

HuD regulates SOD1 expression during oxidative stress in differentiated neuroblastoma cells and sporadic ALS motor cortex

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ARTICLE INFO

Keywords:

Sporadic amyotrophic lateral sclerosis
Oxidative stress
SOD1
ELAVL4/HuD
Motor cortex

ABSTRACT

The neuronal RNA-binding protein (RBP) HuD plays an important role in brain development, synaptic plasticity and neurodegenerative diseases such as Parkinson's (PD) and Alzheimer's (AD). Bioinformatics analysis of the human SOD1 mRNA 3' untranslated region (3'UTR) demonstrated the presence of HuD binding adenine-uridine (AU)-rich instability-conferring elements (AREs). Using differentiated SH-SY5Y cells along with brain tissues from sporadic amyotrophic lateral sclerosis (sALS) patients, we assessed HuD-dependent regulation of SOD1 mRNA. In vitro binding and mRNA decay assays demonstrate that HuD specifically binds to SOD1 ARE motifs promoting mRNA stabilization. In SH-SY5Y cells, overexpression of full-length HuD increased SOD1 mRNA and protein levels while a dominant negative form of the RBP downregulated its expression. HuD regulation of SOD1 mRNA was also found to be oxidative stress (OS)-dependent, as shown by the increased HuD binding and upregulation of this mRNA after H₂O₂ exposure. This treatment also induced a shift in alternative polyadenylation (APA) site usage in SOD1 3'UTR, increasing the levels of a long variant bearing HuD binding sites. The requirement of HuD for SOD1 upregulation during oxidative damage was validated using a specific siRNA that downregulated HuD protein levels to 36% and prevented upregulation of SOD1 and 91 additional genes. In the motor cortex from sALS patients, we found increases in SOD1 and HuD mRNAs and proteins, accompanied by greater HuD binding to this mRNA as confirmed by RNA-immunoprecipitation (RIP) assays. Altogether, our results suggest a role of HuD in the post-transcriptional regulation of SOD1 expression during ALS pathogenesis.

1. Introduction

Post-transcriptional regulation of mRNA stability, localization and translation plays an important role in the nervous system (Di Liegro et al., 2014; Wang et al., 2007). Local translation has been implicated in developing axons and dendrites, and in synapse formation (Thomas et al., 2014; Weiß et al., 2015). Furthermore, activity-dependent mRNA trafficking and local translation underlie long-lasting changes in

synaptic efficacy (Di Liegro et al., 2014; Donnelly et al., 2010). RNA binding proteins (RBPs) are known to orchestrate post-transcriptional regulatory mechanisms via their interaction with consensus sequences primarily located in the 5' or 3' untranslated regions (5' or 3'UTRs) of mRNAs (Bolognani and Perrone-Bizzozero, 2008; Glisovic et al., 2008; Perrone-Bizzozero and Bolognani, 2002; Ray et al., 2013). Mutations of several RBPs have been identified in neurological disorders including Amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disorder

Abbreviations: TPA, 12-O-tetradecanoyl-phorbol-13-acetate; 3' RACE, 3' Rapid Amplification of cDNA Ends; 3'UTR, 3' untranslated region; 5' UTR, 5' untranslated; ARE, adenine-uridine-rich element; APA, alternative polyadenylation; AD, Alzheimer's disease; ALS, Amyotrophic Lateral Sclerosis; ASO, Antisense Oligonucleotide; fALS, Familial ALS; GWAS, genome-wide association study; *Elavl4*^{-/-}, HuD Knock Out mice; HuDOE, HuD overexpressing transgenic mice; NDs, neurodegenerative diseases; OS, oxidative stress; PD, Parkinson's disease; PBMCs, peripheral blood mononuclear cells; polyA, polyadenylation; qRT-PCR, quantitative Real Time PCR; ROS, Reactive Oxygen Species; RBPs, RNA binding proteins; REMSA, RNA electrophoretic mobility shift assay; RIP, RNA immunoprecipitation assay; RNAi, RNA interference; RRM, RNA Recognition Motif; sALS, Sporadic ALS; WB, Western Blot.

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<https://doi.org/10.1016/j.nbd.2020.105211>

Received 26 June 2018; Received in revised form 9 October 2020; Accepted 25 November 2020

Available online 1 December 2020

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affecting motor neurons in the motor cortex (upper motor neurons), brainstem and anterior horn of the spinal cord (lower motor neurons) (Bäumer et al., 2014).

ALS can occur sporadically, without any family history (sALS; 90–95% of patients), whereas a small percentage of ALS cases are considered familial (fALS; 5–10%) (Robberecht and Philips, 2013). Abnormalities in RNA processing and metabolism represent one of the major features of motor neuron loss in both fALS and sALS (Barmada, 2015; Donnelly et al., 2014; Purice and Taylor, 2018; Yasuda and Mili, 2016). Mutations in genes encoding for the RBPs TDP-43, FUS and other members of the hnRNP family have been found in fALS (Ito et al., 2017; Kim et al., 2013). In addition, mutations in Superoxide Dismutase 1 (SOD1) gene account for 10–20% of fALS and are the second most common cause of fALS (Rosen et al., 1993). While the role of SOD1 in sALS is still controversial (Bali et al., 2016; Da Cruz et al., 2017; Hayashi et al., 2016), its mRNA levels were found to be upregulated in peripheral blood mononuclear cells (PBMCs), spinal cord and brain stem of sALS patients (Ferraiuolo et al., 2016; Gagliardi et al., 2010; Milani et al., 2013). Misfolded and aggregated SOD1 has also been associated with prion-like propagation, from in vitro fibril seeding to cell-to-cell and in vivo tissue propagation in fALS (Grad et al., 2014b, 2014a; McAlary et al., 2019) but this does not seem to take place in sALS (Da Cruz et al., 2017).

ELAV-like/Hu proteins are orthologues of the *Drosophila* ELAV (Embryonic lethal abnormal vision) RBP, which is essential for the development of the neurons (Robinow et al., 1988). ELAVL4 (HuD) is one of the neuronal (nELAV) members of the ELAVL family, and is highly expressed in developing neurons, where it is enriched in growth cones and is required for neuronal differentiation (Akamatsu et al., 2005; Smith et al., 2004). In addition, HuD has been linked to neurodegenerative diseases (Amadio et al., 2009; Perrone-Bizzozero and Bird, 2013) and *ELAVL4* was shown to be a susceptibility gene for age-at-onset in Parkinson's disease (PD) (DeStefano et al., 2008; Noureddine et al., 2005). HuD also binds to and regulates mRNAs involved in Alzheimer's disease pathogenesis (e.g., APP, BACE1 and ADAM10), resulting in amyloid beta overproduction (Amadio et al., 2009; Kang et al., 2014). We previously demonstrated the association of HuR, the ubiquitously expressed member of ELAVL family, with increased SOD1 gene expression in PBMCs from sALS patients (Milani et al., 2013). Subsequent studies demonstrated that HuR levels are also increased in spinal cords from ALS patients in association with microglia activation (Matsye et al., 2017). The potential involvement of HuR and HuD in ALS is further supported by the known interactions of these RBPs and the mRNAs of two other RBPs involved in ALS pathogenesis TDP43 and FUS (De Santis et al., 2019; Lu et al., 2014). Finally, Lu and coauthors (Lu et al., 2014, 2009) demonstrated the co-localization of HuR and mutant SOD1 protein, suggesting a toxic effect of mutant SOD1 through RBP sequestration.

The goal of this study was to investigate the potential involvement of HuD in SOD1 regulation during oxidative stress and sALS. We first used human neuroblastoma SH-SY5Y cells exposed to 1 mM H₂O₂ as this cellular model exhibits features of cell damage observed in sALS patients including increased SOD1 expression (Dell'Orco et al., 2016). Here, we found that oxidative stress (OS) increases not only the levels of SOD1 and HuD but also HuD binding to SOD1 mRNA. SOD1 upregulation by OS is dependent on HuD as shown by the suppression of this process upon downregulation of this RBP. In *post-mortem* tissues, both HuD mRNA and protein levels are increased in the motor cortex but not in the cerebellum of sALS patients compared to healthy controls. SOD1 mRNA and protein as well as the interaction of HuD with SOD1 mRNA are also significantly higher in ALS tissues. Altogether, our findings demonstrate that post-transcriptional regulation of SOD1 mRNA by HuD could be involved in ALS pathogenesis, suggesting that this RBP may be a novel neuronal biomarker and/or therapeutic target for this disease.

2. Materials and methods

2.1. Cell culture, differentiation and treatment

Human neuroblastoma SH-SY5Y cells were grown in Dulbecco's modified Eagle/F12 medium (Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 10 mg/mL streptomycin (Life Technologies), at 37 °C in an atmosphere of 5% CO₂ and 95% humidity. Cell medium was changed every 2 days and the cells were subcultured once they reached 80–90% confluence. SH-SY5Y cells were differentiated by treatment with 160 nM 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Sigma-Aldrich, USA) for 16 h (Leli et al., 1992; Linnala et al., 1997; Presgraves et al., 2004). Cells were exposed to 1 mM H₂O₂ (Sigma-Aldrich) in PBS or just PBS for 30 or 60 min.

2.2. Transfection

pcHuD and pcHuD I + II plasmids were generated as previously described (Anderson et al., 2000); cells were also transfected with the empty vector pcDNA3 or left untransfected as control (ctr). Human siHuD sequence and a ctr siRNA were designed according to Kang et al., 2014 (Kang et al., 2014). Human neuroblastoma SH-SY5Y cells were transfected with Lipofectamine 2000 (Life Technologies) according to manufacturer's instruction. After 48 h of transfection, cells were pelleted for both RNA and total protein extractions.

2.3. Human samples

Human *post-mortem* tissues from 5 ALS cases and 5 controls (Table 1) were provided by the Department of Veterans Affairs Biorepository Brain Bank (BBB) and obtained from the Southern Arizona, VA Biorepository Brain Bank (VABBB). The study was reviewed and approved by the University of New Mexico Human Research Protection Office (Study ID #15-001).

2.4. Preparation of total protein extracts

SH-SY5Y cells and human *post-mortem* tissues were lysed using cold Radio-Immunoprecipitation Assay (RIPA) buffer (150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0), incubating on ice for 20 min and the extracts were cleared by centrifugation at 14000 rpm for 10 min. The supernatant represented the total protein extract. Before freezing at −80 °C, protein concentration was determined by Bradford assay (Bio-Rad, USA) using BSA as standard.

2.5. RNA extraction and quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted with Trizol® (Life Technologies) according to the manufacturer's recommendations. RNA quantity was determined using the Qubit (Life Technologies) spectrophotometer. After DNase treatment (New England Biolabs, USA), 1 µg of total RNA was reverse transcribed using the SuperScript II RT (Life Technologies) following the manufacturer's protocol. PCR amplifications were carried out with the Applied Biosystems 7300 Real Time PCR System using SYBR Green (Life Technologies) mix. GAPDH gene was used as the housekeeping gene (hkg) to normalize values. Primer sequences:

Primers: human SOD1 Fw: GGTCCTCACTTTAATCCTCTATCCAG, Rv: CCAACATGCCTCTCTTCATCC; human HuD Fw GGAGGGTC-CATTGCTTGAT, Rv AAGCTCTGCGAGACCCAATA; human GAPDH Fw: CAGCAAGAGCACAAGAGGAAG, Rv: CAACTGTGAGGAGGGGAGATT; human FUS Fw ACCCAAAACACAGGCTATGG, Rv CGTAGCTTTGTTG CTGTCCA.

For multiplex qRT-PCR amplifications were carried out with the

Table 1
Subject demographics and RNA quality of ALS and control tissue samples.

Category	VAB case #	Age	Gender	Tissue RIN ^a	Tissue pH	PMI-cr ^b (hours)	PMI-fzn ^c (hours)
CONTROL	090015	66	M	5.7	6.15	<4.0	83.5
	100,012	81	F	6.2	6.7	<4.0	40
	110,005	62	M	6.6	6.38	2.75	80.75
	110,006	68	M	5.9	6.58	1.5	128
	110,023	71	F	4.4	5.9	2.42	61.92
ALS	100,007	61	M	6.2	6.64	1	76.5
	100,019	62	M	5	6.13	1.75	80.5
	100,034	62	M	5.7	6.28	1.66	39.16
	100,037	64	M	5.4	6.19	1	31
	100,038	57	M	6.4	6.39	3.38	41.72

^a RNA Integrity Number (RIN).

^b PMI-cr post-mortem interval, approximate time from death to body refrigeration (hours).

^c PMI-fzn post-mortem interval, approximate time from death to specimen freezing or 10% formalin fixation (hours).

iCycler PCR Detection System using iQ Multiplex Power Mix (Bio-Rad) and UBC as housekeeping gene (hkg) to normalize values. Primer sequences: UBC Fw GTCTGACTACAACATCCA, Rv ATCTTCGTGAAGCACTC, Probe [HEX]GGTCTCCGTCTCAGAGGTG[BHQ1]; SOD1 tot Fw GCAGAAGGAAAGTAATGG, Rv CACTTTAATCCTCTATCCAG, Probe [6FAM]CAGGCTGTACCACTGCAGGT[BHQ1]; SOD 3'UTR Fw CACA-GATGGGTATTAACCTT, Rv GTATGGCACTTATTATGAGG, Probe [TxD]AATTTCTTTGTCAATCAAGCCTGTGA[BHQ2].

2.6. Western blotting (WB)

WB analysis was performed by SDS–polyacrylamide gel electrophoresis (PAGE). 5 µg of extracted proteins were boiled in Laemmli sample buffer (0.6 g/100 mL Tris, 2 g/100 mL SDS, 10% glycerol, 1% β-mercaptoethanol, pH 6.8) for 10 min, separated on 10% SDS-PAGE gel and transferred to a PVDF membrane (Bio-Rad) using a liquid transfer apparatus (Bio-Rad). The membranes were treated with a blocking solution containing 5% non-fat dry milk in TBS-T buffer (10 mM Tris-HCl, 100 mM NaCl, 0.1% Tween, pH 7.5) for 1 h and incubated overnight with the primary antibodies listed below. Immunoreactivity was detected using the donkey anti-rabbit or anti-mouse (GE Healthcare USA, dilution 1:10,000) secondary peroxidase-conjugated antibodies. The immunoreactive bands were then visualized using the Western Lightning Plus-ECL (PerkinElmer Inc., USA). For subsequent staining, primary and secondary antibodies were removed from the membrane by incubation for 5 min at RT in Restore PLUS Western Blot Stripping Buffer (ThermoFisher Scientific) and, washed with TBS-T (3 times × 10 min); the membranes were then processed as described above. Densitometric analysis of the bands was performed using ImageJ software (<http://rsb.info.nih.gov/ij/>).

Primary antibodies: mouse monoclonal anti-HuD (E1, sc-28299; Santa Cruz Biotechnology, Inc.; dilution 1:5000 in blocking solution); rabbit polyclonal anti-SOD1 (FL-154, sc-11407, Santa Cruz Biotechnology, Inc.; dilution 1:10000 in blocking solution); mouse monoclonal anti-GAPDH (Santa Cruz Biotechnology, Inc.; dilution 1:5000 in blocking solution).

2.7. RNA Immunoprecipitation (RIP)

Samples were collected in polysome lysis buffer (50 mM Tris-HCl pH 7.4; 150 mM NaCl; 0.5% NP-40; 2 mM MgCl₂; 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM Na₃VO₄ and 1 mM DTT) supplemented with 200 U/mL RNaseOUT® Ribonuclease Inhibitor (Invitrogen) and EDTA-free protease inhibitor cocktail (Roche, USA). Protein G-coated Dynabeads (Invitrogen) (40 µL aliquots) were washed 3 times with 200 µL of PBS 1× at RT before adding 4 µg of the primary antibodies mouse monoclonal anti-HuD (E1, sc-28299; Santa Cruz Biotechnology, Inc.) or control mouse IgG. Beads were incubated overnight at 4 °C with constant rotation. After washing the beads 3 times with NT-2 buffer (50 mM Tris-

HCl, pH 7.4, 150 mM NaCl, 0.05% NP-40, 1 mM MgCl₂), lysates were added to each tube with Ab-bound beads and incubated at 4 °C for 4 h. Beads were then washed 6 times with NT-2 buffer supplemented with RNaseOUT (100 U/mL) and resuspended in 150 µL Proteinase K buffer (NT-2 supplemented with 1 mg/mL Proteinase K and 1% SDS). Beads were incubated at 55 °C for 30 min, and Trizol was added directly to the tubes after Proteinase K treatment for RNA extraction. Aliquots for both RNA and proteins were taken before (input) and after the IP.

2.8. 3' Rapid amplification of cDNA Ends (3' RACE)

Aliquots containing 1 µg of total RNA were reverse transcribed for the first strand cDNA synthesis, with Adapter Primer (AP), performed by using SuperScript II RT (Invitrogen) and followed by RNase H treatment. PCR amplifications, with gene specific primers (GSPs) and the Universal Amplification Primer (UAP) were carried out with Pfu Ultra II Fusion HS DNA Polymerase (Agilent Technologies, USA) according to the manufacturer's recommendations starting with 2 µg of cDNA. PCR products were separated and analyzed on a 1.5% agarose gel. GAPDH was used as positive control. Primers sequences: Adapter primer (AP) GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTTTT; Universal Amplification Primer (UAP) CUACUACUACUAGGCCACGCGTCGACTAGTAC; Gene Specific Primers (GSPs): SOD1 GCAGGTCCTCACTTAATCCTCTATCCAG; GAPDH TCCCTGAGCTGAACGGGAAG.

2.9. RNA electrophoretic mobility shift assay (REMSA)

Cy5.5-labeled at 5'-terminus SOD1 and TNFα probes were purchased from Eurogentec (Seraing, Belgium). Probe sequences were as follows: SOD1, 5'-AAACUGAUUUUAUGAUCACUUG GA GAUUUGUAU AGUUU UAUAACACUCAG-3'; TNFα, 5'-AUUAUUUAUUUAUUUAUUUAUU UA-3'. Recombinant HuD protein (250 ng, OriGene Technologies, USA) was incubated with 500 fmol of RNA probes in 1× REMSA binding buffer (10 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM MgCl₂, 1 mM DTT, 4% glycerol and 10 U RNasin Ribonuclease Inhibitor). To reduce nonspecific binding, yeast tRNA (Sigma-Aldrich, Italy; 0.1 µg/µl final concentration) was added. Reaction mixtures were incubated for 30 min at RT in the dark. Binding reactions were then loaded on a non-denaturing 4% polyacrylamide gel (37.5:1 ratio of acrylamide to bis-acrylamide) in 0.5× Tris/borate/EDTA buffer. The gel was pre-run for 30 min prior to loading the samples. Samples were resolved at 80 V at 4 °C in the dark. Probe signals were detected and analyzed using the Odyssey Infrared Imaging System (LI-COR Bioscience, Italy).

2.10. In vitro mRNA decay assay

S100 cytoplasmic extracts were prepared from brain tissue of adult *Elavl4*^{-/-} HuD knockout mice as previously described (Ford et al., 1999; Sokolowski et al., 2008). GST-HuD protein was expressed in BL21 *E. coli*

and purified using the MagneGST™ Protein Purification System (Promega) according to the manufacturer's protocol. Decay reactions were performed as described by (Bolognani et al., 2006). Briefly, 300 ng of total RNA from SH-SY5Y cells treated with H₂O₂ for 60 min were incubated with 60 µg of *Elavl4*^{-/-} (HuD KO) S100 protein and in the presence or absence of 100 ng of purified GST-HuD for 0, 10, 20 and 40 min as indicated in the Figure legend. Differences in decay rates were calculated by Two-Way ANOVAs after transforming the data to a natural logarithm scale (ln).

All animal procedures were approved by the University of New Mexico Institutional Animal Care and Use Committee (IACUC) in compliance with the Guidelines for the Care and Use of Laboratory Animals established by the National Institutes of Health (NIH). *Elavl4*^{-/-} (HuD KO) mice were a gift from Prof. Hideyuki Okano (Akamatsu et al., 2005).

2.11. Sequence analysis

SOD1 3'UTR analysis was performed using a previously published script (Bolognani et al., 2010). This analysis considers both the sequence and the restrictions imposed by the three dimensional structure of the complex between the first two RNA Recognition Motifs (RRMs) in HuD and two different Class I and II AREs (Bolognani et al., 2010; Wang and Tanaka Hall, 2001). Human SOD1 3'UTR sequence variants were downloaded from Ensembl Biomart database (<http://www.ensembl.org/biomart/index.html>) (Gene 81, *Homo sapiens* gene GRCh38.p3 dataset), and non-redundant sequences were obtained using Perl scripts (Bolognani et al., 2010). Specifically, the Perl script searches for the following sequences corresponding to HuD motifs 1, 2 and 3 respectively, allowing one mismatch: [CG][CT][CT]TC[CT][CT]TC[TC]C[TC]C, [TG]TTTGT[TT][GT]TTT, TTTT[TTTT][TA]AAA and [CT]TNN[CT]T [CT].

2.12. RNA sequencing

Total RNA from SHSY-5Y cells after H₂O₂ treatment (60 min) or siHuD/CTR siRNA transfection was used for RNA sequencing (Arraystar, Inc., Rockville, MD). Briefly, polyadenylated RNAs were enriched by oligo (dT) magnetic beads before RNA-seq library preparation using KAPA Stranded RNA-Seq Library Prep Kit (Illumina). Barcoded libraries were captured on Illumina flow cells, amplified in situ, and subsequently pair end sequenced for 150 cycles on an Illumina HiSeq 4000 instrument. The differentially expressed genes and transcripts were filtered using *p* value <0.05 and fold change (FC) > 1.2 as cut offs. Supplementary Fig. 2 shows the volcano plots for T0 vs T60 (Supplementary Fig. 2A) and CTR siRNA vs siHuD (Supplementary Fig. 2B) sequencing respectively. A FC >1.2 cut off was used for the purposes of running pathway and network analyses with >90 genes (Table 2).

2.13. Ingenuity pathway analysis

Molecular functions and cellular pathways regulated by HuD and oxidative stress were identified using Ingenuity Pathway software (IPA, Winter 2019 Release, Qiagen) (Table 3).

2.14. Statistical analysis

All experiments were performed at least three times. Values were expressed as means ± SEM. Statistical analysis was performed using GraphPad Prism version 6 (La Jolla, CA, USA). The data were analyzed by Student *t*-test or analysis of variance (ANOVA) followed by Multiple Comparison tests. Differences were considered statistically significant when *p* values were < 0.05.

Table 2

List of genes regulated by HuD and oxidative stress.

KDM7A	ACVR2B	SMAD6	TP53INP1	MIGA1
SNAI2	ID2	SLC44A5	HECTD2	STAC3
AGPAT4	IVNS1ABP	GALNT1	SPRED1	LSAMP
SLC4A7	NRDE2	PLEKHA6	RIC3	SPIN4
RIMBP2	GPR75	CREB5	CHRFAM7A	IER5L
CDON	SLF2	ZNF711	TMEM170A	SRSF10
HIPK2	PANK3	SHC3	NKX3-1	PTAR1
NUAK1	MSX2	GXYLT1	HOOK3	ARMCX4
RFX3	SV2C	ZFP36L2	FILIP1L	LTN1
STK17B	NBPFL4	SCN3A	GBX2	SACM1L
KLHL42	SLC36A1	LRGUK	SNTB2	SFT2D2
RBM41	SRSF6	LSM11	MECP2	PLXNA4
RGS17	ID1	MMP16	MOB1B	SAPCD1
MAP3K1	DYRK2	DPY19L4	CCDC14	ZNF850
NRP1	CASD1	C8orf37	MTX3	AC008575.2
DICER1	RASL11B	STARD4	KCTD12	
XIAP	EDA2R	PGGT1B	ZBTB18	
ITGB8	MDM2	TBX20	AKAP5	
MYH3	RNF38	GPER1	AC104581.1	

91 genes that were both up-regulated by hydrogen peroxide and downregulated by HuD knockdown in addition to SOD1 (see Fig. 6A).

Table 3

Top disease and biological functions associated with genes regulated by HuD and Oxidative Stress.

	# Molecules	<i>p</i> -value range
Diseases and Disorders		
Developmental Disorders	26	7.9E-03 - 1.23E-05
Organismal Injury and Abnormalities	86	7.9E-03 - 1.35E-05
Neurological Diseases	23	7.9E-03 - 2.04E-05
Molecular and Cellular Functions		
Cell-To-Cell Signaling and Interaction	15	7.9E-03 - 1.39E-05
Cell Death and Survival	25	7.9E-03 - 1.56E-05
Cellular Assembly and Organization	11	7.9E-03 - 1.56E-05
DNA Replication, Recombination and Repair	5	3.08E-03 - 1.56E-05
Cellular Development	30	7.9E-03 - 1.62E-05

Diseases and Disorders, Molecular and Cellular Functions, # molecules in each category and *p*-values were calculated using IPA based on the 91 genes regulated by oxidative stress and HuD.

3. Results

3.1. HuD binds and stabilizes SOD1 mRNA in vitro

HuD-mediated mRNA stabilization requires the binding of this RBP to cis-acting ARE motifs located in the 3'UTR of target mRNAs (Bolognani et al., 2010). To test whether the human SOD1 mRNA is a target of HuD we performed a bioinformatics analysis aimed to identify ARE consensus binding sites specific for HuD interaction. 3'UTR nucleotide composition and length as well as the presence of each ARE subtype were determined using our Bio Perl scripts (Bolognani et al., 2010). As shown in Fig. 1A, two predicted HuD binding motifs are present in the human SOD1 3'UTR.

We previously demonstrated that ELAV-like RBP HuR binds to SOD1 mRNA in PBMCs (Milani et al., 2013). To assess whether HuD could specifically interact with SOD1 mRNA, we used RNA electrophoretic mobility shift assays (REMSA) and an RNA immunoprecipitation assay (RIP) followed by quantitative RT-PCR (qRT-PCR) for SOD1 mRNA. The presence of a shifted band in the REMSA assay, relative to the free probe, confirmed the formation of an RNA-protein complex between recombinant HuD protein and the RNA probe bearing SOD1-ARE sequences (Fig. 1B). A probe with TNFα ARE was used as positive control given that it contains multiple ARE motifs (Milani et al., 2013; Schaljo et al., 2009). RIP assays in human neuroblastoma cells induced to a neuronal-like phenotype with TPA showed a significant increase in the binding of the SOD1 3'UTR in the HuD IP compared to the IgG used as negative

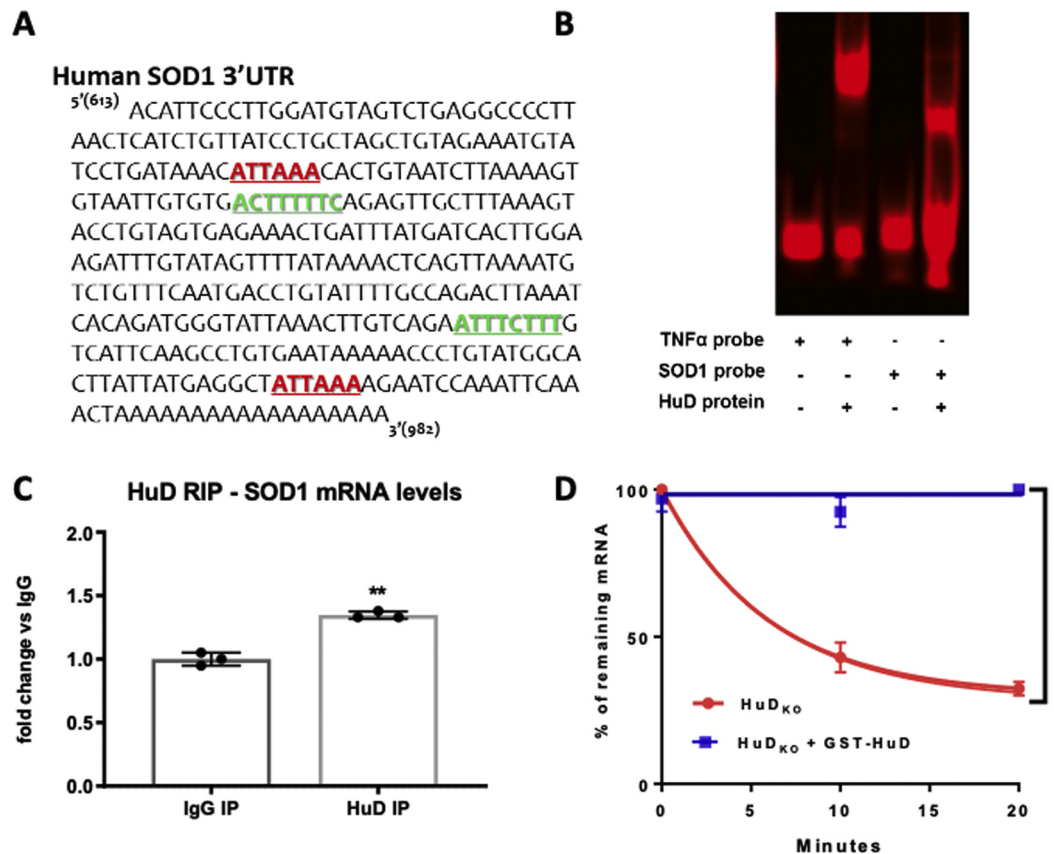


Fig. 1. HuD binds to the human SOD1 3'UTR and stabilizes its mRNA. A) Bioinformatics analysis of SOD1 3'UTR. Predicted HuD binding sequences (green) were determined as previously described (Bolognani et al., 2010). Alternative polyA sites are indicated in red. B) In vitro HuD binding to SOD1-ARE elements. REMSA assay highlighted the formation of a complex between HuD recombinant protein and the probe designed to target SOD1 ARE sequences. REMSA was performed as described in Materials and Methods. The TNFα-ARE probe was used as a positive control. C) HuD RIP followed by qRT-PCR for SOD1 was performed using differentiated SH-SY5Y cells as described in Materials and Methods. Values were expressed as fold change versus the IgG negative control. Data were analyzed by unpaired *t*-test, followed by Welch's correction and are represented as mean ± SEM; ***p* < 0.01, *n* = 3. D) HuD decreases SOD1 mRNA degradation. SOD1 mRNA levels were assessed by qRT-PCR after incubation of total RNA extracted from SH-SY5Y cells treated with H₂O₂ for 60 min with *Elavl4*^{-/-} (HuD KO) S100 extract without (red curve) or with GST-HuD (blue curve). Values were normalized to GAPDH and expressed as percentage of remaining mRNA versus 0 min at 10 and 20 min time points, respectively. Comparisons of the different decay rates were performed by repeated measures Two-Way ANOVAs after transforming the data to ln. Decay curves showing the average results of three separate decay experiments were fitted with a single rate exponential decay curve. **p* < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

control (*p* < 0.001, Fig. 1C, see also western blot validations of the HuD RIP in Supplementary Fig. 1A). Also, we used the mouse SOD1 mRNA for the RIP as a negative control as this does not contain a HuD binding motif in its 3'UTR (Supplementary Fig. 1B-D). Given the role of HuD on mRNA stability, we then investigated whether HuD interaction with ARE elements induces changes in SOD1 mRNA stability using an in vitro decay assay, where total RNA from human neuroblastoma cells was incubated with *Elavl4*^{-/-} S100 extracts in the presence or absence of HuD recombinant protein. Comparisons of different decay rates showed 42.21% of variation (**p* < 0.05) due to HuD protein addition, and 26.8% of variation (***p* < 0.001) between different time points. The lack of HuD protein in *Elavl4*^{-/-} S100 extracts resulted in a rapid decay of SOD1 mRNA with a half-life of 7.5 min (Fig. 1D, red curve), while addition of HuD protein efficiently protected SOD1 mRNA from degradation (Fig. 1D, blue curve).

All members of the ELAVL/Hu protein family have three RNA-recognition motifs (RRMs), with the first two interacting with ARE sequences and the last one, RRM3, binding to the poly(A) tail (Bronicki and Jasmin, 2013) (Fig. 2A). As we have previously shown, RRM3 is required for high affinity binding, target stabilization and HuD function (Anderson et al., 2000; Beckel-Mitchener et al., 2002) (Fig. 2A). To evaluate the direct effect of HuD in SOD1 regulation, we transfected differentiated SH-SY5Y cells, which express endogenous HuD protein,

with two different HuD constructs: pcHuD, expressing the complete protein sequence, and pcHuD I + II, which lacks the RRM3 region and acts as a dominant negative protein. As expected, full-length HuD overexpression lead to increased SOD1 mRNA levels (*p* < 0.05) (Fig. 2B). In contrast, the overexpression of HuD lacking RRM3 significantly reduced SOD1 mRNA levels (*p* < 0.001), suggesting a competition of the truncated dominant negative protein with endogenous HuD (Fig. 2B), as previously described (Anderson et al., 2000).

3.2. HuD regulation of SOD1 mRNA is oxidative stress-dependent

We previously reported that SOD1 mRNA and protein levels are upregulated in H₂O₂-induced OS conditions in SH-SY5Y cells (Dell'Orco et al., 2016; Milani et al., 2013). Here, we confirmed that the SOD1 mRNA increase is maintained in our in vitro model after the induction of a neuronal-like phenotype in SH-SY5Y cells (Fig. 3A). Furthermore, as shown in Fig. 3B, H₂O₂ treatment strongly increases HuD mRNA levels after 60 min (*p* < 0.01) in a similar time-dependent manner as observed for SOD1 mRNA. To test whether this increase in SOD1 mRNA after H₂O₂ treatment was related to changes in HuD binding, we performed RIP assays (Supplementary Fig. 1A) followed by qRT-PCR for SOD1 mRNA. Compared to untreated cells (T0) SOD1 binding to HuD is significantly increased after 30 (T30, *p* < 0.001) and 60 (T60, *p* < 0.001)

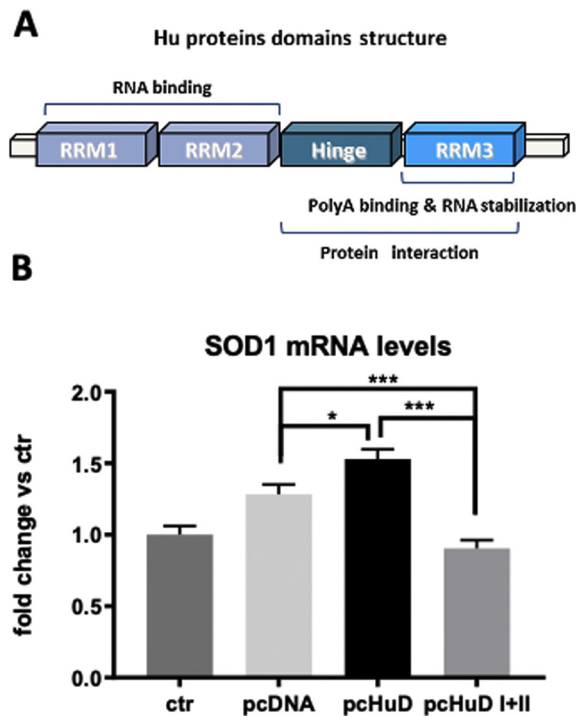


Fig. 2. HuD stabilization of SOD1 mRNA requires the presence of its RRM3 motif. A) Representation of HuD protein domains and functions. B) Plasmids for overexpression of HuD and HuD I + II, a truncated from of the protein lacking RRM3, were transfected in differentiated human neuroblastoma cells as described in Materials and Methods. After qRT-PCR, SOD1 mRNA values were normalized to GAPDH mRNA levels and expressed as fold change versus the untransfected (ctr) cells. Data were analyzed by performing a One-way ANOVA, followed by Tukey's multiple comparison tests and are represented as mean $\pm$ SEM; * p < 0.05, *** p < 0.001, n = 3.

minutes of H_2O_2 treatment showing a time-dependent increase (Fig. 3C). To assess the requirement of HuD in SOD1 mRNA upregulation during OS, we used a specific siRNA to knockdown HuD protein levels to 36% (Fig. 4A). As shown in Fig. 4B, decreasing HuD levels prevents SOD1 mRNA upregulation after hydrogen peroxide treatment (Fig. 4B). In contrast, there was a significant increase of SOD1 mRNA in presence of a CTR siRNA (Fig. 4B), corroborating that HuD is required for OS-induced SOD1 mRNA upregulation.

Two SOD1 transcripts differing in the length of their 3'UTRs, 0.7 kb (S-3'UTR) and 0.9 kb (L-3'UTR) (Sherman et al., 1984) respectively, are generated by APA. Remarkably, since both the ARE binding elements are present after the first polyadenylation (poly A) site (Fig. 1A and sequencing results shown in (Sherman et al., 1984)), only the L-3'UTR transcript contains HuD binding sites. To evaluate the levels of SOD1 S-3'UTR and L-3'UTR transcripts levels during OS, we performed 3' Rapid Amplification of cDNA Ends (3' RACE) and multiplex qRT-PCR analyses. As shown in Fig. 5A, TPA treated-SHSY-5Y cells express both the L and S variants of SOD1 mRNA 3'UTR. In contrast, GAPDH mRNA, used as a negative control, has a single 3'UTR. H_2O_2 treatment increased the percentage of L-3'UTR variant after both 30 (p < 0.05) and 60 min (p < 0.05) compared to S-3'UTR variant (Fig. 5B), indicating that OS induces a shift in the APA towards the longer variant where HuD binding sites are located. In contrast to human SOD1 mRNA, the mouse mRNA only has a short 3'UTR lacking predicted HuD binding sites (Supplementary Fig. 1D). Consistent with this finding, RIP analyses performed in HuD overexpressing transgenic mice (HuD-OE) tissues did not reveal a significant interaction of this RBP with SOD1 mRNA (Supplementary Fig. 1B and C).

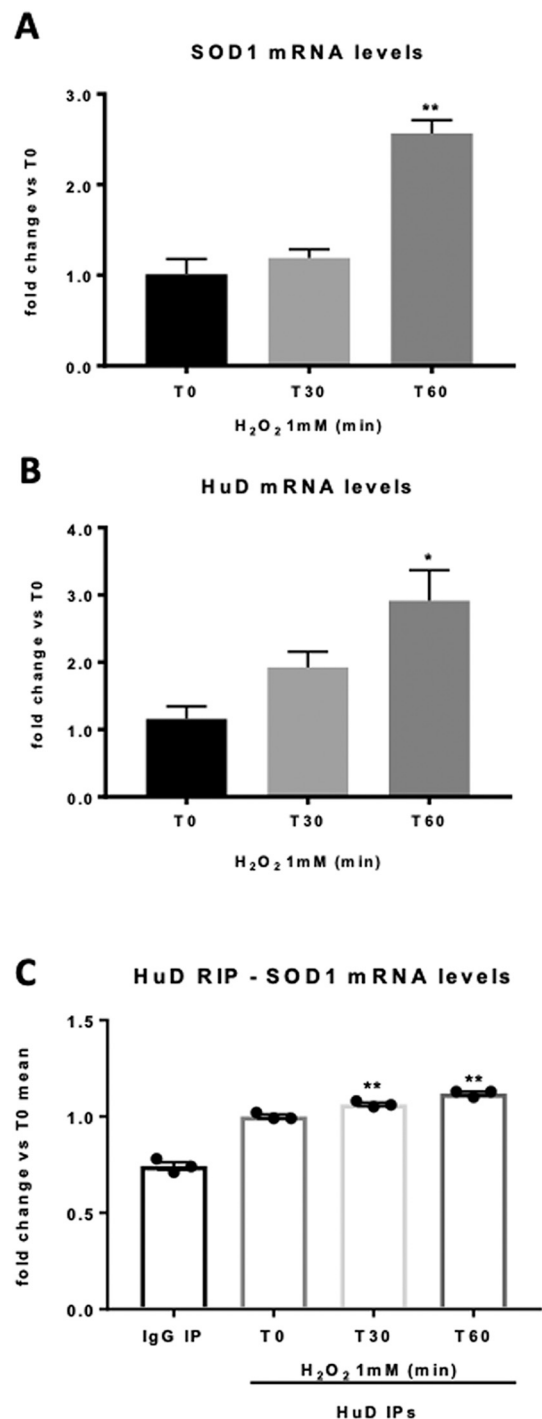


Fig. 3. SOD1 and HuD mRNA levels and HuD-SOD1 mRNA interactions are increased after oxidative stress. A and B) SH-SY5Y differentiated cells were treated with 1 mM H_2O_2 for 30 (T30) and 60 (T60) minutes. SOD1 and HuD mRNA levels were determined by qRT-PCR as described in Materials and Methods. C) Binding of HuD to SOD1 mRNA was analyzed by RIP followed by qRT-PCR for SOD1 as described in Materials and Methods. Normalized levels are expressed as fold change versus the non-treated control (T0). Data were analyzed by ANOVA, followed by Tukey's multiple comparison tests and are represented as mean $\pm$ SEM; * p < 0.05, ** p < 0.01, *** p < 0.001, n = 3.

3.3. HuD regulates ALS-associate pathways under oxidative stress

In previous work we demonstrated that human neuroblastoma cells treated with hydrogen peroxide for 60 min have features similar to sporadic ALS patients; in particular increase ROS and SOD1 high

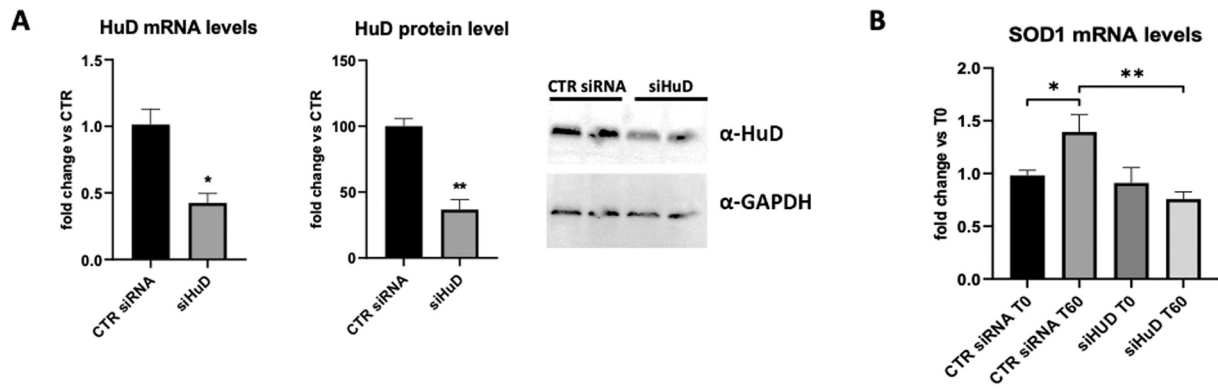


Fig. 4. Oxidative stress induction of SOD1 mRNA requires HuD. A) Human neuroblastoma cells were transfected with 20 pmol siHuD and a CTR siRNA as described in Materials and Methods. After 72 h cells were collected and processed for either total RNA or protein extraction. HuD levels were determined by qRT-PCR and WB. Data were analyzed by unpaired t-test, followed by Welch's correction and are represented as mean $\pm$ SEM; * p < 0.05, ** p < 0.01. B) After siHuD or CTR siRNA 72 h transfection, human neuroblastoma cells were treated with 1 mM H_2O_2 for 30 (T30) and 60 (T60) minutes. SOD1 mRNA levels were analyzed as described in Materials and Methods. Data were analyzed by One-way ANOVA, followed by Tukey's multiple comparison test and are represented as mean $\pm$ SEM; * p < 0.05, ** p < 0.01, n = 3.

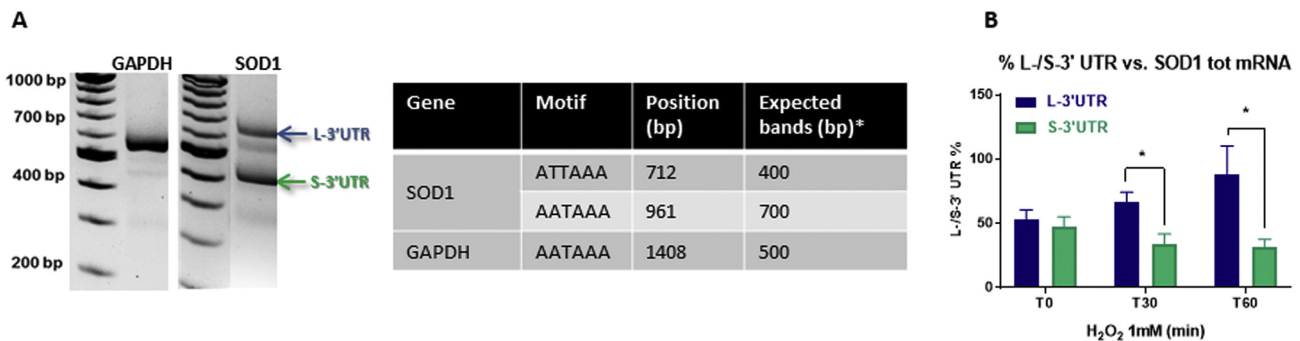


Fig. 5. Oxidative stress increases the levels of the SOD1 mRNA with longer 3'UTR. A) Human SOD1 mRNA is subjected to APA leading to the expression of two mRNA variants with different 3'UTR lengths: S-3'UTR (blue) and L-3'UTR (green). GAPDH transcript was used as a single 3' UTR control. *Bands length is based on Gene Specific Primers (GSP) used in the PCR reaction as described in Materials and Methods. B) Human neuroblastoma cells were treated with 1 mM H_2O_2 for 30 (T30) and 60 (T60) minutes. Multiplex qRT-PCR was performed as described in Materials and Methods. S-3'UTR levels were calculated as the difference between total SOD1 mRNA and L-3'UTR. Values were normalized to GAPDH and expressed as percentage (%) of L-3'UTR and S-3'UTR vs. total SOD1 mRNA. Data were analyzed by One-way ANOVA, followed by Tukey's multiple comparison test and are represented as mean $\pm$ SEM; * p < 0.05, n = 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

molecular aggregates (Dell'Orco et al., 2016). In order to evaluate whether besides SOD1 other changes in HuD regulated gene expression take place during OS, we performed RNA sequencing. Total RNA from SHSY-5Y cells after 1 mM hydrogen peroxide treatment or siHuD transfection was subjected to RNA sequencing as described in Material and Methods. For analyses of biological pathways affected by OS and HuD, transcripts were selected using a cut off 1.2 Fold Change in expression and p -value < 0.05 (Supplementary Fig. 3). To identify genes that were both activated by OS and regulated by HuD we took in consideration only genes upregulated by hydrogen peroxide (T60) and downregulated by HuD knockdown (siHuD). As shown in Fig. 6A, 91 genes matched this criteria (see also Table 2). Using Ingenuity Pathway Analysis (IPA) core expression analysis we discovered the top diseases and biological functions regulated by these 91 genes (Table 3). Not only these genes are involved in Developmental and Neurological disorders but also in Cellular Assembly and Organization, Cellular Development and DNA replication, and Recombination and Repair. Finally, the top 4 biological networks associated to these genes (Fig. 6B 1–4): 1) Nervous System Development and Function, 2) Cellular Development and Cellular Growth and Proliferation, 3) Cell-To-Cell Signaling and Interaction and 4) Cell Death and Survival. Interestingly, these networks contained genes previously shown to be involved in neurological disorders (pink borders) and associated in particular with ALS (blue

symbols) including DICER1, NFkB, PAX6, TGFBI, CD44, BCL2, IL1B, EGFR, TNF and IFNG (Emde et al., 2015; Hu et al., 2017; Matsumoto et al., 2012; Meroni et al., 2019; Prell et al., 2014; Satoh et al., 2015; Seksenyan et al., 2010).

3.4. HuD regulation of SOD1 expression in sporadic ALS

Given our findings that HuD is involved in SOD1 post-transcriptional regulation both in cell-free systems and in culture, we aimed to investigate possible changes of HuD expression in sALS motor cortex, one of the brain regions affected in the disease. HuD expression was assessed by performing qRT-PCR and WB analyses using *post-mortem* tissues from 5 ALS patients and 5 healthy controls (Table 1). The levels of HuD mRNA increased in motor cortex samples of sALS patients (Fig. 7A, left panel, p < 0.05) leading to higher HuD protein expression in the patients' tissues compared to healthy controls (Fig. 7B, left panel, p < 0.001). sALS tissues also had significantly increased levels of SOD1 mRNA (Fig. 5A, right panel, p < 0.05) and protein compared to controls (Fig. 7B, right panel, p < 0.001). The entire western blots of these panels along with the identification number of the samples (Table 1) can be found in Supplementary Fig. 3A. Furthermore, analyses of the cerebellum from the same control and sALS subjects (Supplementary Fig. 4) demonstrate that the observed changes in SOD1 and HuD mRNA and protein levels in the

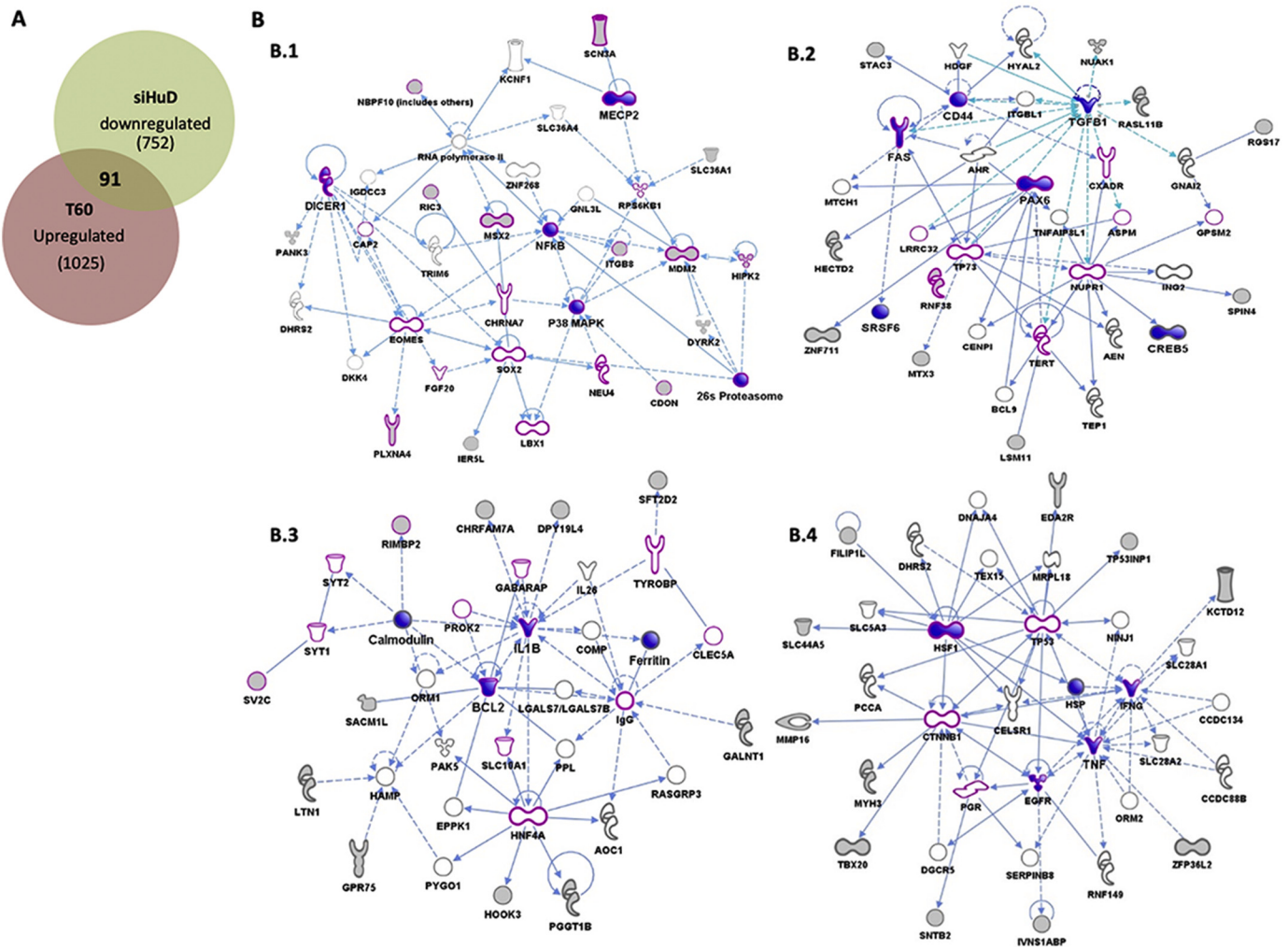


Fig. 6. Hydrogen peroxide-induced Oxidative Stress activates HuD regulated biological pathways associated with ALS. Human neuroblastoma cells were treated for 60 min with 1 mM H_2O_2 and siHuD/CTR siRNA AND Total RNA was isolated and used for RNA-seq analyses as described in Material and Methods. A) Venn diagram showing the number of genes significantly (fold change >1.2 , p -value <0.05) upregulated by hydrogen peroxide (1025) and downregulated by HuD knockdown (752); 91 common genes were identified and further analyzed. B) Top networks activated by oxidative stress and regulated by HuD. B.1, B.2, B.3, B.4. Genes previously associated with neurological disorders are shown with pink borders, while genes in solid blue have been previously linked to ALS as described in Results. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

motor cortex of sALS patients are not present in different motor region that is not affected in ALS. Finally, linking the changes in expression of SOD1 and HuD protein, RIP assays (Supplementary Fig. 3B) demonstrated that HuD binding to SOD1 mRNA is also increased in ALS tissues (Fig. 7C).

4. Discussion

Altered RNA metabolism due to either mutations in RBPs or a dysregulation of their expression and localization has been proposed as a central pathogenic mechanism of motor neuron degeneration in ALS (Barmada, 2015). Previous studies demonstrated the involvement of the ubiquitously expressed member of the ELAVL family, HuR, in the regulation of ALS-related proteins and RBPs such as SOD1 (Milani et al., 2013), FUS and TDP43 (De Santis et al., 2019; Lu et al., 2014). In this study, we sought to assess the potential role of the neuronal HuD/ELAVL4 in SOD1 mRNA regulation and pathophysiology of ALS as this protein has been previously associated with other neurodegenerative disorders (Amadio et al., 2009; DeStefano et al., 2008; Kang et al., 2014; Perrone-Bizzozero and Bird, 2013; Subhadra et al., 2013). Here, we demonstrated that HuD binds to and stabilizes the long 3'UTR variant of

the SOD1 mRNA and that in neuronally-differentiated SHSY-5Y cells this stabilization requires the presence of RRM3 in HuD, which binds to and stabilizes mRNAs with long polyA tails (Anderson et al., 2000). Using a previously characterized model of OS-mediated cell damage (Dell'Orco et al., 2016) and an siRNA specific to human HuD, we further demonstrated that SOD1 mRNA levels increase during oxidative stress and that this upregulation is dependent on HuD. Finally, we showed that SOD1 mRNA and protein levels are increased in sporadic ALS patient motor cortex compared to healthy controls. HuD mRNA and protein levels were also increased in the same tissues as well as HuD binding to SOD1 mRNA.

Oxidative stress and ROS overproduction are two of the major hallmarks linked to motor neuron degeneration and sporadic ALS pathophysiology (Carrì et al., 2015; Rossi et al., 2016). Of interest, besides their role in nervous system development, Hu/ELAVL proteins are known to be pleiotropic proteins involved in various processes where oxidation and inflammation play a primary role (Pascale and Govoni, 2012). Many ROS-regulated mRNAs, whose half-life and/or translational status are controlled in response to oxidative damage, are the targets of RBPs, which can positively or negatively alter their expression (Abdelmohsen et al., 2008). Exposure to oxidative stress and ROS

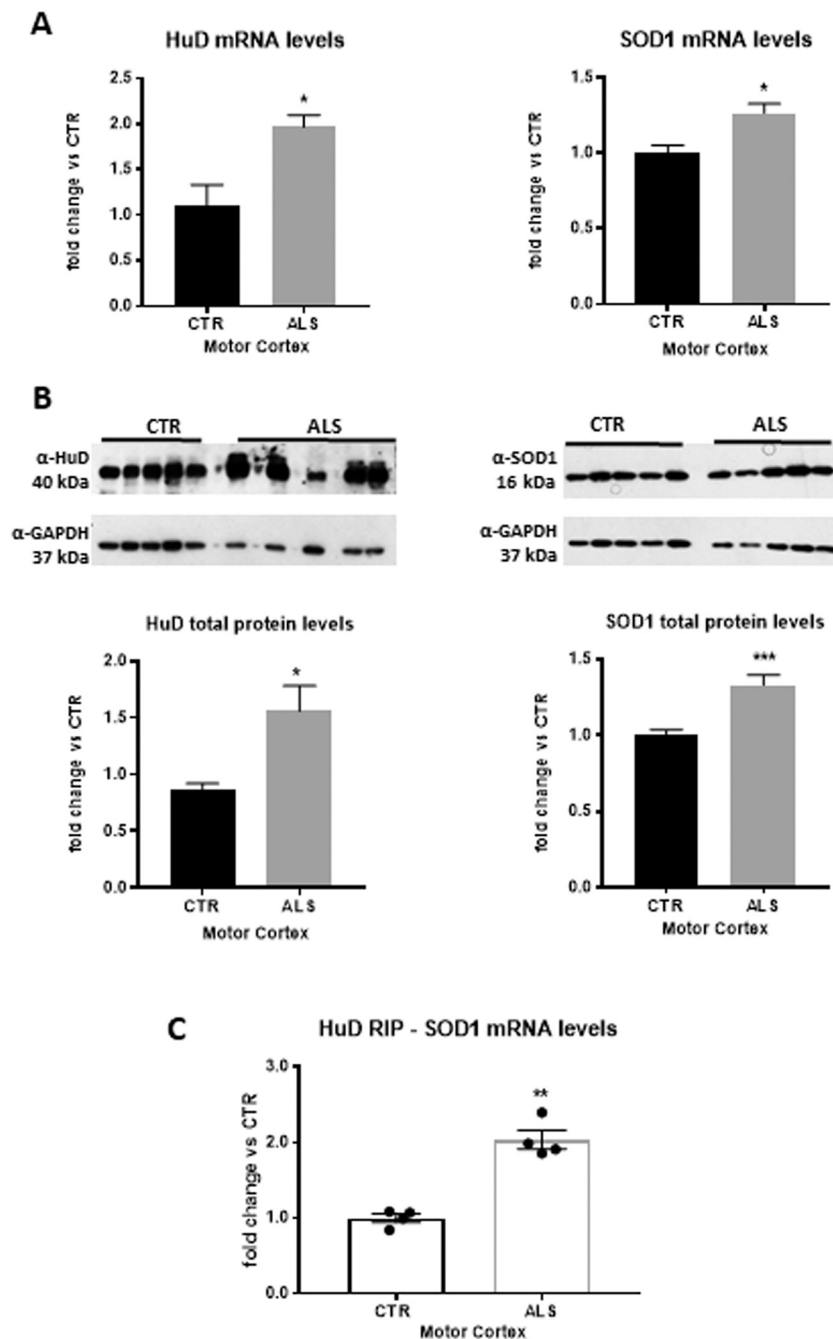


Fig. 7. HuD and SOD1 overexpression in Motor Cortex of sporadic ALS patients A) HuD and SOD1 mRNAs levels were evaluated in *post-mortem* tissues from sALS patients and healthy controls as described in Materials and Methods. Data were analyzed by unpaired t-test, followed by Welch's correction and are represented as mean $\pm$ SEM; * $p < 0.05$, $n = 5$ (5 ALS vs 5 CTR). B) HuD and SOD1 protein levels were quantified by performing western blots. Data were analyzed by unpaired t-test, followed by Welch's correction and are represented as mean $\pm$ SEM; ** $p < 0.01$, *** $p < 0.001$, $n = 5$ (5 ALS vs. 5 CTR). C) RIP followed by qRT-PCR for SOD1 mRNA was performed as described in Materials and Methods. Levels were corrected by the background (IgG non-specific binding) and expressed as fold change versus healthy controls (CTR). Data were analyzed by unpaired t-test, followed by Welch's correction and are represented as mean $\pm$ SEM; ** $p < 0.01$, $n = 4$ (4 sALS vs 4 CTR). See [Supplementary Fig. 3A](#) for complete blots and [Supplementary Fig. 4](#) for WB and qPCR HuD levels in the cerebellum used as negative control.

accumulation, as those found in neurodegenerative diseases, results in the activation of protective pathways through the synthesis of detoxifying/antioxidant enzymes (Calabrese et al., 2009). In this context, HuD stabilization of SOD1 mRNA and the ensuing increase in wild type SOD1 protein could represent an adaptive hormetic response.

Human SOD1 is subjected to APA, with two transcripts of 0.7 and 0.9 kb, respectively, differing in the length of their 3'UTRs (Sherman et al., 1984). APA resulting in longer 3'UTRs is increased in neurons compared to other cell types (Curinha et al., 2014), and it is particularly relevant in neurodegeneration due to the increased presence of RBP and miRNA binding sites (Curinha et al., 2014; Miura et al., 2014). APA has been linked to others neurological conditions such as Parkinson's disease, where a shift to increase the levels of the long 3'UTR of the alpha-synuclein mRNA (SNCA), is associated with risk variants and elevated dopamine levels (Rhinn et al., 2012). In our cellular model, we showed

that hydrogen peroxide treatment significantly altered the ratio between SOD1 L-3'UTR and S-3'UTR isoforms resulting in higher levels of the L-3'UTR containing predicted HuD binding sites. Also, we previously observed that HuD target mRNAs contain on average longer 3'UTRs than non-targeted genes (Bolognani et al., 2010). Although it is not clear whether this effect is due to HuD-induced differential APA or the specific stabilization of the long 3'UTR variant, it is known that *Drosophila* ELAV is required for APA of CNS-specific transcripts leading to 3'UTR extensions (Hilgers et al., 2012; Miura et al., 2014).

4.1. Conclusions and future perspectives

In summary, our results implicate HuD for the first time in the control of SOD1 mRNA in neurons after oxidative stress and in sALS brain tissues. Several studies using mutant SOD1-ALS animal models showed

that decreasing SOD1 levels through RNA interference (RNAi) (Ralph et al., 2005; Raoul et al., 2005), antisense oligonucleotide (ASO) therapy (Miller et al., 2013), or infusion of antibodies against misfolded SOD1 protein (Gros-Louis et al., 2010; Patel et al., 2014) significantly delayed disease onset and progression and extended survival. However, misfolded SOD1 is not associated with sALS. Therefore, it is important to evaluate the regulation of wild-type SOD1 protein in this context. Regarding wild-type SOD1 protein, our findings that HuD increases neuronal SOD1 expression suggest that this RBP may play a role in the cellular anti-oxidant response. Furthermore, our findings that other ALS related-genes and their associated biological pathways are regulated by both HuD and oxidative stress point to additional mechanisms linking HuD post-transcriptional regulation and ALS pathogenesis. Given that the average survival of ALS patients is 3–5 years from the time of diagnosis (Petrov et al., 2017) and current treatments (e.g., Riluzole and Edaravone) only increase patient survival by 3–5 months (Breiner et al., 2020; Hogg et al., 2017; Luo et al., 2019), there is a pressing need for the identification of novel therapeutic targets that could impact both life quality and expectancy in patients.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.nbd.2020.105211>.

Declaration of Competing Interest

None of the manuscript's authors has any relevant financial or nonfinancial relationships that might create a conflict of interest to disclose.

Acknowledgments

This work was supported by NIH 1R01NS089633-01A1, an award from Brain and Behavioral Health Institute (BBHI) at UNM-HSC, a Mentor Engagement Grant (MEG) from the Autophagy, Inflammation and Metabolism (AIM) Disease COBRE center (NIH grant 1P20GM121176) to NIPB and the RC2014-2016 from the Italian Ministry of Health to CC. We are grateful to Drs. Ernestina Trevor and Ian Robey at the VA Biorepository Brain Bank (VABBB) in Tucson AZ for providing us valuable samples for our studies and to the patients and their families for donating tissues for research.

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