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CRYPTIC EXON SPLICING FUNCTION OF TARDBP INTERACTS WITH AUTOPHAGY IN NERVOUS TISSUE

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Keywords: amyotrophic lateral sclerosis; autophagy related 4B cysteine peptidase; biomarker; diagnosis, motor neuron; neurodegeneration; splicing variant

List of abbreviations: ALS: amyotrophic lateral sclerosis; ATG4B: autophagy related 4B cysteine peptidase; AUC: area under the curve; FTLD: frontotemporal lobar degeneration; iPSC: induced pluripotent stem cells; ROC: receiver operating characteristic; TARDBP: TAR DNA binding protein; RT-qPCR: quantitative RT-PCR
Abstract

TARDBP (TAR DNA binding protein) is one of the components of neuronal aggregates in sporadic amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration. We have developed a simple quantitative method to evaluate TARDBP splicing function that was applied to spinal cord, brainstem, motor cortex, and occipital cortex in ALS (n=8) cases compared to age- and gender-matched control (n=17). Then, we quantified the abundance of a TARDBP-spliced cryptic exon present in ATG4B (autophagy related 4B cysteine peptidase) mRNA. Results of these analyses demonstrated that the loss of this TARDBP function in spinal cord, brainstem, motor cortex, and occipital cortex differentiated ALS from controls (area under the curve of receiver operating characteristic: 0.85). Significant correlations were also observed between cryptic exon levels, age, disease duration, and aberrant mRNA levels. To test if TARDBP function in splicing is relevant in ATG4B major function (autophagy) we downregulated TARDBP expression in human neural tissue and in HeLa cells, demonstrating that TARDBP is required for maintaining the expression of ATG4B. Further, ATG4B overexpression alone is sufficient to completely prevent the increase of SQSTM1 induced by TARDBP downregulation in human neural tissue cells and in cell lines. In conclusion, the present findings demonstrate abnormal alternative splicing of ATG4B transcripts in ALS neural tissue in agreement with TARDBP loss of function, leading to impaired autophagy.
Introduction

TARDBP (TAR DNA binding protein) is one of the components of neuronal aggregates in sporadic amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). Whether these aggregates are associated with TARDBP loss of function is not known. TARDBP is involved in the splicing of cryptic exons of selected mRNAs, such as of ATG4B (autophagy related 4B cysteine peptidase). Cryptic exons are considered splicing variants that may introduce frameshifts or stop codons, among other changes in the resulting mRNA. These aberrant mRNA have been demonstrated in motor cortex and middle temporal gyrus of ALS and FTLD patients [1]. However, it was undetermined whether ALS target locations also presented cryptic exons. Further, whether these mRNA anomalies have any impact in translation of the corresponding proteins was unknown. In this brief report, we have developed a simple quantitative method to evaluate this TARDBP splicing function that was applied to samples of various central nervous system regions from ALS cases compared to age- and gender-matched control individuals. Further, we have characterized the effects of TARDBP loss in autophagy role of ATG4B.

Results and Discussion

RNA was extracted from 50-100 mg homogenized nervous tissue. We quantified the abundance of TARDBP-spliced cryptic exons present in ATG4B mRNAs with RT-qPCR. RT-qPCR results agree with the loss of TARDBP function in spinal cord, brainstem, motor cortex, and occipital cortex differentiating ALS from controls (area under the curve of ROC: 0.85, Figure 1A). Next, we observed that the abundance of cryptic exons in the ATGB transcript was mainly influenced by ALS status (p<0.005 for ALS status, p=0.69 for brain region and p=0.1 for interaction in 2-way ANOVA) (Figure 1B). Significant correlations were also observed between bulbar levels of cryptic exons and age (Figure 1C). Indeed, a more severe phenotype of the disease (i.e., shorter duration of disease after diagnosis) was associated with higher level of aberrant mRNAs (Figure 1D).

To demonstrate if TARDBP function is relevant in maintenance of ATG4B mRNA we downregulated TARDB in human nervous tissue cells. The results demonstrate that TARDBP
downregulation increased the amount of cryptic exons to up 20% of ATG4B mRNA (Figure 1E) and causes a loss of 30% of ATG4B protein (Figure 1F). Nonsense-mediated decay of aberrant ATG4B mRNA may explain the hereby observed loss of ATG4B in human cells. The fact that this mRNA anomaly would target mainly motor neurons—a quantitatively minor cell population in these tissues—could account for the low percentage of mRNA affected and the apparent lack of quantitative differences of ATG4B protein in nervous tissue samples observed (data not shown).

Of note, sequence analyses suggest that loss of function of TARDBP on ATG4B cryptic exon introduces a premature stop codon leading to loss of residues 317-393 in the resulting protein. Because these residues contain 2 phosphorylation sites required for ATG4B protein activity [10], their loss induced by cryptic exon inclusion may result in defective autophagy.

To test if TARDBP function in splicing is relevant in ATG4B major cellular function (macroautophagy/autophagy) we downregulated TARDBP expression in HeLa cells. Results showed that TARDBP is required for maintaining the expression of ATG4B, as loss of TARDBP leads to an approximately 50% loss of ATG4B mRNA (Figure 2A) and protein (Figure 2B) in HeLa cells, in association with the presence of cryptic exons in 38% of ATG4B mRNA (Figure 2A). TARDBP is required to orchestrate a physiological autophagy response, as reinforced by the failure of TARDBP-deficient cells to increase ATG4B mRNA in cases of autophagy induction (Figure 2A); TARDBP-deficient HeLa cells also exhibited increased baseline levels of SQSTM1 (Figure 2C), a hallmark of autophagy disturbance [11].

TARDBP deficiency might affect autophagy by other mechanisms, besides ATG4B downregulation, as it modifies the expression of transcripts of approximately 41 genes. However, ATG4B overexpression alone is sufficient to completely prevent the increase of SQSTM1 induced by TARDBP downregulation in HeLa and human neural tissue cells (Figure 2D). Noteworthy, ATG4B loss was not compensated by increased expression of other ATG4B homologs (Figure S6, ESM). Previous data have shown that SQSTM1 interacts with TARDBP aggregates in ALS [12]. Together, the present data point to a novel mechanism implicating autophagy as a relevant factor in the pathogenesis of sporadic ALS, in addition to the involvement of autophagy pathway components in familial ALS including ALS2, OPTN, and SIGMAR1 [13].
Besides ATG4B mRNA, other genes, such as GPSM2 (G protein signaling modulator 2) are also influenced by TARDBP dysfunction [1]. We also designed a RT-qPCR method for analysis of cryptic exon inclusion in its mRNA. The results confirm that abundance of cryptic exons in GPSM2 mRNA could also be useful for ALS diagnosis (area under the curve of ROC for GPSM2 aberrant mRNA: 0.89, Figure 3A). Levels of GPSM2 aberrant mRNA are significantly influenced both by brain region and ALS type (bulbar ALS vs non-bulbar ALS; 2-way ANOVA: p<0.001 for interaction, p<0.0001 for disease status and p=0.0061 for brain region, Figure 3B). Bulbar cases show higher levels of these mRNA in brain stem and occipital cortex, in comparison to spinal cord and frontal cortex, being opposite for non-bulbar cases. These results might be explained by spreading of the disease pathological traits (i.e., loss of TARDBP function) between adjacent anatomical locations, as indicated previously by neuroimaging techniques [14]. Furthermore, levels of cryptic exons in GPSM2 in frontal cortex correlate with those in spinal cord (Figure 3C). Interestingly, and supporting the involvement of the loss of this TARDBP function as a common mechanism for appearance of both cryptic exons, levels of aberrant mRNA in GPSM2 correlate with those in ATG4B mRNAs in every tissue evaluated (Figure 3D).

In conclusion, we show the usefulness of tissue RT-qPCR in searching for cryptic exons as a rapid method to analyze TARDBP function, with potential implications in ALS diagnosis. We recognize that the usefulness for ALS diagnoses, based on this preliminary study, should be further substantiated by employing a larger number of individuals. This is evidenced by the shape of the ROC curve indicated above, showing the impact of a relative low number of specimens analyzed. Nonetheless, the present findings demonstrate abnormal alternative splicing of ATG4B transcripts in ALS neural tissue in agreement with TARDBP loss of function, leading to impaired autophagy.

Materials and Methods

Neuronal tissues

All samples were obtained from the Institute of Neuropathology and the University of Barcelona Brain Bank following the guidelines of the local ethics committees. Extensive
pathological studies were done for ALS diagnosis as previously described [2]. Samples from different anatomical locations were from 5 males and 3 females aged between 50 and 79 years affected with typical neurological and neuropathological characteristics of sporadic ALS. The post-mortem delay between death and tissue processing was between 3 and 16 h. Age- and gender-matched controls with no clinical evidence of neurological disease and with a normal neuropathological study were processed in parallel (see Table S1, Electronic Supplemental Material (ESM)).

**Cell culture and treatments**

HeLa cells were maintained in Dulbecco’s Modified Eagle’s Medium (Thermo Fisher Scientific, 11965), 10% FBS (Thermo Fisher Scientific, 10270), 100 U/ml penicillin-streptomycin (Thermo Fisher Scientific, 15140-122) at 37°C and 5% CO₂. Human neural tissue cells were obtained from Axol (Axol Biosciences, ax0018). Briefly, iPSC-Derived Neural Stem Cells derived from integration-free, induced pluripotent stem cells under fully defined neural induction condition were expanded and differentiated following System A of the manufacturer’s protocol (version 5.0).

*TARDBP* knockdown cells were obtained by transducing cells with a *TARDBP* shRNA lentivirus (see below for further details). Cell media were changed the day after the transduction and the cells were cultured for further 96 h to allow *TARDBP* silencing.

For inducing autophagy by nutrient deprivation, cells were infected with scrambled or *TARDBP* shRNA as indicated above, and for the last 16 h after transduction the medium was replaced with complete fresh medium, as control, or HBSS (Thermo Fisher Scientific, 14025), for nutrient starvation.

**shRNA design**

Primer was designed against the sequence 5′-GACGATGGTGAGTGACTGCAAAC-3′ for human *TARDBP* and cloned in the pSUPER (Oligoengine, pSUPER). Then, an EcoRI-ClaI fragment containing the H1 promoter for RNA polymerase III and the shRNA sequence was cut from pSUPER and subcloned into the pLVTHM plasmid, a gift from Didier Trono (Addgene, 12247) [3]. shRNA vector, the plasmids psPAX2 (Addgene, 12260) and pMD2.G (Addgene, 12259) (both a gift from Dr. Trono) were transfected together using the polyethyleneimine transfection method (Sigma-Aldrich, 408727) into HEK293T cells. The HEK293T medium was collected after 48 h of
transfection and centrifuged at 50,000 × g for 3 h. The final viral pellet was diluted in sterile phosphate-buffered saline (Thermo Fisher Scientific, 10010015) plus 2% bovine serum albumin (Sigma-Aldrich, A4503). The efficacy of the shRNA was demonstrated by western blot.

**RNA extraction, cDNA synthesis, conventional PCR and quantitative RT-PCR (RT-qPCR)**

RNA was extracted from cells and tissue samples using TRI Reagent (Thermo Fisher Scientific, AM9738) following the manufacturer’s instructions. RNA concentrations were measured using a NanoDrop ND-1000 (Thermo Fisher Scientific). One microgram of RNA was used for retrotranscription employing TaqMan Reverse Transcription Reagent using random hexamers (Thermo Fisher Scientific, N8080234).

Primers used to perform conventional PCR reaction were those previously described [1], purchased from Roche Oligo synthesis service (Roche Diagnostics, Barcelona, Spain). For **ATG4B** forward: 5’-TGTGTCTGGATGTGAGCGTG-3’, reverse 5’-TCTAGGGACAGGTTCAGGACG-3’; for **GPSM2** forward: 5’-AGTGGACATGTGGTGGTAAGAA-3’, reverse: 5’-GCTTCAAAGAATGACACGCA-3’; for **GAPDH** (PCR control) forward: 5’-ATCCCATCACCATCTTCCAG-3’, reverse: 5’-CCATCACGCCACAGTTTCC-3’. PCR conditions were as follows: initial denaturation at 98°C for 30 sec, followed by 40 cycles of 98°C for 30 sec, 63°C for 12 sec and 72°C for 30 sec. Gel analyses demonstrated the presence of potential **ATG4B** and **GPSM2** cryptic exons in samples from ALS patients (Figure S1, ESM), whose identity was ensured by Sanger sequencing (>99% sequence homology, E value ranging between 1 and 3e-40) as shown in Figure S2 and Figure S3 (ESM).

In order to quantify the level of cryptic exons we developed a RT-qPCR method. Briefly, RT-qPCR experiments were performed using a CFX96 instrument (Bio-Rad) with SYBR Select Master Mix (Thermo Fisher Scientific, 4472908). Each 20 µL of reaction contained 4 µL cDNA, 10 µL SYBR Select Master Mix, 0.2 nM of forward primer and 0.2 nM of reverse primer solutions and 4 µL PCR grade water. Primers developed for mRNAs quantification by RT-qPCR are listed in Table S2 (ESM). Oligonucleotides for human **GPSM2** and human **ATG4B** were designed with Primer3 [4] (http://primer3.ut.ee). **GAPDH** oligo and **ATG4B** homologs have been previously described [5, 6]. All oligonucleotides were purchased from Sigma-Aldrich. Three technical replicates for all RT-qPCR reactions were conducted. For all RT-qPCR primers, quality control
was performed for their specificity, sensitivity, melting curves and standard curves as shown in Figure S4 and Figure S5 (ESM). The RT-qPCR run protocol was as follows: 50°C for 2 min and 95°C for 2 min, with the 95°C for 15 sec and 60°C for 1 min steps repeated for 40 cycles; and a melting curve test from 65°C to 95 °C at a 0.1°C/sec measuring rate. Cryptic exon abundances were normalized by total mRNA for each gene with the following formula [7]:

$$\Delta C_q = C_q (total \ mRNA) - C_q (cryptic \ mRNA)$$

**Cryptic exon abundance = $2^{-\Delta C_q}$**

For total ATG4b mRNA relative expression, we used the formula as follows:

$$\Delta C_q = C_q (total \ ATG4B) - C_q (GAPDH)$$

$$\Delta \Delta C_q = \Delta C_q (target) - \Delta C_q (control)$$

**ATG4B mRNA fold change = $2^{-\Delta \Delta C_q}$**

**Western blot analysis**

Protein from cells was extracted using radioimmunoprecipitation buffer with 1X Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific, 1861278). After sonication, protein quantification was performed with the Bradford assay [8] using a commercial reagent (Bio-Rad, 5000006). Fifteen micrograms of protein were loaded onto a 12% acrylamide SDS-PAGE gel. Membranes were blocked with I-Block (Thermo Fisher Scientific, T2015) for 1 h and incubated overnight with the antibodies and conditions listed in Table 1.

After primary antibody incubation, membranes were washed 3 times with TBS-T 0.05% (20 mM Tris, 125 mM NaCl, 0.05% TWEEN 20 [Sigma-Aldrich, P7949], pH 7.6) and incubated with secondary antibody for 1 h. Immobilon™ Western Chemiluminiscent HRP Substrate (Merck Millipore, WBKLS0500) was used for immunodetection. Membranes were stained with Coomassie Brilliant Blue G (Sigma-Aldrich, 27815) for normalization. Specific bands were quantified with ImageLab v5.2.1 (Bio-Rad).

**ATG4B overexpression**

HeLa or human neural tissue primary cells derived from iPSC were transfected after 72 h of transduction with shRNA or scrambled with Lipofectamine 2000 (Thermo Fisher, 11668) according to the manufacturer’s protocol. Briefly, 0.5 μl of Lipofectamine were used per 0.5 μg of ATG4B
overexpression plasmid (Sino Biological Inc., HG20407-UT). Complexes were formed in 500 μl of Opti-MEM (Thermo Fisher, 31985070). The mixture was added to the cells grown in complete medium and collected after 24 h post-transfection.

**Immunofluorescence**

HeLa cells (10,000) were seeded in a 24-well plate onto glass coverslips. After 3 h of seeding, cells were transduced and treated as described above. Cells were washed twice with sterile PBS pH 7.4 (Thermo Fisher Scientific, 10010015) and fixed with 4% formaldehyde (Sigma-Aldrich, 252549) solution in PBS for 10 min at room temperature. After fixation, cells were washed twice with PBS, permeabilized and blocked with PBS containing 0.5% Triton X-100 (Sigma-Aldrich, X100) 10% normal goat serum (Abcam, ab7481) for 15 min followed by primary antibody incubation overnight at 4ºC. Cells thereafter were washed 2 times with PBS and incubated with secondary antibody and DAPI (Sigma-Aldrich, D9542) at 1 µg/ml for 1 h at room temperature and mounted with Fluoromount-G (Southern Biotech, 0100-01). Immunofluorescence controls, performed by omitting the primary antibodies, resulted in the abolition of the immunostaining in all cases.

**Statistical analysis**

All statistics and figures were performed with GraphPad Prism (GraphPad Software), unless indicated otherwise. For receiver operating characteristic (ROC) analyses, we employed the Metaboanalyst platform [9]. Student’s t or 2-way ANOVA tests were used to analyze the differences between groups. Associations between variables were studied with non-linear and linear regression analyses. The 0.05 level was selected as the point of minimal statistical significance in every comparison.

**References**


Legends to figures

**Figure 1.** The abundance of TARDBP regulated cryptic exons in ATG4B mRNA is an ALS tissue biomarker and TARDBP knockdown leads to down regulation of ATG4B. (A) Values of ATG4B mRNA analyses (± standard error) indicate that ATG4B cryptic exon levels are higher in samples from ALS when compared with controls. (B) RT-qPCR of ATG4B cryptic exons show different expression levels in spinal cord, frontal cortex, occipital cortex, and brain stem; ATG4B aberrant mRNA levels depend on ALS type (bulbar vs non-bulbar cases). C) ATG4B cryptic exon levels in brainstem of ALS patients have a positive correlation with age and (D) a negative correlation with disease duration. Further, TARDBP knockdown (KD) in human neural tissue cells differentiated from iPSC induced significant increase of the amount of ATG4B cryptic exon levels, as quantified by RT-qPCR (E), with a downregulation of ATG4B protein (F) evaluated by western blot and quantified by densitometry. Bars indicate mean values with standard error. For (B) *** p<0.001 for disease type in a 2-way ANOVA accounting for disease type and region. In (E) and (F)**** p<0.0001 and ** p<0.001 after Student’s t test (E) or post hoc Bonferroni analyses (F). AUC, area under the curve.

**Figure 2.** TARDBP is required for a homeostatic autophagy response. (A) TARDBP knockdown (KD) in HeLa cells leads to decreased ATG4B mRNA levels (left panel) linked to increased amounts of ATG4B mRNA with cryptic exons (right panel); this is associated with a failure to enhance ATG4B mRNA expression as a response to autophagy stimulation by nutrient
deprivation. (B) TARDBP protein loss is associated with ATG4B decrease as shown by western blot (left panel) and densitometry (right panel). (C) Functional analyses reveal that loss of TARDBP in HeLa cells induces increased levels of SQSTM1 as shown by western blot (left panel) and immunofluorescence (right panel), suggesting a functional loss of autophagy flux. (D) The increase in SQSTM1 induced by TARDBP knockdown is rescued by ATG4B overexpression, as shown by densitometry analyses, either in HeLa cells (left panel) or in Human Neural Tissue primary cells (right panel). Blots are representative of different experiments (n=3-5). Bars indicate mean values (± standard error). For (A), (B) and (C) **** p<0.0001, ***p<0.001 after 2-way ANOVA. For (D) *p<0.05 and **p<0.01 in Student’s t test.

**Figure 3.** GPSM2 cryptic exon abundance depends on disease type and region. Values of GPSM2 mRNA analyses (± standard error) indicate that (A) GPSM2 cryptic exon levels are higher in samples from ALS when compared with controls. (B) RT-qPCR of GPSM2 cryptic exons shows different expression levels in spinal cord, frontal cortex, occipital cortex, and brain stem. (C) Linear regression between the amount of cryptic exons in GPSM2 in lumbar spinal cord and motor cortex (Ctx). (D) Tissue concentrations of cryptic exons in ATG4B and GPSM2 correlate positively in a non-linear fashion. For (B), *** p<0.001 and **** p<0.0001 for disease type in a 2-way ANOVA accounting for disease type and anatomic region. AUC, area under the curve.
### Table 1. Antibodies and conditions employed.

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<th>Target</th>
<th>Dilution</th>
<th>Source</th>
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<td>Proteintech, 10782-2-AP</td>
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<tr>
<td>ATG4B</td>
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<td>Sigma-Aldrich, A2981</td>
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<tr>
<td>SQSTM1</td>
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<td>Secondary anti-rabbit, HRP conjugate</td>
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</tr>
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<td>Secondary anti-rabbit, Alexa Fluor® 546 conjugate</td>
<td>1:800 in PBS</td>
<td>Thermo Fisher Scientific, A11010</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2
Figure 3
Dear Dr Klionsky

First of all, on behalf of all authors, let me thank you again for your patience and carefulness in reviewing our paper. We have now separated large figures, previously appearing under the same number, into individual figures. We have also adapted the text to these changes. We have formatted as a Brief Report.

We are thankful for the time you spent in correcting and improving it.

Thanking your time and attention again, most sincerely

M Portero-Otin
University of Lleida-IRBLleida
Table S1. Demographic and pathological characteristics of individuals.

<table>
<thead>
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<th>Age at death</th>
<th>Diagnosis</th>
<th>Disease onset</th>
<th>Disease duration</th>
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<td>F</td>
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<td>ALS</td>
<td>Non-Bulbar</td>
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<td>10 h</td>
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1M: Male; F: Female
Table S2. Primers used for RT-qPCR.

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<td>5'-TTTGGGCTATCTGGTGAATGG-3'</td>
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<tr>
<td>Cryptic ATG4B</td>
<td>5'-CTGAGTGTGCTAGGATGAGT-3'</td>
<td>5'-TTTGGGCTATCTGGTGAATGG-3'</td>
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<td>ATG4A</td>
<td>5'-TTGGCCCGATGACAGCTG-3'</td>
<td>5'-AGGGCCCGTCCACCAATTG-3'</td>
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<td>5'-GTTACCTGCAGAGTCGGGAT-3'</td>
<td>5'-GGCCAGTTCTCAATGTGCAG-3'</td>
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<td>ATG4D</td>
<td>5'-GTCCATGAACTCAGTGTCGC-3'</td>
<td>5'-GTAACCTGCAGAGTCGGGAT-3'</td>
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<tr>
<td>Total GPSM2</td>
<td>5'-GGACGTGCCTTTGGAAATCTT-3'</td>
<td>5'-TTTGGCAATAAGGAGACGCTGC-3'</td>
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<tr>
<td>Cryptic GPSM2</td>
<td>5'-GTGTGTATGAGAGAGAGCGA-3'</td>
<td>5'-AGAAGCTTCCATTCTGTTCATCA-3'</td>
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<td>GAPDH</td>
<td>5'-CCCTTCATTGACCTCAACTACATG-3'</td>
<td>5'-TGGAGTTCTGATGACAGCAAG-3'</td>
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Figure Legends

Figure S1. Conventional PCR shows that ATG4B and GPSM2 cryptic exons are present in several nervous tissue locations from ALS patients, including spinal cord (A), motor cortex (B) and occipital cortex (C). (D, E) Show the trace sequences, demonstrating its identity.

Figure S2. Sequence of band corresponding to cryptic exon in ATG4B mRNA amplified by conventional PCR (excised from gels shown in Figure S1).

Figure S3. Sequence of band corresponding to cryptic exon in GPSM2 mRNA amplified by conventional PCR (excised from gels shown in Figure S1).

Figure S4. The proposed RT-qPCR assay shows high efficiency, sensitivity and specificity for quantitative analyses of cryptic exons in ATG4B mRNA. By using this method total ATG4B mRNA (A) and cryptic exon in ATG4B mRNA (B) show similar values of efficiency and R² similar on standard curve and they show specific peaks in Melt Peak curves.

Figure S5. The proposed RT-qPCR assay shows high efficiency, sensitivity and specificity for quantitative analyses of cryptic exons in GPSM2 mRNA. By using this method total GPSM2 mRNA (A) and cryptic exon in GPSM2 mRNA (B) show similar values of efficiency and R² similar on standard curve and they show specific peaks in Melt Peak curves.

Figure S6. TARDBP regulates only ATG4B mRNA in vitro and positively correlates with ATG4B RNA expression in vivo. In vitro (A, B), ATG4B downregulation induced by TARDBP knockdown (KD) in HeLa cells was not compensated by overexpression of other ATG4B homologs: ATG4A and ATG4C mRNA levels were quantified by RT-qPCR, both in basal conditions or after autophagy induction. ATG4D mRNA levels were not detected in HeLa cells using the indicated primers. Bars show mean values (± standard error) and a 2-way ANOVA analyses was performed (using a Bonferroni post hoc analyses). (C) In vivo expression analyses of TARDBP and ATG4B homologs show that there is a lineal
relationship between TARDBP mRNA levels and ATG4B homologs (except ATG4D), though the highest slope was present in the TARDBP and ATG4B relationship. Levels of mRNAs for different tissues present in the graph were obtained from proteinatlas.org, using the HPA dataset (accessed December 2017).
Figure S1

A Spinal cord

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B Motor cortex

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C Occipital cortex

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<td>GADPH</td>
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</table>
Figure S2

Cryptic ATG4B electropherogram
Figure S3

Cryptic GPSM2 electropherogram
Figure S4

A. Standard Curve Total ATG4B

SYBR E=98.6%
R²=0.983

Cq vs Log Starting Quantity

B. Melt Peak Total ATG4B

-\( \frac{d(RFU)}{dT} \)

C. Standard Curve Cryptic ATG4B

SYBR E=117.2%
R²=0.92

Cq vs Log Starting Quantity

D. Melt Peak Cryptic ATG4B

-\( \frac{d(RFU)}{dT} \)
Figure S5

A

Standard Curve Total GPSM2

SYBR E=122.2%
R²=0.982

B

Melt Peak Total GPSM2

C

Standard Curve Cryptic GPSM2

SYBR E=97.0%
R²=0.980

D

Melt Peak Cryptic GPSM2

Temperature, ºC
Figure S6

A

ATG4A expression (%)

Autophagy induction

- Scr
- TARDBP KD

p=0.07

p<0.0001

B

ATG4C expression (%)

Autophagy induction

- Scr
- TARDBP KD

p=0.07

p<0.01

C

ATG4A

ATG4B

ATG4C

ATG4D

ATG4A mRNA abundance

R=0.12
p=0.03

ATG4B mRNA abundance

R=0.51
p<0.0001

ATG4C mRNA abundance

R=0.38
p<0.0001

ATG4D mRNA abundance

R=0.002
p=0.79