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Review: Transactive response DNA-binding protein 43 (TDP-43): mechanisms of neurodegeneration

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Abstract

Since the identification of phosphorylated and truncated transactive response DNA-binding protein 43 (TDP-43) as a primary component of ubiquitinated inclusions in amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration with ubiquitin-positive inclusions, and the discovery that mutations in the *TDP-43* gene cause ALS, much effort has been directed towards establishing how TDP-43 contributes to the development of neurodegeneration. Although few *in vivo* models are presently available, findings thus far strongly support the involvement of abnormally modified TDP-43 in promoting TDP-43 aggregation and cellular mislocalization. Therefore, TDP-43-mediated neurotoxicity is likely to result from a combination of toxic gains of function conferred by TDP-43 inclusions as well as from the loss of normal TDP-43 function. Nonetheless, the exact neurotoxic TDP-43 species remain unclear, as do the mechanism(s) by which they cause neuronal death. Moreover, little is currently known about the roles of TDP-43, both in the nucleus and the cytoplasm, making it difficult to truly appreciate the detrimental consequences of aberrant TDP-43 function. This review will summarize what is currently understood regarding normal TDP-43 function and the involvement of TDP-43 in neurodegeneration, and will also highlight some of the many remaining questions in need of further investigation.

Keywords

amyotrophic lateral sclerosis; frontotemporal lobar degeneration with ubiquitin-positive inclusions; inclusions; neurodegeneration; neurotoxicity; TDP-43

Introduction

A common feature of many neurodegenerative diseases is the pathological accumulation of proteinaceous aggregates. In 2006, the transactive response DNA-binding protein 43 (TDP-43) was recognized as a primary protein component of intracellular ubiquitinated inclusions in most cases of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U) [1,2]. Previously, a protein of relative obscurity, this discovery has catapulted TDP-43 into the limelight and has initiated a flurry of new research aimed at exposing the scope of TDP-43 pathology in neurodegenerative disease and at understanding the role of TDP-43 in neurotoxicity.

It is now well established that glial and neuronal TDP-43-positive inclusions, the latter taking the form of dystrophic neurites, cytoplasmic inclusions or intranuclear inclusions, are present in both sporadic and familial cases of ALS and FTL-D-U with or without motor neurone disease (MND) [1–13]. It should be mentioned, though, that not all FTL-D-U and ALS cases show TDP-43 pathology [3,5,11,14–18]. With regards to inherited forms of FTL-D-U, TDP-43 inclusions are observed in cases linked to chromosome 9p [3] and in patients with mutations in the genes encoding progranulin (*PGRN*) [3,4] and valosin-containing protein [3,7]. In contrast, no TDP-43 pathology is observed in frontotemporal dementia patients with mutations in the gene encoding charged multivesicular body protein 2B (*CHMP2B*) [3]. As for familial ALS, TDP-43 inclusions are a consistent feature except in those cases resulting from mutations in the genes encoding Cu/Zn superoxide dismutase (*SOD1*) [5,6] or fused in sarcoma (*FUS*) [19,20]. In addition to ALS and FTL-D-U, TDP-43 pathology has been observed in a variety of neurodegenerative disorders including Lewy body disease [21,22], parkinsonism-dementia complex of Guam [23,24], corticobasal degeneration [2,25], Pick's disease [2], Alzheimer's disease (AD) [2,22,25,26], Perry syndrome [27] and hippocampal sclerosis [3,26]. Whether the presence of TDP-43 pathology in these diseases indicates that TDP-43 contributed to their development or progression is not yet known.

Mutations in *TARDBP*, the gene encoding TDP-43, have been discovered in sporadic and familial ALS [28–38], providing evidence of a direct link between TDP-43 abnormalities and neurodegeneration. As with other protein-misfolding diseases, TDP-43-mediated neuronal death likely occurs from the involvement of multiple pathways that ultimately results in a toxic gain of function of TDP-43 species, as well as a loss of normal TDP-43 function. The aim of this review is to summarize what is currently known about TDP-43-mediated neurotoxicity. Also discussed will be TDP-43 functions and how dysfunction of TDP-43 may contribute to the development of neurodegeneration. Finally, this review will highlight some of the many questions in need of further investigation.

Structure of TDP-43

More than 10 years before it made its debut in the neurodegenerative field, TDP-43 was identified as a protein that binds the TAR DNA region of the human immunodeficiency virus type 1 [39]. Subsequently, TDP-43 was found to bind the (TG)_m sequence in the cystic fibrosis transmembrane conductance regulator (CFTR) exon 9 pre-mRNA and to promote exon 9 skipping [40]. Human *TARDBP* is located on chromosome 1p36 and encodes a 414-residue protein of 43 kDa. It contains 5 coding and 2 noncoding exons [32] and can undergo alternative splicing to generate a variety of mRNAs that may express up to 11 different protein isoforms [41]. Although the expression of full-length TDP-43 is generally the only isoform detected in human tissue and cell lines, two additional isoforms have recently been identified in human brain and spinal cord tissue [42]. One of these isoforms varies from full-length TDP-43 in the absence of 6 nucleotides immediately 5' to the stop codon. The other lacks the exon 3 encoded segment and a significant portion of exon 6 encoded amino acids, resulting in a predicted molecular weight of 28 kDa [42]. In addition to alternative splicing, two novel TDP-43 isoforms are reportedly generated by translation at alternate in-frame translation start sites downstream of the natural initiation codon [43]. These two isoforms, termed p35iso (35 kDa) and p25iso (25 kDa), are translated from methionine 85 and amino acids 160–169, respectively [43].

The primary amino acid structure of TDP-43 resembles that of members of the heterogeneous ribonucleoprotein (hnRNP) family, a group of proteins that bind heterogeneous nuclear RNAs and have diverse functions within cells [44]. Like hnRNP members, TDP-43 contains two highly conserved RNA recognition motifs (RRM), which

are involved in RNA and DNA binding, as well as protein–protein interactions [39,45–47] (Figure 1). The RRM2s contain two conserved segments: a RNP2 hexapeptide sequence located at the N-terminus of the domain and a RNP1 octapeptide consensus sequence [48]. TDP-43 from human, mouse, *Drosophila melanogaster* and *Caenorhabditis elegans* are highly homologous up to the carboxyl boundary of the RNP1 in RRM2 [41]. The function of the N-terminal region of TDP-43 is not fully characterized but this region is likely required for normal TDP-43 CFTR exon 9 exclusion as a GFP-tagged N-terminal deletion mutant, GFP-TDP_{76–414}, lacks exon 9 skipping activity even though it contains both RRM2s and localizes to the nucleus [49]. Additionally, TDP-43 is believed to form homodimers within cells [45] and the binding of TDP-43 to other TDP-43 molecules is enhanced by the presence of an intact N-terminus [49]. The glycine-rich region in the C-terminal tail of TDP-43 is involved in the interaction of TDP-43 with other proteins, like hnRNP A1 and hnRNP A2/B1 [50], and is important for CFTR exon-skipping activity [41,51]. Residues 321–366 is the minimal region required for TDP-43 binding with hnRNP A2 and for splicing inhibitory activity [52]. The C-terminus of TDP-43 also influences the solubility and cellular localization of TDP-43 as its deletion decreases TDP-43 mobility between the cytoplasm and the nucleus [53].

TDP-43 is predominantly a nuclear protein but does shuttle between the nucleus and cytoplasm in a transcription-dependent manner [53,54]. That TDP-43 contains a specific bipartite nuclear localization signal (NLS) sequence and a leucine-rich nuclear export signal [42,54], in addition to the RRM2s, suggests that TDP-43 plays a role in nuclear to cytosolic shuttling of mRNAs (Figure 1). Several potential phosphorylation sites are present within TDP-43, including 41 serine, 15 threonine and 8 tyrosine residues (Figure 1). Finally, three putative caspase-3 recognition motifs (DXXD) are present, the cleavage of which is predicted to generate C-terminal fragments of approximately 42, 35 or 25 kDa [55] (Figure 1).

TDP-43-mediated toxicity: toxic gain of function and loss of function

TDP-43-mediated toxicity via toxic gain of function

One of the most striking pathological features of TDP-43 proteinopathies is the cytoplasmic deposition of TDP-43 aggregates, the presence of which is associated with a concomitant loss of nuclear TDP-43. Moreover, pathological TDP-43 is phosphorylated and cleaved to generate C-terminal fragments of approximately 24–26 kDa [1,2]. TDP-43 truncation may be region-specific; an enrichment of C-terminal fragments is observed in TDP-43 inclusions in the hippocampus and cortex of FTLD-U, ALS and AD cases, but lesions in motor neurones are comprised primarily of full-length TDP-43 [56]. In addition to phosphorylation and truncation, TDP-43 ubiquitination is reported in ALS and FTLD-U [1], although this has met with some debate. Using three-dimensional deconvolution imaging, round inclusions in ALS appear to be comprised of a halo of TDP-43 immunoreactivity surrounding an ubiquitin-positive core [57]. As for skein-like inclusions, colabelling of ubiquitin and TDP-43 is observed but some regions show TDP-43 immunoreactivity in the absence of ubiquitin immunoreactivity [57]. These results suggest that TDP-43 need not be ubiquitinated to be present in inclusions and raise the question as to the identity of the protein(s) within the core of round inclusions. Even so, it remains possible that the core contains fragments of TDP-43 that are either masked or not recognized by the TDP-43 antibody used for the study [57].

In a similar fashion to other protein-misfolding diseases, TDP-43-mediated toxicity may result, at least in part, from a toxic gain of function from the cytoplasmic and nuclear TDP-43 inclusions. TDP-43 is an inherently aggregate-prone protein that forms aggregates *in vitro* ultrastructurally similar to TDP-43 deposits in degenerating neurones of ALS and

FTLD-U patients [58]. Traditionally, the toxic potential of protein aggregates is tested in animal models that overexpress the protein of interest. As the involvement of TDP-43 in neurodegeneration is a relatively new discovery, few studies examining TDP-43 overexpression and aggregation in animal models have yet been published. To study the effect of TDP-43 *in vivo*, Ronald Klein's group has taken advantage of an adeno-associated virus vector to deliver human TDP-43 to the substantia nigra of rats. Although TDP-43 is mostly nuclear in this model, diffuse and granular cytoplasmic staining, indicative of preinclusion lesions, is observed in some neurones [59]. Other key aspects of TDP-43 proteinopathies are observed, including ubiquitination, gliosis, neurodegeneration and progressive motor dysfunction. As no mature inclusions are present in rat midbrain neurones following TDP-43 overexpression, neuronal death is not caused by the overt presence of TDP-43 inclusions in this model, suggesting that toxicity can occur even in the absence of TDP-43 aggregation. This is further substantiated by a mouse model of TDP-43 proteinopathy. Wegorzewska and colleagues recently generated transgenic mice expressing a mutant form of human TDP-43 under the control of the mouse prion protein (PrP) promoter [60]. In these PrP-TDP43^{A315T} mice, ubiquitin-positive, but TDP-43-negative, aggregates accumulate in specific neuronal populations and progressive, fatal neurodegeneration is observed [60]. These results again suggest that TDP-43 toxicity does not require the formation of cytoplasmic TDP-43 aggregates. Nonetheless, this does not discount the cytotoxic potential of TDP-43 aggregates nor the possibility that their formation would enhance or hasten TDP-43-mediated neurodegeneration. For instance, in yeast overexpressing full-length human TDP-43 or various TDP-43 truncation products, only those species that form aggregates and contain an intact RRM are toxic, suggesting that TDP-43 aggregation causes a toxic gain of function [61]. In fact, ALS-linked mutations that accelerate aggregation of human TDP-43 *in vitro* promote TDP-43 toxicity in yeast [58]. Further evidence that TDP-43 aggregation is toxic is provided by a model in which C-terminal TDP-43 fragments, corresponding to caspase cleavage products of TDP-43, are overexpressed in differentiated M17 neuroblastoma cells [49]. The aggregation of these fragments is associated with increased cytotoxicity, which likely results from a toxic gain of function as aggregate formation in this model does not markedly alter the nuclear distribution of endogenous full-length TDP-43, nor does it reduce nuclear TDP-43 function, as assessed using a CFTR exon 9 skipping assay [49]. Despite the evidence that TDP-43 aggregates are cytotoxic, the mechanisms of toxicity have yet to be uncovered. As future studies investigate this question, it must be kept in mind that, while TDP-43 aggregates may be toxic in and of themselves, their presence is accompanied by a reduction in nuclear TDP-43 and, consequently, a loss of normal TDP-43 function.

TDP-43-mediated toxicity via loss of function

To fully appreciate the consequences of loss of TDP-43 function, it is essential to understand what functions are normally carried out by this protein. Unfortunately, the exact roles of TDP-43 remain largely unknown at this time. Most studies have so far concentrated on the nuclear properties of TDP-43 and its involvement in specific pre-mRNA splicing and transcription events. Within the nucleus, TDP-43 forms discrete nuclear substructures that bridge other nuclear bodies [62]. It is concentrated in euchromatin domains, specifically in perichromatin fibrils, which are sites of transcription and co-transcriptional splicing [63]. TDP-43 regulates the transcription of human immunodeficiency virus type 1 [39], SP-10 [64] and cyclin-dependent kinase 6 (Cdk6) [65], and also regulates the splicing of CFTR exon 9 [40], apolipoprotein A-II exon 3 [66] and survival of motor neurone 2 exon 7 [67]. Furthermore, TDP-43 may be involved in the biosynthesis of microRNAs as it associates with human and mouse microprocessor complexes [68,69].

In addition to its nuclear activities, it is becoming increasingly evident that TDP-43 carries out important functions within the cytoplasm. For instance, TDP-43 is a component of RNP complexes that consists of mRNAs, noncoding RNAs and RNA-binding proteins [70–73]. Cytoplasmic mRNA transport granules, processing bodies and stress granules are among the various types of RNP complexes. Transport granules are involved in transporting specific mRNA to cellular compartments for localized translation. For example, the delivery of mRNA to dendrites of mature neurones allows local protein synthesis at the synapse, which is required for some forms of activity-dependent synaptic plasticity. Processing bodies are sites for storage of repressed mRNAs and for mRNA degradation, while stress granules, which assemble in response to cellular stressors, prevent mRNAs from initiating translation, perhaps to allow the translation of those proteins involved in cellular recovery [74]. That TDP-43 is present in transport granules, processing bodies and stress granules in rodent and human neurones suggests that TDP-43 plays a role in RNA transport, stabilization, translation and degradation [43,70–73,75].

In primary rat hippocampal neurones, the post-synaptic localization of TDP-43, in the form of RNA granules containing mRNA for β -actin and calcium calmodulin dependent kinase (CaMKII α), is enhanced following KCl-induced depolarization [71]. Furthermore, repetitive depolarization increases the colocalization of TDP-43 with fragile X mental retardation protein and Staufen 1, two RNA-binding proteins that regulate mRNA transport and local translation in neurones, establishing that TDP-43 is a neuronal activity-responsive factor [71]. It is noteworthy that TDP-43 colocalizes strongly with Staufen in human motor neurones from control subjects [73]. In contrast, the colocalization of TDP-43 with exoribonuclease-1, a marker of processing bodies, and with T cell internal antigen-1, a stress granule marker, is observed in motor neurones from ALS patients [73]. When present in processing bodies, TDP-43 may act as a translational repressor as it inhibits translation *in vitro* [71]. The normal function of TDP-43 in stress granules is not yet known but may ultimately serve as a protective mechanism following injury. For example, TDP-43 relocates to stress granules in response to sciatic axotomy in mice [72]. Following axotomy, TDP-43 expression is upregulated in motor neurones with cytosolic TDP-43 colocalizing with Staufen or T cell internal antigen-1 [72]. However, Moisse and colleagues found that the upregulation of TDP-43 expression normally observed in wild-type mice after axotomy is altered in neurofilament light (NFL) knockout mice, a model of NFL mRNA suppression representing an ALS-related abnormality. In NFL(–/–) mice, the upregulation of cytosolic TDP-43 is delayed and associated with caspase 3 activation and neuronal death [76]. As TDP-43 binds and stabilizes NFL mRNA [42], it was proposed that, in the absence of NFL mRNA, TDP-43 is free to interact with and modulate other mRNA substrates, eventually leading to neuronal death [76]. Taken together, these findings suggest that TDP-43 plays a role in RNA processing that may be especially important for proper synaptic function and for enabling a response to, and recovery from, stressful stimuli. However, if the ability of TDP-43 to carry this role is impeded, neurotoxicity may ensue.

Besides TDP-43, various other proteins present in RNP complexes, like fragile X mental retardation protein, survival of motor neurone and FUS, associate with neurological disorders [77], thus emphasizing the involvement of RNA dysregulation in neurodegeneration. In fact, just as mutations in *TARDBP* cause ALS, so do mutations in *FUS* [19,20]. FUS-immunoreactive cytoplasmic inclusions and lower motor neurone degeneration are observed in ALS cases with *FUS* mutations [19,20]. FUS pathology is also observed in atypical FTL-D-U cases with TDP-43-negative inclusions [18]. The absence of TDP-43 pathology in cases with FUS-positive inclusions implies that the neurodegenerative process driven by abnormal FUS is independent of those driven by TDP-43. However, as both proteins are involved in the development of ALS or FTL-D-U, it is most likely that these two proteins, which share structural and functional similarities, would also share

common mechanisms of toxicity. Both TDP-43 and FUS are predominantly nuclear but shuttle to and from the cytoplasm. They are capable of DNA and RNA binding, they regulate RNA transcription and splicing, and they are both found in RNA transport granules in neurones. Like TDP-43 [71], FUS translocates to dendritic spines in hippocampal neurones upon synaptic stimulation, and this is accompanied by an increase in RNA content in dendrites [78].

Based on the above findings, RNA dysregulation is likely to underlie TDP-43-mediated toxicity; nonetheless, the actual RNA targets affected by abnormalities in TDP-43 are not known. The identification of these targets is critical to fully elucidate the normal function of TDP-43 and also to shed light on how loss of TDP-43 function causes neurodegeneration. To identify relevant targets of TDP-43, Fiesel and colleagues performed differential microarray analysis of cells treated with TDP-43 siRNA vs. scrambled siRNA [79]. Using this approach, they found that histone deacetylase 6 (HDAC6) is downregulated by TDP-43 silencing in HEK293E and SH-SY5Y cells. Moreover, TDP-43 knockout in *Drosophila* also leads to reduced levels of HDAC6 mRNA and protein. As HDAC6 plays a role in protein aggregation as well as in autophagy-mediated degradation of misfolded proteins, Fiesel and colleagues examined the effect of TDP-43 knockdown on the turnover rate of polyQ-expanded ataxin-3. They found that TDP-43 knockdown in cells expressing ataxin 3 with an expanded polyQ tract reduces the formation of large aggregates but enhances proteotoxicity in an HDAC6-dependent manner [79]. These results suggest that loss of TDP-43 function may cause neurotoxicity via the down-regulation of HDAC6 and the subsequent impairment in toxic protein turnover. At present, few studies have directly examined how loss of TDP-43 affects neuronal survival. Even so, there is evidence that reducing or preventing TDP-43 expression is indeed neurotoxic. Loss of TDP-43 in human cell lines by RNAi causes aberrant nuclear morphology, misregulation of the cell cycle and apoptotic cell death [65]. These effects occur due to increased Cdk6 expression following TDP-43 depletion, which in turn increases the phosphorylation of the retinoblastoma protein. While these studies were carried out in non-neuronal cells, increased Cdk6 activity and retinoblastoma protein phosphorylation have been linked to neuronal death in a number of cell and animal models of neurodegeneration [80,81]. In *Drosophila*, the depletion of the TDP-43 homologue, TBPH, appears to cause synaptic toxicity. TBPH depletion adversely affects motor neurone presynaptic terminals at neuromuscular junctions, as reflected by a reduced number of axonal branches and synaptic boutons, causes deficits in locomotive behaviours and reduces life span [82]. In addition to playing a role in regulating neuronal axonal structure, TDP-43 likely plays a role in dendritic structural integrity. Loss of TDP-43 decreases dendritic branching in *Drosophila* larvae while the overexpression of either *Drosophila* or human TDP-43 promotes dendritic branching [83]. Similarly, TDP-43 knockdown in differentiated Neuro-2a cells inhibits neurite outgrowth and induces cell death through the dysregulation of Rho family GTPases [84], again demonstrating that loss of TDP-43 function is detrimental.

Pathological TDP-43 modifications associated with disease

Altered cellular distribution and truncation of TDP-43

Both the aggregation of TDP-43 and loss of TDP-43 function are likely to result from the abnormal cellular distribution and post-translational modifications of TDP-43. It is not yet clear what events initiate these changes nor is it entirely understood how TDP-43 mislocalization, truncation and phosphorylation, now considered hallmark features of TDP-43 proteinopathies, contribute to disease pathogenesis. One study has shown that inhibiting autophagic degradation in cultured cells by downregulating the expression of ESCRT (endosomal sorting complex required for transport) subunits leads to the depletion of nuclear TDP-43 and the formation of cytoplasmic, ubiquitin-positive TDP-43 aggregates

[85]. As the ESCRT machinery plays a critical role in the multivesicular body pathway, these findings suggest that multivesicular body dysfunction and/or impaired autophagy are involved in the mislocalization and accumulation of TDP-43. It has also been postulated that TDP-43 mislocalization occurs due to the aberrant cleavage of TDP-43 while it shuttles between the nucleus and cytoplasm. C-terminal TDP-43 fragments lacking a NLS that are produced by proteolytic cleavage could not regain access to the nucleus and would therefore accumulate in the cytoplasm. As C-terminal TDP-43 fragments are recovered in the sarkosyl-insoluble fraction of affected brain regions of ALS and FTL-DU patients [1], these fragments may seed the aggregation of TDP-43 into inclusions. In further support of this, studies have shown that preventing the nuclear import of TDP-43, either by mutating the NLS or by expressing C-terminal fragments that lack the NLS, causes TDP-43 to aggregate into insoluble cytoplasmic inclusions in various cell models [49,54,61,86,87]. Moreover, by sequestering full-length TDP-43, the aggregates thus formed may further deplete the nucleus of TDP-43 [54,87]. The ability of cytosolic TDP-43 inclusions to sequester nuclear TDP-43, however, may be influenced by the specific TDP-43 products present in the cytosolic inclusions.

It is reasonable to believe that more than one species of C-terminal TDP-43 fragments are present in TDP-43 proteinopathies. As TDP-43 contains 3 putative caspase-3 cleavage consensus sites (DXXD) (Figure 1), caspase-cleaved TDP-43 may account for some of the C-terminal fragments observed in disease. Using a variety of proteomic approaches to identify protease substrates following Fas- or staurosporine-induced apoptosis in Jurkat cells, three studies identified TDP-43 as a caspase substrate [88–90]. Incidentally, some hnRNP members were identified in these screens [88–90], and FUS was also recognized as a caspase substrate [88]. The caspase cleavage site (DWFD) in FUS is located at residues 352–355 within its RRM [88]. With regards to TDP-43, Zhang and colleagues have shown that the incubation of recombinant TDP-43 with caspase-3 or caspase-7, but not caspase-8, produces distinct fragments of approximately 42, 35 and 25 kDa *in vitro* [55]. In cultured cells, the activation of caspase 3 by exposure to staurosporine decreases the amount of full-length TDP-43 and increases the level of insoluble 25 and 35 kDa TDP-43 fragments [43,55,91]. Also observed is a 37-kDa TDP-43 product phosphorylated at S409/S410, presumably the phosphorylated form of the 35 kDa fragment [91]. That low levels of 35 kDa TDP-43 are present even in the absence of staurosporine, suggests that caspase-mediated processing of TDP-43 is of physiological relevance [55,91]. Alternatively, this 35 kDa product may represent p35iso, the aforementioned isoform of TDP-43 produced by translation from methionine 85 [43]. Importantly, staurosporine-induced caspase activity leads to TDP-43 cleavage and its redistribution from the nucleus to the cytoplasm in H4 cells overexpressing wild-type TDP-43 but not TDP-43_{D89E,D219E} in which the consensus motifs for caspase cleavage are mutated [55]. Together, these findings indicate that caspase-cleavage of TDP-43 may account, at least in part, for the pathological redistribution of TDP-43 from the nucleus to the cytoplasm. Of interest, the presence of N-terminal caspase-cleaved fragments in Hirano bodies, tangles, reactive astrocytes and neuritic plaques of the AD brain has been demonstrated using an antibody that specifically detects the N-terminal fragment of TDP-43 following cleavage at DVMD²¹⁹ [92].

The molecular basis for altered TDP-43 metabolism remains largely unknown although caspase 3 activation is present in both FTL-DU and ALS [93,94]. Given that proteasome inhibition in cultured cells promotes caspase-mediated cleavage and accumulation of endogenous TDP-43 [32], as well as enhances the aggregation potential of cytoplasmic TDP-43 [95], proteasome dysfunction may be involved in TDP-43 truncation and aggregation, as well as the accumulation of ubiquitinated TDP-43. In addition to proteasome inhibition, caspase 3 activation and subsequent TDP-43 truncation were shown to occur following the downregulation of progranulin in cultured cells [55]. Null mutations in the

gene encoding progranulin are a cause of FTL-D-U with TDP-43-positive inclusions [1,3,4,96,97]. When progranulin haploinsufficiency was modelled in cultured cells using progranulin siRNA, activated caspase-3 levels were increased as was the cleavage of TDP-43 into 25 and 35 kDa fragments, similar to those found in brains of FTL-D-U patients [55]. These findings provide a mechanistic link between decreased progranulin expression and TDP-43 pathology. However, as a second group failed to replicate these findings [91,98], the involvement of progranulin in the cleavage of TDP-43 is presently unclear.

While Nishimoto and colleagues have confirmed that caspase-dependent TDP-43 fragments are produced under apoptosis, they also discovered that the TDP-43 isoforms, p35iso and p25iso, are translated in a caspase-independent fashion [43]. Additionally, other C-terminal TDP-43 products have been identified by two groups that set out to specifically identify the C-terminal fragment observed in FTL-D-U [86,87]. Using cortical urea extracts of FTL-D-U brains, a 22 kDa fragment spanning amino acid residues 208–414 was identified by Dr Lee's group [86]. In a second study conducted by Nonaka and colleagues, two different C-terminal TDP-43 cleavage sites were found: Met218-Asp219 and Glu246-Asp247 [87]. That neither of these two studies identified the same fragments substantiates the notion that multiple TDP-43 truncation products are present in disease. The identity of these products, as well as how they are generated, will certainly be the focus of future studies.

Consequences of TDP-43 truncation—Although many questions remain, much effort has been directed towards understanding how TDP-43 truncation contributes to disease pathogenesis. To study the effects of caspase-cleaved TDP-43, Zhang and colleagues transfected HEK293 cells to express GFP-tagged TDP-43 fragments corresponding to the 35 and 25 kDa C-terminal products generated by cleavage of TDP-43 at residues 89 or 219, respectively (GFP-TDP_{90–414} and GFP-TDP_{220–414}). In contrast to the nuclear localization of full-length GFP-TDP-43, GFP-TDP_{90–414} and GFP-TDP_{220–414} are present in both the cytoplasm and nucleus [49]. A similar pattern of distribution is observed in HeLa cells transfected with V5-tagged TDP-43_{90–414} (TDP_{90–414}-V5) [91] or FLAG-TDP_{90–414}-V5 [43]. While TDP_{90–414}-V5 was not shown to aggregate in the time-frame studied [91], GFP-TDP_{90–414}, GFP-TDP_{220–414} and FLAG-TDP_{90–414}-V5 form compact insoluble cytoplasmic inclusions that are phosphorylated and ubiquitinated [43,49]. GFP-TDP_{220–414} expression is toxic in differentiated neuroblastoma cells and this likely occurs through a gain-of-function of the GFP-TDP_{220–414} aggregates. GFP-TDP_{220–414} binds only weakly to full-length TDP-43 and does not markedly sequester full-length TDP-43 from the nucleus. Furthermore, GFP-TDP_{220–414} does not alter CFTR exon 9 exclusion activity of either endogenous or exogenous full-length TDP-43, indicating that overexpression of this C-terminal fragment is not likely to inhibit normal TDP-43 function through sequestration [49].

As observed with GFP-TDP_{220–414} [49], the overexpression of fragments identified in FTL-D-U brains, TDP_{208–414} [86], GFP-TDP_{219–414} and GFP-TDP_{247–414} [87], results in the formation of ubiquitinated TDP-43 aggregates displaying phosphorylation at S409/S410 in cultured cells. Yet, unlike GFP-TDP_{220–414}, these fragments do modestly decrease endogenous TDP-43 CFTR exon 9 skipping activity, suggesting a loss of function consequence. Whether the decrease in TDP-43 splicing activity results from the sequestration of endogenous TDP-43 by these fragments was not examined but, GFP-TDP_{218–414}, a fragment almost identical to GFP-TDP_{219–414}, does sequester both endogenous and exogenous full-length TDP-43, albeit moderately [87]. Immunoprecipitation experiments show that C-terminal fragments, like GFP-TDP_{220–414} and GFP-TDP_{218–414}, only weakly interact with full-length TDP-43 compared with N-terminal fragments and other full-length TDP-43 molecules [49,87]. In addition to sequestering nuclear TDP-43, it has been proposed that cytoplasmic C-terminal fragments

may adversely affect nuclear TDP-43 function by interacting with hnRNP A/B proteins as they shuttle between the cytoplasm and the nucleus, and thus perturb the equilibrium of available nuclear hnRNP proteins. However, while GFP-TDP₂₂₀₋₄₁₄ does bind strongly to hnRNPA2 [49], hnRNPs A1, A2/B1 and C1/C2 have not been detected in TDP-43-positive inclusions in FTL-D-U brains and no change in their sub-cellular distribution has been observed [99].

Regardless of the inability of some C-terminal fragments to sequester nuclear TDP-43, the cleavage of TDP-43 itself would result in loss of TDP-43 function given that these truncation products lack key functional domains [47]. Various C-terminal fragments, including TDP₂₀₈₋₄₁₄, GFP-TDP₂₁₈₋₄₁₄ and GFP-TDP₂₂₀₋₄₁₄, do not enhance skipping of exon 9 in a CFTR splicing assay as does full-length TDP-43, indicating that the N-terminal region of TDP-43 is required for this function [49,86,87]. Although it could be assumed that loss of activity results from the cytosolic localization of these fragments, that is not necessarily so. As mentioned, the N-terminal deletion mutant, GFP-TDP₇₆₋₄₁₄, localizes to the nucleus but still fails to enhance exon 9 skipping. In fact, when co-expressed with full-length TDP-43, GFP-TDP₇₆₋₄₁₄ inhibits full-length TDP-43 function [49].

TDP-43 phosphorylation

Phosphorylation of full-length TDP-43, resulting in a product of 45 kDa, is observed in FTL-D-U and ALS brains but not in control cases [1,2]. To identify the critical phosphorylation sites of TDP-43, Hasegawa and colleagues generated rabbit polyclonal antibodies against 36 of the 64 potential phosphorylation sites within TDP-43. Of the antibodies produced, the ones that detect the C-terminal sites, pS379, pS403/S404, pS409, pS410 or pS409/S410 are strongly immunoreactive to the ubiquitin-positive inclusions in FTL-D-U and ALS cases, the staining for pS409/S410 being particularly robust [100] (Figure 1). Similar findings using rat or mouse monoclonal antibodies to pS409/S410 have been observed [101,102]. One of the kinases responsible for the abnormal phosphorylation of TDP-43 may be casein kinase 1 (CK1). Twenty-nine CK1 phosphorylation sites have been identified in recombinant human TDP-43, 18 of which are located in the C-terminal glycine-rich region [103]. The *in vitro* treatment of recombinant TDP-43 with CK1 leads to TDP-43 phosphorylation at pS379, pS403/S404 and pS409/S410 and results in a similar electrophoretic mobility to abnormally phosphorylated TDP-43 from FTL-D-U and ALS brains [100]. These sites are only weakly phosphorylated by CK2 and are not phosphorylated by glycogen synthase kinase 3 beta [100].

Phosphorylation-dependent antibodies to pS409/S410 label all types of abnormal inclusions, such as dystrophic neurites, neuronal cytoplasmic and nuclear inclusions, as well as glial cytoplasmic inclusions, in various TDP-43 proteinopathies [100,102]. In some cases, a pS409/S410-specific antibody has been shown to label granular, cytoplasmic, 'pre-inclusions' that are not detectable by anti-ubiquitin, suggesting that phosphorylation at S409/S410 precedes ubiquitination [102]. Importantly, antibodies against pS409/S410 fail to detect nuclear TDP-43 by immunohistochemistry, providing further support that phosphorylation is a disease-specific phenomenon [100–102]. Western blot analysis of sarkosyl-insoluble fractions from FTL-D-U and ALS brains reveal that phospho-specific antibodies for TDP-43 not only stain a 45 kDa band, but also several fragments (approximately 18–26 kDa) and indistinct high molecular weight smears. Hasegawa and colleagues report that the banding pattern of the fragments differs among clinicopathological subtypes (e.g. FTL-D-U, FTL-D-MND and ALS) [100]; however, this has not been replicated by others [102]. In most cases, the TDP-43 fragments present in FTL-D-U and ALS cases are more intensely immunoreactive to antibodies against pS409/S410 compared with full-length TDP-43 [100,102]. Likewise, C-terminal TDP-43 fragments expressed in cultured cells are more strongly immunoreactive to pS409/S410 antibodies than full-length TDP-43

[49,86,87,91]. As full-length TDP-43 is mostly nuclear while C-terminal fragments are cytosolic, the enhanced phosphorylation of TDP-43 fragments may be due to increased accessibility to kinases. However, studies that examined this question using TDP-43 mutants with cytoplasmic expression provide differing results. In one study, insoluble, cytoplasmic aggregates of mutant TDP-43 with a defective NLS did show enhanced phosphorylation at S409/S410 compared with wild-type TDP-43 [86]. In contrast, Nonaka and colleagues found that the cytoplasmic expression of a TDP-43 deletion mutant lacking residues 78–84 (TDP-43 Δ 78–84), which contains a part of the bipartite NLS, does not enhance phosphorylation at S409/S410 compared with the expression of wild-type TDP-43 [95] and similar results were reported by others [49]. Interestingly, if cells expressing TDP-43 Δ 78–84 are treated with the proteasome inhibitor, MG132, cytoplasmic TDP-43 immunopositive for both anti-pS409/S410 and anti-ubiquitin is observed [95], indicating that phosphorylated TDP-43 may be targeted to the proteasome for degradation. The conflicting findings among these studies may be due to differences in cell models, TDP-43 mutations and levels of TDP-43 expression. Nonetheless, it remains possible that the enhanced phosphorylation of C-terminal fragments results, not from its cytoplasmic localization, but rather from changes in conformation that increase its recognition/binding by kinases. *In vitro* phosphorylation experiments of recombinant full-length and truncated TDP-43 will shed light on this matter.

Consequences of TDP-43 phosphorylation—While the pathological significance of TDP-43 phosphorylation is not yet known, it may promote TDP-43 aggregation. Following the *in vitro* phosphorylation of recombinant human TDP-43 by CK1, increased levels of TDP-43 oligomers are observed by Western blot analysis compared with non-phosphorylated TDP-43 [100]. Additionally, abundant filaments, immunopositive for a S409/S410 phosphorylation-specific TDP-43 antibody are seen by immunoelectron microscopy [100]. Of interest, filaments of 15 nm in diameter immunopositive for the S409/S410 phosphorylation-specific TDP-43 antibody are present in the motor neurone inclusions of ALS [100]. Although phosphorylation may enhance TDP-43 oligomerization, it may not actually be necessary for aggregation to occur. For instance, the exogenous expression of GFP-TDP_{220–414} in HEK293 cells causes the formation of S409/S410-phosphorylated cytoplasmic inclusions [49]. Yet, even when phosphorylation of the fragments is prevented by substituting serines 409 and 410 to alanines, a similar extent of aggregation and toxicity is observed [49]. Moreover, findings by Dormann and colleagues suggest that phosphorylation occurs late in the conversion of soluble to insoluble TDP-43, also indicative that phosphorylation is not a prerequisite for TDP-43 aggregation [91].

Aside from influencing the rate of TDP-43 oligomerization, TDP-43 phosphorylation is likely to affect various functions of TDP-43. Once these functions are more fully understood, studies should ascertain if and how they may be altered by aberrant TDP-43 phosphorylation. Additionally, continued research should focus on identifying the kinases, or phosphatases, responsible for altered TDP-43 phosphorylation given the potential involvement of TDP-43 phosphorylation in the progression of TDP-43 proteinopathies.

TARDBP mutations

Extensive mutation analysis of *TARDBP* has led to the discovery of missense mutations in sporadic and familial ALS [28–38,104,105], as well as one frame-shift mutation that creates a premature stop codon (Y374X) leading to the expression of truncated TDP-43 [37] (Figure 1). All reported missense mutations cluster in exon 6 of *TARDBP* except for one, D169G, which is localized in the RRM1 [31]. Even though mutations in TDP-43 have been identified in a relatively small proportion of ALS patients (approximately 2–3%), understanding how these mutations confer toxicity should provide insight on the role of TDP-43 in neurodegeneration.

Given that exon 6 encodes the highly conserved glycine-rich domain of TDP-43, mutations in this region are expected to influence protein–protein interactions and the exon skipping and splicing regulatory activity of TDP-43. Somewhat surprisingly, however, three mutations (Q331K, M337V and G348C) that occur in the 321–366 region required for hnRNP A2 interaction and splicing inhibition, fail to disrupt the binding of TDP-43 to hnRNP A2 and have no effect of TDP-43 CFTR exon 9 splicing inhibition [52]. Nonetheless, compared with human wild-type TDP-43, hTDP-43^{Q331K} and hTDP-43^{M337V} promote dendritic branching to a much lesser extent in *Drosophila* neurones [83].

Many mutations result in substitutions to threonine and serine residues [31,33,34,36,38] and may thus increase TDP-43 phosphorylation, which could adversely impact various TDP-43 functions and the oligomerization potential of TDP-43. Other mutations (Q331K, M337V, Q343R, N345K, R361S and N390D) accelerate the aggregation of TDP-43 *in vitro* and enhance aggregate formation and toxicity in yeast [58]. In contrast, ALS-linked mutations do not facilitate inclusion formation of full-length TDP-43 in SH-SY5Y cells, but they do enhance the aggregation potential of the C-terminal TDP-43 fragment, GFP-TDP_{162–414} [87]. This suggests that pathogenic mutations, in combination with N-terminal truncation, promote abnormal TDP-43 accumulation in mammalian cells [87]. Finally, some mutations may increase the propensity of TDP-43 to become fragmented. Western blot analysis of TDP-43 from frozen lymphocyte lysates revealed that the expression level of an aberrant 30–32 kDa band is elevated in samples from A382T or S393L mutation carriers, compared with samples from patients without missense mutations or from healthy controls [36]. Similarly, when lymphoblastoid cell lines derived from *TARDBP* mutation carriers are treated with the proteasome inhibitor, MG132, a marked increase in the accumulation of detergent insoluble TDP-43 fragments (approximately 25, 28 or 35 kDa), is observed that is not seen in lymphoblastoid cells from control individuals, suggesting that mutations increase TDP-43 truncation and its aggregation potential [31,32]. Biochemical analysis of TDP-43 from spinal cord extracts of autopsied cases also reveal that a 25 kDa TDP-43 fragment, and a 45 kDa TDP-43 product, are increased in the 1% sarkosyl-soluble fraction from a patient with a Q343R mutation, compared with control and sporadic ALS patients [29]. Although ALS-linked TDP-43 mutants fail to show enhanced truncation in SH-SY5Y cells [87], mutant forms of TDP-43 (M337C and Q331K) are fragmented more readily than wild-type TDP-43 in Chinese hamster ovary cells and cause neural apoptosis and developmental delay in chick embryos [28].

Despite the discovery of numerous *TARDBP* mutations in familial and sporadic ALS, many studies failed to show an association between *TARDBP* mutations and pure FTLN [32,106–108]. Recently, however, the N267S mutation initially described in a sporadic ALS case [36] was identified in a patient with the behavioural variant of frontotemporal dementia without MND [109]. Additionally, an A90V mutation was identified in a FTLN-MND patient with a family history of dementia [110], a G295S mutation in exon 6 was identified in two unrelated patients with FTLN-MND [111], and a K263E *TARDBP* variant was identified in a subject who developed frontotemporal dementia, supranuclear palsy and choreiform movements but no signs of MND [112]. The A90V mutation is located between the bipartite NLS sequence of TDP-43. The transient expression of TDP-43-A90V in QBI-293 cells partially disrupts the nuclear localization of TDP-43, thereby increasing its presence in the cytoplasm compared with wild-type TDP-43. Additionally, TDP-43-A90V accumulates, albeit somewhat modestly, in the insoluble urea fraction [110]. While it is speculated that the A90V variant may be a potential genetic risk factor for FTLN, it should be noted that this variant has also been identified in three control cases [28,31,110] so there is the possibility that it represents a rare benign polymorphism.

Taken together, these findings imply that certain missense mutations may cause a toxic gain of function through the intracellular accumulation and aggregation of proteolytically cleaved TDP-43 fragments and may also cause a loss of function through the aberrant phosphorylation and cellular distribution of TDP-43.

Conclusion

Since the recent finding that TDP-43 is a major component of ALS and FTL-DU inclusions, our understanding of this protein has expanded significantly but still much remains unknown. The abnormal phosphorylation and truncation of TDP-43 is believed to trigger its neurotoxic effects by enhancing TDP-43 aggregation and by causing loss of normal TDP-43 function. Nonetheless, the exact neurotoxic TDP-43 species have not been definitively identified, how these species are generated remains speculative and their mechanisms of neurotoxicity have yet to be established. Deciphering the harmful consequences TDP-43 modifications and altered cellular distribution is further complicated by our lack of knowledge of the functions carried out by TDP-43. A growing body of evidence provides support that TDP-43-mediated neuronal death results, at least partially, from RNA dysregulation, a mechanism of toxicity associated with various neurological disorders [77]. As this avenue is investigated in more depth, it should be kept in mind that insight on TDP-43-mediated toxicity may be gleaned by searching for mechanistic commonalities among those neurodegenerative diseases presenting with TDP-43 pathology. Furthermore, given that both TDP-43 and SOD1 are involved in ALS pathogenesis, one could easily anticipate that their mechanisms of toxicity would converge. Oxidative damage, defects in intracellular transport, excitotoxicity, aberrant RNA metabolism and mitochondrial dysfunction have all been implicated in the development of ALS [113]. Given the myriad of roles that TDP-43 is likely to play, both in the nucleus and the cytoplasm, it is quite conceivable that TDP-43 could also cause many of these events. The gaps in our knowledge regarding TDP-43 function and dysfunction, as well as how TDP-43 contributes to neurodegeneration, will surely narrow as new animal models of TDP-43 continue to emerge.

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Figure 1.

A schematic representation of the gene encoding transactive response DNA-binding protein 43 (TDP-43) and full-length TDP-43 protein. The human *TARDDBP* gene is located on chromosome 1p36 and contains 5 coding and 2 noncoding exons (panel **B**) [32]. Coding regions are shown in yellow while noncoding regions are gray. Identified mutations in exons 3, 4 and 6 of *TARDDBP* are shown using the numbering of the 414-amino acid full-length isoform of TDP-43 (panel **A**). Mutations observed in familial and sporadic ALS are indicated in blue and black, respectively. Mutations in red have been reported for both familial and sporadic ALS. The A90V mutation is shown in gray as this mutation has been observed in both affected and control individuals [28,31,110]. Panel **C** depicts the protein structure of full-length human TDP-43. Note the two RNA recognition motifs (RRM1, RRM2), the nuclear localization signal (NLS), the nuclear export signal (NES) and the glycine (Gly)-rich domain. Caspase cleavages sites of TDP-43 are indicated in panel **D** [55], as are the proteolytic sites identified in brain tissue from FTL-D-U patients [86,87]. In panel **E**, TDP-43 sites reportedly phosphorylated in FTL-D-U and ALS are shown [100]. Note that these sites are all present in the C-terminus of TDP-43.