Regulation of neuronal mRNA splicing and Tau isoform ratio by ATXN3 through deubiquitylation of splicing factors

RUNNING TITLE: Neuronal splicing regulation by ATXN3

Andreia Neves-Carvalho1,2, Sara Duarte-Silva1,2, Joana Silva1,2, Bruno Almeida1,2, Sasja Heetveld3, Ioannis Sotiropoulos1,2, Peter Heutink3, Ka Wan Li4 and Patrícia Maciel1,2*

1 Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal
2 ICVS/3B’s – PT Government Associate Laboratory, Braga/Guimarães, Portugal
3 German Center for Neurodegenerative Diseases (DZNE), 72076 Tübingen, Germany
4 CNCR, Amsterdam Neuroscience, Vrije Universiteit, Amsterdam, The Netherlands

CORRESPONDING AUTHOR:
Patrícia Maciel, PhD
Associate Professor
School of Medicine
University of Minho
Braga, Portugal
Telephone: 351-253-604824
Fax: 351-253-604820
E-mail: pmaciel@med.uminho.pt
ORCID ID: 0000-0002-4151-6885

FUNDING
This work has been funded by FEDER funds, through the Competitiveness Factors Operational Programme (COMPETE), and by National funds, through the Foundation for Science and Technology (FCT), under the scope of the project POCI-01-0145-FEDER-007038 and through the project POCI-01-0145-FEDER-016818 (PTDC/NEU-NMC/3648/2014) and fellowships [SFRH/BD/51059/2009 to A. N-C, SFRH/BPD/110728/2015 to B.A, SFRH/BD/78388/2011 to S.D-S, SFRH/BD/88932/2012 to J.M.S and IF/01799/2013 to I.S.]
The ubiquitylation/deubiquitylation balance in cells is maintained by deubiquitylating enzymes, including ATXN3. The precise role of this protein, mutated in SCA3, remains elusive, as few substrates for its deubiquitylating activity were identified. Therefore, we characterized the ubiquitome of neuronal cells lacking ATXN3, and found altered polyubiquitylation in a large proportion of proteins involved in RNA metabolism, including splicing factors. Using transcriptomic analysis and reporter minigenes we confirmed that splicing was globally altered in these cells. Among the targets with altered splicing was SRSF7 (9G8), a key regulator of MAPT (Tau) exon 10 splicing. Loss-of-function of ATXN3 led to a deregulation of MAPT exon 10 splicing resulting in a decreased 4R/3R-Ta ratio. Similar alterations were found in the brain of a SCA3 mouse and humans, pointing to a relevant role of this mechanism in SCA3, and establishing a previously unsuspected link between two key proteins involved in different neurodegenerative disorders.
INTRODUCTION

Ubiquitin signaling is now recognized as a fundamental molecular mechanism tightly regulating a broad range of intracellular events. Ubiquitylation is a highly dynamic biochemical modification in which an ubiquitin (Ub) moiety is attached to a protein. This process is catalyzed by the sequential actions of Ub-activating enzyme (E1), Ub-conjugating enzymes (E2) and Ub-protein ligases (E3) that bind Ub to different lysine (K) residues in the substrate, resulting in mono or poly Ub chains. Ubiquitylated substrates are then recognized by proteins containing ubiquitin binding domains and directed to different fates. For example, K48 usually targets proteins for proteasomal degradation, while K63-linked polyUb regulates protein activation, subcellular localization or degradation in the lysosome (autophagy) and is known to be relevant for DNA repair. Ubiquitylation and proteolysis by the Ubiquitin-proteasome pathway (UPP) are now well-known as important mechanisms in the nervous system as this proteolytic pathway is known to degrade misfolded or short-lived regulatory proteins. Impairment of the UPP has been connected to several neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's diseases. Like most post-transcriptional modifications, ubiquitylation is a reversible signal and is counterbalanced by deubiquitylating (DUB) enzymes, which remove Ub units and play an important role in the modulation of proteins by the proteasome as well as the autophagic pathway, and in Ub signaling in general. Several pieces of evidence have suggested that Ub may also regulate splicing: (i) Ub and Ub-
like proteins have been shown to copurify with splicing complexes \textsuperscript{13,14}, (ii) ubiquitylated splicing factors have been identified in proteomic screenings \textsuperscript{15,16} and (iii) several functional domains related to Ub pathway have been identified in important spliceosome proteins, such as Jab1/MPN domain of the essential U5 SnRNP component Prp8 \textsuperscript{17-20}. Thus, the action of DUBs has a major impact on the ubiquitylated proteome (also known as ubiquitome).

Ataxin-3 (ATXN3) is a protein with DUB activity known to be involved in Spinocerebellar ataxia type 3 (SCA3)/ Machado Joseph disease (MJD), a neurodegenerative disorder of adult onset caused by the expansion of a polyglutamine (polyQ) tract in this protein. Interestingly, in addition to being involved in global protein quality control responses \textsuperscript{21,22}, normal ATXN3 appears to participate in cell adhesion and in the organization of cytoskeleton network \textsuperscript{23-26}, to be involved in transcriptional regulation and DNA repair \textsuperscript{27-29}, and to be required for neuronal differentiation \textsuperscript{25}. Nevertheless, the relative relevance of these functions and the physiological role of this highly conserved but genetically non-essential protein remains to be clarified and its substrates are also not well characterized, particularly in neurons. Given the known relevance of UPP in neuronal function and its link to nervous system diseases, we sought to clarify the precise role of ATXN3. To identify potential candidates for the DUB activity of this protein, we characterized changes in the ubiquitome of neuronal cells lacking ATXN3 (ATXN3\textsuperscript{shRNA} cells) by mass-spectrometry, and identified for the first time a role for ATXN3 in RNA metabolism. We found a global perturbation of splicing in the absence of this protein, among which a mis-splicing of tau exon 10 which perturbs the 4R/3R
tau ratio, a mechanism that may contribute for SCA3 pathogenesis, and links two key proteins involved in different neurodegenerative disorders.

**RESULTS**

Changes in the ubiquitome of cells lacking ATXN3 suggest a link to RNA metabolism

With the goal of identifying substrates of the DUB activity in neuronal cells, we searched for changes in the ubiquitome of neuronally differentiated SH-SY5Y cells lacking ATXN3 (ATXN3<sup>shRNA</sup> cells - Figure EV1A-B)<sup>25</sup>, taking advantage of a recently developed methodology that combines capture of the whole spectrum of polyubiquitylated proteins in a cell extract using an enrichment by Tandem Binding Ubiquitin Entities (TUBEs) followed by mass spectrometry analysis (TUBEs-LC-MS/MS)<sup>30</sup>. Figure 1A summarizes the steps followed for the purification and identification of the polyubiquitylated proteins in ATXN3<sup>shRNA</sup> and scrambled control shRNA (SCR<sup>shRNA</sup>) cells. The integration of the data and the comparison between the proteins identified in ATXN3<sup>shRNA</sup> versus SCR<sup>shRNA</sup> cells resulted in a list of proteins with altered polyubiquitylation in cells lacking ATXN3; among these are potential targets of the DUB activity of ATXN3, i.e., putative ATXN3 substrates. In each pulldown experiment, around 1200-1300 proteins were identified. When the results of all the independent experiments were merged, we observed that many of these proteins were only sporadically detected across the different experiments; for the remaining analysis, these proteins were excluded, reducing the list to 615 consistently identified proteins. From these, 193
proteins showed altered levels in the polyubiquitylated fraction in ATXN3<sup>shRNA</sup> cells when compared to the SCR<sup>shRNA</sup> controls (p<0.05) (Table EV1). The majority of these polyubiquitylated proteins were either not detected (44.04%) or showed decreased levels (24.35%) in ATXN3<sup>shRNA</sup> cells when compared to the controls. These proteins can be grouped into 10 functional networks, the most representative being related to: (i) Gene expression, DNA replication, recombination and repair (17.7%), (ii) RNA post-transcriptional modification (13.3%), (iii) Molecular transport, RNA trafficking (10.8%), (iv) Cell death and survival (8.9%) and (v) Organ morphology (8.9%) (Figure 1B). The fact that a significant proportion of the proteins with altered polyubiquitylation in ATXN3<sup>shRNA</sup> cells are RNA metabolism-related proteins, around 8% of them being splicing factors (Table 1), suggested to us that ATXN3 could be playing a role in mRNA splicing, a previously undescribed role for this DUB.

**Cells lacking ATXN3 show global perturbation of splicing**

We hypothesized that absence of ATXN3 could lead to a deregulation of the pre-mRNA splicing process. To address this, we used three hybrid minigene reporter plasmids: the pyPY minigene, representing splicing events with alternative competing 3′ acceptor sites/splice sites, the AdML minigene, representing constitutive/strong splicing events, and the α-globin minigene, for which the alternative splicing (exon skipping) is indicative of the performance of regulatory splicing factors such as hnRNP and SF proteins<sup>31,32</sup>. As shown in Figure 2A-D, knockdown of ATXN3 significantly altered the processing of the three splicing reporters, suggesting a general deregulation of splicing in the absence of this protein. Interestingly, overexpression of a catalytically inert
version of ATXN3 in SH-SY5Y cells (ATXN3_C14A), previously shown to have a dominant-negative effect \(^{25}\) also yielded similar alterations (Figure 2E).

Consistent with a global deregulation of splicing in the absence of ATXN3, microarray analysis (Figure 2F) using specific arrays containing additional probes for exon/exon junctions, confirmed that 1993 genes (43%), from the 7450 differentially expressed probes in ATXN3\(^{\text{shRNA}}\) cells, presented differentially regulated alternative splicing events (Table EV1). These genes mainly encode components with function in protein degradation systems, cell adhesion, axon guidance and various signaling pathways (Table EV2), the majority of splicing events being decreased when compared to control cells (Table EV3). The most affected alternative splicing event types were exon cassette usage (34%), complex splicing (20%) and intron retention (15%) (Figure 2G). Alternative terminal exon usage, which can be mechanistically linked to alternative splicing, was also seen in 16% of the cases (Figure 2G).

Being a key mechanism in the regulation of gene expression, defects in these regulatory processes may affect a multiplicity of cellular functions related to disease.

**ATXN3 modulates SRSF7 degradation by the proteasome**

Interestingly, among the splicing factors with differential ubiquitylation was the Arginine/Serine-Rich splicing factor SRSF7 (also known as 9G8), known to be involved in the regulation of alternative splicing of the neurodegeneration-related gene \(\text{MAPT}^{33}\), encoding the Tau protein. As shown in Figure 3A-B, we detected decreased levels of ubiquitylated forms of SRSF7 in cells lacking ATXN3 (\(p=0.02\)), validating the data obtained with the proteomic analysis.
Consistent with the hypothesis that the differential ubiquitylation of SRSF7 can have an impact on its degradation, the steady state levels of total (Figure 3A-B) and nuclear (Figure 3C-D) SRSF7 were decreased in ATXN3<sup>shRNA</sup> cells as compared with the SCR<sup>shRNA</sup> control cells (p=0.025 and p=0.001, respectively). This suggests that SRSF7 is indeed being more degraded in the absence of ATXN3, confirming that ATXN3 affects the expression of SRSF7 at the protein and not at the mRNA level (p=0.712) (Figure EV1C).

Based on the fact that SRSF7 was captured using Agarose-TUBEs that have higher affinity for polyubiquitylated than for monoubiquitylated proteins and considering that ATXN3 is a DUB, we asked if ATXN3 could modify ubiquitylation and regulate degradation of SRSF7 through the UPP. Supporting this possibility, the levels of SRSF7 were significantly increased in ATXN3<sup>shRNA</sup> cells upon proteasome inhibition with MG132 (p=0.0016) (Figure 3E-F). Additionally, we detected lower levels of polyubiquitylated SRSF7 presenting Ub-K48 linkages (Figure 3G), a type of Ub chain that usually signals proteins for degradation through the UPP. Protein stability analysis upon inhibition of protein synthesis by cicloheximide treatment showed a decrease in the half-life of SRSF7 in ATXN3<sup>shRNA</sup> cells (Figure 3H), suggesting that ATXN3 normally acts to inhibit SRSF7 degradation in neurons. In order for ATXN3 to modify the ubiquitylation and stability of SRSF7, the two proteins need to interact. Co-immunoprecipitation using protein extracts from differentiated neuron-like SH-SY5Y cells confirmed that an interaction between ATXN3 and SRSF7 occurs in these cells (Figure 3I). This interaction was further confirmed in live cells by the Protein Ligation Assay (Figure 3J,
Globally, our data are compatible with the hypothesis of SRSF7 being a substrate of the DUB activity of ATXN3 in neurons.

**Tau splicing is deregulated in the absence of ATXN3**

Given that one of the splicing factors showing differential ubiquitylation was SRSF7, we next analyzed mRNA levels of Tau isoforms in ATXN3<sup>shRNA</sup> cells to assess the functional impact of the reduction of this splicing factor in Tau splicing. We found a significant decrease in the transcript levels of 4R-Tau isoform (p=7.4x10⁻¹¹) (Figure 4A), but no alteration of the 3R-Tau isoform (p=0.270) in ATXN3<sup>shRNA</sup> cells as compared with SCR<sup>shRNA</sup> control cells (Figure 4B), leading to an altered 4R/3R-Tau ratio (p=2.98x10⁻⁶) and decreased total tau levels (p=1.92x10⁻⁴), both in ATXN3<sup>shRNA</sup> cells (Figure 4D) as well as in cultured primary cerebellar neurons (p=3.23x10⁻⁹) in which ATXN3 expression was silenced by shRNA (Figure EV1D, Figure 4E-H).

Consistently, catalytically inactive (Dominant negative) ATXN3 expression also led to alterations in tau expression and in the isoform ratio similar to those seen in cells with silenced ATXN3: decreased expression of 4R-Tau (p=1.1x10⁻⁵) (Figure 4I), no alterations on the 3R-Tau isoform (Figure 4J), and consequentially a decreased 4R/3R-Tau ratio (p=0.0088) (Figure 4K) and decreased levels of total Tau (p=1.20x10⁻⁶) (Figure 4L). To explore the functional consequences of this reduction in 4R-Tau levels, we performed a morphometric analysis of these cells. ATXN3<sup>shRNA</sup> cultures presented a decreased expression of βIII-tubulin and a decreased average length of the neuritis as compared with the SCR<sup>shRNA</sup> controls, which correlates with a more immature stage of neuronal differentiation (Figure 4M-N).
Decreased SRSF7 expression and Tau splicing perturbation in SCA3 models and patients

To determine if the presence of an expanded polyglutamine (polyQ) tract within ATXN3 would lead to an alteration in this newly identified physiological function, we analyzed a SH-SY5Y cell line expressing a version of ATXN3 bearing 83 glutamines (ATXN3_83Q), and found a globally decreased efficiency of splicing (Figure 5A). We next asked whether the presence of the polyQ tract could also impact MAPT mRNA splicing regulation, altering the 4R/3R-Tau ratio. Interestingly, expression of ATXN3_83Q lead to similar changes in the expression of tau isoforms as observed in cells lacking ATXN3 or expressing a catalytic mutant version of this protein: i) decreased expression of 4R-Tau isoform (p=6.6x10^{-5}) (Figure 5B), ii) no alterations on the 3R-Tau isoform (Figure 5C), iii) a decrease of the 4R/3R-Tau ratio (p=2.4x10^{-4}) (Figure 5D), and (iv) a decrease in the levels of total Tau (p=7.6x10^{-5}) (Figure 5E). Of notice, the overexpression of the wild type ATXN3 (ATXN3_28Q) also caused some (albeit less important) degree of perturbation (Figure 5 A-E), pointing to the importance of a tight regulation of ATXN3 dosage in neurons.

Tau splicing was also analyzed in SCA3 mouse model (CMVMJD135) that reproduces key features of the disease. A decreased expression of the 4R-Tau isoform was seen in the brainstem (affected brain region) of CMVMJD135 mice (Tg) (p=7x10^{-5}) comparing with the Wild type (Wt) controls (Figure 5F), with no alterations of the 3R-Tau isoform expression (p=0.84) (Figure 5G), leading to an altered 4R/3R-Tau ratio (p=1.2x10^{-3}) (Figure 5H) and decreased
levels of total Tau \( (p=1.1 \times 10^{-5}) \) (Figure 5I). In addition, SRSF7 protein levels were also reduced in the brainstem \( (p=0.0093) \), spinal cord \( (p=0.0014) \) and to a lesser extent in the cerebellum \( (p=0.1138) \) (disease-affected brain areas) of Tg mice as compared with the Wt littermate controls (Figure 5J-L, respectively), but no alterations at the mRNA level were detected (Figure EV1F). This indicates that the impact of ATXN3 on tau splicing through the control of SRSF7 levels is also occurring in SCA3 and is corroborated by the decreased levels of SRSF7 protein present in the brains of SCA3 patients as compared with healthy controls (Figure 5M). To explore the functional consequences of this reduction in 4R-Tau levels, an isoform of Tau associated with mature neurons and synaptic function, we performed a morphometric analysis of neurons in this brain region. A decreased mean neurite length \( (p=0.0189) \) (Figure 5N-O) and a decrease in complexity of the dendritic trees \( (p=0.0004) \) (Figure O, P) were observed in neurons from the Medial vestibular nuclei in the Brainstem.

Altogether, these results suggest that the functional interaction of ATXN3 with SRSF7 and its impact on molecular mechanisms regulating tau splicing in neurons may be relevant in the pathogenesis of SCA3.

**DISCUSSION**

Ubiquitylation is a post-translational modification that controls several aspects of neuronal function by regulating protein abundance. Disruption of this signaling pathway has been described in neurodegenerative diseases. Indeed, since many of these diseases exhibit ubiquitylated protein aggregates, loss of ubiquitin homeostasis may be an important contributor for
Considering the importance of DUB enzymes in maintaining the ubiquitylation balance, we focused on the characterization of the ubiquitome of neuronal cells lacking ATXN3. Using TUBEs, that enable the pulldown of polyubiquitylated proteins without further genetic manipulation or inhibition of the proteasome in combination with LC-MS/MS, we were able to consistently identify 615 proteins per condition, a yield comparable to those described in other studies. Among the proteins identified, approximately one third presented altered levels of polyubiquitylation in ATXN3\textsuperscript{shRNA} cells. Curiously, the majority of these proteins presented decreased levels in the polyubiquitin-enriched fraction signal, suggesting that normally ATXN3 might be preventing their degradation, for instance by editing the substrate's ubiquitin chains, as we observed before for ITGA5. Therefore, when ATXN3 is silenced, the polyubiquitin signal is not removed, which may result in an increased degradation of the targeted proteins. The fact that a significant proportion of the proteins with altered polyubiquitylation levels in ATXN3\textsuperscript{shRNA} cells were splicing factors and proteins involved in RNA processing, is suggestive of ATXN3 playing a role in the pre-mRNA splicing process in neuronal cells. This process is particularly important in generating diversity and specificity in the central nervous system, which requires a wide protein repertoire to generate its highly specialized and adaptable neuronal circuits. Given the importance of RNA processing for neuronal function, it comes as no surprise that altered RNA processing is a contributing factor to the pathogenesis of multiple neurodegenerative diseases. Disruption of alternative splicing (AS) regulation, resulting from mutations or abnormal expression of RNA-binding proteins, can lead to an imbalance or an
inappropriate expression pattern of key protein isoforms, with profound consequences on cellular and organismal physiology. Indeed, the relative concentration of splicing factors and hnRNPs, which may be regulated by the Ub pathway, has been shown to regulate AS. For example, it was shown that the abundance of several splicing factors is differentially affected by proteasome inhibition, and abnormal levels of these regulatory proteins were associated with a different AS pattern. Another study showed that the splicing factor SRSF5 is targeted to proteasomal degradation as the cells undergo terminal differentiation. Consistently, assessed general splicing efficacy was decreased in cells depleted of ATXN3, as shown using artificial reporter minigenes, further supporting the involvement of ATXN3 in the regulation of the splicing process. Additionally, genome wide microarray analysis of endogenous splicing events confirmed that absence of ATXN3 leads to a dramatic alteration in the splicing pattern of a large number of genes in neuronal cells, including genes related to protein degradation, adhesion, axon guidance and signaling pathways. This is consistent with our previous findings showing that absence of ATXN3 leads to an impairment in neuronal differentiation and adhesion, and affects the degradation of several target proteins. Significantly, a portion of the predicted splicing regulators of these genes with altered splicing in cells lacking ATXN3 were found to have altered levels of polyubiquitylation in our proteomic analysis, the majority of them being Serine/Arginine (SR) – rich phosphoproteins. Proteins of the SR family are key players in the control of AS, regulating the selection of alternative sites. The fact that SRSF7 (9G8), one of those proteins and a key regulator of MAPT (tau) exon 10 splicing, was captured using the
Agarose-TUBEs, which have high affinity for polyubiquitin chains \(^{30}\), suggested that this splicing factor is polyubiquitylated, and thus might be degraded through the UPP. Indeed, while it has been described that the mature human neurons express approximately equal levels of 4R and 3R-Tau isoforms \(^{50,51}\), whose expression is developmentally controlled and finely-tuned, we found that loss of function of ATXN3 disrupts this balance in neuronal cells leading to a decreased 4R/3R-Tau ratio, that coincides with (and may be caused by) decreased levels of SRSF7. Very recent works have shown imbalanced expression in 3R and 4R-Tau, namely a decreased expression of the 4R isoform as here observed, in the brain of a mouse model of Down syndrome, but also in human AD and PD patients \(^{52-54}\), strengthening the idea that a faulty tau exon 10 splicing might be contributing for the pathogenesis of several neurodegenerative diseases. In addition, the decreased levels of the 4R-Tau isoform presented by ATXN3\(^{\text{shRNA}}\) cells, also correlate with the immature phenotype of these cells, that we previously described \(^{25}\). Indeed Conejero-Godberg and colleagues also assessed the transcriptional profiles related to 3R and 4R-Tau splice variants, revealing that a cluster of genes related to neuronal cell morphology and outgrowth of neurites is upregulated in the presence of 4R isoforms, while genes related to cellular growth and proliferation were downregulated \(^{55}\). This is in agreement with previous work from Sennvik’s team establishing a role for 4R-Tau isoforms in promoting neuronal differentiation \(^{56}\). Taking into account that loss of function of ATXN3 leads to a deregulation in the amount of SRSF7, at the protein level but not RNA level, we hypothesized that this splicing factor could be a substrate of the DUB activity of ATXN3, which could be modulating its
degradation. In line with that, we confirmed that ATXN3 interacts closely with SRSF7 in neuronal cells, suggesting that these proteins are molecular partners in such cells, and establishing a functional link between two key proteins involved in different neurodegenerative diseases. Trying to explore if this interaction could be indicative that ATXN3 is modulating the degradation of SRSF7 through the proteasome, we measured the levels of this protein upon proteasome inhibition; as expected, we saw an accumulation of SRSF7 when the proteasome was inhibited. Interestingly, we also found a decreased 4R/3R-Tau ratio, as well as decreased protein levels of SRSF7, both in cells expressing the mutant protein, in affected brain regions of our mouse model for the disease, at an age when the transgenic animals display an overt phenotype resembling behavioral and pathological characteristics present in human patients. This suggests that the mis-splicing of tau may be contributing to SCA3 pathogenesis, as it does to other neurodegenerative diseases. Considering that we observed a similar phenomenon using cells lacking ATXN3 and cells expressing a catalytic inactive mutant form of the protein, it is reasonable to think that the DUB activity of ATXN3 is important in this cellular process and that the polyglutamine expansion might cause a partial loss of this normal function of the protein (even in presence of 50% of the wild type protein), perhaps through a dominant negative mechanism, thus contributing to SCA3 pathogenesis. Indeed, Winborn and co-workers demonstrated that while expression of ataxin-3 with a non-pathogenic polyQ tract leads to a reduction in total ubiquitylated protein levels in cells, this reduction is not observed upon overexpression of an expanded ataxin-3, which may reflect inefficient deubiquitylation of cellular proteins, pointing to an
overall loss of function. Overall, our data suggest that ATXN3 is involved in splicing regulation through the modulation of the ubiquitylation of splicing factors. This regulatory role may be mediated through the DUB activity of ATXN3 by modulating activation, degradation and/or subcellular localization of the splicing factors, or may be an indirect result of the modulation of E3 ligases specific for these targets.

Recently, several pieces of evidence coming from the use of technologies such as exon arrays and RNA-Seq suggested an association between perturbation of AS and several neurodegenerative diseases including Alzheimer’s disease, Huntington’s disease, Pick’s disease, Spinal Muscular atrophy and Amyotrophic Lateral Sclerosis. Importantly, several ataxia-causing proteins have been described to interact with splicing factors. Also, expression of mutant ATXN3 bearing an expanded polyglutamine tract was previously shown to alter the ability of the subnuclear domains known as Cajal bodies to efficiently participate in small nuclear ribonucleoprotein (snRNP) biogenesis pathway, and to reduce the efficacy of splicing reporter genes. These findings lead us to propose that ATXN3 plays a role in splicing regulation in neurons, a novel function for this protein, and that a perturbation of this physiological function might contribute for SCA3 pathogenesis.

**MATERIALS AND METHODS**

**Cell lines and primary cultures**
**SH-SY5Y cell cultures:** SH-SY5Y human female neuroblastoma cell line (ATCC number CRL-2266) was cultured in Dulbecco’s modified eagle medium: nutrient mixture (DMEM)/F-12 supplemented with 10% (v/v) fetal bovine serum (FBS) (Biochrom), 2mM glutamax (Invitrogen), 100 U/mL penicillin (Invitrogen), 100 µg/mL streptomycin (Invitrogen) and 25 ng/mL puromycin (Sigma Aldrich). Medium was changed every 2 days. Cells were transfected with a shRNA sequence targeting ATXN3 or a scrambled shRNA sequence as described elsewhere. Differentiation of stably infected cell lines was induced by 0.1 µM all-trans-retinoic acid (RA) (Sigma Aldrich) in opti-MEM (Invitrogen) supplemented with 0.5% FBS for 7 days. Medium was replaced every 2 days.

**Primary cultures of cerebellum:** Cerebellar neuron cultures were prepared from P7 Wistar rats. Briefly, upon dissection, cerebella were submitted to a trypsin-based enzymatic digestion followed by mechanical dissociation. Isolated cells were plated on poly-d-lysine (Sigma Aldrich) and lysine (Sigma Aldrich) pre-coated 6-well plates at a density of 1.25x10^6 cells/mL using Neurobasal A (Invitrogen) supplemented with 1x B27 (Invitrogen), 0.1 mg/mL Kanamycin (Invitrogen), 1 mM Glutamax I (Invitrogen) and 10 ng/mL bFGF (Invitrogen) for 7 days. Half of the medium was changed every 4 days.

**Mice**

Female CMVMJD135 (background C57BL/6) mice were used in the study. Transgenic (Tg) and wild-type (Wt) littermates were housed at weaning in groups of 5 animals in filter-topped polysulfone cages 267x207x140 mm (370 cm² floor area) (Tecniplast), with corncob bedding (Scobis Due, Mucedola...
SRL) in a conventional animal facility. The mice were genotyped at weaning by PCR, as we previously described. All animals were maintained under standard laboratory conditions: an artificial 12 h light/dark cycle (lights on from 8:00 to 20:00 h), with an ambient temperature of 21±1°C and a relative humidity of 50-60%; the mice were given a standard diet (4RF25 during the gestation and postnatal periods, and 4RF21 after weaning (Mucedola SRL) and water ad libitum. Animal facilities and the people directly involved in animal experiments were certified by the Portuguese regulatory entity – Direcção Geral de Veterinária. Health monitoring was performed according to FELASA guidelines, confirming the Specified Pathogen Free status of sentinel animals maintained in the same animal room. Humane endpoints for the experiment were defined (20% reduction of the body weight, inability to reach food and water, presence of wounds in the body, dehydration). The animals were euthanized at 45 weeks of age by decapitation, the brain regions of interest were macro dissected and store at -80°C until further processing.

**Pulldown of polyubiquitylated proteins**

Cells treated with RA were lysed by sonication on ice in lysis buffer (50 mM Tris-HCl pH 7.5, 0.15 M NaCl, 1mM EDTA, 1% NP-40, 10% Glycerol, complete protease inhibitors (Roche) and 50 μM UB/UBI protease inhibitor PR-619 (LifeSensors). After lysis, 2 mg of total protein extract was incubated with 100 μL of pre-equilibrated Agarose-TUBEs (LifeSensors), overnight at 4°C on a rocking platform. Sedimented beads were washed 3 times with washing buffer (20 mM Tris pH 8.0, 0.15 M NaCl, 0.1% Tween-20) before
being eluted with 1x SDS sample buffer (62.5mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, Bromophenol Blue). Eluted proteins were immediately boiled at 98°C for 15min and ran in a 10% SDS-PAGE gel. After incubation with the FK2 anti-ubiquitin primary antibody (1:2000, Millipore) overnight at 4°C, membranes were incubated with secondary antibody for 1 hour at room temperature (anti-mouse, 1:10.000, Bio-Rad). Antibody binding was detected by chemiluminescence (Clarity kit, Bio-Rad).

**Digestion of proteins from preparative 1D-PAGE gel**

The 1D PAGE LC-MS/MS approach was used for protein identification as previously described. Eluted proteins were separated using 1.5 mm and 10% SDS-PAGE gels. The quality of purification was controlled by Coomassie Brilliant Blue g-250 (Sigma) staining before MS analysis. Gel image was acquired the Gel Doc™ EZ system (Bio-rad). After Coomassie staining, all the visible blue-stained protein spots were manually excised from the gel. The gel pieces were destained overnight at room temperature using 50% acetonitrile in 25 mM ammonium bicarbonate buffer, pH 8.5, and then dehydrated with 100% acetonitrile. The shrunken pieces were then re-swollen in 50 mM ammonium bicarbonate buffer, dehydrated in 100% acetonitrile and dried in a speedvac® concentrator (Savant) for 30 min. The gel pieces were rehydrated in 60 µL of 20 µg/mL Trypsin (Promega) in 50 mM ammonium bicarbonate solution and incubated for 2h at 55°C. The gel pieces were then incubated with 0.1% trifluoroacetic acid in 50% acetonitrile for 20 min at room temperature in order to extract the remaining peptides from the gel. The tryptic peptides were dried in a speedvac for 2 h.
Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

After re-dissolution in 17 µL 0.1% acetic acid, samples were separated on a capillary C18 column using a nano LC-ultra 1D plus HPLC system (Eksigent) and analyzed on-line with an electrospray LTQ-Orbitrap Discovery mass spectrometer (Thermo Fisher Scientific). MS/MS spectra were searched against a human database (uniprot_sprot, 2010_01) with the ProteinPilot™ software (version 3.0; AB-sciex) using the Paragon™ algorithm (version 3.0.0.0) as the search engine. The detected protein threshold (unused protscore (confidence)) in the software was set to 0.10 to achieve 20% confidence and the proteins identified were grouped to minimize redundancy. Peptides with “unused” values < 2 have low confidence and were excluded from analysis. The “unused” value is defined in the handbook of ProteinPilot as a sum of peptide scores from all the non-redundant peptides matched to a protein. Peptides with confidence of ≥ 99% would have a peptide score of 2. Tryptic peptides shared by multiple proteins were assigned to the winner protein.

RNA extraction and Array hybridization

Total RNA was isolated from ATXN3\textsuperscript{shRNA} and SCR\textsuperscript{shRNA} cells using an miRNeasy mini kit (Qiagen) and quality assessment was achieved using RNA 6000 Nano labchip (Bioanalyzer, Agilent) and by a Nanodrop spectrophotometer (Thermo). Total RNAs RIN values were between 8.7 and 9.3 (average: 9.17). Affymetrix Human Transcriptome Array 2.0 ST arrays were hybridized according Affymetrix recommendations using the Ambion WT
protocol (Life technologies, France) and Affymetrix labelling and hybridization kits. Raw data, transcript data and exon data were controlled with Expression console (Affymetrix).

Microarray data analysis

Affymetrix Human Transcriptome Array 2.0 ST dataset analysis was performed using the GenoSplice technology (www.genosplice.com). Data was normalized using quantile normalization. Background corrections were made with antigenomic probes and probes were selected according to their %GC, cross-hybridization status and potential overlap with repeat region as previously described [PMID:23861464, PMID:23321315, PMID:23284676]. Only probes targeting exons and exon-exon junctions annotated from FAST DB® transcripts (release fastdb_2013_2) were selected [PMID:16052034, PMID:17547750]. Only probes with a DABG P value ≤0.05 in at least half of the arrays were considered for statistical analysis [PMID:23861464, PMID:23321315, PMID:23284676]. Only genes expressed in at least one compared condition were analyzed. To be considered to be expressed, the DABG P-value had to be ≤0.05 for at least half of the gene probes. We performed an unpaired Student’s t-test to compare gene intensities between ATXN3shRNA and SCRshRNA cells. Genes were considered significantly regulated when fold-change was ≥1.5 and P-value ≤0.05 (unadjusted P-value). Analysis at the splicing level was first performed taking into account only exon probes ('EXON analysis) in order to potentially detect new alternative events that could be differentially regulated (i.e., without taking into account exon-exon junction probes). Analysis at the splicing level was also
performed by taking into account exon-exon junction probes (‘SPlicing Pattern analysis) using the FAST DB® splicing pattern annotation (i.e., for each gene, all possible splicing patterns were defined and analyzed. All types of alternative events can be analyzed: alternative first exons, alternative terminal exons, cassette exon, mutually exclusive exons, alternative 5’ donor splice site, alternative 3’ acceptor splice sites and intron retention). EXON and SPlicing Pattern analyses were performed using unpaired Student’s t-test on the splicing-index as previously described [PMID:23861464, PMID:23321315, PMID:23284676]. Results were considered statistically significant for unadjusted P-values ≤ 0.05 and fold-changes ≥ 1.5 for SPlicing Pattern analysis and unadjusted P-values ≤ 0.05 and fold-changes ≥ 2.0 for EXON analysis. Gene Ontology (GO), KEGG and REACTOME analyses of differentially regulated genes were performed using DAVID [PMID:19131956].

Plasmid purification

Hybrid minigene reporter plasmids pyPY, AdML and α-globulin were kindly provided by Prof. Juan Valcárcel (Centre de Regulació Genòmica – CRG, Barcelona). Top10 competent cells (Invitrogen) were transformed with 100 ng of plasmid DNA, according to the recommended protocol. Briefly, the cells were incubated with the constructs on ice for 30 min followed by heat shock at 42ºC for 1 min. After incubation on ice for 2 min, 500 µL of LB medium was added to the cell vial and incubated at 150 rpm for 60 min at 37ºC. Cultures were grown overnight at 37ºC in LB/ampicillin plates. The next day, one colony was inoculated in LB/ampicillin (100 mg/mL) at 37ºC.
overnight. Plasmid extraction was carried out using the ZR Plasmid Miniprep™ (Zymo Research) according with the manufacturer’s protocol. DNA concentration was determined using Nanodrop (Alfagene) and integrity verified by running 200 ng in an agarose gel.

**Cell transfection**

4x10^5 cells per well were plated in gelatin-coated 6 well plates and incubated for 24 h. Before transfection, the culture medium was changed to DMEM/F-12-AA without antibiotics and supplemented with 5% FBS. Cells were transfected with 200 ng of the reporter plasmids using Lipofectamine® 2000 Transfection Reagent (Invitrogen) according with the manufacturer’s instructions. Briefly, reporter plasmid minigenes and the transfection reagents were appropriately diluted in Opti-MEM medium separately and incubated for 5 min at room temperature. The mixed reagents were then incubated at room temperature for 20 min allowing the formation of transfection complexes. The cells were then incubated for 24 h with the transfection mix.

**Semi-quantitative PCR**

PCR amplification of pyPY, AdML and α-globin reporter genes was carried out using Taq DNA Polymerase (Thermo Fisher Scientific) following the manufacturer’s protocol. The cycling conditions were: 95°C for 5 min followed by 24 cycles of denaturing at 95°C for 1 min, annealing at 60°C for 45 sec, extension at 72°C for 1 min and final extension at 72°C for 5 minutes. The primers used are listed in Table S1. The PCR product was ran in a 2%
agarose gel. Gel analyses and splicing efficiency calculations were performed using Image Lab software (Bio-Rad).

**Cell fractionation**

RA-treated pelleted cells were resuspended in ice RSB buffer (10 mM Tris-HCl pH 7.4, 10 M NaCl) and incubated on ice 3 min. After centrifugation for 5 min, 4000 rpm at 4°C, the pellet was resuspended in RSBG40 buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 10% glycerol, 0.5% NP-40, 0.5 mM DTT) and centrifuged for 3 min, 7000 rpm at 4°C. The supernatant was collected as cytoplasmic fraction and stored at -80°C. The nuclear pellet was resuspended in 50 μL B1 buffer (20 mM Tris-HCl pH 7.9, 75 mM NaCl, 5% glycerol, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM PMSF) and 450 μL B2 buffer (20 mM HEPES pH 7.6, 300 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 7.5 mM MgCl₂, 1 M Urea, 1% NP-40). Samples were vortexed for 5 sec, incubated on ice for 10 min and centrifuged for 5 min, 15000 rpm at 4°C. The supernatant was collected as nuclear fraction and stored at -80°C.

**Protein synthesis inhibition and proteasome inhibition**

RA-treated SH-SY5Y cells were incubated with 5 μM cicloheximide (Merck) during 20, 60 and 120 minutes. For proteasome inhibition, RA-treated cells were incubated with 5 μM MG132 (Calbiochem) for 24h prior to lysis.

**High-throughput high-content functional imaging**

SH-SY5Y cells were seeded at a density of 4x10³ cells/well in flat bottom 96-well plates previously coated with Matrigel (BD, Biosciences), and 10 μM all-
trans-retinoic acid (Sigma Aldrich) was added the day after plating in DMEM-F12 with 1% FBS, to induce differentiation. After 5 days, cells were washed in DMEM/F-12 and incubated with 50 ng/mL BDNF (Prepotech) in DMEM/F-12 without serum for 3 days. Cells were then labeled for β-III tubulin (1:1000, R&D Systems), scanned at different locations of each well and the quantitative analysis of neurite length were automatically done using the automatic imaging system Thermo Scientific Cellomics® ArrayScan® VTI.

**Immunoprecipitation**

RA-treated cells were washed in ice-cold PBS and lysed by sonication on ice in NP-40 buffer. Aliquots were taken from protein extracts as 10% inputs. 1 mg of total protein was pre-cleared for 3 h at 4°C by incubation with glutathione-coupled sepharose beads (GE Healthcare) previously equilibrated in Wash buffer 1 (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1x protease inhibitors (Roche)) for 3 times, 10 min at 4°C. Beads were then centrifuged and the supernatant was incubated O/N at 4°C with 50 μL equilibrated beads. After centrifugation, the supernatant was discarded and the beads were washed 2 times with Wash buffer 1 for 10 min at 4°C. Beads were then washed twice with Wash buffer 2 (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.1% NP-40) for 10 min at 4°C, and once with Wash buffer 3 (50 mM Tris-HCL pH 7.5, 0.1% NP-40) for 20 min at 4°C. The supernatant was discarded and the bound proteins were eluted with 1x Laemmli buffer, boiled for 5 min at 98°C and run in a 10% SDS-PAGE gel.

**Proximity Ligation Assay (PLA)**
SH-SY5Y cells were cultured onto polyD-lysine coated cover slips in 24-well plates using D-MEM media as indicated to above. PLA protocol was performed as described in and following manufacturer instructions with adaptations. Cells were fixed with 4% PFA, and permeabilized with 0.5% Triton in PBS at room temperature. Blocking was performed at 37°C in a humidity chamber for 1 hour, using Duolink® PLA Blocking Solution (Sigma). Primary antibodies were incubated overnight at 4°C followed by two washing steps of 5 minutes with Wash Buffer A (Sigma) at room temperature. PLUS and MINUS PLA probes were diluted 1:5 in the Duolink Antibody Diluent (Sigma), added to the cover slips and incubated for 1h at 37°C in a humidity chamber. Washing was performed for 5 minutes two times with Washing Buffer A at room temperature followed by incubation with Ligase solution (Sigma) for 30 minutes at 37°C in a humidity chamber. After washing two times during 2 minutes with Washing Buffer A at room temperature, cover slips were incubated with Amplification solution (Sigma) for 100 minutes at 37°C in a humidity chamber protected from light. Then, cover slips were washed two times during 10 minutes with Washing Buffer B (Sigma) at room temperature followed by an additional washing step of 1 minute using 100X diluted Washing Buffer B. Cover slips were mounted on a glass slide with mounting media and DAPI and cells analyzed by confocal microscopy.

**Immunoblotting**

RA-treated cells were pelleted and frozen in liquid nitrogen. For cellular and brain tissue extracts, 50 µg of total protein isolated in NP-40 buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.6, 0.5% NP-40, protease inhibitors (Roche)) were
resolved in 10% SDS-PAGE gels and then transferred to a nitrocellulose membrane. After incubation with the primary antibodies: Tau-5 (1:2000, Abcam), Tau-4R (1:1000, Millipore), Tau-3R (1:2000, Millipore), Tau (E178) (1:1000, Abcam), Histone H3 (1:10.000, Abcam), SFRS7(9G8) (1:1000, kindly provided by Dr. James Stévine), Actin (1:200, DSHB) overnight at 4ºC, membranes were incubated with secondary antibodies for 1 hour at room temperature (anti-rabbit or anti-mouse, 1:10.000, Bio-Rad, anti-goat, 1:7500, Pierce. Antibody binding was detected by chemiluminescence (Clarity kit, Bio-Rad).

qRT-PCR
1 μg of total RNA purified from differentiated cells or brain tissue samples was reversed transcribed using the One-step SuperScript kit (Bio-Rad). The qRT-PCR reaction was performed in a CFX96 Real-time PCR detection system (Bio-Rad) using the Quantitec SYBR Green kit (Qiagen) and the primers listed on Table S1. Gene expression was normalized to HMBS or B2M levels within each sample. Results are presented as fold change.

Neurostructural analysis
Mice were transcardially perfused with 0.9 % saline (n=9 per group) under deep anesthesia (mixture of ketamine hydrochloride (150 mg/kg) plus medetomidine (0.3 mg/kg)). Briefly, brains were immersed in Golgi-Cox solution for 14 days, processed and cut on a vibratome at 200 μm thick coronal sections. For each selected neuron, all branches of the dendritic tree were reconstructed at x1000 magnification, using a motorized microscope.
(Zeiss, Thornwood) attached to a camera (Sony) and Neurolucida software (Microbrightfield). A three-dimensional analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield).

Quantification and statistical analysis

Continuous variables with normal distributions (Shapiro-Wilk test p>0.05) were analyzed with the Student t-test or two-way ANOVA. Non-parametric Mann-Whitney U-test was used when variables were non-continuous or when a continuous variable did not present a normal distribution (Shapiro-Wilk test p<0.05). All statistical analysis were performed using SPSS 22.0 (SPSS Inc., Chicago, IL). A critical value for significance of p<0.05 was used throughout the study. For real-time quantitative PCR data, the same approach was used and results were presented using the ΔΔCt method, as described before.

AUTHOR CONTRIBUTIONS

Conceptualization, ANC and PM; Methodology, ANC, PM, IS, SH, PH and KWL; Formal analysis, ANC and KWL; Investigation, ANC, BA, SDS, JS, SH and KWL; Writing – original draft, ANC; Writing – review & editing, ANC, PM, IS, PH and KWL; Visualization, ANC; Funding acquisition, PM; Resources, PM, KWL, PH; Supervision, PM

ACKNOWLEDGMENTS

This article has been developed under the scope of the project NORTE-01-0145-FEDER-000013, supported by the Northern Portugal Regional Operational Programme (NORTE 2020), under the Portugal 2020 Partnership
Agreement, through the European Regional Development Fund (FEDER).

This work has been funded by FEDER funds, through the Competitiveness Factors Operational Programme (COMPETE), and by National funds, through the Foundation for Science and Technology (FCT), under the scope of the project POCI-01-0145-FEDER-007038 and through the project POCI-01-0145-FEDER-016818 (PTDC/NEU-NMC/3648/2014) and fellowships [SFRH/BD/51059/2019 to A. N-C, SFRH/BPD/110728/2015 to B.A, SFRH/BD/78388/2011 to S.D-S, SFRH/BD/88932/2012 to J.M.S and IF/01799/2013 to I.S.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES


22. Doss-Pepe, E.W., Stenroos, E.S., Johnson, W.G. & Madura, K. Ataxin-3 interactions with rad23 and valosin-containing protein and its associations with ubiquitin chains and the proteasome are consistent


898

899 64. Nicklas, W., *et al.* Recommendations for the health monitoring of rodent

902 65. Colledge, M., *et al.* Targeting of PKA to glutamate receptors through a

904 66. Shilov, I.V., *et al.* The Paragon Algorithm, a next generation search engine
905 that uses sequence temperature values and feature probabilities to

908 67. Fredriksson, S., *et al.* Protein detection using proximity-dependent DNA

910 68. Glaser, E.M. & Van der Loos, H. Analysis of thick brain sections by
911 obverse-reverse computer microscopy: application of a new, high clarity

913 69. Pfaffl, M.W. A new mathematical model for relative quantification in real-

915

**FIGURE LEGENDS**

**Figure 1.** Experimental design – purification of polyubiquitylated proteins
918 using TUBEs. (A-I) RA-treated SH-SY5Y cells (with silenced ATXN3 or not)
919 were lysed and protein extracts were incubated with TUBEs. (A-II) TUBEs-
920 captured proteins were recovered with Laemmli buffer and analyzed by
comassie blue staining of the polyubiquitylated proteins purified using TUBEs run in a 1-D SDS-PAGE gel or western-blot with anti-ubiquitin FK2 antibody. (A-III) Polyubiquitylated proteins were trypsin-digested and identified by LC-MS/MS. (B) After acquisition, data were processed and integrated in functional networks.

Table 1. RNA metabolism related proteins with altered ubiquitylation in cells lacking ATXN3.

Figure 2. Efficacy of RNA processing assessed by splicing reporter minigenes in ATXN3shRNA cells. (A-E) Semi-quantitative analysis of minigene’ alternative splicing showed a decreased efficiency of splicing in (A-D) ATXN3shRNA cells and (E) ATXN3_C14A cells. Schemes for the splicing products are indicated on the left right. Exons are represented as colored boxes and introns by red lines. The AdML minigene contains one intron, giving rise to two bands: the upper band corresponds to the unspliced transcript, the lower band to the spliced product. The pyPY minigene contains two alternative splice sites originating three bands: an upper band corresponding to the unspliced transcript, a middle band corresponding to the splicing of the weak py tract and a lower band corresponding to the splicing product of the strong PY tract. The pyPY minigene contains two alternative 3’ splice sites associated with polypirimidine (Py) tracts with different strengths. The weak Py tract (py) is represented by thin red lines and the strong Py tract (PY) by a thick red line. The α-globin minigene contains two introns and a set of G triplets in intron 2 that promote the recognition of the 5’ splice site leading
to skipping of exon 2. (F) Experimental design – microarray analysis of alternative splicing using the Affymetrix Human Transcriptome Array 2.0 ST to assess perturbation of global splicing patterns in ATXN3<sup>shRNA</sup> cells. (G) Distribution of the differentially regulated alternative splicing events in ATXN3<sup>shRNA</sup> cells. n≥3 independent biological replicates in all experiments. p<0.05 was taken as the cut-off. T-test was used for comparisons. Asterisks represent mean ± standard deviation. *p<0.05; **p<0.01; ***p<0.001.

**Figure 3.** ATXN3<sup>shRNA</sup> cells showed decreased SRSF7 protein levels. (A-B) Western-blot analysis after capture of polyubiquitylated proteins confirmed the decrease in polyubiquitylated forms of SRSF7 in ATXN3<sup>shRNA</sup> cells. (A-D) Downregulation of both (A-B) total and (C-D) nuclear SRSF7 protein levels in ATXN3<sup>shRNA</sup> cells. (E-F) Protein levels of SRSF7 are increased in ATXN3<sup>shRNA</sup> cells upon proteasome inhibition (MG132 treatment). (G) Western-blot analysis after capture of polyubiquitylated proteins with K48-linked polyubiquitin chains showed decrease levels in K48-polyubiquitylated forms of SRSF7 in ATXN3<sup>shRNA</sup> cells. Relative band density for each protein was analyzed. The results were normalized for Histone H3. (H) Relative amounts of SRSF7 in SCR<sup>shRNA</sup> cells and ATXN3<sup>shRNA</sup> cells at various cycloheximide treatment times. Human ATXN3 co-interacts both (I) in vitro and (J) in situ with SRSF7. Scale bar corresponds to 40 μm. n≥3 independent biological replicates in all experiments. T-test or ANOVA were used for comparisons. Asterisks represent mean ± standard deviation. *p<0.05; **p<0.01, ***p<0.01.
Figure 4. Disruption of 4R/3R-Tau ratio in ATXN3\textsuperscript{shRNA} cells. (A) qRT-PCR analysis showed that silencing of ATXN3 lead to decreased transcription levels of 4R-Tau, (B) no alterations in the mRNA levels of the 3R-Tau isoform, (C) a significant decrease of the 4R/3R-Tau ratio and (D) decreased levels of total Tau, both in ATXN3\textsuperscript{shRNA} cells and in (E-H) cerebellar primary neurons. (I) ATXN3\textsubscript{C14A} cells present decreased mRNA levels of 4R-Tau isoform, (J) no alterations in 3R-Tau expression, (K) decreased 4R/3R-Tau ratio and (L) decreased levels of total Tau. (M) SH-SY5Y ATXN3\textsuperscript{shRNA} cultures presented a decreased expression of βIII-tubulin as assessed by immunostaining. (N) The average length of the neuritis was reduced in ATXN3\textsuperscript{shRNA} cells as compared with the SCR\textsuperscript{shRNA} controls. 4R/3R-Tau ratio was obtained by dividing 4R and 3R-Tau mRNA levels. n≥3 independent biological replicates in all experiments. T-test was used for comparisons. Asterisks represent mean ± standard deviation. ***p<0.001.

Figure 5. PolyQ expansion in ATXN3 affects Tau splicing. (A) Semi-quantitative analysis of minigene' alternative splicing showed a decreased efficiency of splicing in ATXN3_83Q cells. (B) Expression of ATXN3_83Q led to decreased mRNA levels of 4R-Tau isoform, (C) no alterations in 3R-Tau expression, (D) decreased 4R/3R-Tau ratio, and (E) decreased levels of total Tau. (F) Tg mice presented a decrease in the mRNA levels of 4R-Tau, (G) no alterations in expression of 3R-Tau, (H) a decreased 4R/3R-Tau ratio, and (I) a decrease in the levels of total Tau comparing with Wt controls. (J-L) Tg mice showed decreased Sfrs7 protein levels in different disease-affected brain areas. (M) Decreased SRSF7 protein levels in SCA3 patient’s brains.
Decreased total dendritic length and dendritic tree complexity of brainstem neurons in the Tg mice.

mRNA levels were normalized to the HMBS or Hprt genes. Relative band density for each protein was analyzed. The results were normalized for actin levels. n≥3 independent biological replicates in all experiments. T-test or ANOVA were used for comparisons. Asterisks represent mean ± standard deviation. *p<0.05; **p<0.01; ***p<0.001.

Table S1. Differences in polyubiquitylated proteins identified by TUBEs-MS in SH-SY5Y cells lacking ATXN3. List of identified proteins with altered levels of ubiquitylation in RA-treated ATXN3^{shRNA} cells as compared with the SCR^{shRNA} controls (p<0.05). These proteins were present in at least 3 independent experiments. The values are the average of “unused” values given by the Proteinpilot algorithm. The absent “unused” values indicate the (near) complete absence of the polyubiquitylated protein. In red and green are proteins with increased and decreased polyubiquitylated levels, respectively.

$ indicates protein splicing factors.

Figure EV1. No changes in the mRNA levels of SRSF7 in ATXN3^{shRNA} cells and in the CMVMJD135 mice. (A-B) Percentage of ATXN3 silencing using different shRNA clones as compared to the scrambled sequence and empty vector expression. The different generated cell lines showed a similar phenotype. (C) No significant differences were observed in the mRNA levels of SRSF7 between the ATXN3^{shRNA} cells and the SCR^{shRNA} controls. (D) Knockdown of Atxn3 in cerebellar primary neurons. (E) PLA negative control.
(no antibodies) (F) No significant differences were observed in the mRNA levels of Srsf7 in the Tg mice as compared with the Wt-littermates controls. mRNA levels were normalized to the HMBS or Hprt genes. Relative band density for each protein was analyzed. The results were normalized for actin levels. n≥3 independent biological replicates in all experiments. T-test was used for comparisons. Asterisks represent mean ± standard deviation. *p<0.05.

Table EV1. Differentially regulated alternative splicing events in ATXN3<sup>shRNA</sup> cells. List of genes with altered splicing in RA-treated ATXN3<sup>shRNA</sup> cells as compared with the SCR<sup>shRNA</sup> controls.

Table EV2. KEGG pathway analysis of genes with altered splicing in ATXN3<sup>shRNA</sup> cells. The genes identified on the KEGG pathway analysis presented at least one differentially regulated exon/splicing pattern in ATXN3<sup>shRNA</sup> cells.

Table EV3. Changes in alternative types events in ATXN3<sup>shRNA</sup> cells.
Figure 1

A

I. DIFFERENTIATION AND LYSIS

II. PULLDOWN (PolyUb proteins)

III. LC-MS/MS & DATA ANALYSIS

3-6 Biological replicates

Trypsin digestion

LC_MS/MS

Data acquisition

ProteinPlot™ search engine

Data integration and Analysis of the ubiquitome

B

FUNCTIONAL NETWORKS

Cellular development, Cell death and survival, Cell cycle 7%

Gene expression, DNA replication, recombination and repair 18%

Cellular growth and proliferation, Gene expression, Cell cycle 8%

Cellular compromise, Cell death and survival, Neurological disease 8%

Cell cycle, Cell death and survival 8%

DNA replication, recombination and repair, Cell death and survival 8%

Organ morphology 9%

RNA post-transcriptional modification 14%

Molecular transport, RNA trafficking 11%

Cell death and survival 9%
### Table 1

**RNA Metabolism Proteins with Altered Ubiquitylation in ATXN3<sup>−−</sup>RNA Cells**

<table>
<thead>
<tr>
<th>Protein</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upregulated</strong></td>
<td></td>
</tr>
<tr>
<td>RBM10 (RNA-binding protein 10)</td>
<td>4.87E-19</td>
</tr>
<tr>
<td>SNRPB2 (U2 small nuclear ribonucleoprotein B)</td>
<td>3.26E-11</td>
</tr>
<tr>
<td>SFRS2 (Splicing factor arginine/serine-rich 2)</td>
<td>5.36E-11</td>
</tr>
<tr>
<td>RBM8A (RNA-binding protein 8A)</td>
<td>1.56E-06</td>
</tr>
<tr>
<td>PRPF8 (Pre-mRNA-processing-splicing factor 8)</td>
<td>1.00E-03</td>
</tr>
<tr>
<td>SFRS5 (Serine/arginine-rich splicing factor 5)</td>
<td>1.00E-03</td>
</tr>
<tr>
<td>SNRPN (Small nuclear ribonucleoprotein-associated protein N)</td>
<td>5.00E-03</td>
</tr>
<tr>
<td>SRSF9 (Serine/arginine-rich splicing factor 9)</td>
<td>7.00E-03</td>
</tr>
<tr>
<td>RBM12B (RNA-binding protein 12B)</td>
<td>2.00E-02</td>
</tr>
<tr>
<td>AQR (Intron-binding protein aquarius)</td>
<td>2.00E-02</td>
</tr>
<tr>
<td>PRPF40A (Pre-mRNA-processing factor 40 homolog A)</td>
<td>2.00E-02</td>
</tr>
<tr>
<td>HNRPK (Heterogeneous nuclear ribonucleoprotein K)</td>
<td>3.00E-02</td>
</tr>
<tr>
<td><strong>Downregulated</strong></td>
<td></td>
</tr>
<tr>
<td>DDX41 (DEAD-box protein abstrakt variant)</td>
<td>6.22E-06</td>
</tr>
<tr>
<td>DDX56 (ATP-dependent RNA helicase)</td>
<td>2.38E-05</td>
</tr>
<tr>
<td>LUC7L (Putative RNA-binding protein Luc-like 1)</td>
<td>5.00E-04</td>
</tr>
<tr>
<td>SFRS7 (Serine/arginine-rich splicing factor)</td>
<td>8.00E-04</td>
</tr>
<tr>
<td>DDX10 (Probable ATP-dependent RNA helicase)</td>
<td>1.00E-02</td>
</tr>
<tr>
<td>HNRPAL1P10 (Heterogeneous nuclear ribonucleoprotein N)</td>
<td>2.00E-02</td>
</tr>
<tr>
<td>FUS (RNA-binding protein)</td>
<td>3.00E-02</td>
</tr>
<tr>
<td>RMX (Heterogeneous nuclear ribonucleoprotein G)</td>
<td>4.00E-02</td>
</tr>
<tr>
<td>SRRT (Serrate RNA effector molecule)</td>
<td>4.00E-02</td>
</tr>
</tbody>
</table>
Figure 2

A

B

C

D

E

F

G

Retinoic acid (RA) 0.1 µM

3 biological replicates

Data analysis

7450 differentially expressed genes (fold change ≥ 1.5 and p-value ≤ 0.5)

1993 genes with differentially regulated exon usage events

ATXN3
shRNA

SCR
shRNA

py/PY ratio

AdML

α-globin

DN ATXN3

py/PY ratio

SCR

shRNA

ATXN3

shRNA

Types of splicing affected

- Complex: 20%
- Altered terminal exon: 16%
- Intron retention: 15%
- Altered donor splice site: 3%
- Altered acceptor splice site: 3%
- Mutually exclusive exons: 1%
- Exon cassette: 42%
Figure 3

A

B

C

D

E

F

G

H

I

J

**Figure 3**

(A) UbSRSF7

(B) Relative OD (normalized to H3)

(C) Nuclear SRSF7

(D) Relative OD (normalized to H3)

(E) SRSF7

(F) Relative OD (normalized to H3)

(G) K48-Ub

(H) Half-life of SRSF7 protein

(I) IP: ATXN3

(J) DAPI

**Figure 3**

(A) UbSRSF7

(B) Relative OD (normalized to H3)

(C) Nuclear SRSF7

(D) Relative OD (normalized to H3)

(E) SRSF7

(F) Relative OD (normalized to H3)

(G) K48-Ub

(H) Half-life of SRSF7 protein

(I) IP: ATXN3

(J) DAPI
Figure 4

A) 4R-Tau
B) 3R-Tau
C) 4R/3R-Tau
D) Total Tau

SCR
ATXN3

Cerebellar neurons

E) 4R-Tau
F) 3R-Tau
G) 4R/3R-Tau
H) Total Tau

Cerebellar neurons

I) 4R-Tau
J) 3R-Tau
K) 4R/3R-Tau
L) Total Tau

Empty vector
ATXN3_C14A

SH-SY5Y cells

M) DAPI
βIII-tubulin

ATXN3
SCR

SH-SY5Y cells

N) Neurite length (μm)

SCR
ATXN3

not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission. doi: bioRxiv preprint
Figure 5

**A** Expanded ATXN3 py/PY ratio

**B** 4R-Tau

**C** 3R-Tau

**D** 4R/3R-Tau

**E** Total Tau

**F** 4R-Tau

**G** 3R-Tau

**H** 4R/3R-Tau

**I** Total Tau

**J** Spinal Cord

**K** Brainstem

**L** Cerebellum

**M** Human Pons

**N** Mouse Brain

**O** SH-SY5Y cells

**P** Number of Interceptions

---

*Statistical significance:

- **Wt** (Wild Type)
- **Tg** (Transgenic)
- ***p < 0.001
- **p < 0.01
- **p < 0.05

---

Source: Not certified by peer review. All rights reserved. No reuse allowed without permission.

[Link to DOI: https://doi.org/10.1101/711424]
Figure S1

A

ATXN3 shRNA
#1 #2 #3 Scrambled Empty vector

B

ATXN3 silencing

C

SRSF7 mRNA

D

Cerebellar Primary neurons

E

PLA negative control

F

Mouse Srsf7 mRNA

not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.