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Gains or losses: molecular mechanisms of TDP43-mediated neurodegeneration

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Abstract

RNA-binding proteins, and in particular TAR DNA-binding protein 43 (TDP43), are central to the pathogenesis of motor neuron diseases and related neurodegenerative disorders. Studies on human tissue have implicated several possible mechanisms of disease and experimental studies are now attempting to determine whether TDP43-mediated neurodegeneration results from a gain or a loss of function of the protein. In addition, the distinct possibility of pleotropic or combined effects — in which gains of toxic properties and losses of normal TDP43 functions act together — needs to be considered.

TAR DNA-binding protein 43 (TDP43) was identified 5 years ago as the major constituent of the proteinaceous inclusions that are characteristic of most forms of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD, now known as FTLD-TDP; see BOX 1)[1]. Since then, numerous groups have confirmed this finding[2–10] and have shown that dominantly inherited genetic mutations within the gene that encodes TDP43, TAR DNA binding protein (*TARDBP*), are linked with ALS and FTLD-TDP phenotypes[11]. With over 600 studies published on TDP43 and its role in ALS and FTLD, there is now an emerging consensus that TDP43 protein is mechanistically linked to neurodegeneration.

Box 1

Nosology of frontotemporal lobar degeneration

The classification of frontotemporal lobar degeneration (FTLD) has undergone a dramatic transformation over the past decade, mainly driven by remarkable advances in understanding the pathobiology of these disorders. A subset of FTLD cases were initially given the diagnosis of ‘dementia lacking distinctive histopathology’ (DLDH) after post-mortem examination because, although extensive neurodegeneration and reactive gliosis

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Competing interests statement
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were seen throughout the CNS, no specific pathologic features were detected[146]. This was partially rectified when immunohistochemistry for ubiquitin revealed a unique pattern of ubiquitin-positive inclusions in the majority of these DLDH cases[147]. The use of ubiquitin immunohistochemistry resulted from knowledge that many intracellular inclusions that are associated with neurodegenerative diseases are ubiquitylated, including the inclusions formed by tau and α-synuclein. Thus, these enigmatic cases were given the diagnosis of 'FTLD with ubiquitin-positive, tau and α-synuclein-negative inclusions' (also known as FTLD-U). However, when these ubiquitin-positive inclusions were found to be TAR DNA-binding protein 43 (TDP43)-positive by immunohistochemistry and TDP43 was found to be the most sensitive and reliable marker of inclusions in cases such as these that also had a disease specific biochemical TDP43 signature, TDP43 was proposed to be the disease protein in FTLD-U[1]. Accordingly, FTLD cases with prominent TDP43 pathology were renamed FTLD-TDP and those with abundant tau pathology were renamed FTLD-Tau, and the small minority of cases characterized by RNA-binding protein FUS inclusions were designated FTLD-FUS[84,148]. Thus, FTLD-TDP, FTLD-Tau and FTLD-FUS represent 50%, 45% and 5% of all FTLD cases, respectively, at post-mortem examination. Finally, the finding that amyotrophic lateral sclerosis (ALS), the most common motor neuron disease, was often associated with FTLD-TDP prompted evaluation of ALS tissue, which revealed that the ubiquitylated skeins and round inclusions found in ALS were comprised of TDP43 protein in association with a disease-specific biochemical TDP43 signature similar to FTLD-TDP[1]. Furthermore, nearly all sporadic ALS cases and familial ALS cases with mutations of the gene that encodes TDP43 — TARDBP — are TDP43 proteinopathies. Thus, FTLD-TDP and ALS are two seemingly distinct neurodegenerative diseases based on their clinical manifestations, but they are both TDP43 proteinopathies and they therefore represent a spectrum of diseases ranging from relatively pure motor neuron degeneration in ALS to more prominent frontal and temporal neocortical involvement in FTLD-TDP, leading to a variable phenotype ranging from weakness and respiratory failure to psychiatric aberrations, dementia and eventual death.

TDP43 is a 414-amino-acid protein with two RNA recognition motifs (RNA-recognition motif 1 (RRM1) and RRM2) and a carboxy-terminal glycine-rich domain (FIG. 1a). Nearly all of the described ALS-associated TDP43 mutations are dominant missense mutations within the glycine-rich domain, suggesting that altering the function of this domain is sufficient to induce neurodegeneration[11–15].

The initial flurry of studies on TDP43 in ALS and FTLD-TDP described TDP43 pathology and biochemistry in human tissue (FIG.1b,c). In quick succession, TDP43 proteinopathies were documented in a wide range of other neurodegenerative diseases, including Alzheimer's disease, other tauopathies and Lewy body disorders characterized by α-synuclein inclusions[16,17]. The extent of TDP43 pathology in these other diseases is limited in terms of both the amount and distribution of TDP43 compared with cases of primary ALS and FTLD-TDP. Thus, the formation of TDP43 pathology, although a primary event in FTLD-TDP and ALS, may be a secondary event in these other diseases, and it remains to be determined whether abnormal TDP43 exacerbates the extent of neurodegeneration in these patients.

Detailed reviews of the pathology of TDP43 proteinopathies are available elsewhere[16,17]. The major disease-specific findings in FTLD-TDP and ALS — as well as, to a lesser extent, other disorders in which TDP43 pathology is found — include abnormal ubiquitylation and phosphorylation of TDP43, the presence of sarko-syl-insoluble TDP43 inclusions, the presence of truncated 20–25-kDa TDP43 C-terminal fragments (CTFs; particularly in the

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cerebral cortex), mislocalization of TDP43 protein, and loss of normal nuclear TDP43 expression. Careful interpretation of each of these findings is warranted because it is unknown which of these observations are causally linked to neurodegeneration and which are secondary to the disease pathology or are epiphenomena.

A common theme of neurodegenerative diseases is the presence of insoluble aggregates that, if they are intracellular, are often abnormally phosphorylated and ubiquitylated. The unique features of TDP43 proteinopathies are the mislocalization of the cognate protein to the cytoplasm and the loss of its normal nuclear localization. Which of these features are mechanistically linked to neurodegeneration is currently unknown and so all of the observed features of neurodegenerative diseases with TDP43 pathology, referred to as TDP43 proteinopathies, need to be considered when formulating models of disease mechanisms.

Despite the progress towards describing the full spectrum of TDP43 pathology in human neurodegenerative diseases, the fundamental question of whether TDP43 dysfunction mediates neurodegeneration through gain of toxic function or a loss of normal function remains unanswered. To formulate a mechanistic model of TDP43-mediated neurodegeneration, we will review the normal functions of TDP43 and then examine the known abnormal features of TDP43 protein in the diseased human brain. We will use this as a framework for understanding mechanisms of disease in TDP43 proteinopathies. In doing so, we will highlight the most relevant human, animal and tissue culture findings in the context of the pathways that may be responsible for TDP43-mediated neurodegeneration in ALS, FTLD-TDP and related TDP43 proteinopathies. We aim to provide here an in-depth consideration of TDP43; however, it is important to note the growing number of proteins that are linked to ALS and FTLD, information on these proteins is available in other recent reviews[18–20].

Normal TDP43 function

The best-studied functions of TDP43 are those concerning its regulation of RNA (FIG. 2). Evidence from converging lines of research suggests that TDP43 regulates RNA in a variety of ways. The RRM1 domain of TDP43 is critical for its binding to single-stranded RNA[21–24]. TDP43 preferentially binds UG repeats, but is also found to be associated with non-UG repeat sequences[21,24–27]. Indeed, studies using high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) revealed that TDP43 binds to a large proportion of the transcriptome (>6,000 RNA species), preferentially localizing to introns (including deep intronic sites), 3′ untranslated regions (UTRs) and non-coding RNAs[25,27]. The list of TDP43 RNA targets was enriched for long transcripts, and transcripts related to synaptic activity or neuronal development[25,27].

Consistent with its function as an RNA-binding protein, TDP43 associates with members of the heterogenous nuclear ribonucleoprotein (hnRNP) family of proteins that include hnRNP A2/B1, hnRNP A1, hnRNP C1/C2 and hnRNP A3 (REFS 28–30) (FIG. 2a). The interactions of TDP43 with hnRNPs are dependent on its C-terminal glycine-rich domain[28,31]. hnRNP complexes are known to regulate splicing, and TDP43 regulates the splicing of human cystic fibrosis transmembrane conductance regulator[32], survival of motor neuron[33], apolipoprotein A2 (APOA2)[34] and serine/arginine-rich splicing factor 2 (SC35)[35]. Furthermore, transcriptome-wide analysis indicates that the splicing of many additional transcripts is probably mediated by TDP43 (REFS 25,27). In addition to its effects on RNA splicing, TDP43 also influences mRNA turnover: its expression regulates mRNA levels of cyclin-dependent kinase 6 (REF. 36), histone deacetylase 6 (REF. 37), Futsch (the *Drosophila melanogaster* homologue of microtubule-associated protein 1B (MAP1B))[38] and low molecular weight neurofilament[4] (FIG. 2b). The localization of TDP43 in RNA

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granules within neuronal processes also suggests that TDP43 is involved in RNA trafficking[39,40] (FIG. 2c). A variety of stressors cause TDP43 to be redistributed from the nucleus into the cytoplasm, where it resides within stress granules, in which it presumably has a role in trafficking or stabilizing mRNAs[30,39,41–48] (FIG. 2f). Finally, TDP43 is known to interact with the RNA-binding protein FUS (also known as translocated in liposarcoma (TLS)), another RNA-binding protein implicated in motor neuron disease and FTLD[29,30].

Aside from its role in mRNA pathways, TDP43 is also thought to regulate microRNA (miRNA) biogenesis. TDP43 localizes to the perichromatin fibres in which miRNA biogenesis is thought to occur, and binds to the primary miRNA processing Drosha complex. Furthermore, knockdown of TDP43 alters the level of several miRNAs[29,49–52] (FIG. 2d). TDP43 also interacts with DNA — in particular, single-stranded DNA — and was identified as a potential regulator of acrosomal protein SP10 (*ACRVI*; also known as *SP10*) gene expression during spermatogenesis[24,53–56] (FIG. 2e). Indeed, TDP43 derives its name from its discovery in 1995, when it was shown to interact with the HIV-1 TAR DNA sequence motif[22]. Binding of TDP43 to these DNA sequences results in inhibition of transcription through unknown mechanisms.

Post-translational modification of TDP43

Like other proteins that form neurodegenerative disease-related intracellular inclusions, such as tau and α-synuclein, pathological TDP43 aggregates are ubiquitylated and phosphorylated[1]. Under normal circumstances, ubiquitylated and phosphorylated TDP43 is not readily detected in brain tissue. Thus, the very presence of these modified TDP43 species in ALS and FTLD-TDP is abnormal and seems to be disease specific; however, it is not clear whether these modifications lead to aggregate formation and/or neurotoxicity, or whether they represent a normal reaction to the presence of an intracellular aggregate and are therefore indirectly related to TDP43-mediated neurodegeneration. Interestingly, human tissue studies indicate that not all TDP43 inclusions are ubiquitin positive. In particular, so-called ‘pre-inclusions’ (granular, less dense cytoplasmic inclusions) are often not ubiquitin positive, suggesting that ubiquitylation is a relatively late phenomenon in the disease process[2,4]. By contrast, antibodies that specifically detect TDP43 that is phosphorylated at serine residues 409 and 410 seem to recognize most TDP43 inclusions as well as the truncated 25-kDa TPD43 CTFs[57,58]. These studies suggest that phosphorylation precedes ubiquitylation, but they do not address whether these post-translational modifications are mechanistically involved in TDP43-mediated neurodegeneration.

One important recent finding that may help to clarify the role of altered ubiquitylation (and consequently, of impaired protein degradation) in FTLD-TDP and ALS is the identification of rare dominant missense mutations within ubiquilin 2 (*UBQLN2*) that are associated with X-linked ALS[59]. *UBQLN2* is the gene that encodes for UBQLN2 protein, which is thought to be involved in protein degradation. The disease-associated mutations were shown to cause impaired ubiquitin–proteasome degradation in cultured cells. Pathologic examination of CNS tissue from patients with *UBQLN2* mutations showed that the CNS of these patients harbour TDP43 inclusions in addition to novel UBQLN2-positive inclusions, suggesting that perturbation of protein degradation pathways is mechanistically linked to the formation of inclusions, including TDP43 inclusions. These human studies serve as the backdrop for the experimental studies in cultured cells and animals reviewed below.

Ubiquitylation

Cells are thought to dispose of TDP43 protein through the ubiquitin–proteasome system (UPS), consistent with the ubiquitylation of overexpressed TDP43 CTFs in a variety of cell lines and over-expressed full-length TDP43 in a variety of transgenic animal models[60–70].

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Thus, disruption of the UPS might contribute to increased levels of ubiquitylated TDP43 in ALS and FTLD-TDP. Indeed, inhibition of the UPS leads to increased levels of phosphorylated TDP43 aggregates in cultured cells[69]. Full-length TDP43 has an exceptionally long half-life of 12–34 h in most cell types, whereas TDP43 CTFs have a half-life of only ~4 h[29,71] (FIG. 3). As both full-length TDP43 and truncated TDP43 are degraded by the UPS[65,69,70], the differences in their turnover rates may be due to other factors such as their differential interactions with the UPS, particularly as TDP43 CTFs lack the two nuclear localization signal (NLS) motifs and are therefore predominantly cytoplasmic[65,69]. Given the lability of TDP43 CTFs, the accumulation of cytoplasmic TDP43 CTFs in diseased brain and various cellular models is remarkable and may be a consequence of increased cleavage of full-length TDP43 or an impaired ability to degrade TDP43 CTFs.

Autophagosome-mediated degradation — in which cytoplasmic proteins or organelles are targeted to membrane-bound vesicles that are in turn fused with lysosomes — may also be involved in TDP43 protein turnover[72–76]. Evidence for autophagic degradation of TDP43 includes known interactions of TDP43 with a variety of proteins that are involved in autophagy or related pathways, including the endosomal sorting complexes required for transport (ESCRTs)[75], ubiquilin 1 (UBQLN)[73], sequestosome 1 (REF. 74) and STAM-binding protein (STAMBP; also known as AMSH)[77]. ESCRTs are required to traffic proteins into multivesicular bodies, and depleting cells of ESCRT subunits inhibits autophagy and results in increased ubiquitylated, cytoplasmic TDP43 (REF. 75). Notably, mutations of charged multivesicular body protein 2B (CHMP2B), an ESCRT subunit, are linked to FLTD, although these rare cases do not exhibit TDP43 inclusions[78]. Thus, perturbation of autophagy-related pathways results in a clinical phenotype similar to FTLD-TDP, and this may reflect degeneration of similar neuronal populations. Finally, UBQLN is an adaptor protein that functions to deliver ubiquitylated proteins to the proteasome and also regulates autophagy. UBQLN overexpression in HeLa cell cultures results in increased numbers of cytoplasmic TDP43 aggregates that colocalize with both UBQLN and a marker of LC3-positive autophagasomes[73]. However, overexpression of UBQLN reduced survival in *D. melanogaster* and enhanced toxicity in HeLa cell cultures despite reducing TDP43 levels[79].

These studies demonstrate a role for the UPS and autophagosome systems in TDP43 turnover, and the identification of dominant *UBQLN2* mutations suggests that a primary alteration in protein degradation pathways can lead to TDP43-mediated neurodegeneration. However, there is little evidence that a protein degradation defect is primarily responsible for TDP43 dysfunction in sporadic and familial cases without UBQLN2 mutations.

Phosphorylation

TDP43 CTFs or cytoplasmic TDP43 are phosphorylated in a variety of cell lines that are engineered to overexpress TDP43 (REFS 65,67–70). Overexpression of full-length TDP43 in a variety of transgenic animal models also leads to the presence of phosphorylated TDP43 aggregates that are similar to those seen in ALS and FTLD-TDP[60–64,66]. Phosphorylated TDP43 proteins exhibit a longer half-life than non-phosphorylated proteins, suggesting that phosphorylation may inhibit UPS-mediated degradation and thus contribute to aggregate formation[70]. Alternatively, as TDP43 phosphorylation is associated with TDP43 insolublility, it remains possible that aggregation may render TDP43 CTFs less vulnerable to UPS-mediated degradation and that phosphorylation is merely a marker of aggregation (FIG. 3). Another study showed that mutating serine residues 409 and 410 of disease-associated mutant TDP43 to alanines (thereby mimicking non-phosphorylated TDP43) eliminated its neurotoxicity when overexpressed in a *Caenorhabditis elegans* model[80].

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Although wild-type TDP43 was also moderately neurotoxic, introducing the same serine to alanine mutations in wild-type TDP43 did not mitigate neurotoxicity[80].

In summary, phosphorylation seems to be a relatively early event in the onset and progression of ALS and FTLD-TDP. There is some evidence that TDP43 phosphorylation may result in differential degradation and/ or toxicity of the protein, but the precise role of TDP43 phosphorylation in mechanisms of disease remains unclear (FIG. 3). TDP43 phosphorylation is tightly correlated with TDP43 aggregation, and thus it is difficult to know whether phosphorylation is a cause or effect of aggregation. Indeed, phosphorylation is not an absolute requirement for TDP43 cleavage, aggregation or toxicity in cell culture systems[81,82]. Also, disease-associated TDP43 mutations have not been shown to alter TDP43 phosphorylation, and the effects of TDP43 phosphorylation on normal TDP43 function are unknown.

TDP43 insolubility and aggregation

The defining histopathology of FTLD-TDP and ALS is the presence of dense TDP43 aggregates in affected neurons and glia of the CNS[1,83]. The presence of abnormal proteinaceous aggregates is a common theme among most age-related neurodegenerative diseases, although in most cases whether the inclusions are harmful, protective or both at different stages of the disease process remains a matter of debate (FIG. 4). Indeed, the strong correlation between TDP43 aggregation and a host of other pathologic changes (such as nuclear clearance, ubiquitylation, phosphorylation and mislocalization) makes it difficult to definitively designate aggregation as the pathogenic event that culminates in disease.

The lifecycle of TDP43 inclusions has been surmised based on both pathologic and experimental observations. The appearance of granular cytoplasmic TDP43 aggregates that are designated as pre-inclusions has been postulated to be an early event in the formation of TDP43 inclusions[2–4,10]. Notably, neurons that harbour pre-inclusions also exhibit the characteristic absence of normal nuclear TDP43 immunoreactivity. Mature aggregates in ALS are characteristically dense, round inclusions or filamentous skeins in the cytoplasm of affected motor neurons, whereas in FTLD-TDP they are typified by a variety of cytoplasmic, neuritic and intranuclear inclusions in a diverse group of forebrain neurons[1,83–85]. The importance of the diversity of morphologies is unknown.

Purified TDP43 protein is prone to aggregation *in vitro*[86], consistent with the presence of TDP43 inclusions in many cellular and animal models of TDP43 proteinopathy[60–66,68,69]. In cell culture systems, only rare TDP43 aggregates are seen upon overexpression of full-length TDP43. However, overexpression of either cytoplasmically restricted TDP43 (restricted by mutating its NLS motifs) or truncated TDP43 CTFs yields numerous cytoplasmic inclusions[65,68,69]. Transducing recombinant pre-formed TDP43 aggregates into the cytoplasm of TDP43 overexpressing cells can also induce TDP43 aggregates[56], presumably through a ‘seeding’ reaction, similar to that recently observed for tau and α-synuclein[87–90]. Consistent with these data is the recent identification of a C-terminal prion-like domain in TDP43 using a computational algorithm to identify prion-like domains[91]. This prion-like domain has been implicated in aggregation of TDP43 in cultured cells, and it is thought to confer biochemical properties associated with prions including aggregation, protease resistance and cell-to-cell propagation (but not necessarily infectivity)[86,92–94].

The biochemical correlate of TDP43 inclusions is the accumulation of various sarkosyl-insoluble TDP43 species. A sizeable proportion of the 43-kDa TDP43 protein is normally sarkosyl-insoluble, and the amount of this normal TDP43 protein is not appreciably different in normal and diseased brain homogenates. However, other insoluble TDP43 species that are

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found in diseased brains include a phosphorylated 45-kDa species, a high molecular weight ubiquitylated species and CTFs of approximately 20–25 kDa[1] (FIG. 1c).

Examination of patients with sporadic ALS who had the disease for an unusually long duration (10–20 years) showed that the number of TDP43 inclusions was low relative to standard cases of sporadic ALS[95]. The fact that the number of inclusion-bearing neurons is low in cases of ALS with long disease duration suggests that neurons that harbour TDP43 inclusions earlier in the disease process are prone to degeneration. Indeed, TDP43 aggregates are seen in the centre of all neuronophagic cell clusters in the anterior horn of the spinal cord in ALS[96]. The observation that there is selective neuronal degeneration of TDP43 inclusion-bearing cells suggests that TDP43 aggregates are tightly linked with neurodegeneration in a cell autonomous manner. However, these findings do not tell us whether TDP43 aggregates themselves are toxic, or whether they are a response to injury and therefore just a marker of another neurotoxic process.

Transgenic animals that express full-length TDP43 also exhibit TDP43-positive inclusions, although in general they are rare[60–64,66]. Quantification of TDP43-positive inclusions in transgenic mice revealed no correlation between the number of inclusions and neurodegeneration[66]. Thus, TDP43 aggregation itself does not seem to be an absolute requirement for TDP43-mediated neurodegeneration *in vivo*, although it is possible that TDP43 inclusions are sufficient to induce neuron death. Nevertheless, TDP43 inclusions have been postulated to result in a toxic gain of function by virtue of abnormal 'activity' conferred by altered conformation[1]. Finally, several — but not all — TDP43 transgenic mouse models exhibit cytoplasmic inclusions comprised of mitochondrial aggregates that are generally negative for TDP43 (REFS 60–64). Similar mitochondrial inclusions are not observed in human cases and their relevance to human disease is unknown. However, TDP43 toxicity in yeast is partly dependent on mitochondria and oxidative stress[97], suggesting that independent of aggregation, one possible mechanism of neurodegeneration may be abnormal alterations in metabolic pathways.

The effect of TDP43 aggregation on neuronal viability remains unclear. Experimental models have demonstrated that TDP43 aggregates are not necessary for TDP43-mediated neurodegeneration, although this does not preclude the possibility that aggregates may still contribute to neuron dysfunction and death. In particular, the emerging concept of prion-like cell-to-cell transmission remains to be tested with regard to TDP43 proteinopathies, and more clarity on this concept for TDP43 proteinopathies could advance understanding of how TDP43 aggregation and/or fibrilization contribute to the anatomic spread and clinical progression of these diseases.

TDP43 truncation

As described above, the biochemical signature of FTLD-TDP includes the presence of truncated TDP43 protein, evident as 20–25-kDa species in sarkosyl-insoluble brain lysates[1,98]. Amino-terminal sequencing of brain-derived TDP43 fragments demonstrated that at least one of these fragments consists of a CTF beginning at Arg208 of TDP43 (REF. 65). However, there is considerable variability in the number of CTF bands from case to case. Interestingly, inclusions within the brain of ALS and FTLD-TDP patients are readily labelled with antibodies that recognize the C terminus of TDP43, but not with N-terminal TDP43 antibodies[99]. By contrast, spinal cord inclusions are labelled with both N- and C-terminal TDP43 antibodies, suggesting that they are comprised of full-length TDP43 (REF. 99). This regional heterogeneity in terms of CTF formation suggests that it may not be necessary for TDP43-mediated neurodegeneration. Another ~35-kDa fragment was found in lymphoblastoid cell lines derived from TDP43 mutation carriers[13,100], but this species is low

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or absent in brain lysates[1,57,58]. Furthermore, in our experience, it is variably present in ALS and FTLD-TDP human brain homogenates and its presence does not correlate with disease status. These various fragments are presumably generated by proteolytic cleavage of full-length TDP43, although they could reflect splice variants or cryptic transcription start sites[44]. The apoptosis regulator caspase 3 has been proposed as the major protease responsible for generating the 25-kDa fragment in cultured cells[44,81,82,101] (FIG. 5). As TDP43 contains three caspase 3 cleavage consensus sequences, and caspase 3 is able to cleave TDP43 into 25- and 35-kDa fragments both *in vitro* and in cultured cells, this suggestion is plausible[101].

TDP43 contains two NLS motifs that lie between the N terminus and RRM1 (REF. 69). As TDP43 CTFs lack these motifs, they are predominantly cytoplasmic (FIG. 5). Furthermore, the C-terminal domain of TDP43 was found to be critical for aggregation *in vitro*[86]. Thus, TDP43 cleavage by caspase 3 or another protease may be one factor that accounts for the cytoplasmic localization and aggregation TDP43. Indeed, overexpression of truncated TDP43 results in cytoplasmic aggregates that are phosphorylated and ubiquitylated[44,65,68,81,82,101,102]. However, although expressing TDP43 CTFs consistently leads to cytoplasmic aggregates, *de novo* intranuclear cleavage of full-length TDP43 yields distinct results[71]; introducing a tobacco etch virus protease cleavage site within TDP43 induced cleavage of TDP43 within the nucleus, generating TDP43 CTFs that were efficiently translocated into the cytoplasm and rapidly degraded. In contrast to direct expression of TDP43 CTFs, these *de novo* CTFs aggregated only in the presence of a second event or 'hit' such as the introduction of natively misfolded CTFs[71].

The presence of TDP43 CTFs has been evaluated in several rodent models overexpressing TDP43, many of which exhibit some level of lower molecular weight TDP43 fragments[61–64,103]. The first TDP43 transgenic mouse model was found to exhibit CTFs before onset of gait abnormalities or neurodegeneration, and levels of these CTFs increased with disease progression[62]. This initial report was confirmed by other groups that also reported a correlation between disease progression and the accumulation of TDP43 CTF[61,63]. However, several biochemical differences have been found between TDP43 CTFs in transgenic mice and human brains. 35-kDa fragments, when present, tend to be found at much higher levels in the brains of transgenic mice than in human brains[61–63]. TDP43 CTFs are highly soluble in mice extracts[62–64,103], and partition into nuclear, as opposed to cytoplasmic, fractions[63]. Furthermore, not all TDP43 transgenic mice exhibit TDP43 CTFs despite having very similar neurodegenerative phenotypes, indicating that CTFs may not be absolutely necessary for TDP43-mediated neurodegeneration[60,66]. A transgenic mouse expressing TDP43 CTFs in the absence of full-length protein has yet to be described.

Few studies have addressed whether TDP43 CTFs confer a gain or loss of function. One group found that TDP43 CTFs impair neurite growth during differentiation of cultured rodent neurons, and that full-length TDP43 rescues this phenotype, suggesting that TDP43 CTFs act by a dominant-negative mechanism[104]. However, expression of full-length TDP43 in *D. melanogaster* was toxic to a variety of neuronal cell types, whereas expression of TDP43 CTFs did not result in neurotoxicity[105,106]. Importantly, TDP43-mediated toxicity in *D. melanogaster* and chick motor neurons required RNA binding activity[106]. As TDP43 CTFs are deficient in RNA binding, this suggests that TDP43-mediated neurodegeneration is not due to abnormal TDP43 cleavage but instead is due to a toxic gain of function of full-length TDP43. Clearly, additional mechanistic studies are required to determine the effect of TDP43 CTFs on normal TDP43 function.

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TDP43 mislocalization

Although TDP43 is predominantly nuclear, interspecies heterokaryon assays have shown that it shuttles between nuclear and cytoplasmic compartments, which is consistent with studies that have been able to detect low levels of cytoplasmic TDP43 in normal cells[21–23,69,107] (FIG. 2). A striking feature of TDP43 proteinopathies is the reversal of this partitioning such that most TDP43 proteins are mislocalized to the cytoplasm or neurites in the form of aggregates[1]. Although most TDP43 inclusions are extranuclear, some are also seen within the nucleus where they exhibit a characteristic 'cat eye' or lenticular morphology[1,83–85] (FIG. 1b). TDP43 is normally localized to euchromatic regions of the nucleoplasm, including perichromatin fibrils and nuclear speckles where transcription and cotranscriptional splicing occurs[49,108]. Thus, although some TDP43 inclusions are nuclear, these can still be considered mislocalized. Four subtypes of FTLD-TDP have been described, and although intranuclear TDP43 inclusions can be seen in multiple subtypes, they are particularly prevalent in FTLD-TDP type D, the subtype that is associated with mutations in the gene encoding the valosin-containing protein (VCP)[83–85]. Little is known about why VCP mutations result in increased numbers of intranuclear inclusions.

TDP43 contains two NLS motifs between the N terminus and RRM1, and one nuclear export signal (NES) within RRM2, and these are the major *cis* elements regulating nuclear localization[69,107] (FIG. 1a). The A90V ALS-associated mutation of TDP43 results in increased cytoplasmic partitioning, presumably because this mutation is located between the two NLS motifs[109]. Rare cases of FTLD-TDP have been linked to mutations in the 3′ UTR of sigma non-opioid intracellular receptor 1 (SIGMAR1) that increase SIGMAR1 expression and are thought to increase the ratio of cytoplasmic to nuclear TDP43 through unknown mechanisms[110]. A *D. melanogaster* model of mutant VCP-mediated neurodegeneration has shown that VCP and TDP43 interact genetically and that disease-associated VCP mutations result in a redistribution of TDP43 from the nucleus to the cytoplasm[111]. In addition to these genetic determinants, a variety of cellular stressors increase cytoplasmic TDP43 levels. For example, axotomy results in a time-dependent increase in cytosolic TDP43 expression[41–43]. Additionally, in response to a variety of other stressors, TDP43 or its 35-kDa CTFs redistribute to the cytoplasm and colocalize with stress granules[30,39,44–47] (FIG. 5). Thus, both genetic and environmental factors seem to influence TDP43 localization. The fact that cytoplasmic TDP43 pathology is found to be secondary to a variety of genetic mutations, and the prevalence of cytoplasmic TDP43 pathology in a variety of neurodegenerative diseases other than ALS and FTLD-TDP suggest that there are a variety of stressors or insults that may lead to cytoplasmic localization and aggregation.

Several cell or animal model studies have attempted to address whether cytoplasmic TDP43 causes neurodegeneration. We, and others, have found that mutation of one or both NLS motifs renders TDP43 predominantly cytoplasmic, resulting in cytoplasmic aggregates both in cultured cells and transgenic mice[66,67,69]. These cytoplasmic aggregates were also shown to recruit non-mutated nuclear TDP43 into the cytoplasm of cultured cells[69]. Others have used automated time-lapse microscopy to show TDP43-mediated toxicity in primary rat neurons overexpressing fluorescently tagged TDP43. They found that the disease-associated A315T mutation resulted in increased neurotoxicity compared to the neurotoxicity in wild-type TDP43, and that neurotoxicity correlated with the amount of cytoplasmic TDP43 expression irrespective of inclusion bodies or even the presence of the A315T mutation[112]. Consistent with these findings, expression of human TDP43 in the developing *D. melanogaster* eye resulted in mild eye abnormalities (disruption of ommatidia and few necrotic patches) that were worsened by mutating the NLS motifs[113]. However, the same authors found that expressing human TDP43 in an adult fly using an inducible pan-neuronal system reduced lifespan to a greater extent than expressing mislocalized human TDP43 (that

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is, TDP43 with NLS or NES mutations)[113]. Finally, transgenic mice expressing TDP43 with an NLS mutation resulted in cytoplasmic TDP43 and rare cytoplasmic inclusions[66]. However, similar patterns of neurodegeneration were observed in mice expressing wild-type human TDP43, albeit to a lesser extent (due to lower TDP43 expression levels).

Thus, overexpression of both cytoplasmic and nuclear TDP43 seems to be toxic in animal models. Although cytoplasmic localization of TDP43 is not an absolute requisite for TDP43-mediated neurotoxicity, mislocalized TDP43 could still potentially contribute to neurodegeneration. In theory, TDP43 may retain its biological activity such as RNA binding but may do so in an abnormal location, resulting in a toxic gain of function. Although several cell culture models indicate that cytoplasmic TDP43 is toxic, animal models have yielded mixed results, and further work is required to determine the mechanistic importance of cytoplasmic versus nuclear TDP43.

Nuclear clearance and autoregulation

The most intriguing pathologic change that is associated with ALS and FTLD-TDP is the loss of normal nuclear TDP43 immunoreactivity in inclusion-bearing cells[1]. This nuclear clearance phenomenon seems to be a very early event in ALS and FTLD-TDP, occurring in cells that harbour pre-inclusions or ubiquitin-negative inclusions[2–10]. A distinction between mislocalization and nuclear clearance should be made, as the presence of an abnormally localized inclusion does not necessarily translate into the reduction of a normal protein in its normal location. For example, clearance or absence of normal huntingtin protein is not seen in the neurons bearing huntingtin inclusions that are associated with Huntington's disease[114]. Presumably, the localization of TDP43 within specific nucleoplasmic domains is required for its normal function, and therefore nuclear clearance is expected to disrupt normal function. Remarkable progress has been made towards understanding the normal functions of TDP43, partly driven by the hypothesis that a loss of normal function may play a role in ALS and FTLD-TDP.

As described above, TDP43 exhibits a diverse array of normal functions. Thus, understanding which pathways may be important in terms of mediating neurotoxicity is difficult. Indeed, genome-wide analyses thus far have indicated that a large number of genes and transcripts are regulated by TDP43 expression making identification of disease-relevant pathways difficult[25,27,60,66,115]. However, one finding is of particular relevance to the phenomenon of autoregulation. As is the case for many RNA regulatory proteins, TDP43 downregulates its own mRNA transcript levels[25,27,116] (FIG. 6a). Thus, overexpression of exogenous TDP43 leads to a decrease in endogenous TDP43 mRNA and protein in cultured cells and transgenic mice[66,69,116]. Furthermore, TDP43 protein was found to physically bind to its own mRNA transcript within the 3′ UTR, providing a mechanism of autoregulation[25,27,116]. Studies disagree with regard to what happens after TDP43 binds to its cognate mRNA (nonsense-mediated decay versus other mechanisms leading to exosome-mediated decay)[25,116]. Whether this autoregulatory mechanism contributes to nuclear clearance in affected human brain has not been definitively demonstrated. However, in transgenic mice overexpressing human TDP43 protein, endogenous TDP43 expression is reduced[66]. Perhaps over-abundant TDP43 protein in inclusion-bearing neurons can similarly reduce endogenous TDP43 expression leading to nuclear clearance. Furthermore, neurodegeneration in transgenic mice correlates better with the loss of normal endogenous TDP43 expression than with post-translational modification of TDP43, aggregation, truncation or subcellular localization, indicating that the dysregulation of normal activity is linked to neuron loss[66].

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To more definitively demonstrate that a loss of TDP43 function results in neurodegeneration, multiple cellular and animal models have been generated in which TDP43 expression is reduced or eliminated. Knockdown of TDP43 in Neuro 2a cells inhibits neurite growth and diminishes cell viability, possibly through inactivation of RHO family GTPases[117], whereas knockdown in HeLa or U2OS cells induces apoptosis through the CDK6–etino-blastoma pathway[36]. Knockout of TDP43 in embryonic stem cells inhibits proliferation[115]. Disrupting TDP43 in *D. melanogaster* has yielded conflicting results with one group reporting early lethality[37], and others describing a semi-lethal phenotype with reduced survival into adulthood, reduced lifespan, locomotor defects, abnormal neuromuscular junctions and reduced dendritic branching[118]. Interestingly, in *Danio rerio,* both overexpression of human TDP43 and knockdown of zebrafish TARDBP resulted in the same swimming behaviour defects that are associated with short motor neuron axons that have premature and excessive branching[119]. Finally, several groups have generated TDP43 knockout mice, and in each instance all TDP43 knockout mice die during embryonic stages owing to defective outgrowth of the inner cell mass[120–122]. Even postnatal knockout of TDP43 using a tamoxifen-inducible knockout mouse model resulted in rapid lethality[115]. Although these mouse studies clearly demonstrate that TDP43 is required for development and even postnatal viability, they are unable to address whether a loss of TDP43 function is toxic to postmitotic neurons. This question awaits analysis of postnatal brain or spinal cord-specific knockout mice. However, taken together, the studies that are summarized above make it clear that TDP43 regulates a wide range of cellular processes and that the loss of normal TDP43 function is likely to be harmful. Thus, nuclear clearance of normal TDP43 in ALS and FTLD-TDP is likely to play an important role in TDP43 mediated neurodegeneration.

TDP43, and ALS and FTLD genetics

There has been a renaissance in the human genetics of ALS and FTLD. The list of genes that are associated with either ALS or FTLD include *TARDBP*[12–15], *FUS*[123], superoxide dismutase 1 (*SOD1*)[124], *UBLN2* (REF. 59), optineurin (*OPTN*)[125], granulin (*GRN*)[126,127], valosin containing protein (VCP)[128], ataxin 2 (*ATXN2*)[129], sigma non-opioid intracellular receptor 1 (*SIGMAR1*)[110,130] and *C9ORF72* (REFS 131,132). Despite the variety of genes that are implicated in ALS and FTLD, there is remarkable homogeneity in terms of downstream pathology: nearly every patient with ALS or FTLD that is linked to a genetic mutation exhibits TDP43 pathology. Thus, in the vast majority of cases, dysregulation of TDP43 is the common downstream mechanism of neurodegeneration. Exceptions to this rule are the absence of TDP43 pathology in cases that are linked to SOD1 or FUS mutations[123,133]. Although TDP43 inclusions are not observed in FUS mutant cases, FUS is an RNA-binding protein that is found in complex together with TDP43, and ALS-associated TARDBP mutations promote the interaction between TDP43 and FUS[29,30]. Thus, there is strong speculation that dysregulation of TDP43 and FUS may be mechanistically linked at the level of RNA processing. Indeed, FUS also exhibits a prion-like domain[91,134] and forms inclusions in affected neurons[123]. Furthermore, in *D. melanogaster* models of neurodegeneration, two independent groups have found that TDP43 and FUS act together within a common genetic pathway[135,136].

Moreover, it was recently shown that an intronic hexa-nucleotide repeat expansion of C9ORF72 can cause ALS-FTLD[131,132]. Nearly nothing is known about the protein product of C9ORF72. However, the mutation leads to differential splicing of the C9ORF72 transcript and the formation of nuclear RNA foci[131]. The presence of nuclear RNA foci is reminiscent of myotonic dystrophies that are linked to nucleotide repeat expansions in which abnormal RNA foci are thought to sequester RNA-binding proteins and alter the biogenesis of other cellular RNAs[137]. Similar RNA foci are observed in other nucleotide repeat

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disorders, and so the association between ALS and intermediate-length polyglutamine repeats in ATXN2 is intriguing (although RNA foci have not been reported in association with ATXN2 expansions)[129]. Notably, ATXN2 polyglutamine repeats that are associated with ALS are all interrupted by between one and three CAA codons within the CAG expansion[138]. Together, these findings suggest that toxic RNA species associated with an abnormal RNA primary structure may be one mechanism related to downstream dysregulation of TDP43 and RNA biogenesis.

In summary, several of the genetic causes and modulators of ALS and FTLD support the emerging theme of abnormal RNA processing as central to the molecular mechanisms of neurodegeneration in ALS and FTLD. The plethora of genes and proteins that are associated with TDP43 pathology provides support and plausibility for the notion that common neurodegeneration pathways that are linked to TDP43 and RNA mis-metabolism can be identified that underlie sporadic as well as familial ALS and FTLD including those autosomal and X-linked dominant forms of these disorders that are caused by distinctly different genetic mutations.

Models of TDP43-mediated neurodegeneration

Although enormous advances have been made in demonstrating that pathological TDP43 is linked to neurodegeneration in ALS and FTLD-TDP, there is still much progress to be made to understand how the normal functions of TDP43 relate to the mechanisms of TDP43-mediated neurodegeneration. We now have an idea of the key steps that occur from the onset of TDP43-mediated neurodegenerative disease through to the culmination of these events in dysfunction and death of affected neurons in ALS, FTLD-TDP and related TDP43 proteinopathies (FIGS 3,4). Advances in TDP43 research have been extremely rapid considering that 7 years elapsed following the discovery of amyloid-β as the building block of vascular amyloid in Alzheimer's disease[139] before mutations linked to Alzheimer's disease were found in the amyloid-β precursor protein (APP) gene in 1991 (REFS 140–142). It took four more years to generate animal models of Alzheimer's disease-like amyloid-β plaque pathology[143,144], and more than 25 years later the mechanisms of amyloid-β-mediated neurodgeneration remain incompletely understood[145].

As is often the case for disease processes, multiple pathways are likely to be involved, and the downstream consequences of TDP43 phosphorylation, aggregation, cleavage, mislocalization and clearance from the nucleus are unclear. Perhaps it is easiest to conjecture that the absence of normal nuclear TDP43 implies that there is a loss of normal TDP43 function. Given the plethora of normal nuclear TDP43 functions, this alone might lead to neurodegeneration. Alternatively, perhaps TDP43 retains its ability to bind RNA, and therefore its cytoplasmic localization acts as a peripheral sink, thereby implicating a toxic gain of function. Or maybe TDP43 CTFs act in a dominant negative fashion by occupying hnRNP binding sites without being able to bring along its usual RNA cargo.

In an attempt to synthesize the literature on the various potential mechanisms of neurodegeneration, we will consider the normal autoregulation of TDP43 (FIG. 6a) and two hypothetical models of TDP43-mediated neurodegeneration (FIG. 6b,c). The first model, which we term the 'loss of autoregulation' model, was proposed by two groups independently and is updated here[25,116] (FIG. 6b). Although the initial insult is generally unknown (aside from cases that are linked to genetic mutations), one possible initiating event may be the presence of abnormal, toxic RNA species. Cellular stress is thought to cause a redistribution of TDP43 from the nucleus to the cytoplasm. This is akin to the formation of stress granules that has been observed experimentally, although there is currently little evidence showing an upregulation of stress granules in ALS and FTLD.

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Alternatively, RNA transport granules that are usually localized within peripheral neurites may be abnormally distributed. According to this model, this redistribution results in the aggregation of TDP43 into pre-inclusions that are phosphorylated, variably ubiquitylated and difficult to degrade. These cytoplasmic inclusions lead to further redistribution of normal TDP43 from the nucleus as protein is sequestered into inclusions, accounting for nuclear clearance. If TDP43 autoregulation occurs within the nucleus as suggested[25], there would be a loss of TDP43 autoregulation and increased TDP43 expression, leading to increased aggregate formation. This would feed forward through a vicious cycle leading to cell death.

Alternatively, a 'gain of autoregulation' model can be imagined (FIG. 6c). According to this model, a stressor can lead to redistribution of TDP43 into the cytoplasm resulting in aggregation (similar to the loss of autoregulation model). If autoregulation of TDP43 mRNA is a cytoplasmic event and TDP43 aggregates retain the ability to bind RNA and therefore autoregulate its cognate RNA, then an increase in cytoplasmic TDP43 may lead to increased autoregulation, thereby decreasing TDP43 mRNA and decreasing newly synthesized TDP43 protein in affected cells. This decrease in synthesis may account for the nuclear clearance phenomenon (that is, aggregates represent the existing pool of TDP43 protein and not newly synthesized protein). Given the myriad of normal TDP43 functions, loss of normal nuclear TDP43 may further increase cellular stress, again resulting in a vicious cycle leading to cell death.

Conclusions

The exact mechanisms by which cell death occurs are not known. However, using the pathological and biochemical signature of abnormal TDP43 in human disease as a guide, many advances in our understanding of TDP43 biology have been made. We now speculate that many pathways may be involved in TDP43-mediated neurodegeneration, including gains of functions (mediated by aggregates or abnormal cytoplasmic function) and losses of functions (mediated by nuclear clearance, CTFs, RNA dysregulation or even UPS dysfunction). Indeed, the two proposed models highlight the possibility that gains and losses of function together may affect neuronal viability and are not mutually exclusive, highlighting the complexities of understanding neurodegenerative diseases, and in particular TDP43 proteinopathies. The fact that TDP43 pathology is found in nearly all cases of sporadic and familial ALS or FTLD (excluding ALS caused by SOD1 or FUS mutations and FTLD due to primary tauopathies) speaks to the centrality of TDP43 in the pathogenesis of these diseases. Furthermore, the mechanisms of TDP43-mediated neurodegeneration is likely to involve abnormal RNA processing, either in the form of abnormal splicing, differential RNA stability or the presence of toxic RNA species. The current challenges in understanding TDP43 proteinopathies are largely due to the extraordinary complexity of TDP43 functions, including the large number of mRNAs with which TDP43 interacts in potentially important pathways and networks linked to normal physiological or metabolic processes. Equally daunting is identifying the disease-relevant RNAs that are mechanistically linked to neurodegeneration, with which TDP43 interacts or regulates, so this remains an imposing challenge due to the vast number of RNAs targeted by TDP43 protein. Also, understanding how the diverse genetic (and presumably environmental) factors lead to downstream TDP43 pathology is a major obstacle to mechanistic understanding across the field of neurodegenerative diseases in general, and this is true for TDP43 proteinopathies as well. However, these challenges also represent compelling and largely tractable opportunities to identify shared molecules and pathways. There are also opportunities in understanding TDP43 proteinopathies within the context of neurodegenerative diseases in general, such as the role of prion-like cell-to-cell transmission in the progression and spread of protein misfolding diseases. Thus, despite the considerable

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progress in elucidating the mechanisms of TDP43-mediated neurodegeneration, much remains to be discovered and the opportunities to dissect disease-relevant gains and losses remains a dominant focus of TDP43 biology. However, given the amazing progress in understanding ALS and FTLD since the discovery of TDP43 as the disease protein in these disorders in 2006, there is a sense of optimism among scientists in this field. There is now belief that we have the ideas, the tools and the skills to advance our understanding of ALS, FTLD and related TDP43 proteinopathies to the point at which we can translate this understanding into improvements in the diagnosis and treatment of these disease, if these efforts were appropriately resourced.

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Glossary

Dominant missense mutation	An alteration of a single nucleotide within a gene, resulting in a codon that encodes for an amino acid that is different from normal. Thus, a change in a single allele is sufficient to result in the mutation-associated phenotype
Epiphenomena	Secondary processes or events that occur in parallel to a primary event and that may even be a result of the primary event. However, epiphenomena are in addition to the course of a disease and are not necessarily causally related to the primary mechanisms of disease
High-throughput sequencing of RNA isolated by crosslinking immunoprecipitation	(HITS-CLIP). A method to identify the RNA-binding sites of a given protein in which protein–RNA interactions are stabilized by UV crosslinking. The protein of interest is immunoprecipitated, and the interacting RNA species are identified using high-throughput next-generation sequencing platforms
RNA granules	Macromolecular structures in neurons enriched with RNA and RNA-binding proteins. They are thought to be involved in the preservation and transport of mRNA
Stress granules	Dense cytosolic protein and RNA aggregations that appear under conditions of cellular stress. The RNA molecules are thought to be stalled translation pre-initiation complexes
Multivesicular bodies	Endosomal intermediates in which small membrane vesicles are enclosed within a limiting membrane. The internal vesicles are thought to form by invagination and budding from the limiting membrane
Adaptor protein	A protein that contributes to cellular function by recruiting other proteins to a complex. Such molecules often contain several protein–protein interaction domains

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Neuronophagia	The process in which dying neurons are cleared by phagocytic cells including microglia
Interspecies heterokaryon assays	A test that is performed on cells that contain multiple genetically different nuclei from different species. The test is capable of demonstrating low levels of dynamic nucelo–cytoplasmic shuttling by measuring the transport of a nuclear protein from a donor nucleus to a receptor nucleus

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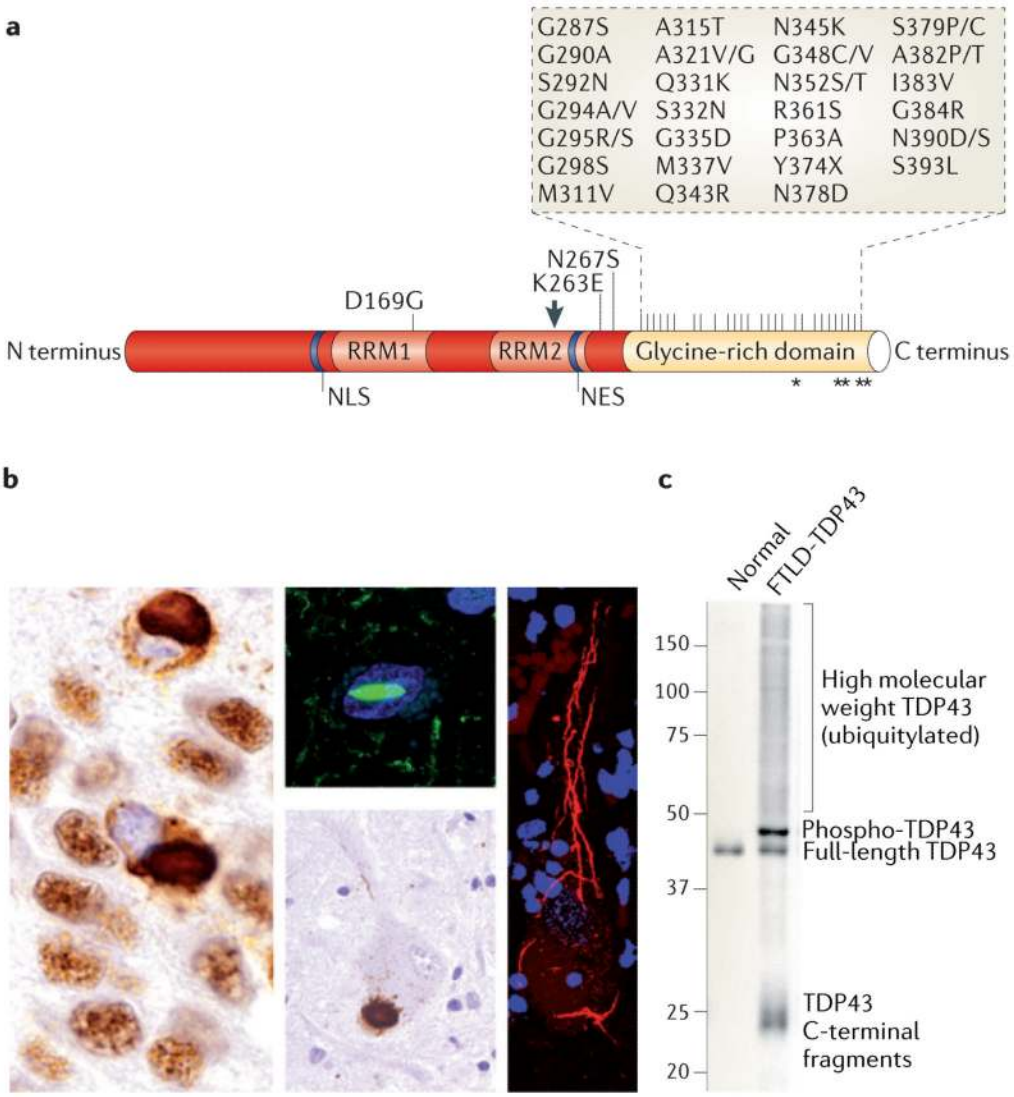

Figure 1. The genetics, pathology and biochemistry of TDP43 proteinopathies

a | TAR DNA-binding protein 43 (TDP43) protein contains two RNA-recognition motifs (RNA-recognition motif 1 (RRM1) and RRM2), a carboxy-terminal glycine-rich domain, a bipartite nuclear localization signal (NLS) and a nuclear export signal (NES). Numerous mutations (shown by short vertical lines) have been linked to sporadic and familial forms of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). These are almost exclusively found within or immediately adjacent to the glycine-rich domain with the exception of an Asp169Gly mutation within exon 4 (the site at which TDP43 cleavage putatively occurs is shown by an arrow). Known TDP43 phosphorylation sites (Ser 379, Ser403 + Ser404, and Ser409 + Ser 410), when heavily phosphorylated, also contribute to the disease-specific TDP43 biochemical signature (these sites are indicated by asterisks)[58]. **b** | TDP43 immunohistochemistry of human FTLD brain reveals intracytoplasmic inclusions affecting neurons. Dystrophic neurites and glia also exhibit TDP43 inclusions (not shown). An image of the dentate gyrus stained with an anti-TDP43 antibody shows cells without inclusions with normal nuclear immunoreactivity, whereas inclusion-bearing cells show a loss of normal nuclear staining that leads to a presumptive loss of TDP43 function in the nucleus. TDP43 immunofluorescence (shown in green) of an intranuclear inclusion in

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FTLD-TDP43 is shown. TDP43 immunostaining using a phospho-specific anti-TDP43 antibody of human ALS spinal cord shows characteristic round inclusions (shown by brown immunohistochemistry) and skeins (shown by red immunofluorescence) in lower motor neurons. **c** | Biochemical analysis demonstrates the distinct biochemical disease-specific TDP43 signature characterized by the accumulation of sarkosyl-insoluble TDP43 species comprised of phosphorylated TDP43, ubiquitylated high molecular weight TDP43 and truncated C-terminal fragments.

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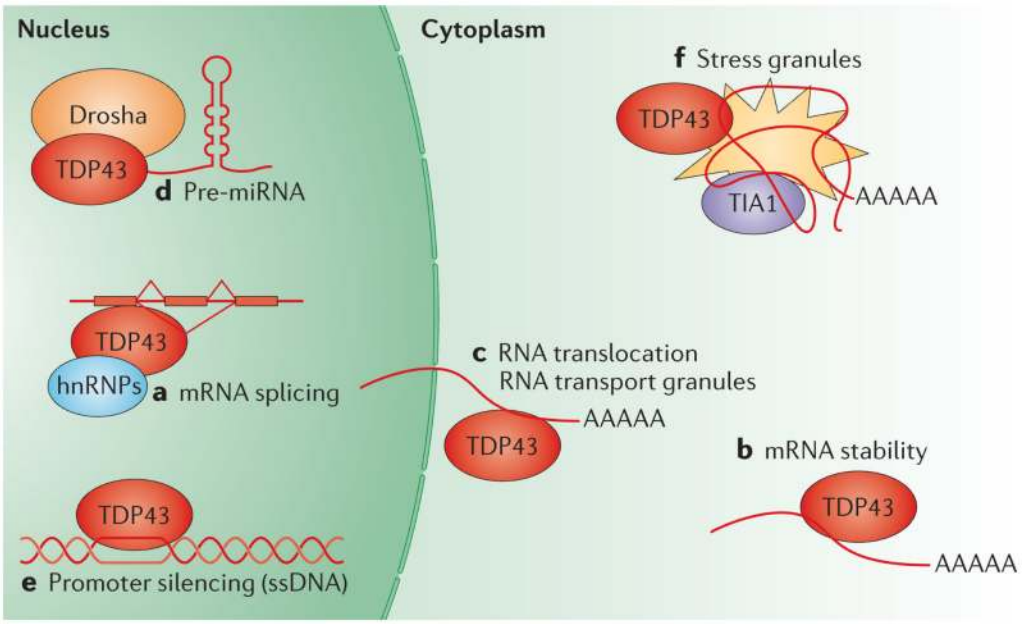

Figure 2. Normal functions of TDP43

TAR DNA-binding protein 43 (TDP43) exhibits multiple normal biological functions, predominantly those that regulate RNA pathways. **a** | TDP43 is a component of heterogenous nuclear ribonucleoprotein (hnRNP) particles, which regulate splicing of pre-mRNA species. **b** | TDP43 also binds to mRNA sequences, particularly within the 3′ untranslated region, and affects mRNA stability and turnover. **c** | TDP43 is thought to play a part in mRNA trafficking, as TDP43 undergoes rapid nucleo–cytoplasmic shuttling and is localized within dendritic RNA granules. **d** | TDP43 is also a component of the Drosha complex, which functions to process primary microRNAs. **e** | TDP43 can act as a transcriptional repressor by binding to single stranded DNA (ssDNA) promoter sequences. **f** | TDP43 also colocalizes with stress granules that are thought to sequester and protect mRNAs under conditions of stress.

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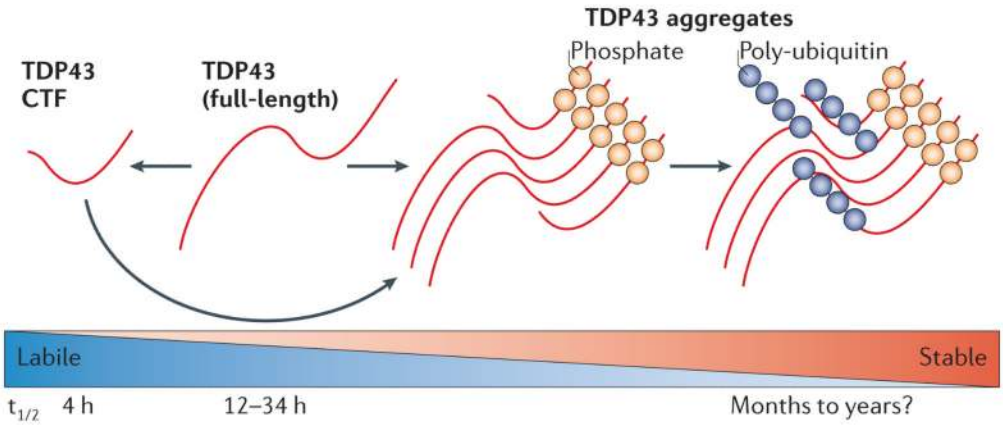

Figure 3. TDP43 modification, stability and turnover

Both proteosomal and autophagasomal degradation of TAR DNA-binding protein 43 (TDP43) protein has been described. We found that full-length TDP43 is a long-lived protein with a half-life of greater than 34 hours, although other studies have reported that it has a half-life ranging from 4 to 12 hours depending on the cell type[29,71]. Truncation of TDP43 results in the production of carboxy-terminal fragments (CTFs) that are rapidly translocated to the cytoplasm and degraded. TDP43 aggregates can form under various conditions in which CTFs and full-length TDP43 protein seem to co-aggregate, and TDP43 aggregation is tightly linked with the presence of phosphorylated TDP43. Ubiquitylation of TDP43 aggregates seems to occur late in the lifecycle of an inclusion. Given that TDP43 aggregates are resistant to degradation, different TDP43 isoforms and conformers exhibit different turnover rates, ranging from the labile soluble CTFs to stable insoluble aggregates. $t_{1/2}$, half-life.

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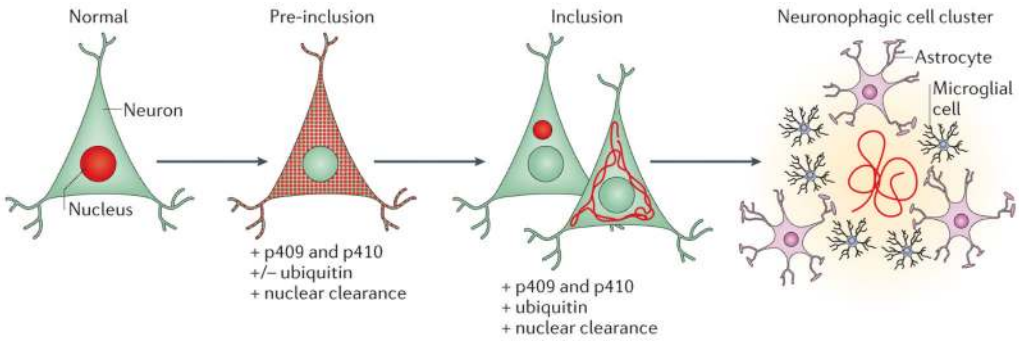

Figure 4. Lifecycle of TDP43 pathology

Normal neurons show robust intranuclear TAR DNA-binding protein 43 (TDP43) immunoreactivity (shown in red) with little cytoplasmic TDP43. So-called 'pre-inclusions' have been described, and these consist of granular cytoplasmic aggregates that are positive for phospho-TDP43 epitopes (p409 and p410) but that are often negative for ubiquitin. Neurons with pre-inclusions show characteristic loss of normal nuclear staining. Bona fide inclusions exhibit a variety of morphologies ranging from dense round inclusions and skeins in motor neurons to dystrophic neurites, cytoplasmic inclusions or intranuclear 'cat eye' inclusions in other neurons. Neuronophagia can rarely be seen in amyotrophic lateral sclerosis, and neuronophagic cell clusters have been reported to be associated with TDP43 inclusions[96].

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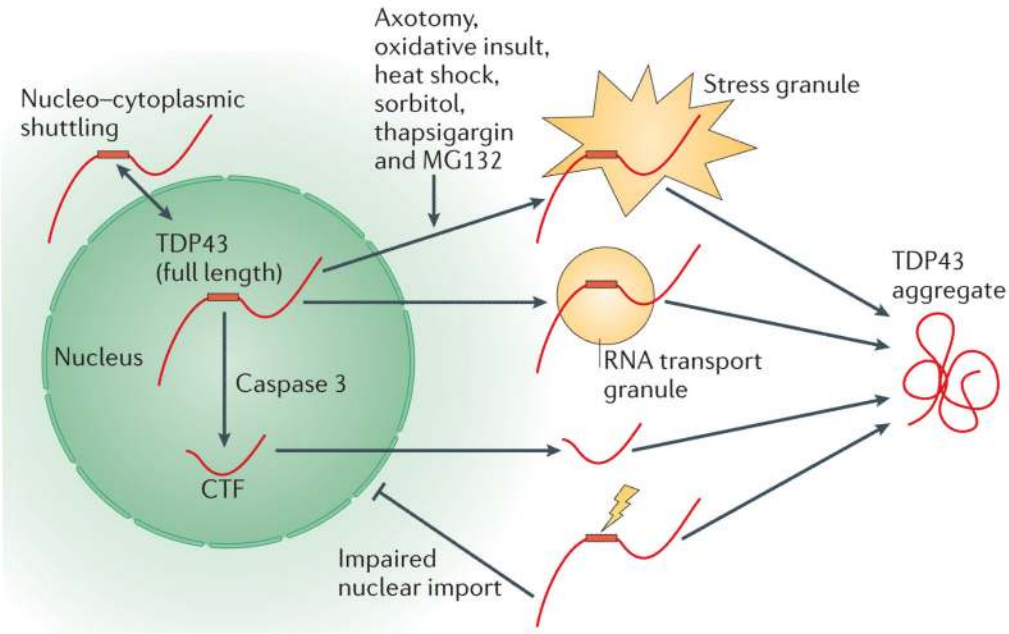

Figure 5. Subcellular localization of TDP43 protein

TAR DNA-binding protein 43 (TDP43) shows a predominantly nuclear localization, although nucleo–cytoplasmic shuttling of TDP43 has been shown and low levels of cytoplasmic TDP43 can be demonstrated. TDP43 accumulation in the cytoplasm can be induced by a variety of cellular stressors that result in the formation of TDP43-positive stress granules. TDP43 protein has also been found in RNA transport granules within neuronal processes. TDP43 protein is thought to undergo proteolytic cleavage by caspase 3 to generate a carboxy-terminal fragment (CTF). As the CTF no longer contains the bipartite nuclear localization signal (NLS, shown by a red rectangle), CTFs translocate into the cytoplasm, where they may participate in aggregate formation. Experimentally generated mutations of the NLS and disease-associated mutations (shown by a yellow lightning bolt) have been found to increase the amount of cytoplasmic versus nuclear TDP43. MG132 is a proteasome inhibitor.

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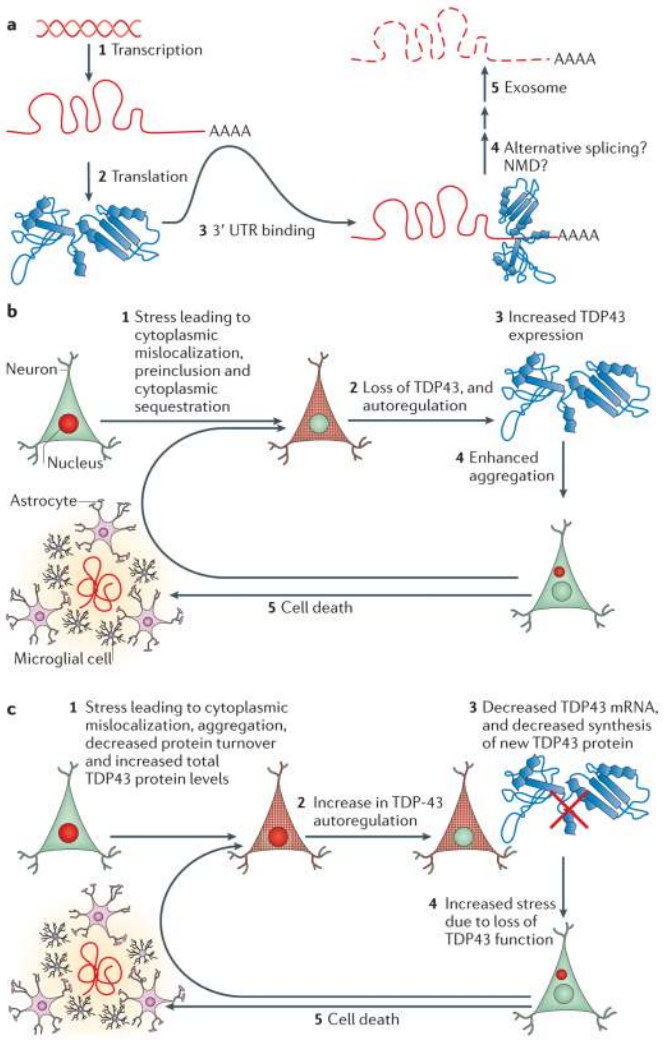

Figure 6. Autoregulation of TDP43 and models of TDP43 toxicity

a | Normal TAR DNA-binding protein 43 (TDP43) autoregulation. TDP43 expression is tightly regulated under normal conditions, and overexpression of exogenous TDP43 results in a reduction of endogenous TDP43 expression. This autoregulation of TDP43 expression by itself is mediated at the level of mRNA stability. Within the 3′ untranslated region (UTR) of TDP43 mRNA is a binding site for TDP43 protein that is critical for autoregulation. TDP43 binding to this 3′ UTR site promotes degradation of TDP43 mRNA, at least partly through the exosome. There are conflicting reports about the mechanism of autoregulation, in particular whether alternative splicing leading to nonsense-mediated decay (NMD) is the mechanism of TDP43 autoregulation. **b** | Loss of autoregulation. According to this model, the exposure of neurons to as-yet-unidentified stressors can lead to cytoplasmic mislocalization of TDP43 (**1**), and this is perhaps related to the stress granule response. Given TDP43's propensity to aggregate, TDP43 forms phosphorylated pre-inclusions within the cytoplasm that sequester free TDP43 protein. This cytoplasmic sequestration leads to a loss of normal nuclear TDP43. If autoregulation occurs within the nucleus, a loss of TDP43 autoregulation ensues (**2**), which results in increased TDP43 mRNA and protein (**3**). This further exacerbates TDP43 aggregation (**4**). This vicious cycle leads to cell death (**5**) possibly through a variety of gain and loss of functions. **c** | Gain of autoregulation. In this model, neurons that are exposed to unknown stressors can undergo cytoplasmic

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mislocalization of TDP43 protein (**1**), and this is perhaps related to the stress granule response. Given the propensity of TDP43 to aggregate, TDP43 forms phosphorylated pre-inclusions within the cytoplasm which are resistant to degradation. If autoregulation occurs within the cytoplasm, an increase in cytoplasmic TDP43 may result in an increase in TDP43 autoregulation (**2**) that would decrease TDP43 mRNA and therefore decrease synthesis of new TDP43 protein (**3**). This reduction in TDP43 protein synthesis leads to a loss of normal nuclear TDP43 protein. Given the plethora of normal nuclear TDP43 functions, the absence of nuclear TDP43 is detrimental to neuronal viability, increasing the stress response (**4**) and leading to cell death (**5**). Again, this model allows for the possibly of a variety of gain and loss of functions that coordinately result in toxicity.