The Debated Toxic Role of Aggregated TDP-43 in Amyotrophic Lateral Sclerosis: A Resolution in Sight?

Rudolf C. Hergesheimer[1]*, Anna A. Chami[1]*, Denis Reis de Assis[1], Patrick Vourc'h[1,2], Christian R. Andres[1,2], Philippe Corcia[1,3], Débora Lanznaster[1#], Hélène Blasco[1, 2]

1 UMR 1253, iBRAIN, Université de Tours, INSERM, Tours, France.

2 CHU de Tours, Service de Biochimie et Biologie Moléculaire, Tours, France.

3 CHU de Tours, Service de Neurologie, Tours, France.

* These authors contributed equally to the manuscript

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[#]Correspondance to: Dr. Débora Lanznaster, INSERM UMR1253, Team 2, University of Tours, 10 Bd Tonnellé, 37044 - Tours, France; Tel: + 33 2 47 36 74 45. Email: debora.lanznaster@univ-tours.fr

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Abstract

Transactive Response DNA-Binding Protein-43 (TDP-43) is an RNA/DNA binding protein that forms phosphorylated and ubiquitinated aggregates in the cytoplasm of motor neurons in Amyotrophic Lateral Sclerosis (ALS), which is a hallmark of this disease. ALS is a neurodegenerative condition affecting the upper and lower motor neurons. Even though the aggregative property of TDP-43 is considered a cornerstone of ALS, there has been major controversy regarding the functional link between TDP-43 aggregates and cell death. In this review, we attempt to reconcile the current literature surrounding this debate by discussing the results and limitations of the published data relating TDP-43 aggregates to cytotoxicity, as well as therapeutic perspectives of TDP-43 aggregate clearance. We point out key data suggesting that the formation of TDP-43 aggregates and the capacity to self-template and propagate among cells as a "prion-like" protein, another pathological property of TDP-43 aggregates, are a significant cause of motor neuronal death. We discuss the disparities among the various studies, particularly with respect to the type of models and the different forms of TDP-43 utilized to evaluate cellular toxicity. We also examine how these disparities can interfere with the interpretation of the results pertaining to a direct toxic effect of TDP-43 aggregates. Furthermore, we present perspectives for improving models in order to better uncover the toxic role of aggregated TDP-43. Finally, we review the recent studies on the enhancement of the cellular clearance mechanisms of autophagy, the ubiquitin proteasome system, and endocytosis in an attempt to counteract TDP-43 aggregation-induced toxicity. Altogether, the data available so far encourage us to suggest that the cytoplasmic aggregation of TDP-43 is key for the neurodegeneration observed in motor neurons in ALS patients. The corresponding findings provide novel avenues toward early therapeutic interventions and clinical outcomes for ALS management.

Keywords: ALS, TDP-43, aggregation, neurodegeneration, therapeutics

Abbreviations: ALS = Amyotrophic Lateral Sclerosis; CMA = chaperone-mediated autophagy; CTF = C-terminal fragment; ELP = endosomal-lysosomal pathway; fALS = familial ALS; FTLD = Frontotemporal Lobar Dementia; GFP = green fluorescent protein; GOF = gain of function; hnRNP = heterogeneous nuclear ribonucleoprotein; IB = immunoblot; IF = immunofluorescence; iPSC = induced pluripotent stem cell; LLPS = liquid-liquid phase separation; LOF = loss of function; NES = nuclear export sequence; NLS = nuclear localization sequence; PABPN1 = poly-A binding protein 1; PrP = prion protein; PTM = post-translational

modification; RRM = RNA recognition motif; sALS = sporadic ALS; SUMO = small ubiquitin-related modifier; TDP-43 = Transactive Response DNA-Binding Protein 43; UPS = ubiquitin proteasome system; wtTDP-43 = wild-type TDP-43

1. Introduction

Transactive Response DNA Binding Protein 43 (TDP-43, 43 kDa) is a ubiquitous protein encoded by the *TARDBP* gene that is highly conserved throughout different species (e.g., *C. elegans, Drosophila melanogaster,* mammals, etc.). TDP-43 is essential for the development of the central nervous system (CNS) from the earliest stages of embryonic life to adulthood (Huang *et al.*, 2010; Sephton *et al.*, 2010) and preferentially binds RNA UG motifs (Tollervey *et al.*, 2011; Xiao *et al.*, 2011). TDP-43 belongs to the heterogeneous nuclear ribonucleoprotein (hnRNP) families and is implicated in multiple steps of transcriptional and post-transcriptional regulation (Nakielny and Dreyfuss, 1997; Krecic and Swanson, 1999; Dreyfuss *et al.*, 2002; Prasanth *et al.*, 2005; Martinez-Contreras *et al.*, 2007; He and Smith, 2009; Busch and Hertel, 2012). Under physiological conditions, the majority of TDP-43 is nuclear, while a small proportion is continuously shuttled between the nucleus and cytoplasm. In the nucleus, the functions of TDP-43 include the repression of gene expression, pre-mRNA splicing, and autoregulation of its own mRNA (Ayala *et al.*, 2011a; Ayala *et al.*, 2011b). TDP-43 also regulates miRNA biogenesis through its interaction with the Drosha-containing protein complex (Kawahara and Mieda-Sato, 2012). In the cytoplasm under stress conditions, TDP-43 controls mRNA stability, translation, and nucleocytoplasmic transport by forming cytoplasmic ribonucleoprotein complexes, termed stress granules (Zhao *et al.*, 2018).

An ever-increasing number of research groups report the presence of TDP-43-enriched cytoplasmic aggregates in diverse neuropathological conditions. Since the 1990s, it has been shown that the presence of ubiquitin-positive and tau-negative cytoplasmic aggregates is a common pathological feature in the motor neurons of patients suffering from Amyotrophic Lateral Sclerosis (ALS) and in the frontal and temporal lobes in patients with Frontotemporal Lobar Degeneration (FTLD) (Okamoto *et al.*, 1990; Kwong *et al.*, 2007). Pathological TDP-43 has been found to be depleted from the nucleus and sequestered in insoluble, cytoplasmic aggregates in post-mortem neural tissue, suggesting that the nucleo-cytoplasmic relocation is involved in pathogenic aggregation (Arai *et al.*, 2006; Neumann *et al.*, 2006; Winton *et al.*, 2008). Full-length and fragmented TDP-43 are the major components of these aggregates in the brains and motor

neurons of ALS patients. Currently, TDP-43 aggregates are considered the histopathological hallmark of ALS.

However, it has remained very debatable as to whether this aggregation, indeed, causes the motor neuronal degeneration. In this review, we explore the main findings that put the spotlight on the cytotoxicity of TDP-43 aggregation. Moreover, we discuss the structural properties of TDP-43 that underlie its propensity to aggregate and suggest novel therapeutic interventions that could decrease TDP-43 aggregation and mitigate the debilitating neurodegeneration of ALS.

2. Aggregated TDP-43 is a Hallmark for ALS - Patient Case Studies

Aggregates of wild-type TDP-43 (wtTDP-43) are present in both sporadic (sALS) and familial (fALS) cases of ALS (Duan *et al.*, 2010). ALS is characterized by the progressive loss of the upper motor neurons in the brain and of the lower motor neurons in the brainstem and spinal cord (Robberecht and Philips, 2013). The disease presents a very poor prognosis, and patients usually die within two to five years after the onset of symptoms, primarily due to respiratory failure. An estimated 30 genes have now been identified to be involved in ALS (Chia *et al.*, 2018). Importantly, around 60% (19) of these genes are described as also involving TDP-43 aggregation in postmortem analyses of patient samples, cell culture, and animal models of ALS (**Table 1)** and are extensively reviewed elsewhere (Scotter *et al.*, 2015; Chia *et al.*, 2018; Takeda, 2018). Of equal importance, TDP-43 aggregation was demonstrated not to occur for only four of these genes, while there is no study to date indicating either the absence or the presence of these aggregates for the other seven genes. Mutated TDP-43 accounts for 3% of familial and 1.5% of sporadic cases of ALS (Lagier-Tourenne and Cleveland, 2009), meaning that around 95% of patients presenting TDP-43-positive aggregates do not carry any mutation in this pathogenic protein (Smethurst *et al.*, 2015). These aggregates consist of aberrantly phosphorylated and ubiquitinated full-length TDP-43, as well as 35 and 25-kDa C-terminal fragments of the protein (Arai *et al.*, 2006; Neumann *et al.*, 2006; Mackenzie *et al.*, 2007).

It has been suggested that the presence of ubiquitinated, phosphorylated, aggregated TDP-43 observed in degenerated neurons could trigger almost all of the pathogenic alterations observed in ALS patients. In fact, studies suggest that the severity of motor neuron degeneration may be proportional to aggregated TDP-43 levels (Brettschneider *et al.*, 2013; Brettschneider *et al.*, 2014). Stages of sequential phospho-TDP-43 spreading have been described in certain ALS cases,

and this spreading pattern follows axonal projections throughout different regions in the CNS (Brettschneider *et al.*, 2013). Similarly, clinical data has pushed neurologists to propose that motor neuron degeneration in ALS begins at a focal point and subsequently spreads throughout the CNS, correlating with disease progression and the severity of motor symptoms (Ravits *et al.*, 2007; Ravits and La Spada, 2009).

3. TDP-43 Structure

TDP-43 belongs to the family of hnRNPs that play important roles in RNA regulation. While the complete 3D structure of TDP-43 remains unresolved, the separate domains of this 414 amino acid-long protein have been structurally characterized. These include the N-terminal domain, two RNA recognition motifs (RRM1 and RRM2), and the C-terminal domain (**Fig. 1**) (Sun and Chakrabartty, 2017). Additionally, the protein harbors a nuclear localization sequence (NLS) and a nuclear export sequence (NES) (Blokhuis *et al.*, 2013). The N-terminal domain (residues 1-78) contains a ubiquitin-like fold with six β-sheets and one α-helix (Mompean *et al.*, 2016). It is involved in regulating the TDP-43 self-interaction. Recently, multiple studies have suggested that functional TDP-43 is most likely a homodimer and that the first ten residues of the N-terminus seem to mediate this homodimerization (Wang *et al.*, 2013b; Zhang *et al.*, 2013; Sun *et al.*, 2014).

The RRMs (RRM1: residues 106-176; RRM2: residues 191-262) regulate the interactions with RNA (Buratti and Baralle, 2001) and single-stranded DNA, presenting high affinity for UG-rich and TG-rich sequences, respectively (Kuo *et al.*, 2009; Lukavsky *et al.*, 2013). Due to its affinity for UG-rich motifs, TDP-43 plays a significant role in the regulation of RNA, including mRNA splicing and transport (Buratti and Baralle, 2001). We recently identified the first ALS-related mutation in the RRM2 domain at residue 259 (N259S). Our structural analyses revealed a close proximity of residue 259 to a uracil base in UG-rich RNA motifs, suggesting not only an important role of RRM2 in RNA regulation but also a role in ALS pathogenesis (Maurel *et al.*, 2018b).

The C-terminal domain (residues 277-414) controls the protein-protein interactions and the solubility of TDP-43 (Ayala *et al.*, 2008). This domain is low in complexity and is particularly rich in glycine, glutamine (Q), asparagine (N), and polar residues, while poor in aliphatic and charged residues (**Fig. 1**). The C-terminus is a dynamic and flexible region that is capable of adopting transient secondary structures, ranging from α-helices to β-sheets. As a result, the C-

terminal sequence resembles that of prion proteins. Prions are aggregation-prone conformers of certain proteins, namely prion protein (PrP), primarily consisting of β-sheets. These proteins are capable of self-templating and transmitting themselves among cells and organisms, usually causing neurodegenerative diseases, such as Creutzfeldt-Jakob disease (Collinge and Clarke, 2007; King *et al.*, 2012). Hence, the prion-like properties of the C-terminus of TDP-43 could provide the protein with pathogenic potential.

The C-terminus also attributes TDP-43 with a liquid-liquid phase separation property (LLPS), which involves its oligomerization into functional stress granules. These are transient complexes, or "membrane-less organelles", that contain mRNA and other RNA-binding proteins. Forming these granules allows TDP-43 to halt the translation of its mRNA targets and to protect them from degradation during cellular stress (Sun and Chakrabartty, 2017; Li *et al.*, 2018a; Li *et al.*, 2018b). Interestingly, a recent study showed that the physiological oligomerization is regulated by the N-terminus that helps maintain the physical separation between the C-terminal domains of each monomer (Afroz *et al.*, 2017). It has been found that certain ALS-related TDP-43 mutants demonstrate altered LLPS properties and stress granule dynamics in comparison to the wild-type form, which could promote pathological TDP-43 aggregation (Conicella *et al.*, 2016; Li *et al.*, 2018a; Li *et al.*, 2018b). In fact, approximately 60 mutations in the different TDP-43 domains have already been described (**Supplementary Table 1**), and almost all the ALS-related mutations occur on the C-terminal domain. Therefore, it is this domain that is the most suspect in TDP-43 aggregation.

4. Intrinsic Characteristics of Pathological TDP-43 and Factors Influencing its Propensity to Aggregate

Given the intrinsic characteristics of the structure of TDP-43, researchers have wondered how this ultimately leads to the accumulation of the pathogenic, aggregated form in ALS patients. Here, we summarize and attempt to reconcile the current disputed literature surrounding the involvement of the aggregation of pathogenic TDP-43 in neurodegeneration. Factors that influence its aggregation include its post-translational modifications, cytoplasmic accumulation, CTF, mutant, and wild-type forms.

4.1 Post-Translational Modifications (PTMs)

TDP-43 is a target for several PTMs, which can change its structure, localization, overall functions, and its aggregative propensity (Kametani *et al.*, 2016; Buratti, 2018). As previously mentioned, TDP-43-positive aggregates found in ALS brains are well recognized to be ubiquitinated and phosphorylated (Neumann *et al.*, 2006).

Analyses of aggregated TDP-43 from ALS patients reveal ubiquitination of the Lys79 residue (Kametani *et al.*, 2016). Several different lysine residues have also been described as potential ubiquitination sites (Seyfried *et al.*, 2010; Wagner *et al.*, 2011; Dammer *et al.*, 2012). Ubiquitin is a small signal protein that is used by the ubiquitin-proteasome system (UPS) to designate proteins for degradation. However, the UPS regulatory factor ubiquilin-2, when overexpressed or mutated, has been seen to promote TDP-43 mislocalization, aggregation, and neurodegeneration *in vitro* and *in vivo* (Kim *et al.*, 2009; Hanson *et al.*, 2010; Ceballos-Diaz *et al.*, 2015; Picher-Martel *et al.*, 2015; Osaka *et al.*, 2016). Therefore, ubiquitinated TDP-43 aggregates in ALS brain could signify a species that is not correctly degraded by the UPS. As a result, TDP-43 becomes prone to aggregation and nuclear depletion. Even though, these studies rather demonstrate a toxic role for aberrant ubiquilin-2, the changes that occur for TDP-43 should have their own toxic outcomes; the depletion from the nucleus would prevent TDP-43 from carrying out its critical nuclear functions, and its cytoplasmic aggregation would also inhibit cytoplasmic function and provoke abnormal interactions.

As for phosphorylation, Ser409 and Ser410 are well-documented targets, as seen in ALS patients (Hasegawa *et al.*, 2008; Neumann *et al.*, 2009). Other phosphorylated residues of pathological TDP-43 have also been described, including Ser379, 403, 404, 409, and 410 (Hasegawa *et al.*, 2008; Inukai *et al.*, 2008; Gu *et al.*, 2018). In fact, *in vitro* results suggest that all serine and threonine residues in the C-terminal domain are phosphorylatable (Kametani *et al.*, 2009). Phosphorylation may either increase the propensity of TDP-43 to aggregate or to be hydrolyzed into C-terminal fragments (Goh *et al.*, 2018). For example, by the action of a hyperactive, C-terminally truncated form of the kinase CK1ε, the tendency of TDP-43 to aggregate increased, paralleled by a decreased cell viability *in vitro* (Nonaka *et al.*, 2016). Yet, studies such as this cannot unequivocally attribute the toxicity to TDP-43 aggregation, since the decreased viability could be due to other actions of the aberrant CK1ε.

On the other hand, aberrant TDP-43 phosphorylation could represent a cellular defense mechanism. For instance, expression of a TDP-43 construct bearing hyperphosphorylation-mimetic mutations in Neuro2A cells restored neurite extension and cell viability to control levels, followed by a decrease in the number of cells with cytoplasmic aggregates (Li *et al.*, 2011). Given

these data and the numerous studies also showing that neurite extension is inhibited by pathogenic TDP-43 (Wachter *et al.*, 2015; Tian *et al.*, 2017; Baskaran *et al.*, 2018), the phosphorylation of TDP-43 could reflect a pathological role of its aggregation in decreased neurite integrity.

One example of a less studied PTM of TDP-43 is cysteine oxidation. It is well known that oxidative stress causes physiological TDP-43 to re-localize to the cytoplasm and coalesce to form stress granules (Ayala *et al.*, 2011a; Cohen *et al.*, 2012; Feiler *et al.*, 2015; Liu *et al.*, 2015). However, this process can promote aggregation if prolonged. This is probably due to aberrant disulfide bridges formed by Cys residues 173, 175, 198, and 244, turning the physiological complexes into cytotoxic aggregates. Other studies have shown that N-terminal Cys39 and Cys50 form disulfide bridges that reinforce the dimerization of the N-terminal domain *in vitro*, which is thought to prevent the protein from aggregating (Jiang *et al.*, 2017). Mutating these residues to serine residues appears to strongly reduce TDP-43 oligomerization in response to oxidation in motor neuron-like cells (Bozzo *et al.*, 2016). Moreover, splicing activity was diminished in the same type of mutant (Jiang *et al.*, 2017). These results indicate that the tertiary structure of TDP-43 is particularly sensitive to the oxidation state of Cys39 and Cys50. Therefore, their oxidation seems to promote the initial oligomerization steps of the aggregating process. In turn, the fundamental splicing function of TDP-43 could be diminished, reflecting a loss of function, which would be detrimental to the cell.

Another less commonly studied PTM is acetylation that can occur on Lys145 and Lys192. It has been shown that TDP-43 acetylation in the region K145-149 within the RRM1 domain is associated with a loss in RNA-binding ability and with increased TDP-43 aggregation in the spinal cord of ALS patients (Cohen *et al.*, 2015). Similar to cysteine oxidation, acetylation appears to promote aggregation and diminish TDP-43 functionality.

Lastly, SUMOylation by small ubiquitin-related modifier (SUMO), a ubiquitin-like protein, is a covalent and reversible PTM. SUMOylation of TDP-43 aggregates was elucidated following CTF overexpression in mouse primary neurons (Seyfried *et al.*, 2010). Although the direct SUMOylation of TDP-43 has not yet been demonstrated, we have described a putative site for TDP-43 SUMOylation at K136 (Dangoumau *et al.*, 2013). However, the role of TDP-43 SUMOylation in its propensity to aggregate or in its toxicity has not yet been explored.

4.2 TDP-43-ΔNLS: Investigating Cytoplasmic Mislocalization and Accumulation

As mentioned in the introduction, the pathogenic TDP-43 aggregates detected in ALS patients' motor neurons are usually located in the cytoplasm, partly reflecting a defect in TDP-43 nucleo-cytoplasmic trafficking (Neumann *et al.*, 2006; Winton *et al.*, 2008). To specifically analyze the effects of cytoplasmic accumulation, it is common to genetically alter the nuclear localization sequence (NLS) of TDP-43, which restricts the exogenous protein to the cytoplasm (Winton *et al.*, 2008; Urushitani *et al.*, 2010). Many of the studies using this overexpressed construct *in vitro* and *in vivo* report an overall low number of aggregate-positive cells, compared to the high cytotoxic toll (**Table 2**). For instance, one study demonstrated progressive motor dysfunction caused by cortical atrophy and neuromuscular denervation in mice overexpressing TDP-43-ΔNLS in the brain and spinal cord, while only a small population of motor neurons displayed TDP-43 aggregation (Walker *et al.*, 2015a). Furthermore, another group found that the accumulated TDP-43-ΔNLS rarely aggregated in the cytoplasm of murine primary neurons but was cytotoxic to most cells, partly by increasing the activation of caspase-3, a protease involved in apoptosis (Sasaguri *et al.*, 2016). As a result, these authors argue that TDP-43 aggregation is not essential to the cytotoxicity of the pathological protein that is accumulated in the cytoplasm (Barmada *et al.*, 2010; Igaz *et al.*, 2011; Walker *et al.*, 2015a; Sasaguri *et al.*, 2016)

Nonetheless, other studies still suggest a certain level of cytotoxicity attributed to aggregated TDP-43. For example, Winton and others (2008) revealed in QBI-293 cells overexpressing TDP-43-ΔNLS the sequestration of endogenous TDP-43 from the nucleus to the cytoplasm. In addition, the presence of ubiquitinated, insoluble, endogenous TDP-43 intensified with time, as well as the apparition of 25-kDa CTFs. In agreement with this, Zhang and others (2013) demonstrated that overexpressed TDP-43-ΔNLS aggregated in primary neurons and sequestered co-overexpressed wtTDP-43 from the nucleus. These effects were paralleled by decreased neurite outgrowth. Remarkably, inhibiting the ability to aggregate by deleting residues 1 - 10 of TDP-43-ΔNLS abolished the sequestration/mislocalization of wtTDP-43, and neurite outgrowth was almost completely unaltered.

Taken together, these results regarding TDP-43-ΔNLS overexpression show that the cytoplasmic accumulation of TDP-43, whether diffuse or aggregated, is highly toxic to cells and can provoke ALS motor phenotypes in mice. Even though several publications claim that the aggregation is not crucial to TDP-43 cytotoxicity, this argument is biased due to the overexpressed, therefore necessarily accumulated, cytoplasmic construct. This is not reflective of ALS pathogenesis. However, the studies that do analyze the effect of the cytoplasmic aggregation of TDP-43-ΔNLS show the increased nuclear depletion, insolubility, and co-aggregation of wtTDP-43 over time, which is indeed part of the TDP-43 pathology found in ALS patients.

Therefore, the cytoplasmic aggregation of TDP-43 seems to become cytotoxic by preventing either nascent TDP-43 from entering the nucleus or shuttled TDP-43 from returning to the nucleus to perform its normal functions.

4.3 C-Terminal Fragments (CTFs) of TDP-43

Apart from full-length TDP-43, abnormal 35- and 25-kDa CTFs of TDP-43 are also found in the aggregates of ALS patients (Neumann *et al.*, 2006). Overexpressed 25 kDa CTF is able to drive the most cytoplasmic aggregation out of all the forms of TDP-43. Foci of fluorescently tagged CTFs form in at least 50% of several transfected mammalian cell types (**Table 2**). Likewise, the CTFs decrease cell viability in roughly twice the number of cells overexpressing wtTDP-43, suggesting a highly toxic character for the CTFs (Zhang *et al.*, 2009; Fallini *et al.*, 2012; Chou *et al.*, 2015; Chang *et al.*, 2016). Moreover, the majority of the overexpressed CTF tends to be recovered in the detergent-insoluble fraction of lysates (Yamashita *et al.*, 2014*;* Chang *et al.*, 2016). In fact, one study demonstrated that fusing an NLS to overexpressed CTF in Neuro2A cells, thereby forcing it into the nucleus, dramatically decreased the rate of cell death while still revealing aggregated CTF species by immunoblot (Kitamura *et al.*, 2016). This suggests that specifically the CTF aggregation in the cytoplasm is toxic to the cell. Altogether, these data show that a high proportion of transfected cells display aggregated, detergent-insoluble CTFs that is always accompanied by a decrease in viability.

Also, it is important to recognize that there exists a variety of "25-kDa" C-terminal fragments, whose lengths are shown in **Table 2**. Remarkably, the different fragments appear to possess different aggregative and toxic properties. For instance, $CTF^{162-414}$ contains all of RRM2, the C-terminal end of RRM1, and has a theoretical molecular weight of 27 kDa. This fragment generated many cells with foci, while GFP-wtTDP-43 virtually showed no foci in SH-SY5Y cells. Yet, the cytotoxicity of GFP-CTF was unexpectedly lower than that of GFP-wtTDP-43 (Yamashita *et al.*, 2014). $CTF^{220-414}$ has a theoretical mass of 20 kDa and includes the last 40 residues of RRM2. This fragment generated aggregates in only 11.2% of transfected Neuro2A cells and demonstrated 15% cellular mortality of similar proportion (Kitamura *et al.*, 2016).

Given the variable levels of cytotoxicity and aggregation of the different CTFs, further studies taking into account the structural differences among the fragments could yield valuable insight into the mechanistic basis of the toxicity of TDP-43 aggregation. For example, by overexpressing several constructs in yeast, Johnson and others (2008) observed that $CTF^{237-414}$,

starting from the last 20 residues of RRM2, formed cytoplasmic aggregates without becoming cytotoxic. However, the $CTF^{188-414}$, containing the full RRM2, both aggregated and caused cytotoxicity. This implies a toxic gain of function for RRM2 in cytoplasmic TDP-43 aggregation.

4.4 Mutant Full-Length TDP-43

Interestingly, differentiated motor neurons from human induced pluripotent stem cells (iPSCs) that are derived from fALS patients carrying mutant TDP-43 have revealed a role for TDP-43 aggregation in neurodegeneration (**Table 2**). Multiple studies have shown an increased mislocalization of mutant TDP-43 forms, including $TDP\text{-}43^{G298S}$ (Sun *et al.*, 2018) and $TDP\text{-}43^{Q343R}$ (Egawa *et al.*, 2012), in the cytoplasm and subsequent aggregation, compared to motor neurons from control patients with exclusively nuclear wtTDP-43. Even though in a certain study differentiated motor neurons from a patient with $TDP\text{-}43^{M337V}$ did not show evident signs of aggregate formation in the cytosol, its insolubility augmented with time, compared to control motor neurons (Seminary *et al.*, 2018). Moreover, ALS-derived motor neurons exhibit a hindered survival against oxidative stress (Egawa *et al.*, 2012; Seminary *et al.*, 2018) and inhibited protein degradation (Sun *et al.*, 2018).

Nonetheless, recently one group did not find increased cytoplasmic localization for $TDP\text{-}43^{A382T}$ in motor neurons from one ALS patient, at least during the time of their experiments (Bossolasco *et al.*, 2018). However, they did not present data regarding the solubility of TDP-43 or cell viability. Also, the overall nuclear localization could be attributed to the specific mutant (A382T) used in the study, which was not used in those previously mentioned.

Moreover, in yeast several overexpressed mutants cause cytoplasmic aggregation in significantly more cells than wtTDP-43. For example, $TDP\text{-}43^{Q331K}$ was seen to produce cytoplasmic aggregates in roughly 27% of transformed yeast cells, compared to 4% in those overexpressing wtTDP-43. Spotted growth assays showed that the mutant was approximately twice as cytotoxic as wild-type form (Johnson *et al.*, 2009; Armakola *et al.*, 2011). It was also found in transfected mouse primary neurons and HEK293 cells that the majority of $TDP\text{-}43^{A315T}$ was recovered in the detergent-insoluble fraction of the lysate, while the majority of wtTDP-43 was detergent-soluble. These cells also exhibited a lower survival rate while expressing the mutant, compared to wild-type (Guo *et al.*, 2011).

These findings regarding ALS-related mutant TDP-43 indicate that pathological TDP-43 has a tendency to, over time, transition from the nucleus to the cytoplasm and become more and

more detergent-insoluble, ultimately forming aggregates. This has negative implications on cellular proteolysis, defense mechanisms against oxidative stress, and overall cell survival.

4.5 Wild-Type (WT) Full Length TDP-43

Because at least 95% of ALS cases include patients who possess the wild-type form of TDP-43 in post-mortem brain samples (Xu and Yang, 2014), wtTDP-43 is a very relevant species for the analysis of the neurotoxicity of TDP-43 aggregates. In bacteria and yeast, the overexpression of the protein readily reveals its incorporation into cytoplasmic aggregates in many cells. In parallel, there is an acute drop in growth rate, morphological changes, vacuolar fragmentation, and cell death in yeast. (Johnson *et al.*, 2009; Armakola *et al.*, 2011; Prasad *et al.*, 2016; Liu *et al.*, 2017; Park *et al.*, 2017; Leibiger *et al.*, 2018). Regarding *in vivo* models, wtTDP-43 overexpression causes animals to experience motor dysfunction leading to death, resembling ALS pathology in humans (**Table 2**).

However, overexpressing wtTDP-43 in mammalian models has illustrated very rare aggregation, a primarily nuclear localization, and a disproportionately high cytotoxicity, downplaying the toxic role of aggregates (Zhang *et al.*, 2009; Watanabe *et al.*, 2013; Yamashita *et al.*, 2014; Kitamura *et al.*, 2016; Baskaran *et al.*, 2018). Furthermore, Barmada and others (2010) reported cytoplasmic aggregation in a minority of transfected rat primary cortical neurons that did not show significant neurotoxicity. Another group found neither aggregation nor toxicity in the same cell type (Guo *et al.*, 2011) (**Table 2**). These data have enticed researchers to doubt the hypothesis that aggregated wtTDP-43 is a toxic species in ALS.

Nevertheless, the results of Capitini and others (2014) bring wtTDP-43 aggregation back into the spotlight. Instead of generating aggregated protein from overexpression, the authors directly transfected SH-SY5Y cells with purified wtTDP-43 aggregates from *E. coli*. This method permits a more direct observation of aggregated TDP-43 alone. Indeed, the cells revealed the presence of the purified aggregated TDP-43 in the cytosol without altered nuclear levels of endogenous murine TDP-43. Capitini and others observed a striking drop in viability compared to cells transfected with control inclusion bodies from *E. coli*. The decreased viability was linked to heightened levels of reactive oxygen species and caspase-3 activation. Interestingly, the endogenous TDP-43 was not sequestered from the nucleus into the cytoplasmic aggregates. So, the cellular toxicity seemed to be tightly linked to the aggregated TDP-43 in the cytoplasm.

Taken together, these data illustrate that overexpressed wtTDP-43 is capable of inducing cellular toxicity and ALS-like features. But, unlike the mutant, CTF, and ΔNLS forms of TDP-43, the wild-type form mostly remains nuclear and rarely aggregates. Even though it still generates cellular toxicity and ALS-like symptoms in transgenic animals, the overall localization of the overexpressed protein does not represent the pathological hallmark of ALS. However, directly transfecting cells with pre-aggregated wtTDP-43 (Capitini *et al.*, 2014; Cascella *et al.*, 2016; Cascella *et al.*, 2017) does show direct, toxic effects, including increased oxidative stress and caspase-3 activation. Thus, the cytoplasmic aggregation of wtTDP-43, when it occurs, seems intrinsically toxic to cells by gaining deleterious functions.

4.6. Aggregated TDP-43: Gain and Loss of Function

The results presented throughout section 4 have suggested certain toxic characteristics to wild-type, mutant, and CTFs of TDP-43. It has been hypothesized that the cytotoxicity originates from a combination of gain and loss-of-function (GOF and LOF, respectively) mechanisms (Sun and Chakrabartty, 2017). A handful of studies have displayed a comparable association between TDP-43 aggregation and a number of ALS-related, deregulated pathways in motor neurons that could result from either GOF or LOF. For example, TDP-43 dysfunction is known to be associated with disturbances in energy metabolism, protein transport, mitochondrial dysfunction, aggravated oxidative stress, glutamatergic excitotoxicity, calcium dysregulation, and impaired axonal outgrowth. These pathological mechanisms involved in TDP-43 pathology are reviewed comprehensively elsewhere (Scotter *et al.*, 2015; Shenouda *et al.*, 2018).

More specifically, a gain of function could be acquired by sequestering off-target proteins and mRNA in the environment. For instance, the RNA-binding proteins RBM14, NonO, and PSF having roles in pre-mRNA splicing and transcriptional repression were found enriched in the insoluble fraction of overexpressed TDP-43 (Dammer *et al.*, 2012). One study also showed an indirect GOF by reporting the mislocalization of the nuclear transport factor THOC2 in the cytosol of HEK293T cells transfected with a CTF of TDP-43. As a result, mRNA aberrantly accumulated in the nucleus (Woerner *et al.*, 2016). Aggregated TDP-43 in the cytosol has also been seen to sequester its own nuclear counterpart (Cascella *et al.*, 2016). This nuclear depletion would then disallow TDP-43 to carry out its functions in the nucleus, thereby causing a simultaneous LOF. These findings show that aggregated TDP-43 is not an inert product of other pathological mechanisms. Rather, it represents a pathological species of TDP-43 that breaks down cellular homeostasis through a combination of loss and gain-of-function.

5. *In vitro* and *in vivo* Models: Limitations and Insights for Improvement

As seen in the preceding section and **Table 2**, various *in vitro* and *in vivo* models have been established to investigate the possible neurotoxicity of TDP-43 aggregation. Instead of yielding consistent data that would definitively characterize the relationship between TDP-43 aggregates and the neuronal death observed in ALS, the data vary considerably from one model to the next that only partially respond to the question at hand. Therefore, better models and approaches are required. In order to undertake this challenge, we must understand the advantages and limitations that each current model presents.

5.1. The Yeast Model

The yeast model has been one of the most important tools for the study of the functions of mammalian proteins, especially in the case of diseases (Botstein *et al.*, 1997). Even though some consider it an *in vivo* model since it is a unicellular organism (Johnson *et al.*, 2008), the budding yeast *Saccharomyces cerevisiae* is perceived by most as an *in vitro* model when used for the study of TDP-43 pathology.

As previously mentioned, most studies using yeast support the hypothesis that TDP-43 aggregation is cytotoxic by showing that the exogenous expression of different forms of TDP-43 leads to the formation of cytoplasmic aggregates and a parallel decrease in cell viability. The majority of investigations are based on overexpression, because this leads to cytoplasmic aggregation, whereas decreasing the expression reveals physiological, nuclear localization. However, the overexpression is inherently toxic, which clouds the relationship between TDP-43 aggregation and yeast viability. Moreover, yeast do not possess a TDP-43 homolog, and the intracellular environment of a yeast cell does not represent that of the neuron. Thus, the introduction of a completely foreign protein in a non-neuronal environment adds an important confounding factor to the understanding of the pathology of TDP-43 aggregation in human motor neurons. Nevertheless, this organism has been useful in genetic screens for the prediction of potential modifiers of TDP-43-mediated toxicity (Armakola *et al.*, 2011).

5.2 Mammalian Neuronal Models

In order to shorten the gap between the different intracellular environments of the human motor neuron and the yeast cell, a range of mammalian neuronal cell types has been utilized to overexpress the different forms of TDP-43 (**Table 2**). Section 4 illustrated that despite the relative consistency that yeast show regarding cytoplasmic aggregation and cellular demise, the results vary considerably not only among different neuronal models but also among different research groups that have employed the same model (**Table 2**). These discrepancies encompass a large part of the long-lasting debate regarding the neurotoxicity of TDP-43 aggregates, and understanding the corresponding limitations could help put an end to the debate.

Firstly, as previously mentioned, the overexpression that usually occurs in these models is inherently toxic and makes it challenging to attribute the cellular effects to TDP-43 itself. But, endogenous levels of pathological protein can be generated with human iPS cells derived from ALS patients and differentiated into motor neurons (Egawa *et al.*, 2012; Bossolasco *et al.*, 2018; Seminary *et al.*, 2018; Sun *et al.*, 2018). Not only does this approach omit the bias from overexpression conditions but an advantage of these cultures is the ability to observe the possible, initial pathological events that take place in TDP-43 aggregation, because one can follow the fate of the protein from the beginning of motor neuronal differentiation and expression.

Secondly, the various results in neuronal models could also be due to the fact that studies usually focus on isolated cultures of neurons, which is not representative of the CNS where neurons interact with glial cells. In fact, there is accumulating evidence arguing that ALS can be a cell non-autonomous disease, in which the given affected cell type inflicts its pathology onto other types that would otherwise be unaffected. Moreover, TDP-43 aggregation has also been demonstrated in astrocytes, microglia, oligodendrocytes, and muscle fibers (Ilieva *et al.*, 2009; Yan *et al.*, 2014; Wachter *et al.*, 2015). Therefore, the co-culturing of neurons with the other cell types mentioned above has gained more importance in the determination of the toxic effects of TDP-43 aggregation. However, its involvement in cell non-autonomy is currently debatable (Haidet-Phillips *et al.*, 2013; Serio *et al.*, 2013; Wachter *et al.*, 2015; Ditsworth *et al.*, 2017). Nonetheless, reproducing the neuron-glial network through co-culture could be a way to render the results of TDP-43 aggregation and toxicity more relevant to ALS in patients.

5.3 *In vivo* Models

In general, *in vivo* studies rely on the overexpression of TDP-43 in neurons and display symptoms reminiscent of ALS, notably muscle denervation, decreased motor performance, and

loss of body mass. Upon inspection of the affected tissues, intense neurodegeneration is apparent, while the exogenous TDP-43 is seen to be partly delocalized in the cytoplasm in the form of punctate aggregates. Multiple studies also show that subsequently silencing this TDP-43 overexpression results in clearance of TDP-43 aggregates, improved motor function, and increased longevity in comparison to the unsilenced group (Walker *et al.*, 2015a; Walker *et al.*, 2015b). *In vivo* models such as these primarily suggest that high amounts of TDP-43 are toxic *in vivo*, but it remains very difficult to unravel the link between TDP-43 aggregation and neurodegeneration. This is due to several limitations.

First, as previously discussed, the potent toxicity of overexpression might accelerate pathogenesis and overload the animal with high amounts of TDP-43, causing it to die prematurely from mechanisms that could be unrelated to TDP-43 aggregation and ALS, such as intestinal occlusion (Wegorzewska *et al.*, 2009; Joyce *et al.*, 2011; Herdewyn *et al.*, 2014). Although, one recent study managed to create a mouse model with a more gradual manifestation of ALS symptoms, as seen in human patients, by expressing TDP-43 at endogenous levels, in which mice experienced both a pre-symptomatic (three months) and symptomatic phase (nine months) (Gordon *et al.*, 2019). Remarkably, analyses of the brain and spinal cord demonstrated the gradual increase of the detergent-insoluble fraction of TDP-43 in tissue extracts between both disease phases. Yet, immunohistochemical analyses revealed the absence of visible aggregates in the duration of the study. This observation compels us to reconsider the implied assumptions that most studies make when evaluating TDP-43 aggregation, which is that the aggregates are detergent-insoluble species with respect to immunoblots and visible as fluorescent foci with respect to immunohistochemical/fluorescence techniques.

If an aggregate of TDP-43 is defined as a detergent-insoluble species, then the findings of Gordon and others (2019) demonstrate the importance of comparing the insolubility of TDP-43 in detergent to its formation of foci viewed under the microscope, which is not consistently described in the literature. Since Gordon and others did not detect any visible aggregation, the insoluble TDP-43 must have had the appearance of a diffuse species, assuming that the insolubility was not affected by the method of protein extraction. Therefore, studies that do not compare quantitatively the detergent-insolubility with the visible aggregation could be underestimating the number of aggregate-positive, degenerating cells. Thus, the association between aggregate formation and cellular toxicity could be tighter than certain studies recommend.

Second, the common approach of knocking out TDP-43 following its expression (Ke *et al.*, 2015; Walker *et al.*, 2015a; Walker *et al.*, 2015b; Spiller *et al.*, 2016) does not necessarily

specifically target the inherent cytotoxicity of its aggregation due to the possibility of off-target effects. *In vivo* models should target TDP-43 aggregation more specifically. For example, a transgenic mouse model overexpressing wtTDP-43 showed severe pathology, including TDP-43 aggregates, with a very mitigated lifespan. However, transgenic mice that also underwent a complete knockout for ataxin-2, an RNA-binding protein that forms stress granules with TDP-43, exhibited an extremely reduced pathology and survived much longer than TDP-43 transgenic mice with normal ataxin-2 expression (Becker *et al.*, 2017). Furthermore, while showing no change in TDP-43 protein levels, mice with the ataxin-2 co-knockout revealed fewer signs of TDP-43-positive stress granules and aggregates when analyzed at the same time point as transgenic mice carrying one or two copies of ataxin-2. This model suggests that TDP-43 aggregation, possibly originating from irregular stress granular dynamics, is neurotoxic due to a gain of toxic function that depends on the presence of ataxin-2. Notwithstanding, ataxin-2-negative mice still showed eventual motor impairment, which is probably due to the inherent toxicity of the knockout condition. Therefore, the *in vivo* models that rely solely on transgenics often yield results that cannot be thoroughly interpreted.

Finally, another reason behind the lack of interpretive power of current *in vivo* models is the inability of the experimenter to follow the evolution of TDP-43 in real time. This poses a major limitation, since aggregation is not a two-step reaction but a sequence of events, including misfolding, oligomerization, and eventual formation of large aggregates. *In vivo* models, such as *Drosophila melanogaster*, mice, and rats only offer snapshots of what occurs during the time course of TDP-43 pathology. Recently, however, a zebrafish model has been developed that permits the observation of fluorescently tagged TDP-43 in real time in individual, degenerating motor neurons (Svahn *et al.*, 2018). Given that UV light can be used to induce neurodegeneration in individual motor neurons of zebrafish through the activation of caspases (Soustelle *et al.*, 2008), the authors reported that zebrafish transgenic for wtTDP-43 demonstrated cytoplasmic mislocalization of the protein in UV-injured motor neurons. Furthermore, a population of the TDP-43 pool became fragmented and localized in the axons, which became deformed. However, the lack of immunoblot analyses prevents the understanding of TDP-43 solubility and oligomerization in this study. Nonetheless, this transparent model permitting the observation of pathological TDP-43 in real time could reveal substantial information regarding the dynamics and effects of its aggregation.

6. The Prion-Like Characteristics of Aggregated TDP-43

In cell culture, TDP-43 displays similar characteristics to those of prion protein (Ayers and Cashman, 2018; Brauer *et al.*, 2018; Nonaka and Hasegawa, 2018). A considerable number of studies suggest that ALS is a "prion-like" disease as a consequence of its hallmark, the TDP-43-containing aggregates. The C-terminal region is capable of forming stable β-sheets, structures with a tendency to form amyloid-like fibrils, initiating the seeding mechanism similar to prions (Guo *et al.*, 2011; Jiang *et al.*, 2013; Sun and Chakrabartty, 2017).

Full-length and cleaved cytoplasmic, phosphorylated, ubiquitinated TDP-43 isolated from ALS brain extracts activate and seed cytoplasmic aggregation in the otherwise mainly nuclear, soluble, recombinant wtTDP-43 in neuronal and glial cultures (Nonaka *et al.*, 2013; Smethurst *et al.*, 2016; Ishii *et al.*, 2017). In these cases, the recombinant TDP-43 is recovered in the detergent-insoluble fraction and reveals phosphorylation and ubiquitination, reproducing the hallmark phenotype recognized in the brain of ALS patients. Remarkably, this seeding effect is specific for TDP-43, because it has been shown that TDP-43 is not seeded by superoxide dismutase-1 (SOD-1) fibrils (Furukawa *et al.*, 2011) or α-synuclein fibers (Nonaka *et al.*, 2013). The seeding effect is paralleled by a decrease in cellular proliferation, indicating a tie between TDP-43 aggregation and cellular demise.

Furthermore, TDP-43 from transfected cells can propagate to naive, neighboring cells (Ding *et al.*, 2015; Smethurst *et al.*, 2016; Ishii *et al.*, 2017; Zeineddine *et al.*, 2017). Further evidence of TDP-43 spreading comes from the higher levels of free and exosomal TDP-43 identified in the cerebrospinal fluid (CSF) of ALS patients compared to control groups (Kasai *et al.*, 2009; Sproviero *et al.*, 2018). TDP-43 aggregates were also discovered in serum leukocytes from ALS patients (Foulds *et al.*, 2008; Corrado *et al.*, 2009; Foulds *et al.*, 2009; De Marco *et al.*, 2011; Verstraete *et al.*, 2012; Alquezar *et al.*, 2016; De Marco *et al.*, 2017). Although pathological TDP-43 has the property to penetrate nearby cells, it remains unclear as to how this phenomenon occurs. Some theories have been proposed concerning the involvement of exosomes, tunneling nanotubes, endocytosis, and even passive diffusion (Smethurst *et al.*, 2015).

However, observations made *postmortem* suggest that aggregated TDP-43 does not spread to neighboring cells in the brain, because aggregation was not found throughout the somata of degenerated neurons. Rather, the aggregates were seen to be dispersed along the axons, affecting the downstream oligodendrocytes and neurons (Brettschneider *et al.*, 2013). As TDP-43 is actively transported in motor neuron axons (Fallini *et al.*, 2012), it could be received by oligodendrocytes through zones of axonal contact. In support of this hypothesis, ALS is known to

spread throughout the neuraxis in both upper and lower motor neurons, similar to prion diseases (Beekes and McBride, 2007; Ravits *et al.*, 2007; Brettschneider *et al.*, 2013).

Taken together, these investigations suggest that insoluble, aggregated TDP-43 presents prion-like properties that seem to contribute to ALS pathogenesis. Although *in vivo* experiments in this regard have not yet been published, *in vitro* studies demonstrate that the aberrant structure of TDP-43 forms fibrils that inhibit the proper function of otherwise normal TDP-43 proteins. Further studies are required to clarify the role of TDP-43 aggregation in the cell-to-cell spreading of ALS.

7. Clearance Mechanisms of Aggregated TDP-43 and Therapeutic Perspectives

If a protein, such as TDP-43, becomes abnormal by misfolding, the cell possesses several clearance mechanisms to dispose of it. Of the multiple pathways that exist in the cell, the most studied pathways regarding TDP-43 clearance have been autophagy, the ubiquitin proteasome system, and the endosomal-lysosomal pathway. A plethora of efforts has been made to manipulate these pathways to clear the cell of the misfolded, aggregated TDP-43 in ALS models. It has been found that the stimulation of these degradation pathways in cells overexpressing TDP-43 results in the decrease of aggregated TDP-43 and improved cell viability, as we will discuss below.

7.1 Autophagy

Autophagy is responsible for the clearance of dysfunctional organelles and large protein aggregates (Rubinsztein, 2006). TDP-43 regulates autophagy by increasing Atg7 mRNA stability (Bose *et al.*, 2011), whose translated product assists in autophagosome formation (**Fig. 2**). Interestingly, several mutations in genes involved in autophagy have been identified to be involved in ALS pathogenesis, notably *FIG4, OPTN, VCP, C9orf72, SQSTM1, UBQLN2,* and *TBK1*. All cases, except mutated *FIG4,* include the detection of TDP-43 aggregates in post-mortem samples (**Table 1)** (Maurel *et al.*, 2018a). Moreover, one study demonstrated the accumulation of poly-ubiquitinated and aggregated endogenous TDP-43 (Filimonenko *et al.*, 2007), and another showed the accumulation of 25-kDa CTFs upon autophagy inhibition (Wang *et al.*, 2010). Indeed, transfecting murine Neuro2A and NSC-34 cells with pre-formed TDP-43 aggregates results in there degradation by way of autophagy (Cascella *et al.*, 2017). These findings strongly suggests that autophagy deregulation could be involved in pathological TDP-43

aggregation and that enhancing autophagy could counteract this pathology. With the goal of exploiting autophagy in a therapeutic manner, several chemical activators of autophagy have been tested *in vitro* and *in vivo*.

Trehalose, a non-reducing disaccharide found in the hemolymph of invertebrates (Sarkar *et al.*, 2007), has been observed to stimulate autophagy and the selective clearance of overexpressed CTF in HEK293 cells, while wtTDP-43 is cleared to a lesser extent (Wang *et al.*, 2010; Scotter *et al.*, 2014). The mechanism of trehalose remains poorly understood, but it seems to activate transcription factors that regulate lysosome and autophagosome biogenesis, as well as lysosome-autophagosome fusion (Wang *et al.*, 2018) (**Fig. 2**). Nonetheless, since autophagy preferentially attacks aggregated species, such as the highly aggregation-prone CTFs of TDP-43, this can explain the significant efficiency of CTF clearance as opposed to wtTDP-43. However, no tests on cell viability were conducted in the corresponding studies.

In addition, a handful of research groups have shown that inhibitors of the mammalian target of Rapamycin (mTOR), a central kinase complex that negatively regulates autophagosome formation (Zarogoulidis *et al.*, 2014), induce autophagy with therapeutic potential against TDP-43 accumulation and aggregation, as is the case for rapamycin, berberine, tamoxifen, and the antipsychotic drug fluphenazine (FPZ) (**Fig. 2**). Essentially, these inhibitors induce the clearance of TDP-43 aggregates formed by overexpression, followed by improvement in neuronal survival and motor symptoms *in vitro* and *in vivo*, respectively (Caccamo *et al.*, 2009; Barmada *et al.*, 2010; Wang *et al.*, 2012; Wang *et al.*, 2013a; Barmada *et al.*, 2014; Cheng *et al.*, 2015; Chang *et al.*, 2016; Li *et al.*, 2016).

Still, some reports challenge these current findings regarding the therapeutic ability of certain autophagy stimulants. For example, Scotter and others (2014) reported on the ability to induce TDP-43 aggregate formation by inhibiting the UPS but not autophagy. After testing the effect of rapamycin, they observed no effect on wtTDP-43, mutant, or CTF clearance in HEK293 cells. Moreover, Liu and others (2017) reported only a modest effect on the clearance of similar forms of TDP-43 and found no reduction in toxicity in yeast. Finally, Leibiger and others (2018) surprisingly reported that yeast displayed a detrimental effect of autophagy stimulation by rapamycin in the presence of overexpressed wtTDP-43. To sum up, it remains debatable whether autophagy would be a promising therapeutic tool against TDP-43 pathology.

7.2 The Ubiquitin Proteasome System

Unlike autophagy, the ubiquitin proteasome system (UPS) targets mostly soluble, misfolded proteins in the nucleus and cytoplasm (Rubinsztein, 2006). This pathway essentially consists of the covalent tagging of proteins with ubiquitin (**Fig. 2**), targeting them for proteasome degradation (Rubinsztein, 2006; Maurel *et al.*, 2018a). Similar to autophagy, the involvement of UPS-associated genes in ALS has been documented, such as *SOD-1*, *FUS*, *VCP*, *UBQLN2*, and *CCNF* (Maurel *et al.*, 2018a). TDP-43 aggregation has been recorded in post-mortem samples of ALS patients involving these mutated genes, except *CCNF* for which the data is absent (**Table 1**). The specific inhibition of the UPS resulted in heightened levels of ubiquitinated TDP-43 aggregates (Wang *et al.*, 2010; Scotter *et al.*, 2014; Walker *et al.*, 2015b). Furthermore, Tashiro and others (2012) found that the conditional knockout of the proteasome subunit Rpt3 in mouse motor neurons led to the development of TDP-43-positive aggregates accompanied by motor decline. On the contrary, the conditional Atg7 (autophagy factor) knockout in the same study only produced ubiquitin and p62-positive aggregates without any TDP-43 pathology. Upon transfection of purified TDP-43 aggregates in Neuro2A and NSC-34 cells, it was found that the fraction of aggregates in equilibrium with the monomeric form of TDP-43 was primarily degraded by the UPS (Cascella *et al.*, 2017). Therefore, utilizing the UPS in a therapeutic sense is also an intriguing possibility.

DNAJB1, an Hsp40 chaperone, provides a potential avenue to boost the UPS. It accelerates the ATPase activity of Hsp70 chaperones that act on misfolded proteins destined for the UPS (Rauch and Gestwicki, 2014; Maurel *et al.*, 2018a). DNAJB1 improved the viability of primary neurons transfected with WT and A315T TDP-43 (Park *et al.*, 2017). Given that the UPS targets species that appear before the large aggregates, DNAJB1 might promote the UPS-directed degradation of smaller, early-stage aggregates.

Moreover, poly-A binding protein (PABPN1), a direct binding partner of TDP-43 in mammalian neural tissue, reduced pre-formed TDP-43 aggregates and cell death in a yeast model overexpressing mutant and CTF TDP-43, as well as in primary neurons. Remarkably, PABPN1 did not target functional, endogenous TDP-43 for degradation, as it preserved its solubility and nuclear localization while targeting pathological TDP-43 for degradation (**Fig. 2**). Inhibition of the UPS, but not autophagy, undermined the function of PABPN1, strongly suggesting that its function in degradation is associated with the UPS. However, it remains to be studied how PABPN1 directs TDP-43 toward the UPS (**Fig. 2**) (Chou *et al.*, 2015).

Lastly, Tamaki and others (2018) engineered an intrabody expressing only the VL and VH domains of the complementarity determining region of their previously conceived antibody (Shodai *et al.*, 2012) that specifically bound to cytoplasmic, aggregated TDP-43. The VH domain

naturally possessed a PEST sequence that served as a target for the proteasome, and the C-terminus contained an artificial, chaperone-mediated autophagy signal sequence (Heymsfield *et al.*) for autophagy-directed degradation. The VH-VL-CMA intrabody (**Fig. 2**) prevented the increase in the number and size of cytoplasmic aggregates of overexpressed TDP-43 compared to the untreated control in HEK293 cells and following *in utero* electroporation in the cerebral cortex of murine fetuses. In Neuro2A cells, the authors observed an increase in cell viability and a decrease in cell death, compared to controls without intrabody. This intrabody suggests that exploiting both the UPS and autophagy could be a valuable therapeutic method.

7.3 The Endosomal-Lysosomal Pathway

Finally, the endosomal-lysosomal pathway (van der Zee *et al.*) also takes part in TDP-43 clearance. This pathway comprises the formation and trafficking of vesicles, such as endosomes and phagosomes, by ESCRT protein complexes that fuse with the lysosome to degrade cargo (**Fig. 2**) (Hu *et al.*, 2015). It is unclear as to which TDP-43 species this pathway targets and whether it coordinates with autophagy. Two different studies suggest that the ELP assumes a more significant role in TDP-43 elimination than autophagy (Liu *et al.*, 2017; Leibiger *et al.*, 2018). In fact, Leibiger and others (2018) found that deleting ELP-related genes significantly aggravated cellular toxicity induced by TDP-43 expression. Therefore, it is important to consider the ELP as an outlet for TDP-43 degradation.

Rab5 is a regulatory GTPase that associates with early endosomes and plays a role in endosome membrane fusion reactions (**Fig. 2**) (Woodman, 2000). One study showed that aggregated TDP-43 co-localized with Rab5 in the cortex of five ALS patients and in HEK293 cells overexpressing TDP-43. In the same study, Rab5 overexpression following endogenous-level expression of either WT, mutant, or CTF TDP-43 abolished aggregation through activation of ELP-mediated clearance while increasing cell viability in HEK293 cells. The overexpression of Rab5 also improved locomotor function in *Drosophila* expressing WT or mutant TDP-43 in motor neurons (Liu *et al.*, 2017). Therefore, Rab5 is an attractive therapeutic target for the ELP-mediated clearance of pathological TDP-43.

8. The Verdict: Innocent or Toxic?

In the present review, we have scrutinized the current research dedicated to deciphering the role that TDP-43 aggregation plays in the neurodegeneration of ALS. We have found a large amount of evidence supporting the hypothesis that TDP-43 aggregation is a key factor behind the motor neuronal death in this disease. TDP-43 aggregation leads to a combination of loss and gain of functions that bring about toxic consequences, including but not limited to decreased neurite outgrowth, hindered survival against oxidative stress and stress granule dynamics, nucleo-cytoplasmic transport, pre-mRNA splicing, mitochondrial dysfunction, and glutamatergic excito-toxicity. Important insights into the central role of TDP-43 aggregates in ALS pathology stem from observational studies performed in ALS patients, as almost all cases of sporadic ALS present cytoplasmic aggregates of TDP-43 in degenerated motor neurons. In addition, diverse studies show a convergence of the key proteins involved in ALS pathogenesis, including TDP-43, SOD-1, and FUS. These proteins interact indirectly in such a way to induce the others to aggregate (Ling *et al.*, 2010; Kabashi *et al.*, 2011; Kryndushkin *et al.*, 2011; Pokrishevsky *et al.*, 2016; Lin *et al.*, 2017). This further implies that neuronal death observed in ALS is connected to TDP-43 proteinopathy.

Notwithstanding, a significant number of *in vitro* and *in vivo* studies show an apparent low amount of aggregation while revealing a high level of neurotoxicity, downplaying the noxious nature of TDP-43 aggregates for certain researchers. At the same time, they could be overlooking the upstream aggregating species of TDP-43 that could be too small to be detected by the microscopy methods employed in such studies. These include misfolded and oligomerized forms that could have different toxicities depending on solubility. This oversight could be avoided by targeting mislocalized and misfolded TDP-43 in addition to the final aggregate, which has indeed already been demonstrated (Shodai *et al.*, 2012).

As TDP-43 seems to be a determinant for the neurodegeneration observed in ALS patients, the clearance of TDP-43 aggregates during the early stages of the disease could open new doorways to therapeutic interventions. The data showing that TDP-43 clearance and the resulting lowering of aggregation improves cell survival and motor symptoms in cellular and animal models of ALS reinforces this therapeutic strategy. Two ongoing clinical trials are evaluating this strategy, one using rapamycin and the other employing tamoxifen.

Conclusion. This review supports the hypothesis that cytoplasmic TDP-43 aggregates play a central role in the neurodegeneration observed in ALS patients, which is sustained by numerous studies performed in cultured cells, animal models, and autopsies of patients. This consideration

has strong implications for the development of therapeutic strategies. We encourage fellow researchers to reinforce the collective effort to uncover the mechanisms that lead to TDP-43 aggregation to begin to make larger strides towards an effective treatment for ALS.

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Main Figures

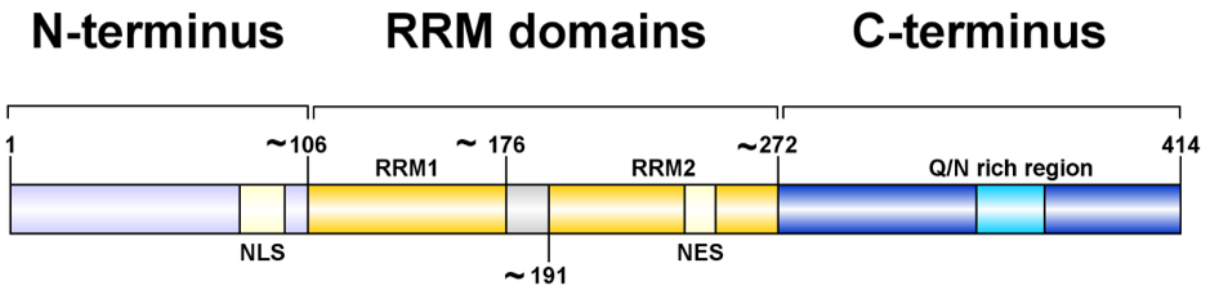

Figure 1. Schematic Diagram of the TDP-43 Regions and Domains. NLS: Nuclear Localisation Signal; NES: Nuclear Export Signal; RRM: RNA Recognition Motif; Q/N: Glutamine/Asparagine.

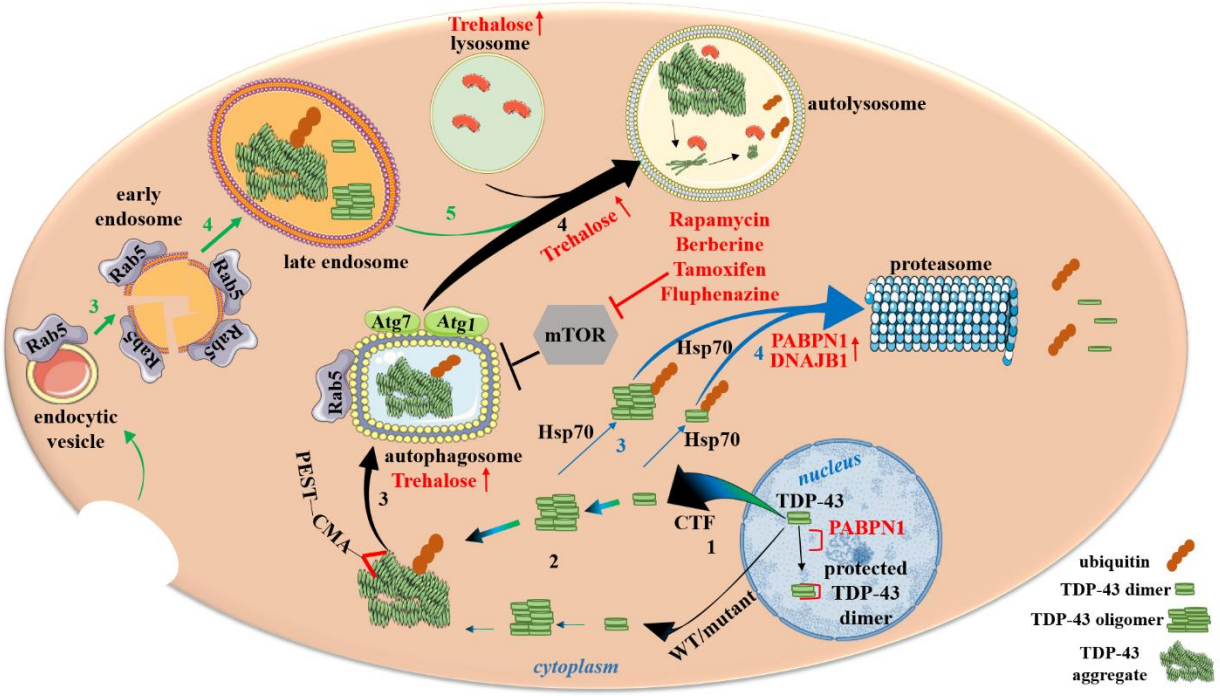

Figure 2. Mechanisms of TDP-43 Degradation and Proposed Therapeutic Interventions.

Autophagy (black sequence) - 1: Nuclear, dimeric TDP-43 aberrantly accumulates in the cytoplasm. Degradation can occur to produce C-terminal fragments. 2: All TDP-43 forms enter the aggregation pathway (multi-color arrows), in which the CTF is more active (thicker arrows), forming an oligomer and, finally, a poly-ubiquitinated aggregate. 3: Autophagy responds by engulfing the aggregate in an autophagosome, whose construction is essentially directed by Atg1 and Atg7 proteins. 4: The autophagosome fuses with the lysosome, creating the autolysosome in which the proteases degrade the aggregate, releasing peptides and free ubiquitin. In red: rapamycin, berberine, tamoxifen, fluphenazine, trehalose stimulate the corresponding autophagic processes. The CMA sequence of the intrabody bound to the aggregate directs it to autophagy. UPS (blue sequence) – Steps 1 and 2 are identical to those of autophagy. 3: The misfolded dimer and oligomer are now ubiquitinated and maintained by Hsp70 chaperones. 4: The ubiquitinated species are directed to the proteasome, where they are degraded. In red: DNAJB1 stimulates Hsp70 activity. PABPN1 protects nuclear TDP-43 from cytoplasmic sequestration and increases UPS flux by an unknown mechanism. The PEST sequence of the intrabody directs the aggregate to the UPS. ELP (violet sequence) – Events 1 and 2 are the same as in autophagy and the UPS. 3: The endocytic vesicle forms an early endosome that is largely regulated by Rab5. 4: This leads to the late endosome that can harbor material destined for degradation. For the sake of simplicity,

we consider every form of TDP-43 to be a target for ELP. 5: The late endosome fuses with the lysosome, where the material is degraded. Autophagy and ELP have the ability to cooperate, because their pathways converge. Figure designed using image templates from Servier Medical Art https://smart.servier.com/image-set-download/.

Main Tables

Mutation	**TDP inclusion**	**Studies**
TARDBP	yes	Van Deerlin *et al.* (2008); Kabashi *et al.* (2008)
SOD-1	yes	Sumi *et al.* (2009); Okamoto *et al.* (2011); Sabatelli *et al.* (2015); Jeon *et al.* (2018)
NEFH	NR	--
SETX	yes	Bennett *et al.* (2018)
ALS2	NR	--
DCTN1	yes	(in Perry Syndrome); Wider *et al.* (2009)
VABP	NR	--
ANG	yes	Kirby *et al.* (2013)
CHMP2B	no	Holm *et al.* (2007) ; Ghazi-Noori *et al.* (2012)
FUS	no	Vance *et al.* (2009)
ELP3	NR	--
FIG4	NR	--
C9orf72	yes	Simon-Sanchez *et al.* (2012); Murray *et al.* (2011); Stewart *et al.* (2012) ; Al-Sarraj *et al.* (2011)
SQSTM1	yes	van der Zee *et al.* (2014)
UBQLN2	yes	Deng *et al.* (2011); Williams *et al.* (2012)
VCP	yes	Neumann *et al.* (2007)
OPTN	yes	Kamada *et al.* (2014)
ATXN2	yes	Elden *et al.* (2010)
SPG11	no	(in spastic paraplegia); Denora *et al.* (2016)
PFN1	yes	Wu *et al.* (2012)
GRN	yes	Mackenzie (2007)
HNRPA1	yes	Kim *et al.* (2013)
HNRNPA2B1	yes	Kim *et al.* (2013)
CHCHD10	yes *	Woo *et al.* (2017); Genin *et al.* (2018)
MATR3	yes	Johnson *et al.* (2014)
TUBA4A	no	Smith *et al.* (2014)
TBK1	yes	Gijselinck *et al.* (2015)
C21orf2	NR	--
NEK1	NR	--
CCNF	yes *	Williams *et al.* (2016)

Table 1: Genes related to ALS and presence of TDP-43 aggregation.

* Findings from cell or animal models of ALS; no studies performed in postmortem samples in these cases.

Table 2: The Diverse Models to Explore the Association of TDP-43 Aggregation with Toxicity.

Study	Model	Specific TDP-43 form	Cytoplasmic Aggregates	Toxicity
WT TDP-43 *in vitro* Models				
Park *et al.* (2017) Baskaran *et al.* (2018)	rat primary cortical neurons	*WT	yes	yes
Barmada *et al.* (2010); Park *et al.* (2017)	rat primary cortical neurons	WT	yes	no
Fallini *et al.* (2012)	mouse primary motor neurons	WT	no	yes
Guo *et al.* (2011)	mouse primary cortical neurons	WT	no	no
Yamashita *et al.* (2014)	SH-SY5Y	WT	no	yes
Watanabe *et al.* (2013) Kitamura *et al.* (2016)	Neuro2A	WT (stabilized by fusion protein) WT	no no	yes yes
Zhang *et al.* (2009)	M17 Neuroblastoma	WT	no	yes
Johnson *et al.* (2009) Armakola *et al.* (2011) Prasad *et al.* (2016) Liu *et al.* (2017) Park *et al.* (2017) Leibiger *et al.* (2018)	yeast	WT	yes	yes
Nonaka *et al.* (2016)	yeast	WT	no	no
Nonaka *et al.* (2016)	yeast	WT + CK1δ1-317 (kinase)	yes	yes
WT TDP-43 *in vivo* Models				
Becker *et al.* (2017)	mouse pan-neuronal expression	WT	yes	yes
Wang *et al.* (2013b)	mouse FTLD-U brain	mouse WT	yes	yes
Choksi *et al.* (2014)	*Drosophila* pan-neural expression	WT	no	yes
Mutant TDP-43 *in vitro* Models				

Bossolasco *et al.* (2018)	iPSC-derived motor neurons from ALS patient	A382T	no	no
Seminary *et al.* (2018)	iPSC-derived motor neurons from ALS patient	M337V	no	no
Barmada *et al.* (2010) Park *et al.* (2017)	rat primary cortical neurons	A315T	yes	yes
Baskaran *et al.* (2018)	rat primary cortical neurons	Q331K M337V	yes yes	yes yes
Guo *et al.* (2011)	mouse primary cortical neurons	A315T	yes	yes
Johnson *et al.* (2009)	yeast	Q331K	yes	yes
Mutant TDP-43 *in vivo* Models				
Choksi *et al.* (2014)	*Drosophila*	Q331K	yes	yes
Choksi *et al.* (2014)	*Drosophila*	M337V	no	yes
ΔNLS/NES TDP-43 *in vitro* Models				
Sasaguri *et al.* (2016)	mouse primary cortical neurons	FL ΔNLS	yes	yes
Zhang *et al.* (2013)	mouse primary cortical neurons	FL ΔNLS	yes	yes
Winton *et al.* (2008)	mouse hippocampal primary cortical neurons tsBN2 cells	FL ΔNLS	yes	N/A
Yamashita *et al.* (2014)	SH-SY5Y	FL ΔNLS	no	no
Kitamura *et al.* (2016)	Neuro2A cells	NLS -CTF	no	no
Armakola *et al.* (2011)	yeast	FL ΔNLS	yes	yes
***ΔNLS/NES TDP-43 in vivo* Models**				
Walker *et al.* (2015a)	mouse (expression in brain/spinal cord)	FL ΔNLS	yes	yes
Sasaguri *et al.* (2016)	mouse (pan-neuronal)	FL ΔNLS	yes	yes

Igaz *et al.* (2011)	mouse (forebrain)	FL ΔNLS	yes	yes
Miguel *et al.* (2011)	*Drosophila* neurons retina	 FL ΔNLS FL ΔNES FL ΔNLS FL ΔNES	 no no no no	 yes yes yes yes
***CTF TDP-43 in vitro* Models**				
Fallini *et al.* (2012)	mouse primary motor neurons	$CTF^{208-414}$	yes	yes
Chou *et al.* (2015)	mouse primary cortical neurons	$CTF^{208-414}$	yes	yes
Yamashita *et al.* (2014)	SH-SY5Y	$CTF^{162-414}$	yes	yes
Kitamura *et al.* (2016)	Neuro2A cells	$CTF^{220-414}$ $NLS\text{-}CTF^{220-414}$	yes no	yes no
Zhang *et al.* (2009)	M17 Neuroblastoma	$CTF^{220-414}$	yes	yes
Liu *et al.* (2017)	HEK293A	$CTF^{220-414}$	yes	yes
Chou *et al.* (2015)	yeast	$CTF^{208-414}$	yes	yes
***CTF TDP-43 in vivo* Models**				
Walker *et al.* (2015a)	mouse cortex, hippocampus	$CTF^{208-414}$	yes	yes
***Other Forms of TDP-43 in vitro* Models**				
Zhang *et al.* (2013)	mouse primary cortical neurons	TDP 10-414 ΔNLS	no	no

WT = wild-type, FL = full length, ΔNLS = artificial defective NLS, ΔNES = artificial defective NES, CTF = 25 kDa C-terminal fragment.

* If not specified, the species of TDP-43 is human.

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Supplementary material.

Supplementary Table 1. Identified Mutations in the structure of TDP-43.

Mutation	**Localisation**	**Reference**
D23A	N-terminal	ALS Data Browser
A66A	N-terminal	Daoud *et al.* (2009)
A90V	N-terminal - NLS	Winton *et al.* (2008); Brouwers *et al.* (2010); Vanden Broeck *et al.* (2015)
S92L	N-terminal - NLS	ALS Data Browser
P112H	RRM1	Moreno *et al.* (2015)
D169G	RRM1	Kabashi *et al.* (2008)
K263E	RMM2	Kovacs *et al.* (2009)
N259S	RRM2	Maurel *et al.* (2018)
N267S	C-terminal	Borroni *et al.* (2009); Corrado *et al.* (2009)
G287S	C-terminal	Kabashi *et al.* (2008); Corrado *et al.* (2009)
G290A	C-terminal	Van Deerlin *et al.* (2008)
S292N	C-terminal	Xiong *et al.* (2010); Zou *et al.* (2012)
G294A	C-terminal	Sreedharan *et al.* (2008)
G294V	C-terminal	Corrado *et al.* (2009); Del Bo *et al.* (2009)
G295C	C-terminal	van Blitterswijk *et al.* (2014)
G295R	C-terminal	Corrado *et al.* (2009); Ticozzi *et al.* (2011)
G295S	C-terminal	Benajiba *et al.* (2009); Corrado *et al.* (2009); Del Bo *et al.* (2009)
G298S	C-terminal	Van Deerlin *et al.* (2008)

M311V	C-terminal	Lemmens *et al.* (2009)
A315E	C-terminal	Fujita *et al.* (2011)
A315T	C-terminal	Gitcho *et al.* (2008); Kabashi *et al.* (2008)
A321G	C-terminal	Baumer *et al.* (2009)
A321V	C-terminal	Kirby *et al.* (2010)
Q331K	C-terminal	Sreedharan *et al.* (2008)
S332N	C-terminal	Corrado *et al.* (2009)
G335D	C-terminal	Corrado *et al.* (2009)
M337V	C-terminal	Rutherford *et al.* (2008); Sreedharan *et al.* (2008); Corrado *et al.* (2009)
M339I	C-terminal	ALS Data Browser
Q343R	C-terminal	Yokoseki *et al.* (2008)
N345K	C-terminal	Rutherford *et al.* (2008)
G348C	C-terminal	Kabashi *et al.* (2008); Kuhnlein *et al.* (2008); Daoud *et al.* (2009); Del Bo *et al.* (2009)
G348V	C-terminal	Kirby *et al.* (2010); Zou *et al.* (2012)
G348R	C-terminal	Ticozzi *et al.* (2011)
N352S	C-terminal	Kuhnlein *et al.* (2008)
N352T	C-terminal	Ticozzi *et al.* (2011)
G357S	C-terminal	Iida *et al.* (2012)
G357R	C-terminal	Chiang *et al.* (2012)
G357D	C-terminal	ALS Data Browser
M359V	C-terminal	Kabashi *et al.* (2008)

R361S	C-terminal	Kabashi *et al.* (2008)
R361T	C-terminal	Chiang *et al.* (2012)
P363A	C-terminal	Daoud *et al.* (2009)
G368S	C-terminal	Chio *et al.* (2010); De Marco *et al.* (2011)
Y374X	C-terminal	Daoud *et al.* (2009)
G376D	C-terminal	Conforti *et al.* (2011); Czell *et al.* (2013)
N378D	C-terminal	Ticozzi *et al.* (2011); Tsai *et al.* (2011); Soong *et al.* (2014)
N378S	C-terminal	Huang *et al.* (2012)
S379C	C-terminal	Corrado *et al.* (2009)
S379P	C-terminal	Corrado *et al.* (2009)
A382T	C-terminal	Kabashi *et al.* (2008); Corrado *et al.* (2009); Del Bo *et al.* (2009)
A382P	C-terminal	Daoud *et al.* (2009)
I383V	C-terminal	Rutherford *et al.* (2008)
G384R	C-terminal	Millecamps *et al.* (2010); Ticozzi *et al.* (2011)
W385G	C-terminal	Millecamps *et al.* (2010)
S387delinsTNP	C-terminal	Solski *et al.* (2012)
N390D	C-terminal	Kabashi *et al.* (2008)
N390S	C-terminal	Kabashi *et al.* (2008)
S393L	C-terminal	Corrado *et al.* (2009); Origone *et al.* (2010)

NLS: Nuclear Localization Signal; RRM: RNA Recognition Motif. ALS Data Browser available at alsdb.org.

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