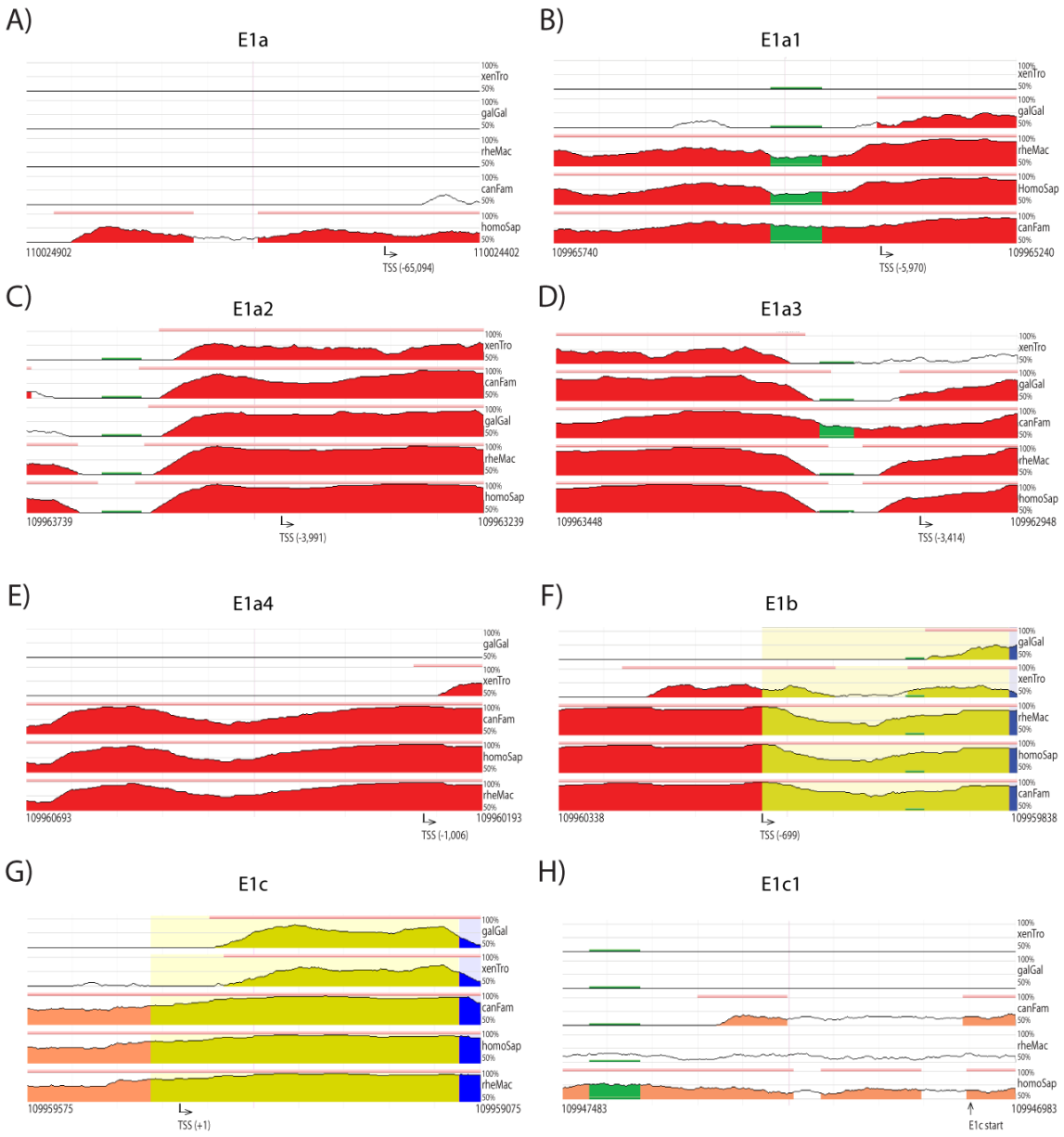


Figure 3.8 Sequence identity amongst metazoan species of *HuD* E1 variants and their 5'RRs

Evolutionary Conserved Region (ECR) browser (Ovcharenko, M.A et al. 2006) was used to produce a schematic showing the DNA percent identity of *HuD* **(A)** E1a, **(B)** E1a[1], **(C)** E1a[2], **(D)** E1a[3], **(E)** E1a[4], **(F)** E1b, **(G)** E1c and **(H)** E1c[1] variants between *Mus musculus, Homo sapiens, Rhesus macaque, Canis farmiliaris, Gallus gallus and Xenopus tropicalis* species. Pink horizontal lines indicate well-conserved regions (>70%). Red, green, salmon, yellow and blue regions represent intergenic regions, transposons and simple repeats, intronic regions, UTRs and coding regions, respectively. Also indicated are nucleotide locations of shown regions within chromosome 4 and the predominant TSS of each *HuD* E1 relative to the TSS of E1c.

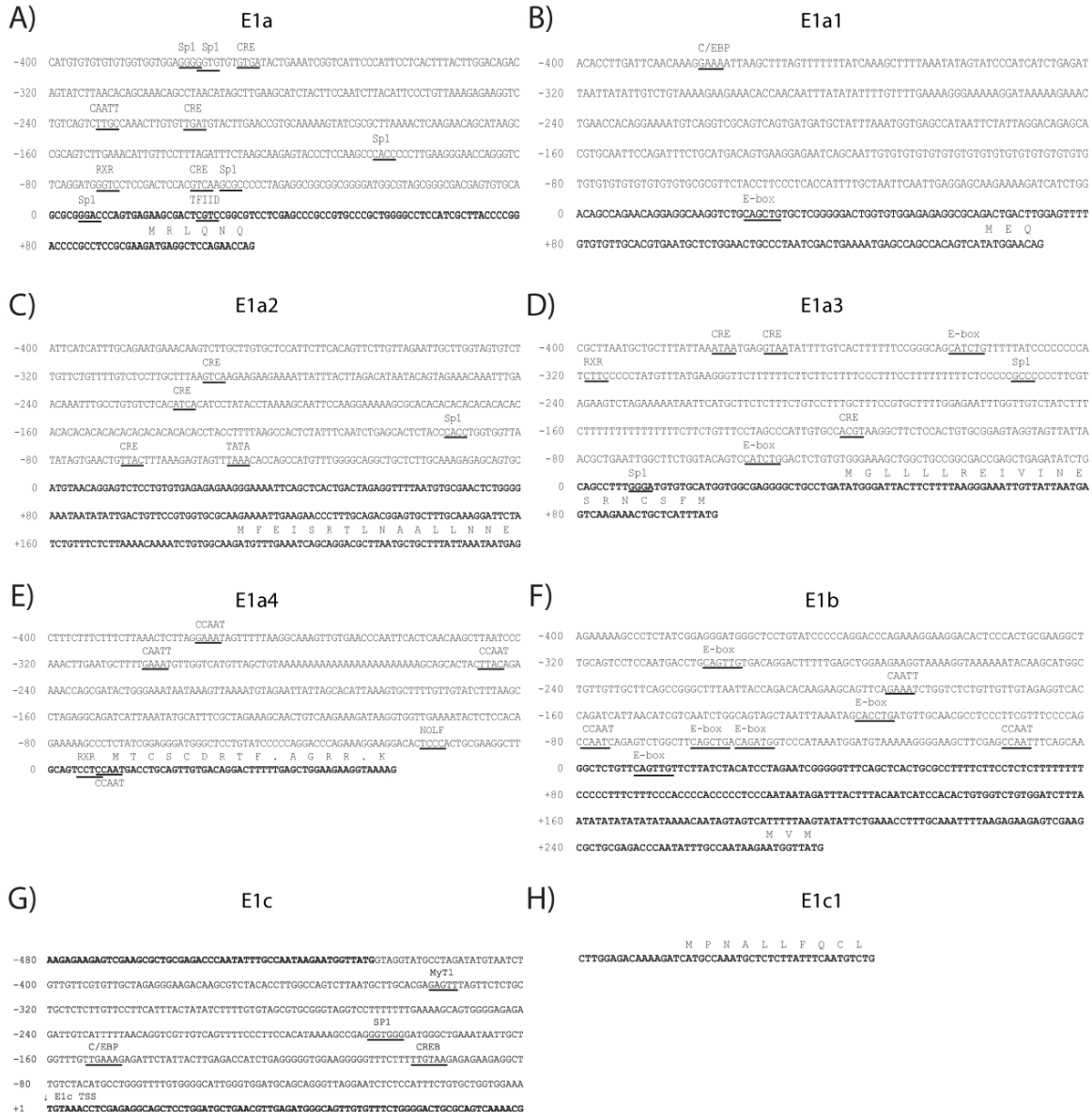


Figure 3.9 Complete 5'RR sequence of each E1 variant

The upstream sequences for the *HuD* **(A)** E1a, **(B)** E1a[1], **(C)** E1a[2], **(D)** E1a[3], **(E)** E1a[4], **(F)** E1b, **(G)** E1c and **(H)** E1c[1] variants depicting the intronic (regular font), exonic (bold font) and putative *cis*-elements (underlined). +1 designates the major transcription start site identified through 5'RACE.

alternate N-terminals of the HuD protein (Figures 3.7C and 3.9). The exception to this is E1a^4, since it contains two stop codons downstream of the AUG (Figure 3.7C and 3.9E). Collectively, these results demonstrate that the mammalian *HuD* gene contains eight conserved leader exons, indicative of complex transcriptional regulation and alternate 5' ends in *HuD* transcripts.

We confirmed expression of all eight E1 variants by RT-PCR on RNA samples from P19 cells (Figure 3.10), embryonic (E14.5) and adult murine brains (~6 weeks; Figure 3.11). During P19 differentiation, we found that all eight E1s were up-regulated by 4 days in neurons and we detected minor levels of most variants in mesodermal cells (Figures 3.10). These findings illustrate that although these E1 variants are detectable in non-neuronal cells, they are predominantly expressed in neurons. Interestingly, we found a developmental decrease in some E1 containing transcripts (E1a, E1a^1, E1a^2 and E1b) between mouse embryonic and adult brains developmental time points (Figures 3.11). The reduced expression of these four E1 variants could account for the decrease in pan (all isoforms) *HuD* transcript levels known to occur from late mouse embryonic development to adulthood (Hambardzumyan et al., 2009; Abdelmohsen et al., 2010).

Analysis of the mouse and human spliced EST sequences suggested that E1c, along with E1b, are two of the most abundant HuD E1 variants (Table 3.1). In agreement with this, we found that the majority (74%) of 5'RACE products encoded E1c. Based on these findings, we focused hereafter on characterizing the expression pattern and regulation of E1c-containing *HuD* transcripts.

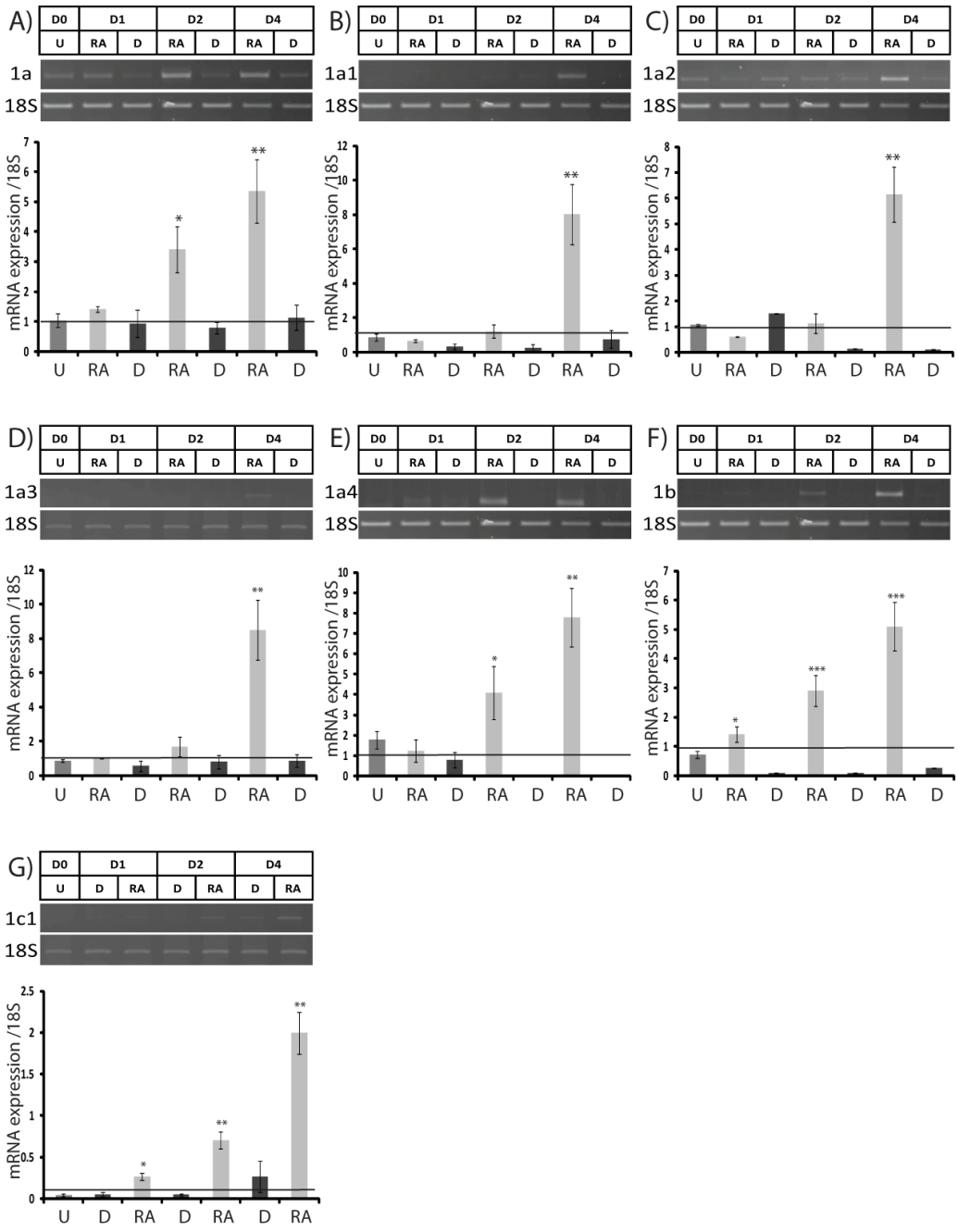


Figure 3.10 Temporal expression profiles of seven *HuD* E1 variants in differentiating P19 cells

Top: Ethidium bromide-stained 1% agarose gels showing RT-PCR product intensity of bands representing *HuD* **(A)** E1a, **(B)** E1a[1], **(C)** E1a[2], **(D)** E1a[3], **(E)** E1a[4], **(F)** E1b, **(G)** E1c[1] and *18S* at different time points of P19 cell differentiation. Primers spanning the E1-E2 boundary of *HuD* mRNA were used for PCR amplification of each E1 isoform. *Bottom:* Graphs representing the quantification (Image J) of RT-PCR product intensity, normalized to 18S. Data are means ± SEM, n=3. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ns, $p>0.05$ (Student's t-test).

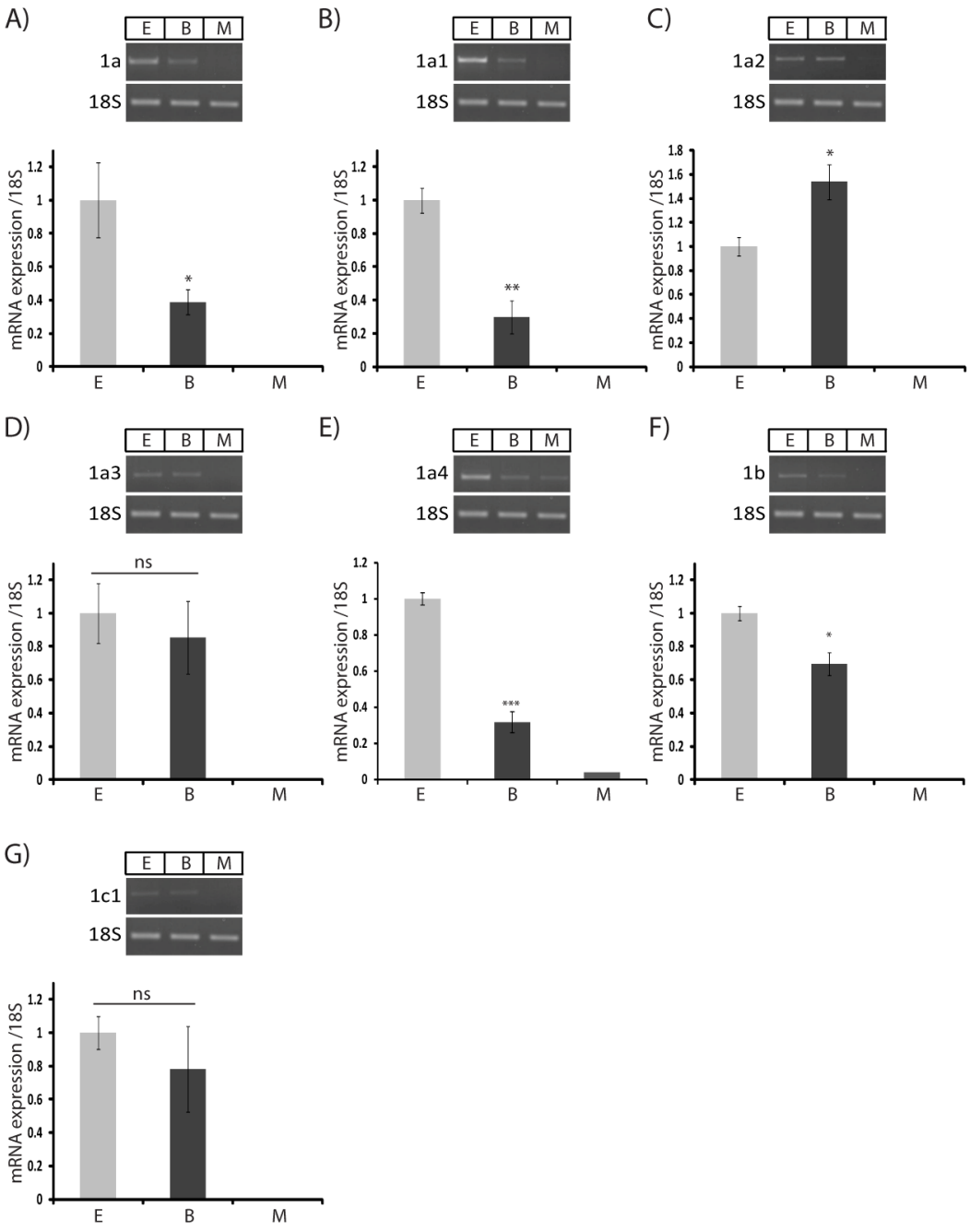


Figure 3.11 Expression of seven *HuD* E1 variants in embryonic and adult brain

Top: Ethidium bromide-stained 1% agarose gels showing RT-PCR product intensity of bands representing *HuD* **(A)** E1a, **(B)** E1a[1], **(C)** E1a[2], **(D)** E1a[3], **(E)** E1a[4], **(F)** E1b, **(G)** E1c[1] and *18S* at different stages of P19 cell differentiation. Primers spanning the E1-E2 boundary of *HuD* mRNA were used for PCR amplification of each E1 isoform. *Bottom:* Graphs representing the quantification (Image J) of RT-PCR product intensity, normalized to 18S. E= Embryonic brain, B= adult brain and M= skeletal muscle. Data are means ± SEM, n=3. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ns, $p>0.05$ (Student's t-test).

3.4 Spatial and temporal expression of the *HuD* E1c variant

For our fourth objective, we set out to determine cell-type specific expression of E1c-containing *HuD* mRNA in neurons. We first performed semi-quantitative RT-PCR with exon-specific forward primers and a common E2 reverse primer. The abundance of *HuD* mRNAs containing E1c significantly increased after 1 day of neuronal differentiation ($p<0.05$), with the highest levels measured at day 4 ($p<0.05$; Figures 3.12A). Notably, this pattern of expression parallels that of pan *HuD* mRNA (Figure 3.4A). E1c mRNA levels remained relatively stable during mesodermal P19 differentiation (Figures 3.12A).

Subsequently, we set out to establish the pattern of E1c expression in mouse embryonic (E14.5) and adult (~6 weeks) brains. Similar levels of E1c-bearing mRNA abundance were found in embryonic and adult brains, whereas no expression was detected in adult skeletal muscle (used as a control; Figures 3.12B). These *in vivo* results parallel our cell culture findings, which show that E1c is highly abundant in neuronal cells but is barely expressed or not detectable in a mesodermal lineage.

In order to examine the spatial distribution of *HuD* E1c-containing mRNAs in mouse embryonic brain, we performed *in situ* hybridization experiments with probes specific to E1c. Dispersed expression of E1c was observed at E14.5 throughout the brain, including the developing amygdala, hippocampus, and cerebral cortex (Figure 3.12C). In addition, we detected strong expression of E1c in the olfactory bulb and retina (Figure 3.12C). The localized expression of E1c-containing transcripts in the embryonic brain parallels the previously reported

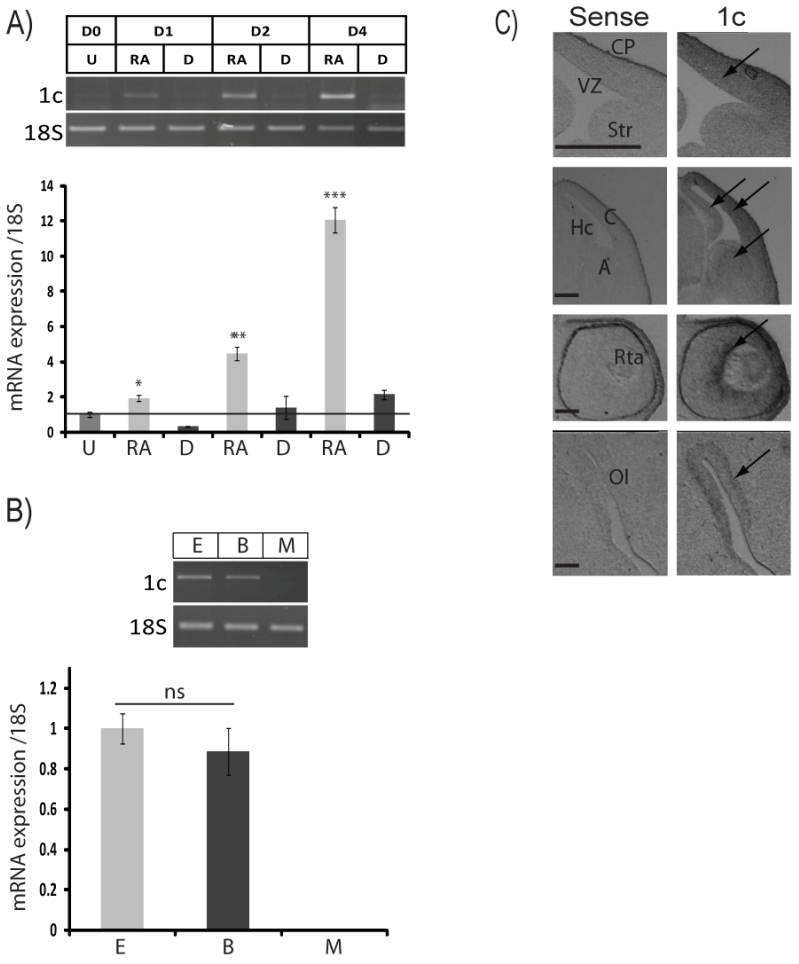


Figure 3.12 Expression profile of *HuD* E1c-containing transcripts in differentiating P19 cells, developing mouse brain and mouse skeletal muscle

Top: Ethidium bromide-stained 1.5% agarose gels showing RT-PCR product intensity of the *HuD* E1c variant and *18S* at different stages of **(A)** P19 cell differentiation and **(B)** mouse brain development. Primers spanning the E1c-E2 boundary of *HuD* mRNA were used for PCR amplification of each E1 isoform. *Bottom:* Graphs representing the quantification (Image J) of RT-PCR product intensity, normalized to *18S*. Data are means ± SEM, n=3. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ns, $p>0.05$ (Student's t-test). E- embryonic brain, B- adult brain and M- adult skeletal muscle. **(C)** *In situ* hybridization using a S^{35} radiolabelled RNA probe against E1c on coronal sections of embryonic day 14.5 mouse head (C57*Bl*6). Representative micrographs showing hybridization of *HuD* E1c sense and antisense probes on E14.5 mouse midbrain sections. Arrows point to amygdala (A), cerebral cortex (C), cortical plate (CP), hippocampus (Hc), olfactory bulb (OB), retina (Rta), striatum (Str) and ventricular zone (VZ). Scale bar= 100µm.

expression of pan (all isoforms) *HuD* mRNA, indicating that E1c is present in most, if not all, HuD-expressing neurons in the developing brain (Okano and Darnell, 1997; Clayton et al., 1998).

3.5 Role of the 1c 5'UTR in controlling HuD translation

To obtain some insight into the function of E1c, we wanted to assess the effect of the 5'UTR encoded by E1c on HuD protein levels. Alternate leader exons have been shown to differently affect translation of A disintegrin and metalloprotease domain 22 (Adam22; Godde et al., 2007) and peroxisome proliferator-activated receptor (PPARβ/δ; Larsen et al., 2002). For these experiments, we generated chimeric reporter constructs with the entire 1c or 1a (used as a control) 5'UTRs fused to the *firefly luciferase* ORF (Figure 3.13A). In addition, these constructs contained the full-length *HuD* 3'UTR attached to the *luciferase* 3' end. We transfected these constructs into undifferentiated P19 cells and harvested the cellular lysates the following day to establish reporter activity. Our results indicate that the 1c 5'UTR decreased reporter activity as compared to the 1a 5'UTR and the parental control (Figure 3.13B). These findings suggest that in undifferentiated P19 cells, the 1c 5'UTR is under negative post-transcriptional and/or translational control thereby producing reduced HuD protein levels.

3.6 Identification of functional regulatory regions upstream of *HuD* E1c

Since E1c is one of the major *HuD* leader exons expressed in developing

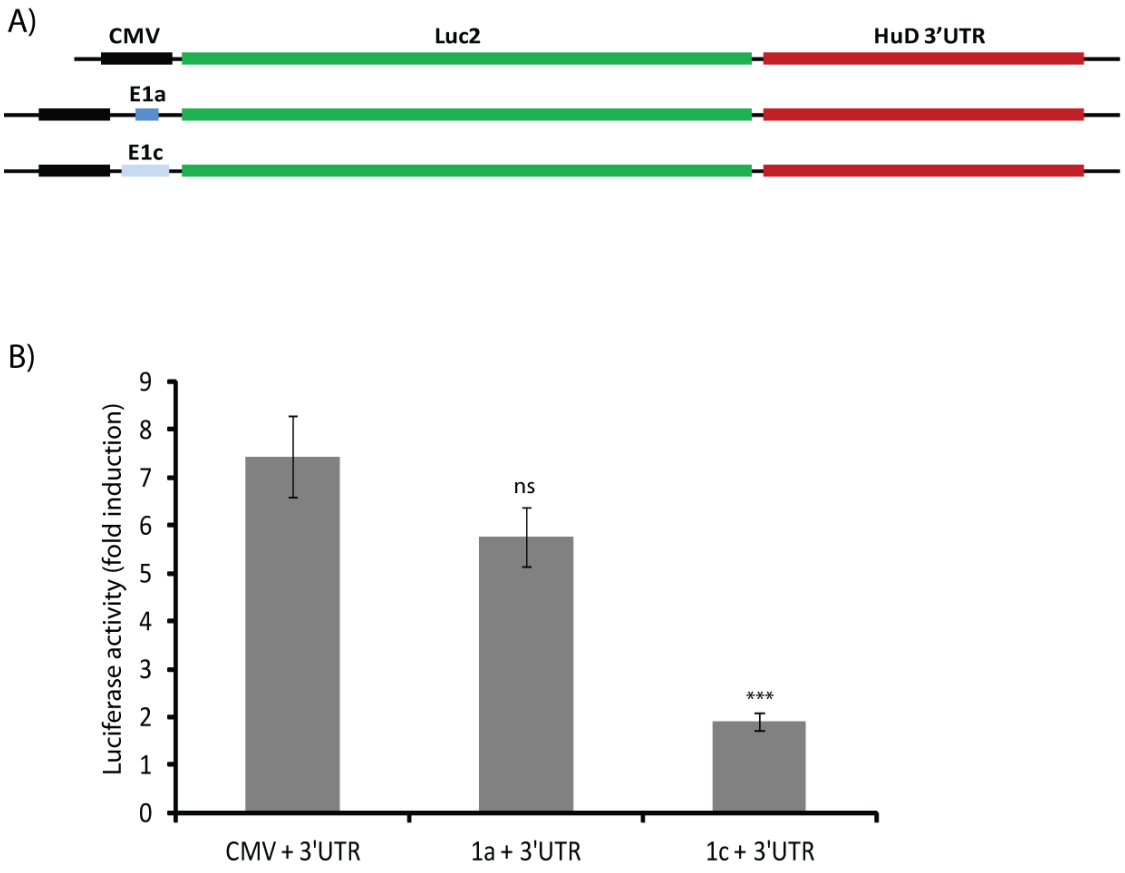


Figure 3.13 Effect of the 1c 5'UTR on HuD protein expression

(A) Schematic of chimeric constructs containing a CMV promoter, *HuD* 1a or 1c 5'UTR, *Luciferase2* ORF and full-length *HuD* 3'UTR. **(B)** Constructs were transfected into undifferentiated P19s and cells were harvested 24hrs later. Construct lacking a 5'UTR served as control (CMV + 3'UTR). Values were standardized to renilla luciferase (phRG) activity. Data are means ± SEM, n=2. ***, $p<0.001$; ns, $p>0.05$ (Student's t-test).

neurons, for our fifth objective we set out to establish whether its 5' RR contains a functional promoter and *cis*-elements that regulate neuron-specific expression. For this purpose, we first analyzed and aligned the mouse and human genomic sequences proximal to E1c using the Genomatix MatInspector (Cartharius et al., 2005) and Evolutionary Conserved Region browser (Ovcharenko et al., 2004) software. A high degree of homology between various metazoan species was found in several regions (Figure 3.8G), suggesting the presence of functionally conserved regulatory elements. Because E1c is one of the most expressed E1 variants, its transcription start site (TSS) was numbered +1 based on the longest exon sequence obtained in our 5' RACE, and the surrounding nucleotides were numbered accordingly (Figure 3.9G). Although no consensus TATA box was found around -30bp upstream of E1c TSS, a few conserved putative binding sites in the proximal promoter region were identified, including CREB (-93/-98), C/EBP (-148/-154), Sp1 (-181/-187), and MyT1 (-321/-326; Figure 3.9G).

To assess if the region upstream of *HuD* E1c contains a functional neuron-specific promoter and/or *cis*-regulatory elements, *luciferase* promoter-reporter constructs harbouring different length fragments of the E1c putative upstream regulatory region were generated (Figure 3.14A). These constructs were transiently transfected into undifferentiated P19 cells and the cells were then simultaneously differentiated into neuronal and mesodermal lineages. Initially, we isolated and tested a ~3.7kb fragment (pLUC3.4; -3,414/+328) of the *HuD* E1c 5'RR and found that it produced significantly higher reporter activity ($p<0.05$) in P19 neurons compared to mesoderm (Figure 3.14B). In order to

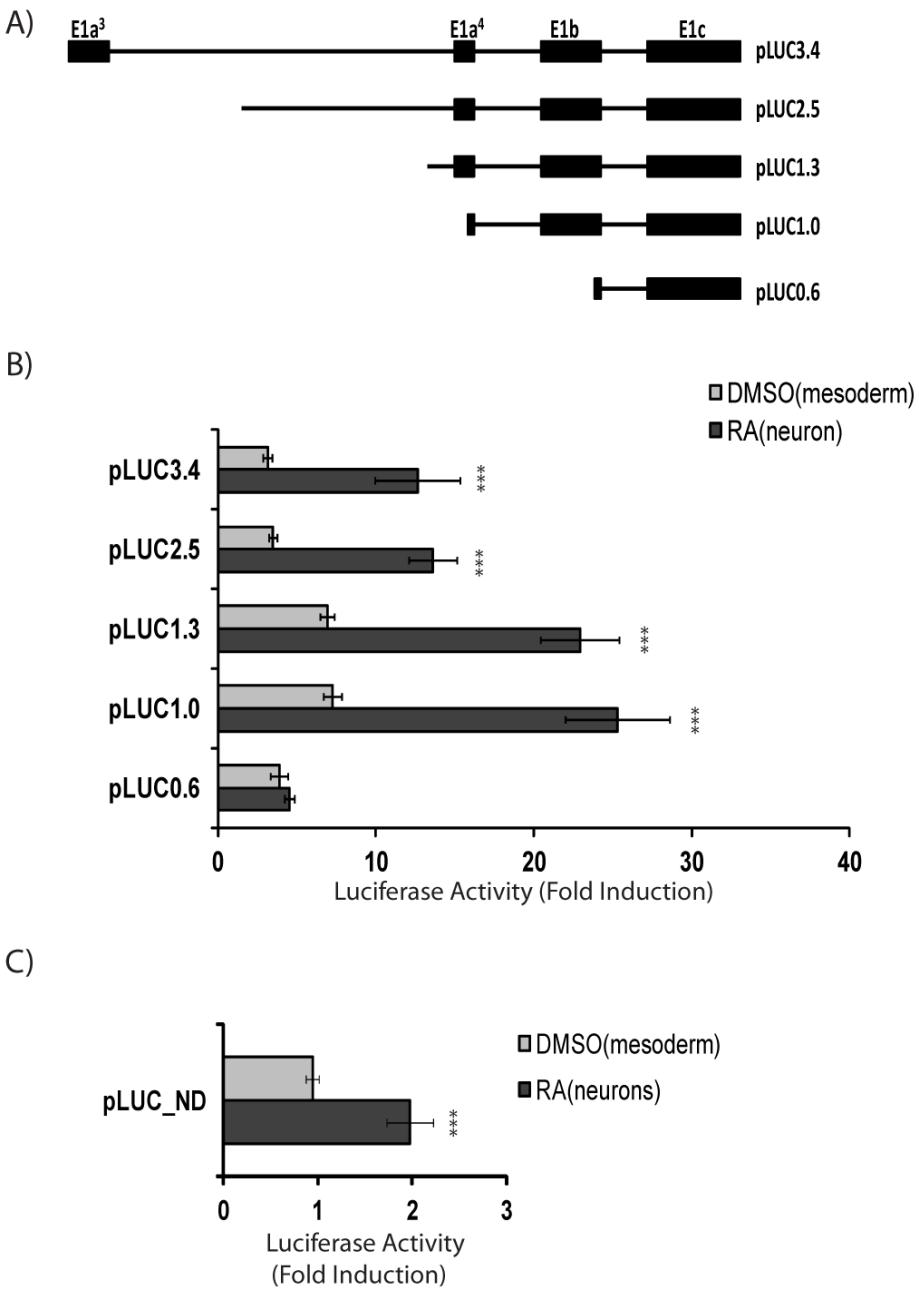


Figure 3.14 A 400bp region enhances expression of HuD specifically in P19 neurons

(A) Schematic representation of different sized *HuD* E1c 5'RR fragments that were used for promoter reporter assays (below). Luciferase activity produced by **(B)** various sized E1c 5'RR fragments or **(C)** the *NeuroD* promoter (control) in neuronal (RA) and mesodermal (DMSO) differentiated P19 cells, standardized to parental renilla activity (phRGTK). Data are means ± SEM, n=4. ***, $p<0.001$ (Student's t-test).

locate a RR that restricts expression of HuD to neurons, we created successive 5' deletion fragments of the pLUC3.4 construct, resulting in pLUC2.5 (-2,514/+328), pLUC1.3 (-1,342/+328), pLUC1.0 (-1,002/+328) and pLUC0.6 (-606/+328). The former three constructs also significantly augmented (p<0.05) luciferase activity specifically in neuronal versus mesoderm cells, with pLUC1.0 producing the greatest increase (Figure 3.14B). Conversely, the pLUC0.6 construct generated similar levels of luciferase activity in both neuron and mesoderm cells (p<0.05), suggesting that this promoter fragment lacks the *cis*-elements required to induce expression of HuD specifically in neuronal cells (Figure 3.14B). In these experiments, pLUC1.0 produced higher reporter activity in neuronal compared with mesodermal cells, whereas pLUC0.6 generated similar reporter activity in both cell types. These findings therefore indicate that the ~400 bp region (-1002/-606) contains *cis*-elements that promote neuron-specific transcription of the *HuD* E1c variant. As a positive control for these neuron-specific promoter-reporter experiments, we examined reporter activity driven by the *NeuroD* promoter. We found that the *NeuroD* promoter generated increased luciferase activity only in 4-day differentiated neurons, indicating neuron-specific transcriptional regulation (Figure 3.14C).

To identify potential TF binding sites within the 400bp fragment, we conducted *in silico* analysis with the MatInspector software. Examination of this region revealed a high degree of conservation amongst vertebrates (93%) and the presence of several putative *cis*-elements (Figure 3.15A). We sorted through the list of putative motifs and filtered out non-targets of documented neuron-

specific regulators to narrow possible candidates. The most notable *cis*-elements included five putative E-box sequences, E-box 5 (-994/-988), E-box 4 (-810/-804), E-box 3 (-756/-750), E-box 2 (-749/-743) and E-box 1 (-685/-679), four of which are highly conserved amongst vertebrates (Figure 3.15A).

Subsequently, we set out to identify *trans*-acting factors that bind to these E-boxes. In neurons, the E-box motif is a target of proneural basic helix loop helix (bHLH) TFs, such as Ngn, NeuroD, and Mash family members (Bertrand et al., 2002). Each TF displays preference for specific E-boxes, particularly the two central nucleotides in the hexamer sequence, onto which they typically heterodimerize with E-proteins (Seo et al., 2007). To initially test whether bHLH TFs can *trans*-activate HuD transcription via these E-boxes, luciferase activity was examined following overexpression of full-length cDNAs of *Mash-1 (Ascl1), NeuroD or Neurogenin 2 (Ngn2/MATH4a)* in undifferentiated P19 cells. Co-transfection with plasmids containing the cDNA of a bHLH heterodimerizing partner was not performed because some co-factors (e.g. E47) are already abundant in P19 cells (Kim et al., 2004). For these experiments, we tested the pLUC1.3 construct since it contains the 400bp region and similarly promotes higher reporter activity in neurons compared to mesoderm P19 cells (Figure 3.14B). Our results revealed that over-expression of MASH-1 did not modulate luciferase expression ($p>0.05$) whereas over-expression of NeuroD generated a small but significant increase in reporter activity ($p<0.05$) compared to control (Figure 3.15B and C). Remarkably, the largest increase in luciferase activity

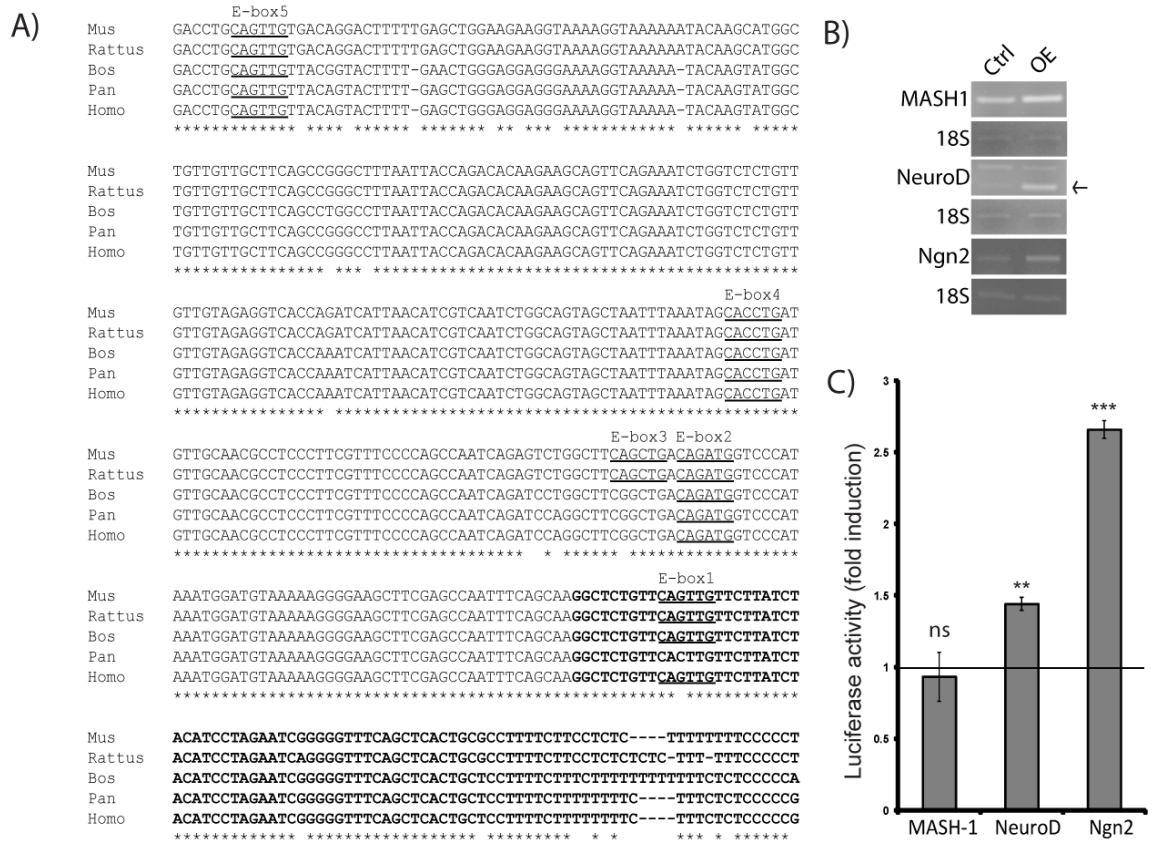


Figure 3.15 bHLH transcription factors promote reporter activity via the *HuD* E1c 5'RR

(A) Alignment of the mouse, rat, cow, monkey and human 400bp DNA sequences upstream of *HuD* E1c (-1,002/-606) with putative E-boxes (underlined) and 5'end of E1b (bold). Asterisks represents nucleotide conservation amongst all species **(B)** Ethidium bromide-stained 1% agarose gels showing RT-PCR product intensity of bands representing expression of *Mash-1*, *Ngn2*, *NeuroD* and *18S* mRNAs in parental or bHLH over-expressing cells. **(C)** Relative luciferase activity produced by promoter-reporter constructs harbouring a 1.7kb fragment upstream of the E1c 5'RR region (pLUC1.3) when co-transfected with *MASH-1*, *NeuroD* or *Ngn2* in P19 cells. Values were standardized to renilla luciferase (phRG) activity. Data are means ± SEM, n=3. **, $p<0.01$; ***, $p<0.001$; ns, $p>0.05$ (Student's t-test).

(~2.5X; $p<0.05$) was observed following NGN2 over-expression (Figure 3.15C).

3.7 NGN2 promotes transcription of *HuD* through two E-boxes upstream of E1c

NGN2 belongs to the neurogenin family (NGN 1/2/3) of TFs and plays a central role in neuronal commitment, differentiation and specification by heterodimerizing with co-factors, such as E47, onto E-boxes in gene promoters (Ross et al., 2003; Guillemot, 2007). To determine the NGN2-targeted motifs upstream of E1c, we first tested the ability of NGN2 to increase endogenous expression of *HuD* mRNA. To this end, we transiently transfected constructs harbouring the full-length *Ngn2* cDNA into undifferentiated P19 cells and harvested global RNA for RT-PCR analysis the next day. As expected based on our previous reporter assays results (Figure 3.15B and C), over-expression of NGN2 increased endogenous *HuD* mRNA levels (Figure 3.16A).

To determine which of the 5 E-boxes in the 400bp region upstream of E1c are putative targets of NGN2, we screened their hexamer sequences for NGN2 consensus motifs. Both E-boxes 2 and 3 contained the preferential motifs bound by NGN2 (Figure 3.16C). Accordingly, we examined whether these two motifs are necessary for NGN2-mediated reporter activity by transfecting promoter-reporter constructs containing mutated or deleted E-boxes 2 and 3 in parallel with a construct harbouring *Ngn2* (PC1G2:*Ngn2*) or a parental plasmid (PC1G2) into P19 cells (Figure 3.16B). In agreement with our previous reporter assays

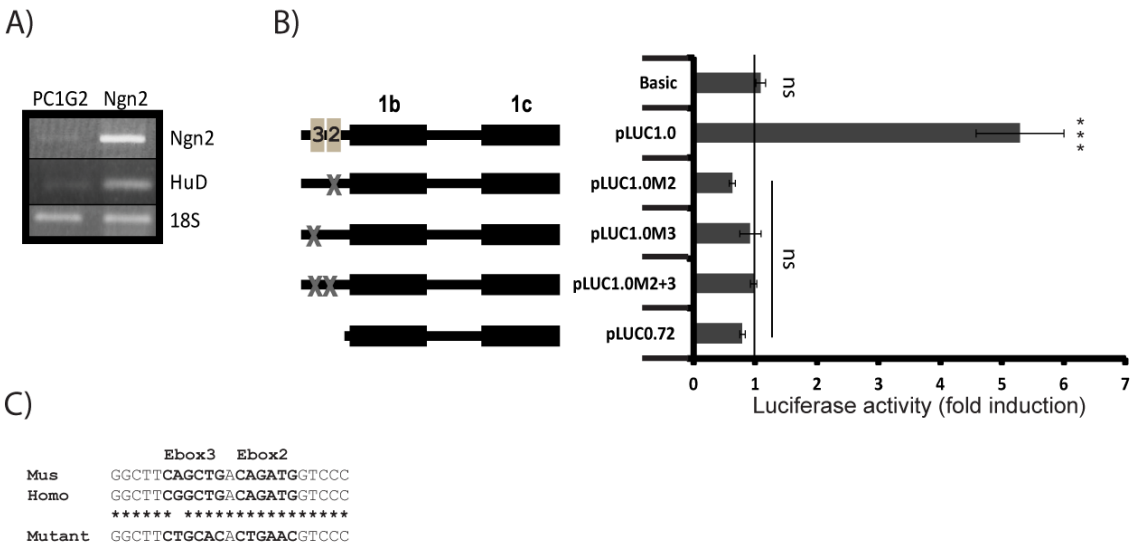


Figure 3.16 NGN2 promotes reporter activity via two E-boxes in the *HuD* E1c 5'RR

(A) Over-expression of NGN2 (PC1G2:Ngn2) in undifferentiated P19 cells increases endogenous *HuD* mRNA expression, compared to parental (PC1G2). Data are means ± SEM, n=3. **(B)** Relative luciferase activity produced by promoter-reporter constructs harbouring different fragments and mutations of the *HuD* E1c 5'RR region in NGN2 (PC1G2:Ngn2), or parental vector (PC1G2), over-expressing P19 cells. Values were standardized to renilla (phRG) luciferase activity. Data are means ± SEM, n=3. ***, $p<0.001$; ns, $p>0.05$ (Student's t-test). **(C)** Nucleotide sequence of wild-type and mutated mouse and human E-boxes 2 and 3.

(Figure 3.14B), the wild-type pLUC1.0 construct containing the 400bp region generated a significant (p<0.05) increase in luciferase activity in the presence of exogenous NGN2, compared to control (Figure 3.16B). On the other hand, mutating either E-box 2, 3, or both (pLUC1.0M2, pLUC1.0M3 and pLUC1.0M2+3, respectively), or deleting the region containing these two E-boxes (pLUC0.72), completely prevented the induction of relative luciferase activity observed following NGN2 over-expression (Figure 3.16B). Collectively, these studies indicate that NGN2 can promote transcription of *HuD* E1c by acting through two tandem E-boxes.

We used two parallel methods to examine the interaction of NGN2 to E-boxes 2 and 3. In the first approach, EMSAs were conducted. A 291bp probe (-1002/-711) was generated and incubated with *in vitro* transcribed/translated NGN2 and/or its co-factor E47. The formation of a major complex on the EMSA gel was only observed when the probe was incubated with both recombinant NGN2 and E47 (Figure 3.17A, lane 4). An additional, slower migrating, complex was detected when recombinant E47, but not NGN2, was incubated alone with the probe (Figure 3.17A, lane 3). This result is in agreement with the previous finding showing that E47 proteins can homodimerize on E-box motifs (Shen and Kadesch, 1995). Complex formation with NGN2 -E47 was concentration dependent (Figure 3.17A, lanes 4-6) and could be reduced by adding a cold probe competitor (Figure 3.17A, lanes 7-8). Moreover, mutation of E-boxes 2 and 3 completely abolished complex formation with NGN2 and E47, further demonstrating NGN2 specificity for E-boxes 2 and 3 (Figure 3.17A, lane 9-11).

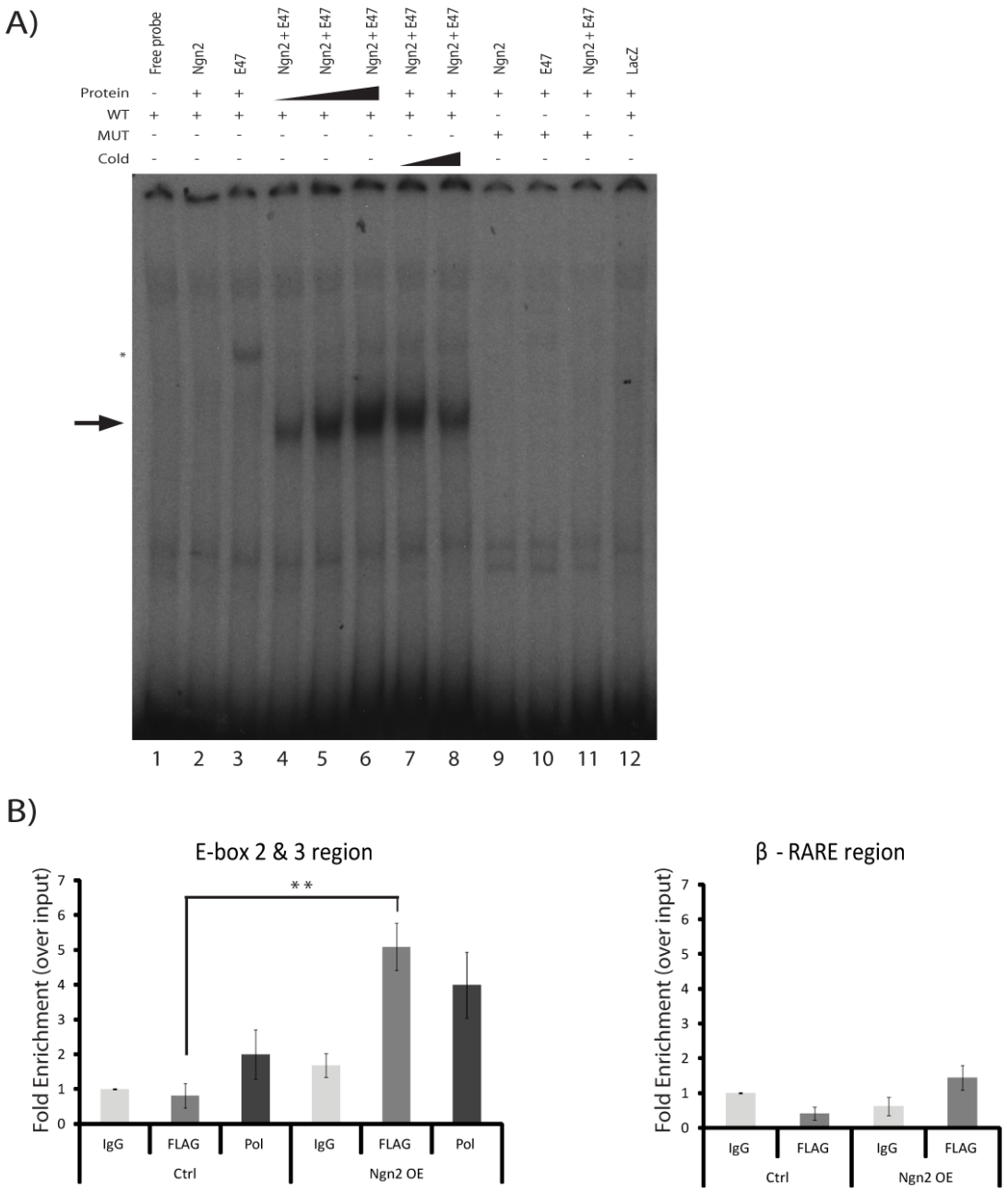


Figure 3.17 NGN2-E47 heterodimers bind to E-boxes 2 and 3

(A) Representative image of an EMSA gel showing that NGN2 -E47 heterodimers (lanes 4–6), but not NGN2 alone (lane 2), can bind to E-boxes 2 and 3. This interaction is specific as shown through sequence mutation (lanes 9–11), addition of nonspecific protein (lane 12), and addition of 5–10X molar excess of a cold probe (lanes 7–8). Arrow points to the NGN2-E47 heterodimer and asterisk indicates the E47 homodimers. n=5. (**B)** Results from ChIP assays showing occupancy of the DNA region harboring E-boxes 2 and 3 in FLAG-tagged *Ngn2* (NGN2 OE) or parental plasmid (Ctrl) overexpressing cells. Immunoprecipitations were performed with anti-control (IgG), tag (FLAG), or RNA Polymerase II (Pol) antibodies. The **β**-RARE site was used as a negative control. Data are means SEM, *n*=3. ***p*<0.01 (Student's t-test).

In our second approach, we determined whether NGN2 is recruited to the DNA region containing E-boxes 2 and 3 *in vivo*. For this, chromatin immunoprecipitation (ChIP) experiments were performed in undifferentiated P19 cells transiently transfected with a construct containing a FLAG-tagged *Ngn2* cDNA or a control vector. Our results revealed increased binding to the DNA region containing both E-boxes ($p<0.05$), but not to a non-specific region (β-RARE; $p>0.05$), in cells over-expressing the *Ngn2 trans*-gene (Figure 3.17B). We also detected a ~2X enrichment, although not significant ($p>0.05$), of RNA polymerase II binding to the same region in P19 cells over-expressing NGN2 (Figure 3.17B). Together, these findings show that the NGN2-E47 heterodimer binds to two E-boxes upstream of E1c both *in vitro* and *in vivo* to promote *HuD* transcription.

3.8 The NGN2 co-factor retinoic acid receptor

Recently, a study by Lee et al (2009) has demonstrated that the alpha retinoic acid receptor (αRAR) promotes motor neuron differentiation in the presence of RA by heterodimerizing with the NGN2-E47 complex onto E-boxes upstream of proneuronal genes (Lee et al., 2009). Classically, RARs are known to bind RA response elements (RAREs) in the RRs of proneural genes as homodimers or heterodimers with retinoic x receptors (RXRs; Venepally et al., 1997; Arima et al., 2005; Maden, 2007). In view of this indirect αRAR function, we performed a series of experiments to establish whether RA-treatment potentiates NGN2-dependent transcription of *HuD* through a αRAR-NGN2-E47 complex on E-boxes 2 and 3. We first set out to test whether RA enhances the

NGN2-mediated induction of *HuD* transcription. For these experiments we developed a promoter-reporter construct harbouring a ~300bp region upstream of E1c that encompasses the NGN2-targeted E-boxes 2 and 3 (Figure 3.18A). We co-transfected pLUC0.3 with a plasmid harbouring the *Ngn2* cDNA or parental vector and treated undifferentiated P19 cells in monolayer with RA or vehicle (ethanol) for two days. Our results show that RA treatment produced a significant increase in reporter activity in the presence of ectopic NGN2, compared to vehicle treatment (Figure 3.18A). These findings indicate that NGN2 activity on the *HuD* E1c 5'RR is enhanced by RA treatment.

To assess if αRAR physically interacts with NGN2, we conducted co-IP experiments. To this end, we overexpressed FLAG-tagged RAR and His-tagged NGN2 in C_2C_{12} cells, which do not contain endogenous NGN2, and used anti-FLAG or His antibodies to IP the complex. Our results show a band corresponding to the appropriate size of FLAG-RAR in the anti -FLAG and -His lanes of the western blot, clearly demonstrating association between αRAR and NGN2 (Figure 3.18B).

We then wanted to establish whether αRAR complexes with NGN2 onto E-boxes 2 and 3 in the *HuD* E1c 5'RR using two complementary techniques. First we conducted EMSAs, where we *in vitro* radiolabeled the ~0.3kb HuD DNA fragment encompassing E boxes 2 and 3 and incubated it with *in vitro* synthesized αRAR, E47 and/or NGN2 (Figure 3.19A). Addition of NGN2 and E47 retarded migration of the probe on the EMSA gel (arrow, Figure 3.19A; lane 2),

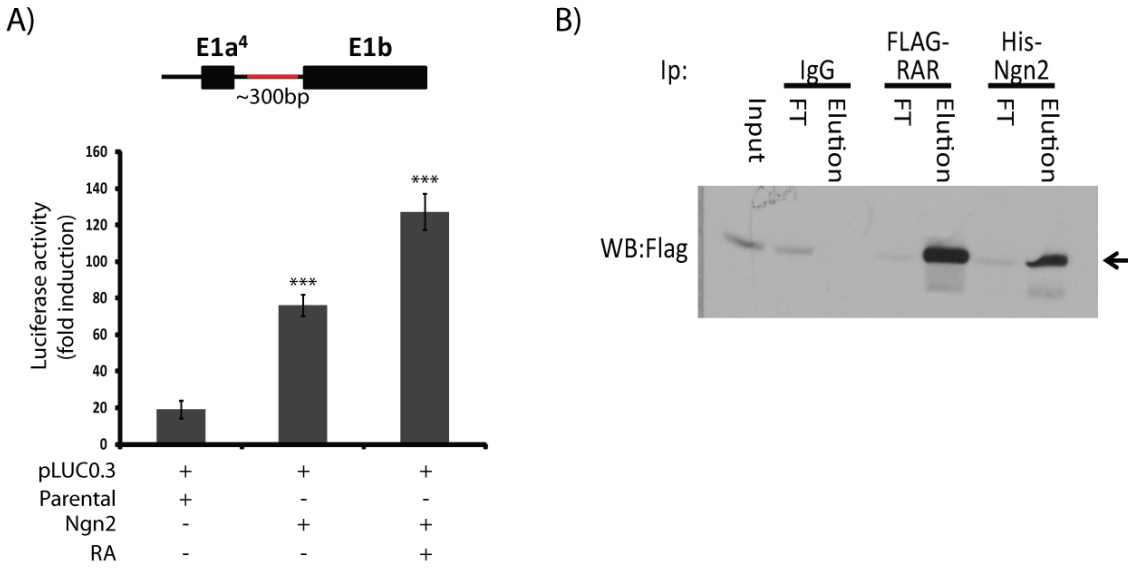


Figure 3.18 Retinoic acid receptor (RAR) interacts with NGN2 and RA enhances NGN2-mediated HuD E1c transcription

(A) Undifferentiated P19 cells were transfected with the indicated constructs, treated with RA and reporter activity was assayed 48hrs later. Graph showing reporter activity produced by pLUC0.3 following vehicle or RA treatment. Data are means ± SEM, n=3. ***, $p<0.001$ (Student's t-test). **(B)** Co-immunoprecipitation experiment showing the interaction of FLAG-hRAR with His-NGN2 in C_2C_{12} cells. n=2.

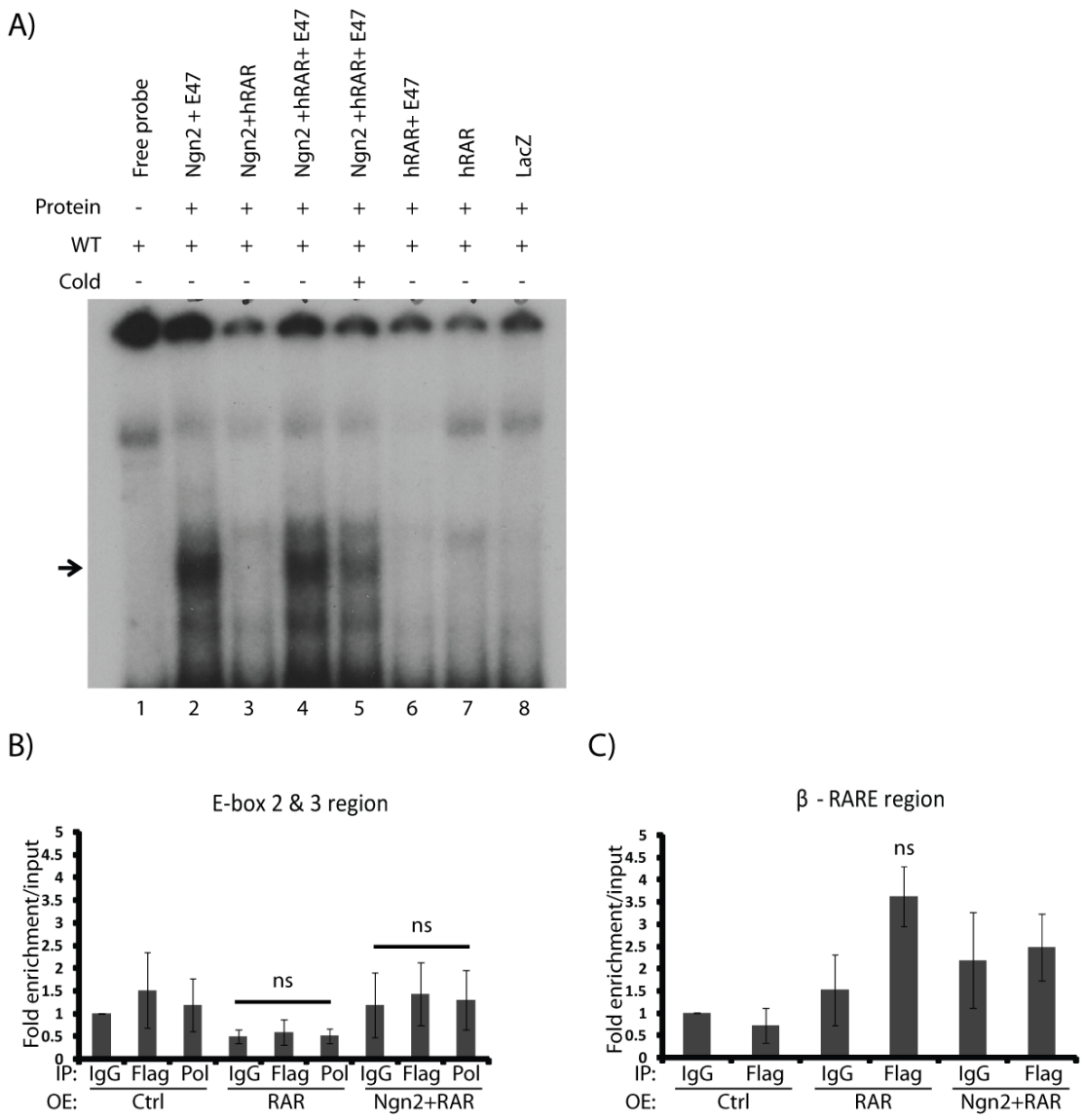


Figure 3.19 αRAR does not heterodimerize with NGN2 on E-boxes 2 and 3 *in vitro* or *in vivo*

(A) Representative image of an Electrophoretic Mobility Shift Assay gel showing that NGN2-E47 heterodimers can bind to E-boxes 2 and 3 in the probe but hRAR does not bind to this complex. Arrows point to the Ngn2-E47 heterodimer. n=1. **(B and C)** ChIP assays showing no enhancement at the DNA region harbouring E-boxes 2 and 3 of αRAR in cells transfected with vehicle, FLAG-hRAR alone or FLAG-hRAR and PC1G2-Ngn2. RNA polymerase II (Pol) and β - RARE were used as positive and negative controls, respectively. Data are means ± SEM. n=2. ns, $p<0.05$ (Student's t-test).

as previously shown (Figure 3.17A). However, incubation with αRAR alone or NGN2 +E47 did not shift or supershift, respectively, probe migration (Figure 3.19A; lanes 3, 4, 6 and 7). In our second approach, we performed ChIPs to determine whether αRAR is enriched in the same E-box region as NGN2 (Figure 3.19B). Ectopic expression of FLAG-RAR in undifferentiated P19 cells demonstrated an enrichment of RAR in a control DNA region (not in the *HuD* locus) containing a β-RARE site (Figure 3.19C). However, over-expression of αRAR alone or in combination with NGN2 did not enrich its binding to the genomic region upstream of *HuD* E1c encompassing E-boxes 2 and 3 (Figure 3.19B). Together, these results imply that in undifferentiated P19 cells αRAR does not heterodimerize with NGN2 on NGN2-targeted E-boxes upstream of *HuD* E1c.

3.9 Identification of other important regions in the *HuD* E1c 5'RR

Subsequently, we set out to further characterize the *HuD* E1c 5'RR and to determine whether other molecular mechanisms act upstream of E1c to control neuron-specific transcription of *HuD*. For these experiments we used N2a cells since they are already committed to the neuronal lineage (neuroblasts) and therefore do not need to be differentiated (Tremblay et al., 2010). Importantly, transfection of the pLUC1.0 and pLUC0.6 constructs into N2a cells produced an analogous 4-5X difference in reporter activity (Figure 3.20B), as detected in P19 cells (Figure 3.14B). This result suggests that undifferentiated N2a cells mimic 4-day differentiated neuronal P19 cells at least in regards to reporter expression

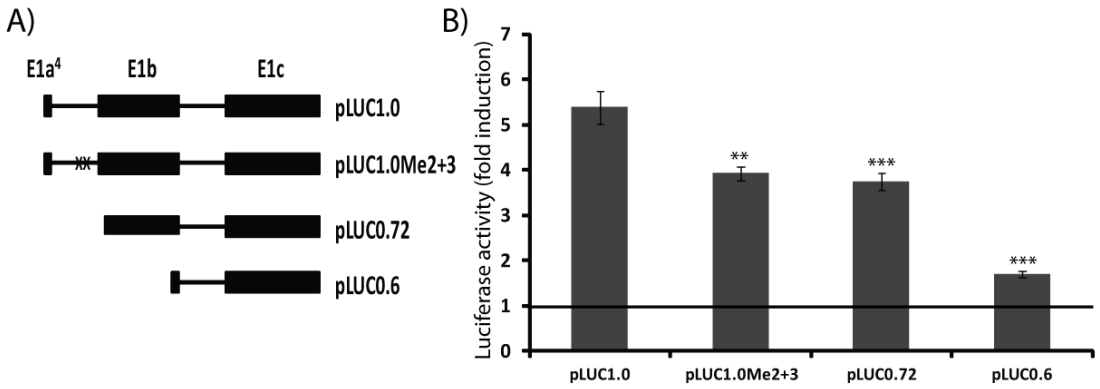


Figure 3.20 E-boxes 2 and 3 only partially responsible for transcription of *HuD* in neurons.

(A) Schematic representation of different sized E1c 5'RR fragments and mutations that were used for these promoter reporter assays. **(B)** Luciferase activity produced by various sized E1c 5'RR fragments in N2a cells, standardized to parental renilla activity (phRGTK). Data are means ± SEM, n=3. ***, $p<0.001$; ns, $p>0.05$ (Student's t-test).

levels driven by the *HuD* E1c 5'RR. In light of this finding, we transfected N2a cells with constructs that contain mutated (pLUCMe2+3) or deleted (pLUC0.72) E-boxes 2 and 3. Both constructs produced significantly decreased reporter activity in N2a cells compared to pLUC1.0 (Figure 3.20A and B); however, the luciferase activity generated by these constructs did not return to the level produced by pLUC0.6 (Figure 3.20A and B). These findings indicate that although E-boxes 2 and 3 are necessary, there are other *cis*-element(s) in the ~120bp region between pLUC0.72 and pLUC0.6 that are also critical for transcription of *HuD*.

3.10 Additional *cis*-elements driving neuronal *HuD* expression

To identify the other *cis*-acting elements that are important for E1c expression, we employed the MatInspector software to search for putative motifs targeted by neuronal TF. Since the first half of the 120bp region is more conserved amongst higher metazoans than the second half, we started out by screening the former. Within this ~70bp region, five putative motifs were found, including another E-box (E-box1), a CCAAT box, Krueppel-like factor (KLF), GATA and nuclear deformed epidermal autoregulatory factor-1 (DEAF-1)-related (NuDR) motifs (Figure 3.21A). Importantly, all the TFs suggested by MatInspector to bind these putative motifs, namely NeuroD (Miyata et al., 1999), CCAAT/enhancer binding proteins (C/EBPs; Cortes-Canteli et al., 2002), KLFs (Moore et al., 2011), GATAs (Nardelli et al., 1999) and NuDR (Hahm et al., 2004), respectively, have been implicated in neuronal development. We mutated

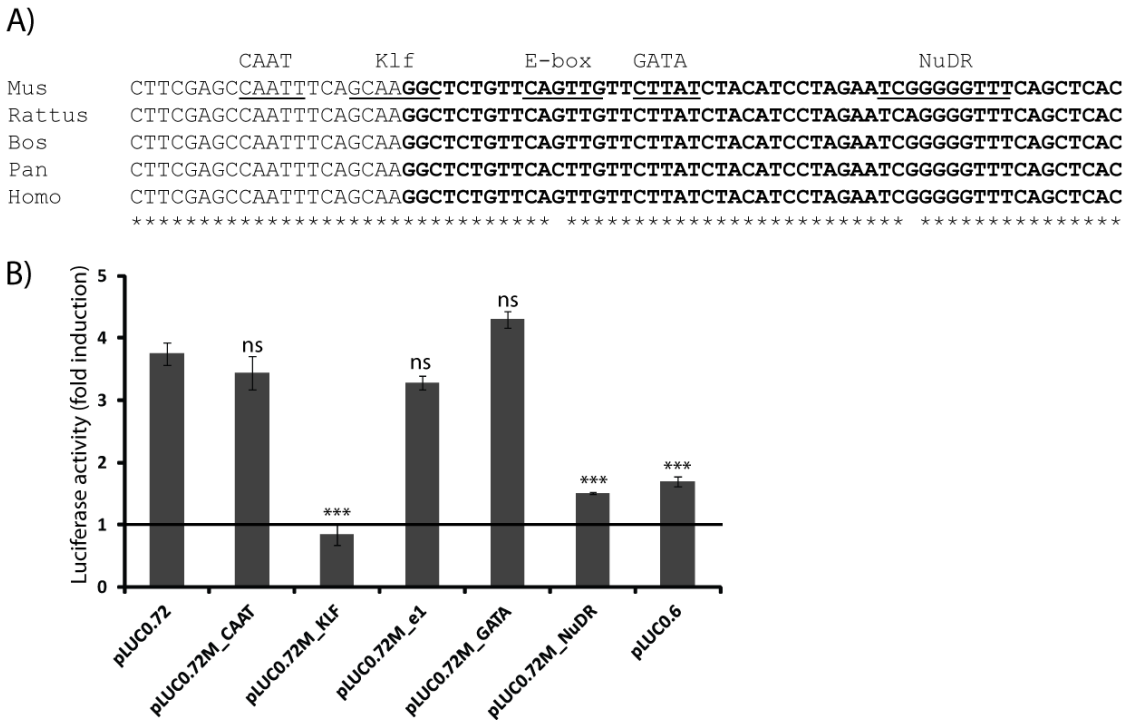


Figure 3.21 Additional functional *cis*-acting elements upstream of *HuD* E1c

(A) Nucleotide sequence of a ~70bp DNA fragment upstream of E1c that contains additional *cis*-acting elements (underlined) necessary for neuronal expression of HuD. **(B)** The effect of different deletion and mutation fragments of the *HuD* E1c 5'RR on producing luciferase activity in different N2a cells. Values were standardized to renilla luciferase (phRG) activity. Data are means ± SEM, n=3. ***, $p<0.001$; ns, $p>0.05$ (Student's t-test).

each of these *cis*-elements individually in pLUC0.72 and transfected the constructs, along with controls, into N2a cells. Our results show that mutation of the CCAAT and E-boxes did not significantly (p>0.05) alter luciferase activity compared to pLUC0.72 (Figure 3.21B). Interestingly, we found that mutation of either the putative KLF or NuDR motifs reduced reporter activity to levels produced by pLUC0.6 (Figure 3.21B). Together, our results suggest that *trans*-acting factors targeting these *cis*-elements positively regulate transcription of *HuD*.

Based on these findings, we initiated experiments to identify TFs that bind to these *cis*-elements and control expression of *HuD* E1c. Since the KLF family of TFs contains 17 isoforms, we searched for variants that are expressed early during neuronal development and/or have been implicated in neuronal differentiation. Our search revealed that KLF6 and 7 have been shown to have important developmental functions in neurons (Moore et al., 2011). We subcloned the full-length cDNA of *KLF6* into pcDNA3 and obtained the NuDR1 overexpression construct as a kind gift from Dr. Paul Albert (OHRI, Ottawa). Next, we individually transfected these constructs, along with pLUC0.72, into N2a cells and assessed luciferase activity 24hrs later. Our results demonstrate that despite successfully overexpressing these genes, at least at the mRNA level, neither KLF6 nor NuDR1 increased reporter activity (Figure 3.22A and B). In fact, ectopic expression of NuDR1 decreased reporter activity, which is in agreement with its dual activator/repressor functions in neurons (Czesak et al., 2006).

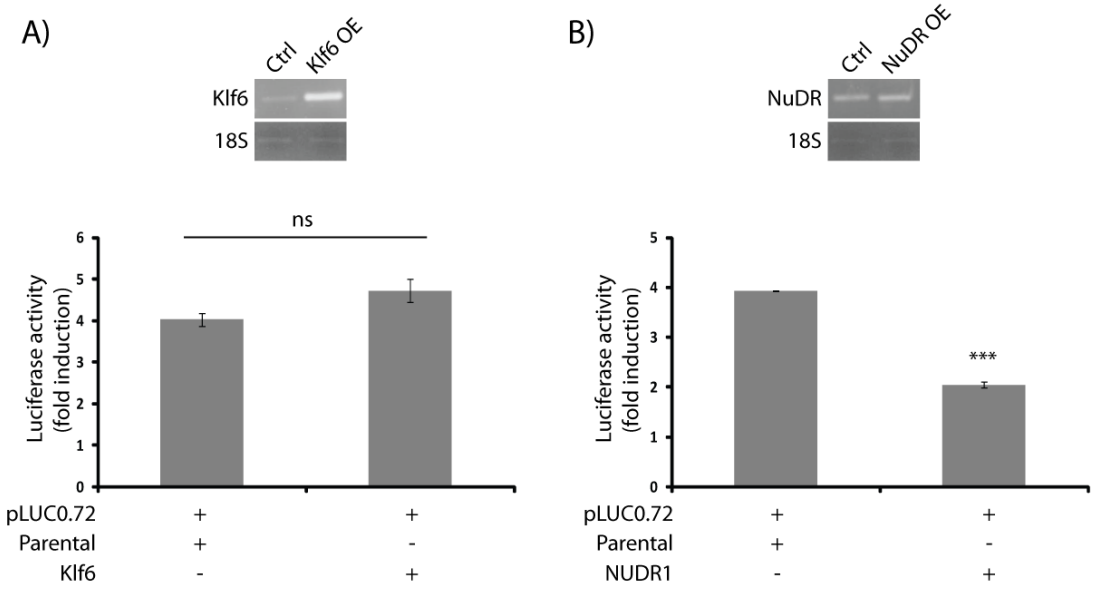


Figure 3.22 The transcription factors KLF6 and NuDR do not promote transcription of *HuD* in undifferentiated N2a cells

Relative luciferase activity produced by promoter-reporter constructs harbouring a 1.1kb fragment (pLUC0.72) upstream of E1c when co-transfected with plasmids containing the full-length cDNAs of **(A)** *KLF6* or **(B)** *NuDR* into N2a cells. Values were standardized to renilla luciferase (phRG) activity. Data are means ± SEM, n=3. ***, $p<0.001$; ns, $p>0.05$ (Student's t-test).

Further studies are necessary to establish the TFs, and their specific isoforms, that bind *cis*-elements upstream of E1c to regulate expression of *HuD*.

Chapter 4

4: DISCUSSION

Despite its broad spectrum of roles in regulating mRNA metabolism, including processing, stability and translation, the molecular mechanisms that govern the expression of *HuD* in mammals remained largely unexplored until now, particularly at the transcriptional level (Bronicki et al., 2012). Previous work demonstrated that the mammalian *HuD* gene contains at least three leader exons and only a few studies had uncovered transcriptional and post-transcriptional events that so far have only been shown to negatively control *HuD* transcript levels (Abdelmohsen et al., 2010; Lee et al., 2012; Cuadrado et al., 2003). In our studies, we identified five additional leader exons in the 5' genomic region of mammalian *HuD*, indicating that a total of eight E1 variants exist, which may encode unique N-termini. Using the multipotent P19 differentiation model, we established that expression of *HuD* mRNA is primarily under transcriptional control in neurons. We found that neuron-specific expression of *HuD* mRNA is dependent on NGN2 binding to two E-boxes upstream of the predominantly expressed E1c. Furthermore, we identified two other putative *cis*-acting elements proximal to these two E-boxes, potentially targeted by KLF and NuDR/DEAF-1 TFs, that also positively control transcription of *HuD*. To our knowledge, our findings are the first to elucidate molecular events that positively regulate expression of *HuD* transcripts.

4.1 Expression of *HuD* mRNA in different neuronal development models

The temporal expression pattern of HuD during mouse embryonic brain development and in cultured cells differentiated into neurons has been previously established; however, the molecular events responsible for the induction of *HuD* mRNA are unknown (Okano and Darnell, 1997; Steller et al., 1996; Abdelmohsen et al., 2010; Mansfield and Keene, 2011). To decipher these mechanisms, we first assessed the abundance of *HuD* mRNA in three different neuronal differentiation models. In neurospheres we did not detect augmented expression of *HuD* or *GAP-43* mRNAs following six days of neurosphere differentiation. Around 80-90% of spheres are able to generate a mixed population of cells that includes neurons, astrocytes and oligodendrocytes, indicating that neurons may not be the prevalent cell-type found in differentiated neurospheres (Reynolds and Weiss, 1996). Thus, our findings could be a consequence of inefficient neurosphere differentiation into the neuronal lineage or the low relative amount of neurons produced in the heterogeneous cell population of differentiated neurospheres (Reynolds and Weiss, 1996).

We also determined the expression of *HuD* mRNA at three different time points (day 10, 14 and 18) of mouse embryonic brain development. Although we detected augmented mRNA expression of the neuronal differentiation markers *GAP-43* and *tau*, we did not detect a significant increase in *HuD* mRNA levels at these time points of embryonic brain development. However, we did observe a trend that suggests *HuD* mRNA levels are increased at E14 and significantly reduced at E18, compared to E10. This pattern of expression is consistent with

previous findings showing that *HuD* mRNA levels are detectable by E10, peak at E16 and decline into adulthood during murine brain development (Hambardzumyan et al., 2009; Abdelmohsen et al., 2010). These results corroborate HuD as an early marker of the neuronal phenotype and suggest that high levels of *HuD* mRNA (between E14-18) are important for proper neurogenesis in the brain. The augmented expression of HuD overlaps with critical stages of nervous system development, such as neurogenesis of intermediate zone and cortical plate neurons in the cerebral cortex (Sansom and Livesey, 2009). The importance of this embryonic stage is further emphasized by studies showing that prenatal infection at E16 in mice, or the mid-second trimester in humans, is linked to the development of neuropathies later in life (Brown et al., 2004; Fatemi et al., 2008). Based on this data, it seems evident that timely peak expression of HuD and the other nELAVs during embryogenesis is critical for proper development of nervous system.

In addition, we assessed expression of *HuD* mRNA in the well-established embryonal carcinoma P19 cell line. These cells originated from transplantation of 7.5 day embryo into the testis of a mouse (McBurney and Rogers, 1982). As a result a teratocarcinoma formed from which cultured cell lines with stem cell-like properties were derived. One of these cell lines, P19, shares many physiological and biochemical properties with embryonic stem cells, such as expressing the Oct 3/4 TF (Okamoto et al., 1990) in the undifferentiated state. In addition, P19 cells reliably differentiate into a neuronal lineage following treatment with the vitamin A metabolite, RA, which also plays an important role in promoting

neuronal differentiation *in vivo* (Maden, 2007; Olson and Mello, 2010). Neuronal differentiation of P19 cells closely mimics murine neuroectoderm development (Staines et al., 1994) and requires diffusible signals (RA) and cell adhesion, similar to ES cells (Bain et al., 1995; Kumar et al., 2007; Wang et al., 2006; Chen et al., 2007b). After 6 days of RA-treatment, the heterogeneous P19 cell population contains up to 85% neurons (McBurney et al., 1988) which exhibit various properties of murine adult forebrain neurons, such as neurotransmitter expression, action potentials (McBurney, 1993; Magnuson et al., 1995a; Magnuson et al., 1995b), responses to neurotransmitters (Magnuson et al., 1995a) and calcium fluctuations (Morley et al., 1995). Furthermore, neuronal P19 cells have been shown to express various neuronal phenotype markers, including high levels of *AChE* (Coleman and Taylor, 1996), *tau* (Heicklen-Klein et al., 2000), *GAP-43* (Zhao et al., 2012; Chiu et al., 1995) and *nELAV* (Steller et al., 1996; Mansfield and Keene, 2011) mRNAs.

Another advantage of using P19 cells is their pluripotency, a feature that facilitates the study of neuron-specific regulation of gene expression (McBurney et al., 1982; McBurney, 1993). Treatment of aggregated P19 cells with DMSO promotes skeletal and cardiac muscle differentiation, with both cell types exhibiting various physiological and biochemical characteristics similar to their embryonic counterparts, including expression of the mesodermal marker Brachyury T (Vidricaire et al., 1994; Rudnicki et al., 1988; Rudnicki et al., 1990; Skerjanc et al., 1994; Wobus et al., 1994; Skerjanc, 1999). In our study, we confirmed differentiation of P19 cells into a mesodermal lineage by

demonstrating mRNA expression of the TF *Brachyury T* (Vidricaire et al., 1994). Interestingly, we also detected faint levels of *HuD* mRNA during mesodermal P19 differentiation. This finding, along with our promoter-reporter and nuclear run-on assays, indicate that the *HuD* gene is transcribed at low levels in mesodermal cells. In support of our data, low levels of *HuD* mRNA have been previously detected in skeletal and cardiac muscle, two tissues derived from mesoderm (Abdelmohsen et al., 2010). Thus, although we acknowledge that care must be taken when interpreting results obtained from this cultured cell line, these studies indicate that P19 cells are a suitable model to investigate the molecular mechanisms controlling mRNA expression of *HuD*.

The ability to simultaneously differentiate P19 cells into neurons and mesoderm cells enabled us to detect neuron-specific induction of *HuD* and *GAP-43* transcripts within 24hrs after RA treatment. The early augmentation of *HuD* mRNA levels during P19 neurogenesis is consistent with our *in vivo* findings and other studies that describe HuD as one of the earliest markers of neuronal phenotype (Hambardzumyan et al., 2009; Wakamatsu and Weston, 1997). Importantly, the early expression of *HuD* emphasizes its role as a central factor in promoting the neuronal differentiation program. During neuronal differentiation of P19 cells we detected increasing *HuD* mRNA levels that peaked at 4 days, indicating that neuron-specific transcriptional and/or post-transcriptional events govern its induction. Slightly different temporal mRNA expression patterns have been described for the two other nELAV members, *HuB* and *HuC*, during neuronal development (Hambardzumyan et al., 2009; Mansfield and Keene,

2011), suggesting that at least some unique mechanisms regulate *HuD* mRNA abundance.

4.2 Regulation of *HuD* during neurogenesis

In our study we used several complementary approaches to demonstrate that expression of *HuD* is transcriptionally regulated in differentiating P19 neurons. Nuclear run-on assays showed that more *de novo* synthesis of *HuD* mRNAs occurs in neuronal as compared to mesodermal cells. Promoter-reporter assays using upstream *HuD* E1c 5' RR fragments demonstrated higher luciferase activity in neuronal versus mesodermal cells. Together, these results indicate that the induction of *HuD* mRNA during P19 neurogenesis is subject to transcriptional events.

Control of *HuD* transcription has been previously demonstrated in only two other studies. In neuronal precursor cells, high thyroid hormone (T3) levels were determined to negatively regulate expression of *HuD* mRNA, and therefore presumably neuronal differentiation, through an undefined transcriptional mechanism (Cuadrado et al., 2003). This data produces a paradox since T3 was shown to promote neuronal differentiation *in vivo* (Chen et al., 2012; Kapoor et al., 2012). The discrepancy between these findings could be due to T3 having variable effects at different developmental time points or the experimental models used (cultured cells versus mouse embryonic development). The presence of HuD in pancreatic beta cells lead to the detection of another transcriptional mechanism controlling its expression. In these cells, low glucose levels were found to activate the TF FoxO1, resulting in the transcriptional repression of *HuD*

(Lee et al., 2012). Interestingly, FoxO1 is also abundant in neurons where it may play a role in PD (Dumitriu et al., 2012) and proliferation of neuronal precursors in the DG (Yuan et al., 2008; Paik et al., 2009). These latter findings signify that FoxO1 might also negatively control transcription of *HuD* in neuronal precursors to maintain them in a proliferative state.

Transcription is a fundamental process driving mRNA expression during neurogenesis and has been described for several other proneural genes, including *GAP-43* and *AChE* (Zhao et al., 2012; Greene and Rukenstein, 1981). However, post-transcriptional mechanisms, such as increased mRNA stability, also play a critical role in the expression of both of these genes later in neuronal development (Mobarak et al., 2000; Bronicki and Jasmin, 2012; Deschenes-Furry et al., 2003; Bolognani and Perrone-Bizzozero, 2008). In our studies, actinomycin D and *HuD* 3'UTR-reporter experiments produced similar *HuD* mRNA half-lives between neuronal and mesodermal P19 cells. The absence of distinct regulation of *HuD* mRNA observed in our studies between neuronal and mesodermal cells implies that in 4-day differentiated P19 cells *HuD* mRNA is not under neuron-specific post-transcriptional control. Given that *HuD* mRNA is targeted by its own protein, other ELAV members (Bolognani et al., 2010) and miR-375 (Abdelmohsen et al., 2010), post-transcriptional events likely also affect expression of HuD at some point of neuronal development and/or under other contexts. For instance, HuD and the other nELAVs may bind to the 3'UTR of *HuD* to negatively control its translation and therefore maintain proper HuD abundance in mature neurons, as suggested for the *Drosophila* ELAV mRNA

(Samson, 1998). On the other hand, miR-375 may function as a negative regulator of HuD expression in mature non-neuronal cells, such as skeletal muscle and liver, to prevent aberrant levels of HuD in other tissues (Abdelmohsen et al., 2010).

4.3 Characterization of the 5' genomic region of *HuD*

Through bioinformatics and 5' RACE analysis, we found that the *HuD* gene contains eight alternate leader exons in its 5' genomic region, most of which are conserved among higher mammals. The identification of multiple E1 variants is consistent with the very large 5' genomic region of the mammalian *HuD* gene, which spans ~100kb of DNA. Moreover, comparing nucleotide sequences of this region among mammalian *HuD* genes and examination of DNase hypersensitivity clusters (indicative of actively transcribed region; UCSC genome browser) implies that other E1 variants exist, particularly within the large intron separating E1a and E1a[1]. Analysis of published human ESTs in the UCSC Genome Browser revealed that *HuB* and *HuC* also contain multiple leader exons, a finding that is not surprising given the homology between *nELAV* genes (Okano and Darnell, 1997). Additionally, multiple leader exons are a common feature of many genes that encode proteins with important functions in the brain, such as *AChE* (Meshorer et al., 2004), nerve growth factor (*NGF*; Bertaux et al., 2004) and adenosine A_{2A} receptors (Kreth et al., 2008).

The presence of several *HuD* leader exons raises questions regarding their expression and function. Expression of multiple E1 variants is typically a result of developmental and/or cell-type specific alternate promoter usage,

leading to the notion that *HuD* E1 variants may be under neuron-specific transcriptional control during neurogenesis and/or in the developed nervous system (Beltcheva et al., 2003; Kimura et al., 2006; Tsukamoto et al., 2007; Ishii et al., 2010; Jacox et al., 2010). Examination of the core (roughly +/- 100bp) and proximal promoter regions (up to 300bp upstream of TSS) with Genomatix MatInspector (Cartharius et al., 2005) revealed common and unique putative motifs surrounding the TSS of each *HuD* leader exon (Sandelin et al., 2007). The common putative motifs include regulatory promoter elements (CCAAT and GC boxes) and target motifs for TF implicated in driving gene expression during neurogenesis (C/EBP, E-box and CRE), indicating that some *trans*-acting factors can regulate the transcription of multiple *HuD* leader exons simultaneously (Bertrand et al., 2002; Cortes-Canteli et al., 2002; Carninci et al., 2006; Dworkin and Mantamadiotis, 2010). Conversely, the less commonly found putative motifs, such as RXR, could have a role in controlling transcription of individual E1 variants. The presence of assorted putative *cis*-acting elements supports the idea that distinct transcriptional events may drive expression of each *HuD* leader exon at different time points and to different levels during neurogenesis and/or in alternate neuronal subtype populations (Figure 4.1). Moreover, expression of these E1 variants could be altered in learning and memory or neuronal diseases, as previously observed for pan (all isoforms) *HuD* mRNA abundance.

In line with this view, we found that the relative abundance of transcripts containing these E1 variant varies at different time points of P19 neuronal and

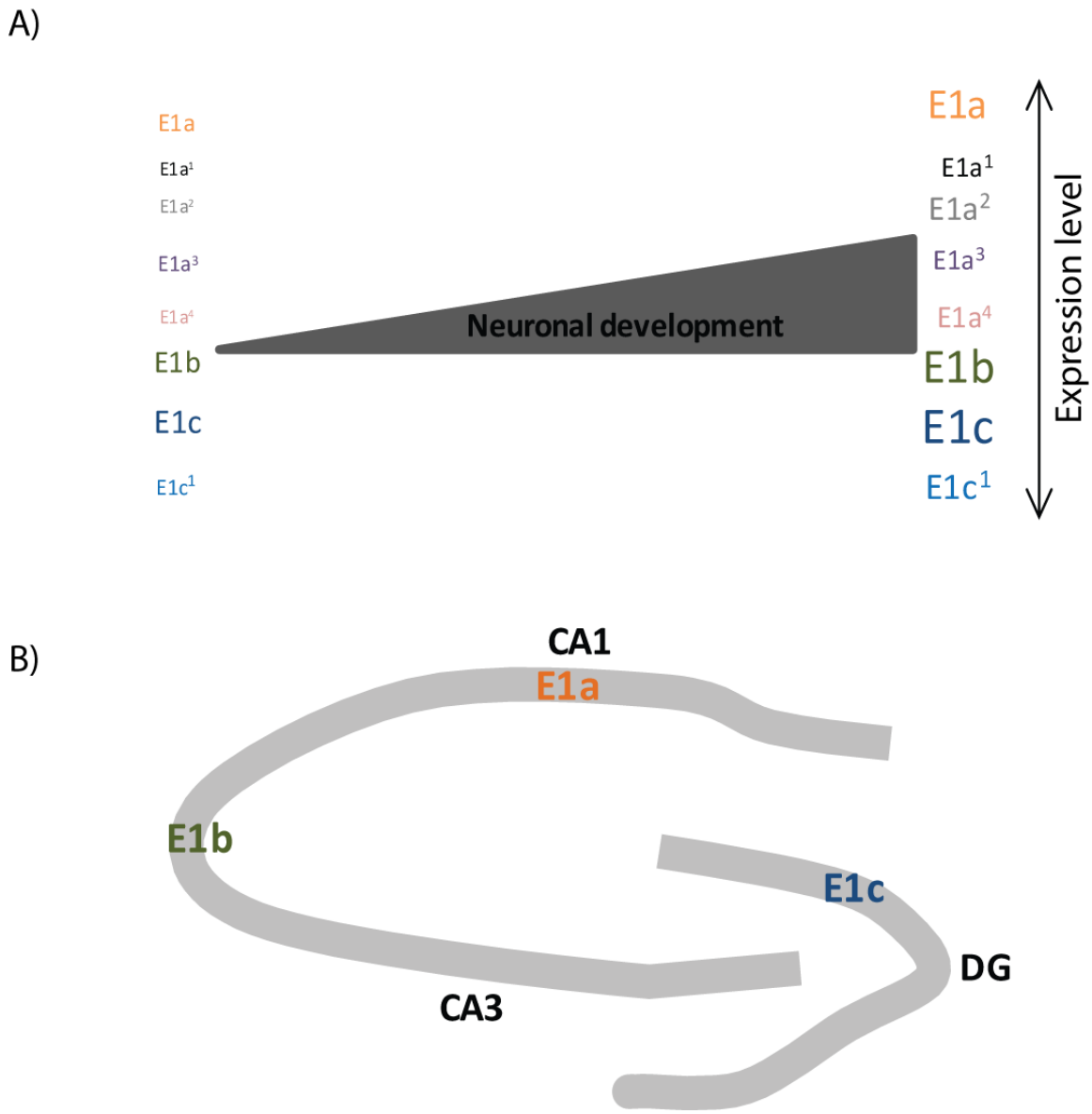


Figure 4.1 Spatiotemporal expression of *HuD* E1 mRNA variants in neurons

A) Schematic depicting the hypothetical variation in relative *HuD* E1 levels during neuronal development. Size of text represents relative expression of each exon 1 variant. **B)** In addition, *HuD* E1s may be variably expressed in neuronal subpopulations, as depicted here in different neural subtypes of the hippocampus.

mouse brain development, indicating that expression of *HuD* E1s is temporally regulated. In further corroboration of our findings, examination of the 5' flanking genomic region of *Xenopus elav-like ribonucleoprotein D gene (elrD),* a *HuD* homologue, showed that it contains two promoters that drive expression of two distinct E1 variants (Nassar and Wegnez, 2001). Moreover, these variants encode transcripts exhibiting unique temporal expression during development (Nassar, 2011). Remarkably, the *elrD* leader exons, E1 and E'1, partially align with mouse E1b and E1c, respectively, implying that the developmental regulation of these two mammalian exons is evolutionarily conserved (Figure 4.2).

4.4 Developmental expression and function of the *HuD* E1c variant

In the present study, through a combination of experiments, namely 5'RACE and EST analysis, we revealed that E1c is one of the principal *HuD* E1 variant expressed in neurons. We found high levels of E1c in the mitral cells of the olfactory bulb, ganglion cells of the retina, and all three cortical epithelial cell layers at E14.5 of mouse brain development. The spatial and temporal expression of E1c-containing *HuD* mRNA parallels that of pan *HuD* mRNA since pronounced expression of the latter has been previously detected in all of these regions (Okano and Darnell, 1997; Clayton et al., 1998). These findings indicate that E1c encodes a *HuD* 5'UTR and/or N-terminal peptide that has/have an important function(s). Possible functions could include intercellular localization or

Figure 4.2 Alignment of part of the *HuD* 5' genomic region sequence between different species

Nucleotide alignment of the *Xenopus ElrD* and mouse and human *HuD* 5' genomic regions encompassing exons 1b and 1c. Sequences for *Xenopus* E1 and E'1 and *mouse* E1b and E1c are in bold. In the consensus rows capital (letters), miniscule (letters) and periods represent conservation between all three, only two and none of the species, respectively.

translation regulation and depend on *cis*-acting or structural elements in the 5'UTR, which are targeted by *trans*-acting factors (Davuluri et al., 2008).

Examining the roles of E1c and E1a 5'UTRs in controlling the translation efficiency of HuD revealed that the former negatively regulates HuD protein synthesis in proliferating P19 cells. Additional biochemical assays, such as polysome profiling, are necessary to substantiate these findings. Similar effects were described for the Adam22 transcript, whose protein product is involved in neurogenesis and highly expressed in the brain. Adam22 mRNA also contains distinct 5'UTRs with one variant causing reduced Adam22 protein synthesis (Godde et al., 2007). In addition to influencing expression of HuD at the post-transcriptional and translational levels, the E1c variant may encode, for example, a different N-terminal that alters the binding specificity or binding strength of HuD to target mRNAs (Park-Lee et al., 2003).

An indirect role of E1c may be related to the spatiotemporal expression of an uncharacterized intermediate length ncRNA. ncRNAs belongs to a group of untranslated RNAs with functions in most cellular and developmental processes (Matera et al., 2007; Amaral and Mattick, 2008). A recent study using human foetal brain to search for intermediate sized ncRNAs uncovered a novel ncRNA that maps directly within *HuD* E1c (Yan et al., 2011). The specific function of this particular ncRNA is unknown; however, its high abundance in the foetal brain implies a developmental function (Yan et al., 2011).

4.5 *Cis*-acting elements in the *HuD* E1c 5'RR

Since we detected predominant expression of the E1c variant and transcriptional regulation of *HuD* in developing neurons, we examined the E1c 5'RR for putative *cis*-acting elements and *trans*-acting factor(s) that govern transcription of *HuD*. Initially, we determined the E1c TSS based on the longest clone obtained from our 5'RACE analysis; however, it should be noted that other TSSs in this vicinity were previously identified for the *HuD* gene (Inman et al., 1998). Based on these findings, E1c likely contains multiple TSSs with a particular preference for one strong site, indicative of a mixed mode promoter (Juven-Gershon and Kadonaga, 2010). These types of promoters contain a combination of focused (TSS at a single nucleotide or within a narrow range) and dispersed (weak TSS spread over 50 to 100 nucleotides) transcription initiation sites (Juven-Gershon and Kadonaga, 2010; Lenhard et al., 2012). Our bioinformatic analysis of the E1c 5'RR implies that no DNA sequence elements, such as TATA-box, Initiator element or CpG island, are present in the core promoter. TATA-boxes and Initiator elements are often located in promoters that contain focused TSSs- usually found in tissue-specific and regulated genes. On the other hand, CpG islands are situated in promoters that contain dispersed TSSs, often found in genes that are ubiquitously and constitutively expressed (Sandelin et al., 2007; Lenhard et al., 2012). Given that HuD E1c contains a mixed mode promoter without any DNA elements typically associated with focused or dispersed TSS, this indicates that the E1c 5'RR harbours uncommon *cis*-acting elements that initiate transcription.

Further upstream of the E1c TSS in the proximal promoter region, we identified Sp1, non-classical C/EBP, MyT1 and CREB motifs. The TFs that bind these sites have been implicated in transcriptional regulation of multiple neuronal genes. For example, by binding to their DNA motifs upstream of proneural genes, C/EBPβ (Menard et al., 2002) and CREB (Finkbeiner et al., 1997) play essential roles in the differentiation of various neuronal populations. Some of these sites are also present in the mammalian *HuB* and *zebrafish HuC* promoters, raising the possibility that certain evolutionarily conserved transcriptional mechanisms are shared among nELAV members (King, 1996; Park et al., 2000a).

We demonstrated that the *HuD* E1c proximal promoter region was sufficient to promote basal expression in both neuronal and mesodermal cells. These results are supported by ChIP experiments coupled with mass sequencing demonstrating that RNA Polymerase II binds upstream of *HuD* E1c in a mesodermal lineage cell line (GM19099; ENCODE Project Consortium, 2011). In addition, we identified a well-conserved ~400bp region that enhanced gene expression specifically in neurons further upstream of E1c. This 400bp region shares a high degree of homology with one of the *elrD* promoter fragments that was shown to induce neuron-specific reporter expression during *Xenopus* development (Nassar and Wegnez, 2001; Nassar, 2011). These findings imply that the 400bp fragment harbours *cis*-elements, potentially conserved in vertebrates, that are important for transcription of *HuD*.

4.6 NGN2-dependent control of *HuD* transcription

A striking feature of the 400bp DNA region is the presence of several putative *cis*-elements, of which the most notable are five E-boxes (5' CANNTG 3'). In neurons, E-boxes are often targeted by basic helix-loop-helix (bHLH) TFs that play essential roles at different stages of neuronal development (Bertrand et al., 2002; Ross et al., 2003). For instance, the proneural MASH1, NGN 1/2/3 and MATH1 are important for neuronal progenitor commitment, cell cycle exit and neuronal development while NEUROD1/2 promote neurogenesis at later stages (Guillemot, 2007). Similar to our findings, the *zebrafish HuC* gene was found to contain several E-boxes that play a critical role in maintaining its neuron-restricted expression (Park et al., 2000a). In this study, we demonstrate that the 400bp region, containing the five E-boxes, is particularly responsive to NGN2. Analysis of these E-boxes revealed that two of them (E-box 2 and 3) are necessary for NGN2-dependent transcription of *HuD*.

Our results are in agreement with converging lines of evidence correlating the functions and spatiotemporal expressions of these two proteins. In the developing nervous system, NGN2 regulates the generation of progenitor cells, cell cycle exit, neuronal lineage commitment, cell migration, axon guidance, terminal neuronal differentiation and survival (Guillemot, 2007; Fode et al., 1998; Fode et al., 2000; Cai et al., 2000; Seibt et al., 2003; Britz et al., 2006). NGN2 also inhibits glial cell differentiation and promotes neuronal subtype identity, including neurotransmitter specification (e.g. cortical glutamatergic neurons; Bertrand et al., 2002; Kele et al., 2006). Several of these functions match the

diverse repertoire of roles performed by HuD, including control of cell proliferation, cell cycle exit, commitment, axogenesis, glutamatergic identity and neuronal survival (Hinman and Lou, 2008; Pascale et al., 2008; Deschenes-Furry et al., 2006; Ince-Dunn et al., 2012). Complimentary to conducting similar functions in the nervous system, the temporal expression of *Ngn2* mRNA precedes (detected as early as E8.5 in mice) and overlaps that of *HuD* mRNA during embryonic development (Fode et al., 1998; Thoma et al., 2012). Furthermore, *Ngn2* transcripts are localized to several similar embryonic brain neuronal populations as *HuD* E1c, including the ventricular zone of the cerebral cortex, hippocampus, spinal cord, olfactory bulb and retina (Clayton et al., 1998; Yan et al., 2001; Schuurmans et al., 2004; Yan et al., 2005; Galichet et al., 2008; Henke et al., 2009b; Shaker et al., 2012; see also Allen Developing Mouse Brain Atlas; http://developingmouse.brain-map.org). Lastly, studies involving misexpression of NGN2 corroborate our finding that it positively regulates transcription of *HuD*. For instance, NGN2 over-expression in cultured cells or knockout in mice results in increased or decreased HuD protein levels, respectively (Farah et al., 2000; Mattar et al., 2004). Together with our findings, the parallel expression and functions of these two proneural genes indicates that NGN2-dependent expression of HuD may play an instrumental role in several neuronal contexts.

4.7 The role of NGN2-dependent expression of HuD in different neuronal contexts

As stated above, ectopic expression of NGN2 leads to transcription of various proneural genes resulting in precocious neuronal differentiation in cultured stem-like cells (Farah et al., 2000), neural progenitor cells (NPCs; Serre et al., 2012) and embryonic stem cells (ESCs; Thoma et al., 2012). Thus, together with our study and previous reports describing augmented expression of nELAV proteins following over-expression of NGN2 (Farah et al., 2000), these findings strongly suggest that HuD is a major downstream effector of NGN2-dependent neuronal development (see section 4.10).

Based on the implications of NGN2 and HuD in neuronal function and plasticity, it is tempting to speculate that NGN2-dependent expression of HuD is also an important event in learning and memory, neuronal recovery following injury and neuronal disease. Studies on developing NGN2 knockout mice showed that they contain fewer neuronal progenitors and display neurogenesis defects in the DG, a region in the hippocampus that has a central function in learning and memory (Galichet et al., 2008; Roybon et al., 2009). NGN2 may perform similar functions in adult mice since it is expressed in subgranular zone (SGZ) progenitor cells of the adult DG (Ozen et al., 2007; Hodge et al., 2008). In line with its role in neuronal plasticity, NGN2 expression has been detected in adult monkey DG progenitors following ischemia (Tonchev and Yamashima, 2006), suggesting that it is involved in generating new neurons following certain brain insults (Liu et al., 1998). Also of particular interest, transplantation of human

embryonic precursors ectopically expressing NGN2 to a severe spinal injury site in rats promoted axonal sprouting and functional recovery (Perrin et al., 2010).

Emerging studies are also demonstrating that NGN2 has a causal or casual role in neuropathies. For example, *Ngn2* mRNA abundance was found to be directly proportional to $A\beta_{42}$ levels, the peptide presumed to initiate the pathological cascade in AD (Uhrig et al., 2009). In addition to AD, deletion of the *Ngn2* gene in mice showed that it is required for proper development of dopaminergic precursors and mature dopaminergic neurons, implying that NGN2 is also involved in PD (Kele et al., 2006; Andersson et al., 2006). Although a recent cohort genetic analysis of the *Ngn2* gene found no mutations associated with PD (Deng et al., 2010), NGN2 expression and/or function could potentially be misregulated in PD patients. Also of particular interest, increased levels of NGN2 have been found in certain neuroendocrine cancers, such as pituitary adenomas and medulloblastomas, indicating that it plays a role in malignant cells (Rostomily et al., 1997; Fratticci et al., 2007). Taken together, this data implicates NGN2 in similar neuroplasticity circumstances and neuropathies as HuD, signifying that regulation of *HuD* transcription by NGN2 may be directly involved in plasticity-dependent and pathological contexts.

4.8 Molecular events regulating NGN2 expression and function

To better understand the role of NGN2-dependent regulation of HuD in neurogenesis and other contexts, it is important to explore the molecular events that control NGN2 expression and function. For instance, in embryonic NSCs the positive and negative control of *Ngn2* transcription by Pax6 and Hes family TFs,

respectively, produce oscillations in NGN2 protein abundance (Scardigli et al., 2001; Scardigli et al., 2003; Shimojo et al., 2008). These fluctuations in NGN2 protein levels result in progression of neuronal differentiation when *Ngn2* levels are high and maintenance of NSCs in an undifferentiated and self-renewing state NGN2 levels are reduced (Guillemot, 2007; Guillemot et al., 2006). Additionally, molecular mechanisms controlling stability of *Ngn2* mRNA also play an important role in its expression and, consequently, in neuronal fate. A recent study has found that the microprocessor Drosha binds to hairpin structures in the *Ngn2* 3'UTR and, through a miRNA-independent mechanism, decreases its mRNA stability and protein abundance (Knuckles et al., 2012). In turn, this process inhibits accumulation of NGN2 protein, its downstream effectors and consequently neurogenesis, resulting in maintenance of NSCs in an undifferentiated state. Analysis of the *Ngn2* mRNA using an ARE database (http://rna.tbi.univie.ac.at/cgi-bin/AREsite.cgi) indicates that one of the prominent targets of the HuD protein is *Ngn2* mRNA (Perrone-Bizzozero et al., 2011). The *Ngn2* 3'UTR contains six putative ARE sites raising the possibility that HuD binds to these motifs in the *Ngn2* 3'UTR, consequently antagonizing Drosha binding, to stabilize the *Ngn2* mRNA and form a positive feedback loop that drives neuronal differentiation.

Post-translational modification of NGN2 and its association with co-factors also alters the expression and role of NGN2, thereby altering different cell fate. For instance, multisite phosphorylation of NGN2 reduces the affinity of NGN2 for E-boxes in proneural gene promoters and thereby prevents neurogenesis (Ali et

al., 2011; Hindley et al., 2012). On the contrary, un(der)phosphorylated NGN2 associates onto E-boxes of proneural genes with several co-factors, such as E12/E47 and presumably LMO4 (Asprer et al., 2011) and BRG1 (Seo et al., 2005) at different time points of development to drive proper sequential progression of neurogenesis. NGN2 protein function and expression is also regulated following interactions with ASCl1 (Henke et al., 2009a), $p27^{Kip1}$ (Nguyen et al., 2006) and MTGR1 (Aaker et al., 2009; Aaker et al., 2010). Additionally, phosphorylation of NGN2 on two specific serine residues and subsequent interaction with LIM co-factors, RA-activated RARs and the histone acetyltransferase CREB-binding protein (CBP) on E-boxes upstream of specific genes is necessary for spinal motor neuron differentiation (Lee et al., 2009; Ma et al., 2008).

In our studies, we found that NGN2 binds to RARα and that RA enhances NGN2 -dependent transcription of *HuD*. However, we were not able to demonstrate that RARα along with NGN2 is recruited to the two NGN2 -targeted E-boxes upstream of E1c. There are two likely explanations for our seemingly contradicting findings. First, RAR may not form a complex with NGN2 on these two E-boxes under any context. In this case, the enhanced transcription of E1c that we detected following RA treatment could be a consequence of other mechanisms activated by RA. The second possibility is that another RAR isoform (s) (β and γ) complexes with NGN2 on these E-boxes, which would explain our inability to detect the RARα interaction with NGN2 on this DNA region.

4.9 Additional putative motifs controlling transcription of *HuD*

Our studies show that although NGN2 is central for optimal induction of *HuD* transcripts, other TFs are also involved. Deletion and mutation of E-boxes 2 and 3 only partially reduced reporter activity, indicating that upstream *cis*-acting motif(s) also promote expression of *HuD* mRNA. Through further mutational analysis, we identified putative motifs targeted by KLF and NuDR/DEAF-1 TFs as regulators of *HuD* expression. Importantly, specific members of the KLF family and NuDR/DEAF-1 have been documented to be involved in neuronal development (Hahm et al., 2004; McConnell and Yang, 2010).

KLFs are zinc-finger-containing *trans*-acting factors that share homology with Sp1 (Kadonaga et al., 1987) and *Drosophila* KRUPPEL (Schuh et al., 1986) TFs and control cell proliferation, development and apoptosis (McConnell and Yang, 2010). Out of the 17 members in the KLF family, there is strong evidence that KLF6 and 7 are implicated in embryonic neuronal development and adult neuronal plasticity (Veldman et al., 2007; Moore et al., 2009; Blackmore et al., 2012). Moreover, KLF6 is expressed in various neural subtypes in the adult mouse forebrain (Jeong et al., 2009). The similar role of these two factors in axon outgrowth raises the possibility that they share common downstream targets and are thus functionally redundant. In particular, KLF6/7 have been demonstrated to promote transcription of genes whose mRNA products are incidentally targeted by HuD, including $p21^{WAF}$, *p27* and *GAP-43* (Shie et al., 2000; Benzeno et al., 2004; Laub et al., 2005; Kajimura et al., 2007). Based on these findings, it is within reason to speculate that KFL6/7 promote(s) the expression of genes

controlling neurogenesis directly through transcriptional and, indirectly by inducing expression of HuD, post-transcriptional mechanisms. In our studies we found a functional *cis*-acting motif for KLF TFs upstream of E1c. However, we were unable to demonstrate altered transcription of *HuD* by overexpressing KLF6. These findings do not rule out the possibility that other KLF members or TFs target the putative KLF motif.

The TF NuDR/DEAF-1 contains a DNA-binding SAND domain (Bottomley et al., 2001) and shares nucleotide similarity with the *Drosophila Deaf-1* gene (Huggenvik et al., 1998). Expression of NuDR/DEAF-1 occurs early in development and the protein is abundant in the adult rat brain (Huggenvik et al., 1998; LeBoeuf et al., 1998). Although initial examination of NuDR/DEAF-1 knockout mice that develop into adulthood did not reveal any gross morphological changes in the brain (Czesak et al., 2012), the described functions of NuDR/DEAF-1 in neural tube formation (Hahm et al., 2004) and cellular proliferation (LeBoeuf et al., 1998) warrants more detailed analysis. In light of these functions and based on our identification of a functional putative NuDR site upstream of E1c, we tested the effect of NuDR/DEAF-1 on transcription of *HuD* in neurons. Our findings demonstrate that ectopic expression of NuDR/DEAF-1 did not increase transcription of *HuD*. In fact, our results indicate that NuDR/DEAF-1 negatively controls expression of HuD. This unexpected finding could be a result of transcriptional squelching and is supported by the known dual functions of NuDR/DEAF-1 as an enhancer and repressor, as previously demonstrated for the 5-HT1A receptor in different neuronal subtypes (Czesak et

al., 2006). Thus, NuDR/DEAF-1 may require additional co-factors or post-translational modification to activate transcription of HuD.

4.10 Model of HuD induction in different neuronal contexts

Our findings together with the other molecular events known to regulate expression of HuD generate a model, best exemplified in neurogenesis, where the abundance and function of HuD are intricately controlled at multiple levels (Figure 4.3). In this model, extrinsic signals (such as RA and neurotrophic factors) act on NSCs to activate intracellular molecular cascades, resulting in increased expression of proneural TFs and co-factors (e.g. NGN2 and potentially KLF6 and NuDR; Maden, 2007; Kumar et al., 2007; Sharpe and Goldstone, 2000; Tang et al., 2002; Ribes et al., 2008). These TFs subsequently target other proneural genes, which in the case of NGN2 include *NeuroD* and *HuD*, and induce their expression. In parallel, the abundance and/or activity of negative regulators of HuD are reduced, such as TR and FoxO1. Extrinsic signals may also directly induce expression of HuD through, for example, RA-RAR-dependent mechanisms (Lee et al., 2009).

In addition to controlling transcriptional mechanisms, RA and neurotrophins (through PKC) have been recently shown to decrease CARM1 protein abundance, a factor that post-translationally controls HuD and other RBPs (Hubers et al., 2010; Blackwell and Ceman, 2012). As a consequence, augmented neural gene expression and prevention of CARM1-mediated methylation of HuD at the onset of neuronal differentiation permits RA or neurotrophin signalling to function as a molecular switch promoting cell-cycle exit

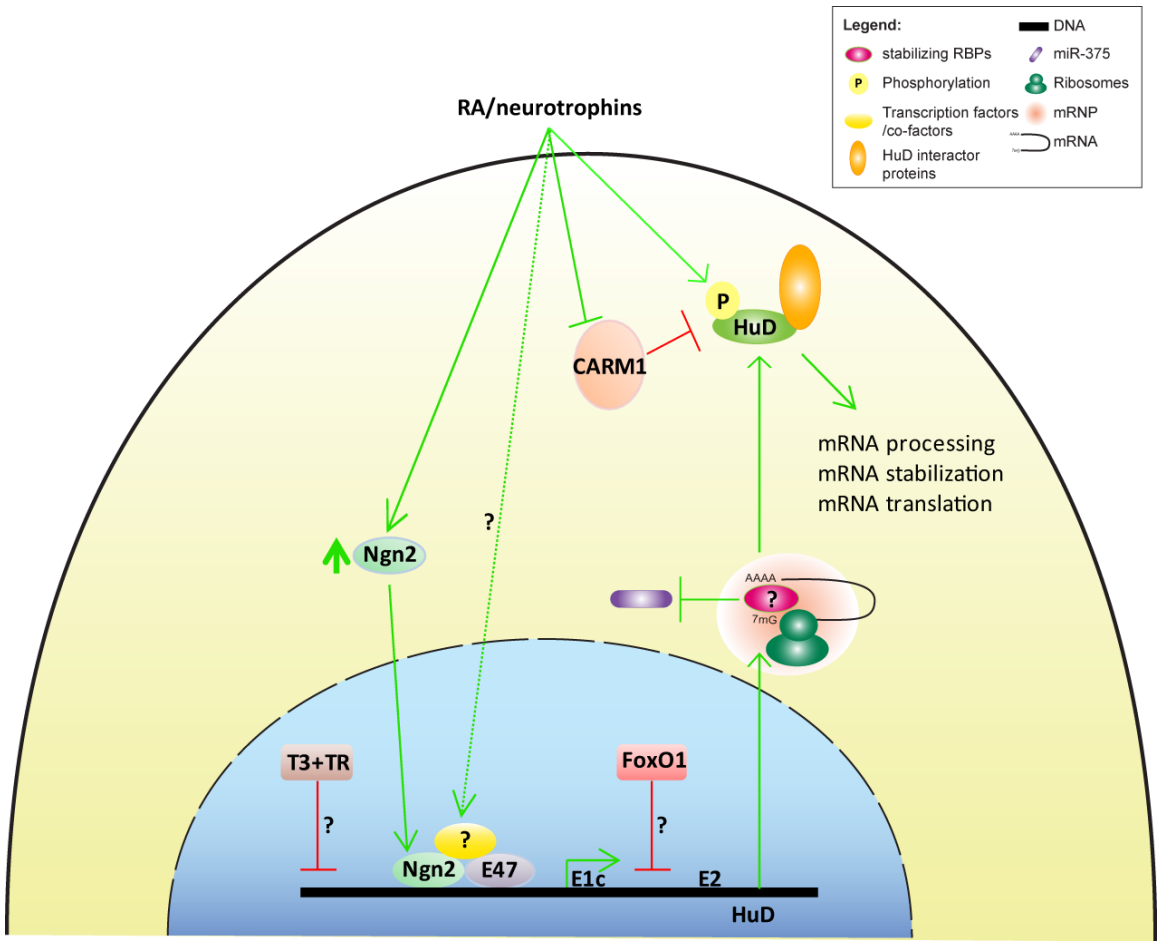


Figure 4.3 Simplified model of HuD expression during neuronal differentiation

In NSCs, the abundance and activation of specific proteins is increased or decreased (e.g. NGN2 and CARM1, respectively) by RA or neurotrophin signalling to promote neurogenesis. Subsequently, transcription factors such as the proneural NGN2 bind to the *HuD* 5' genomic region and enhance its transcription. In contrast, the expression and/or activity of *trans*-acting factors that negatively control HuD are reduced, including TR and FoxO1. During neurogenesis, *HuD* mRNA is likely subject to post-transcriptional control by proneural RBPs that prevent destabilizing RBP and miR binding. The eventual increase in unmethylated HuD protein levels (due to a reduction in CARM1 levels), its post-translational modification (e.g. phosphorylation) and interaction with other proteins allows HuD to positively control mRNA metabolism of proneural genes at multiple levels, resulting in neurogenesis. Green and red lines represent pathways that promote and inhibit neuronal differentiation, respectively.

and neuronal commitment (Hubers et al., 2010). The essential role of HuD in this cascade is further highlighted by the finding that misexpression or altered function of HuD severely inhibits the ability of RA or neurotrophins to induce neurogenesis (Kasashima et al., 1999). As neurogenesis progresses, expression of HuD is likely controlled at post-transcriptional and translational levels by *trans*-acting factors, which in part prevent binding of destabilizing *trans*-acting factors to HuD mRNA. At later stages of neurogenesis, post-translational modification of HuD (e.g. phosphorylation) and its interaction with other proteins, such as AKT1 and MAP1B, promotes morphological neuronal differentiation (Fujiwara et al., 2011b; Fujiwara et al., 2006; Pascale et al., 2005; Fujiwara et al., 2011a). From this model, it is evident that the timely and multilevel control of HuD expression and proneural activity enables commencement and proper progression of neuronal differentiation. In addition to significantly contributing to our current understanding of neurogenesis, similar molecular events controlling the abundance and function of HuD could also transpire in other contexts, including learning and memory and axonal regeneration.

4.11 Conclusion and future perspectives

In light of the key roles of HuD in developing and mature neurons, its implications in learning and memory as well as its involvement in several neurological diseases, it appears crucial to define the molecular machinery that promotes its expression. Leading up to this work, the 5' genomic region of *HuD* was largely uncharacterized. Our findings showing that the mammalian *HuD*

gene contains eight leader exons that are expressed in neurons shed new light on the complex regulation of *HuD* but concurrently raised questions regarding their expression and functions. Based on previous work outlining pan HuD expression, it seems reasonable to postulate that the E1 variants have specific functions in neuronal development (Anderson et al., 2000; Mobarak et al., 2000; Anderson et al., 2001; Akamatsu et al., 2005), learning and memory (Quattrone et al., 2001) and/or nerve regeneration (Deschenes-Furry et al., 2007; Anderson et al., 2003). Along these lines, the expression and/or function of these variants may also be specifically affected in neurological diseases (Hubers et al., 2010; Tiruchinapalli et al., 2008b; Bolognani et al., 2007a; Amadio et al., 2009; Noureddine et al., 2005).

In addition, prior to our studies, knowledge of the molecular mechanisms that control expression of *HuD* mRNA, particularly the *trans*-acting factors and *cis*-acting elements that affect its transcription, were also rather limited. Only two TFs, T3 (presumably bound to TR) and FoxO1, were known to play integral roles in controlling expression of HuD in mammalian cells, albeit in a negative manner (Lee et al., 2012; Cuadrado et al., 2003). Our studies address a substantial lack in understanding of the events regulating expression of HuD by determining that HuD is primarily under transcriptional control during early neurogenesis. Additionally, we were the first to identify *trans*-acting factors that induce *HuD* mRNA synthesis, namely the NGN2 and potentially KLF and NuDR TFs. These findings, in the context of the current literature, suggest that expression of HuD is controlled by a balance of negative and positive molecular events, presumably at

multiple regulatory levels. Consequently, it can be envisaged that a shift towards the former maintains neuronal stem cell identity whereas the latter promotes neurogenesis and potentially neuronal plasticity. On the other hand, a disruption in the balance of these factors may be associated with various neurological disorders, such as AD and PD. Thus, based on our findings and the multifunctional roles of HuD, it becomes evident that a thorough knowledge of the molecular machinery that promotes expression of this master regulator is critical for understanding not only neuronal development and function, but also various neuronal disease states.

Although these conclusions are derived from a well-established neuronal cell culture model (P19 cells) and mouse embryonic brain, future studies should address the developmental and neuron-/neuron subtype-specific pattern of expression of *HuD* E1 variants, especially E1c, using a transgenic mouse approach (e.g. by fusing *HuD* E1 5'RRs to the *enhanced green fluorescent protein* gene). The transgenic mice could also be employed to determine the expression pattern of *HuD* E1c, and other E1 variants, in mouse models of neuropathies, including PD and AD. Furthermore, prospective studies should also define the role of NGN2 in controlling expression of *HuD* E1c during *in vivo* neuronal development, in synaptic plasticity models and in disease states. The same transgenic mice (expressing the E1c 5'RR) can be used in combination with lentiviral-mediated over-expression or down-regulation of NGN2 levels to address these questions. Equally important, further studies are necessary to unravel the role of TFs and co-factors, such as KLF, NuDR and LMO4, in

regulating neuronal expression of *HuD in vitro* and *in vivo* under different contexts. The ‘reverse ChIP’ technique, for example, could be employed for this purpose.

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5: APPENDIX A- PERMISSION TO REUSE PUBLISHED MATERIAL

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SfN Central Office

Journal of Neuroscience

SfN Article:

Lucas M. Bronicki*, Guy Bélanger, and Bernard J. Jasmin*

Characterization of Multiple Exon 1 Variants in Mammalian HuD mRNA and Neuron-Specific Transcriptional Control via Neurogenin 2

The Journal of Neuroscience, 15 August 2012, 32(33):11164-11175; doi:10.1523/JNEUROSCI.2247-12.2012

6: CURRICULUM VITAE

Lucas Bronicki

Education

Ph.D. in Neuroscience/Cellular and Molecular Medicine **2005-2012**

University of Ottawa, Ontario

Thesis: Characterization of multiple exon 1 variants and neuron-specific transcriptional control of mammalian *HuD*.

Advisor: Dr. Bernard J. Jasmin, Full professor and Vice Dean of research (cellular and molecular medicine), University of Ottawa.

B.Sc. Biology with biotechnology option **2001-2005**

University of Ottawa, Ontario

Thesis: Brain-derived neurotrophic factor (BDNF) induces serum response factor-mediated immediate early gene up-regulation in neurons.

Teaching Experience

Genetics laboratory teaching assistant **2012**

University of Ottawa, Ontario

- Instructed fundamentals of genetics to undergraduate students
- Effectively explained and illustrated concepts to students
- Managed students' productivity, quality of experiments and grades

General and organic chemistry laboratory teaching assistant **2006-2012**

University of Ottawa, Ontario

- Demonstrated general and organic chemistry laboratory techniques to undergraduate students
- Supervised and assisted chemistry laboratory experiments
- Managed students' productivity, quality of experiments and grades

Anatomy and physiology marker **2010-2012**

University of Ottawa, Ontario

- Marked quizzes and mid-term and final exams for anatomy and physiology courses

Relevant experience

- Extensive knowledge of DNA and RNA based assays, including DNA and RNA extraction methods, reverse transcriptase polymerase chain reaction (RT-PCR), real-time quantitative PCR, *in situ* hybridization (ISH), RNAse protection assay and 5' rapid amplification of cDNA ends (RACE)
- Planning, designing, executing and troubleshooting molecular experiments
- Presenting my own research to other scientists
- Collaborating with other researchers on various projects
- Writing reports, including grants and peer-reviewed articles
- Completed select courses: Chemistry (general, organic and physical chemistry), Biochemistry (introduction to biochemistry, general intermediary metabolism), Biology (cell biology, genetics, genomics, molecular genetics) and Statistics (probability and statistics for natural sciences) at the University of Ottawa

Research Interests

- Cellular and molecular neuroscience
- Clinical molecular genetics
- Molecular diagnostics
- Nutrition and exercise physiology

Peer reviewed papers

- **BRONICKI L**. BELANGER G. and JASMIN BJ. *Characterization of multiple exon 1 variants in mammalian HuD mRNA and neuron-specific transcriptional control via Neurogenin 2.* Journal of Neuroscience. 2012.
- **BRONICKI, L.**, JASMIN, BJ. *Trans-acting factors governing expression of AChE mRNA.* Review. Frontiers in molecular neuroscience. 2012.

Abstracts

- **BRONICKI L**., DESCHENES-FURRY J., JASMIN, BJ. *Molecular mechanisms regulating the RNA-Binding Protein HuD during neurogenesis.* Poster presented at the Neuroscience convention, Atlanta, GA 2006.
- CHOPARD A., **BRONICKI L**., HILLOCK S., LUNDE J., JASMIN BJ. *Expression of RNA-Binding Proteins that control RNA stability in atrophying or hypertrophying skeletal muscle cells.* Poster presented at the Myology convention, France 2008.

Awards and Scholarships

Ontario Graduate Scholarships in Science and Technology	**2009-2010**
University of Ottawa excellence scholarship	**2009-2010**
CIHR Pfizer/IMHA/Rx&D summer studentship	**2005-2006**

Professional memberships and certifications

American Association of Clinical Biochemists	**2012-present**
Can Fit Pro Personal Trainer Specialist certified	**2009-present**
CPR level A	**2009-present**

Volunteer

Brain health day (poster judge)	**2012**
Brain Awareness Week (presented to elementary students)	**2010**
Cellular and molecular medicine graduate student council (VP social and athletic)	**2006-2008**
The Ottawa Civic Hospital continuing care center (motivator and assistant to staff)	**2004**

Languages

- English- fluent written and spoken
- French- conversant in written and spoken
- Polish- conversant in written and spoken

References:

Available upon request.