Triad of TDP43 control in neurodegeneration: autoregulation, localization and aggregation

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Abstract | Cytoplasmic aggregation of TAR DNA-binding protein 43 (TDP43; also known as TARDBP or TDP-43) is a key pathological feature of several neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). TDP43 typically resides in the nucleus but can shuttle between the nucleus and the cytoplasm to exert its multiple functions, which include regulation of the splicing, trafficking and stabilization of RNA. Cytoplasmic mislocalization and nuclear loss of TDP43 have both been associated with ALS and FTD, suggesting that calibrated levels and correct localization of TDP43 — achieved through an autoregulatory loop and tightly controlled nucleocytoplasmic transport — safeguard its normal function. Furthermore, TDP43 can undergo phase transitions, including its dispersion into liquid droplets and its accumulation into irreversible cytoplasmic aggregates. Thus, autoregulation, nucleocytoplasmic transport and phase transition are all part of an intrinsic control system regulating the physiological levels and localization of TDP43, and together are essential for the cellular homeostasis that is affected in neurodegenerative disease.

Heterogeneous nuclear ribonucleoprotein (hnRNP). An RNA-binding protein involved in several aspects of nucleic acid metabolism such as transcriptional and translational regulation, alternative splicing and mRNA stabilization.

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TAR DNA-binding protein 43 (TDP43) is a heterogeneous nuclear ribonucleoprotein (hnRNP) initially discovered for binding to the *trans*-active response element in the HIV-1 DNA sequence[1]. TDP43 was subsequently identified as a regulator of the splicing of the RNA encoding human cystic fibrosis transmembrane conductance regulator (CFTR)[2] and as the major component of intracellular proteinaceous inclusions and pathogenic aggregates in patients with amyotrophic lateral sclerosis (ALS) or frontotemporal dementia (FTD)[3,4]. Of note, both familial and sporadic forms of ALS and FTD are characterized by proteinaceous inclusions enriched in ubiquitylated, hyper-phosphorylated and fragmented forms of TDP43, which are mainly found in the cytoplasm of affected neurons and glial cells[3,4]. These inclusions are often accompanied by nuclear clearance of TDP43 (REF.[5]), suggesting that both the loss of nuclear TDP43 function and its cytoplasmic mislocalization contribute to the onset and progression of disease[6].

TDP43 abnormalities have been found in all cases of ALS except those carrying mutations in the genes encoding SOD1 and FUS, and in approximately 45% of patients with FTD[7]. TDP43 pathology has also been observed in other neurodegenerative diseases, revealing a whole spectrum of TDP43 proteinopathies characterized by the presence of hyper-phosphorylated, fragmented and aggregated TDP43 protein with reduced solubility[8]. For example, TDP43 cytoplasmic inclusions constitute the third most prevalent proteinopathy in the brains of patients with Alzheimer disease, appearing in up to 57% of cases[9–13], and are also present in many cases of Parkinson disease[14,15] and Huntington disease[16,17].

The fact that TDP43 pathology characterizes a large spectrum of neurodegenerative diseases with similar co-morbidities has led to the suggestion that they might share pathogenic mechanisms[18]. The features of TDP43 pathology (cytoplasmic accumulation of insoluble aggregates and nuclear depletion of TDP43) suggest that the physiological levels and correct localization of TDP43 must be under tight control to retain its normal function as required for cellular homeostasis. Here, we review current knowledge of and new insights into three key mechanisms regulating TDP43: autoregulation, nucleocytoplasmic transport and phase transitions. We consider how their dysfunction can lead to cytoplasmic accumulation and nuclear loss of TDP43, ultimately causing the onset and progression of neurodegenerative disease.

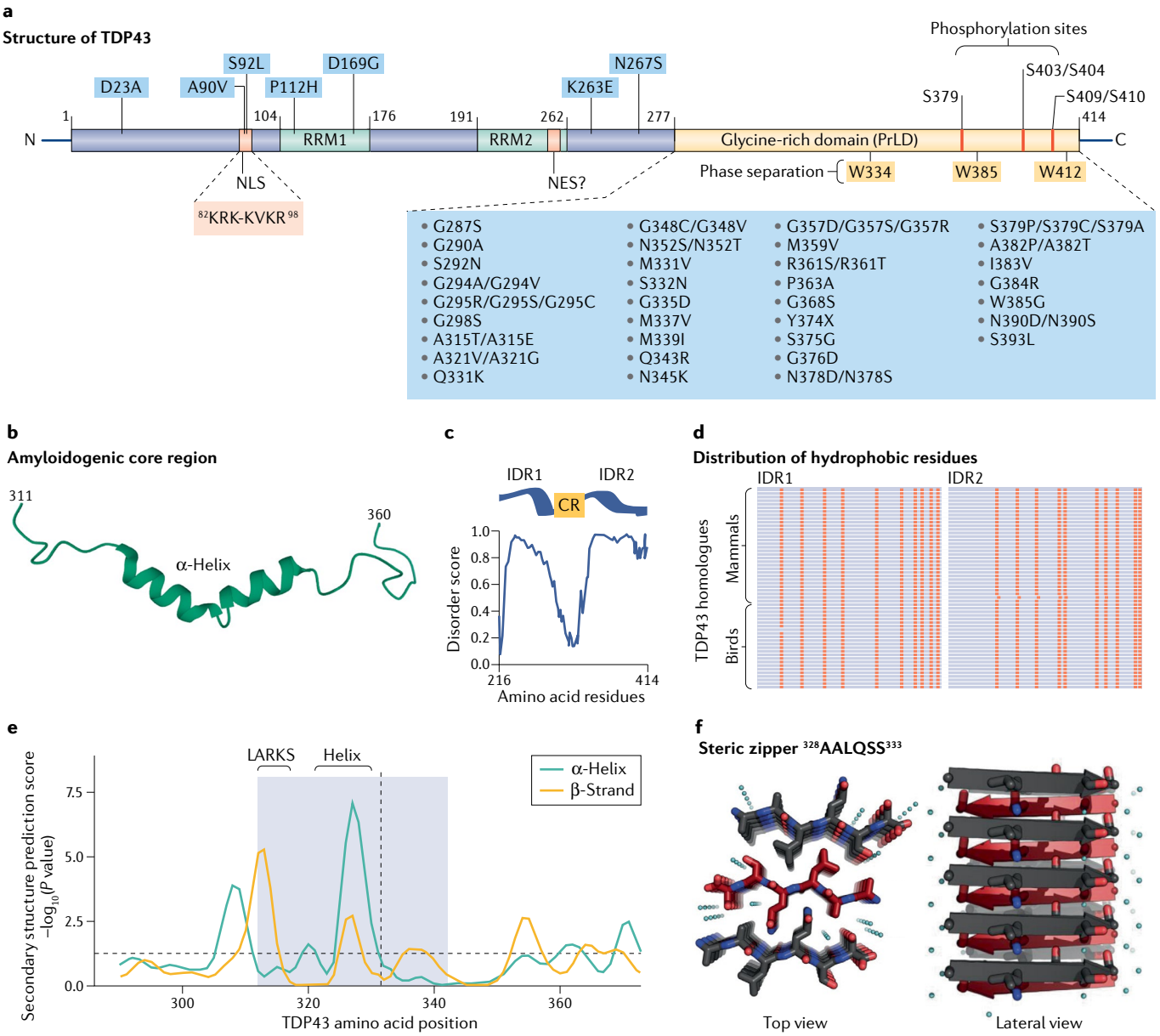
TDP43 structure and function

TDP43 is an evolutionarily conserved protein of 414 amino acids encoded by the *TARDBP* gene on human chromosome 1 (REF.[1]). Through its two RNA-recognition motifs — RRM1 and RRM2 — TDP43 acts as a

Splicing
A form of processing of a newly synthesized precursor mRNA transcript during which introns are removed and exons are joined together, thereby transforming it into a mature mRNA.

Proteinaceous inclusions
Aggregates of one or more proteins found in the nucleus and/or the cytoplasm, a main characteristic of many neurodegenerative diseases.

DNA-binding and RNA-binding protein[19] (FIG. 1a). The binding of TDP43 to RNA predominantly depends on RRM1 (REFS[1,20]) and usually involves its recognition of UG repeats in the target RNA[2], with the specificity of binding increasing with the UG repeat length[21]. Initial studies in human hepatoma cells suggested that TDP43 binding to *CFTR* target RNA required a minimum of five UG repeats[2]; however, later genome-wide identification of TDP43 RNA targets showed that UG-rich repeats were neither necessary nor sufficient to specify a TDP43 binding site[22]. TDP43 targets more than 6,000 RNA species in mammals[23] and binds preferentially to the 3′ untranslated regions (UTRs) and introns of both coding and non-coding RNAs[22,24]. In addition to its RRM1 domain, the DNA or RNA binding of TDP43 is also dependent on its glycine-rich carboxy terminus[25,26], a low-complexity domain that is also responsible for the recruitment of TDP43 into stress granules and cytosolic aggregates[27,28]. This glycine-rich domain shows sequence similarities to yeast prion proteins[29,30] (and is therefore also known as the prion-like domain (PrLD)) and has a dynamic structure inside which secondary elements can form, including an α-helix within the amyloidogenic core of residues 311–360 (REF.[31]) (FIG. 1b). The amyloidogenic core is part of a highly conserved region flanked by two intrinsically disordered regions (IDR1 and IDR2)[32] (FIG. 1c) that are involved in phase separation and aggregation of TDP43 (REFS[33–35]). Phylogenetic comparison of the C-terminal domain across vertebrate TDP43 homologues identified hydrophobic residues (valine, leucine, isoleucine, methionine, phenylalanine, tyrosine, tryptophan) occurring at conserved intervals within IDR1 and IDR2 (FIG. 1d) that are important for the propensity of TDP43 for liquid demixing and aggregation, as demonstrated by site-directed mutagenesis in yeast and human embryonic kidney cells[32,36]. Simulation studies of the TDP43 low-complexity region further suggest that a helix–turn–helix structure can form between amino



Aggregates
Accumulations of misfolded and/or intrinsically disordered proteins that form amyloid-like fibrils and insoluble depositions in the nucleus and/or the cytoplasm and are highly associated with neurodegeneration and pathology.

Low-complexity domain
An intrinsically disordered protein sequence that is unable to form orderly 3D structures, such as α-helices or β-strands, and is thus prone to intermolecular interactions and involved in phase separation and the formation of pathological aggregates.

Stress granules
Membraneless organelles formed upon cellular stress by liquid demixing and accumulation of RNA and proteins.

acid residues 320 and 343, whereas residues 341–366 can form two antiparallel β-sheets (FIG. 1e).

Structural studies have identified numerous additional features of TDP43 that may be linked to disease pathology. These include segments of the TDP43 low-complexity domain that contain low-complexity aromatic-rich kinked segments (LARKs)[37] (FIG. 1e), which drive labile amyloid-like interactions characteristic of protein hydrogels and the proteins found in membraneless organelles (including stress granules)[38]. Other segments of the TDP43 PrLD contain steric zippers (FIG. 1f), stretches of very short peptide sequences that fold into self-complementary β-sheets that are characteristic of the amyloid fibrils seen in Alzheimer disease, FTD and ALS[39–41]. Additional cryo-electron microscopy studies showed that residues 311–360 (the amyloidogenic core region) of TDP43 can also form three polymorphs, all with dagger-shaped folds, that represent irreversible fibril structures and that residues 286–331 can form R-shaped folds and may participate in both reversible and irreversible fibrils[42]. Moreover, nuclear magnetic resonance studies investigating the assembly of stress granules and the role of ALS-associated mutations in disease revealed that an α-helical structure[34] and hydrophobic residues[32] in the low-complexity domain mediate phase separation of TDP43.

The significance of the C-terminal low-complexity domain for TDP43 pathology was further corroborated by structure–function analyses that showed that, under physiological circumstances, TDP43 forms oligomers in a manner that is dependent on its amino-terminal domain[43]. This study identified homo-oligomers of physiological nuclear TDP43, derived from human cells and brain tissue, that can adopt dynamic, solenoid-like structures that are resistant to cellular stress. This oligomerization spatially separates the C-terminal low-complexity domains of TDP43 monomers, thereby antagonizing their intermolecular interactions and the formation of pathological aggregates[43]. Moreover, the study revealed that TDP43 oligomerization is essential for its function in RNA splicing regulation. Thus, transient expression of oligomerization-deficient forms of human TDP43 in mouse cells that are deficient for endogenous TDP43 failed to rescue the mis-splicing of specific RNA targets, including *Sortilin 1* (*Sort1*) and polymerase δ-interacting protein 3 (*Poldip3*) in these cells[43].

As emphasized by these findings, one of the key functions of TDP43 is the regulation of alternative splicing of various target RNAs. For example, experiments using human cell culture revealed direct binding of TDP43 to a stretch of RNA within intron 2 of apolipoprotein A2 (*APOA2*) that contains 16 GU repeats and negatively regulates splicing of exon 3 (REF.[44]). Further, TDP43 regulates the splicing of the RNA encoding mammalian tau, by directly binding to six binding sites in intron 9, thereby promoting exon 10 inclusion and the expression of the 4R-tau isoform[45]. As alterations in the ratio of 3R-tau to 4R-tau occur in tauopathies[46–50], changes in TDP43 expression could affect tau expression and function in these disorders. TDP43 also regulates the splicing of risk genes associated with ALS, including those encoding ataxin 2 (REF.[51]), hnRNP A1, hnRNP A2/B1, hnRNP A3 and hnRNP C1/C2 (REFS[26,52,53]), SORT1 (REF.[54]) and POLDIP3 (REF.[55]). With the exception of hnRNP A1 (REF.[56]), hnRNP A2 (REF.[57]) and POLDIP3 (REF.[55]), however, a role for the direct binding of TDP43 in splicing regulation has not been unambiguously demonstrated for these and other target RNAs, many of which have been linked to TDP43 on the basis of UV cross-linking, immunoprecipitation and sequencing[22,24,58–61].

TDP43-mediated splicing regulation was also shown to include skipping of constitutive exons leading to changes in gene expression[62]. Moreover, its regulation of splicing also extends to the repression of cryptic exons, with one study showing that TDP43 utilizes UG microsatellites, DNA motifs typically repeated 5–50 times, to repress incorporation of cryptic exons into mRNA[63]. This study further showed that loss of TDP43 in mouse embryonic stem cells results in splicing of cryptic exons into mRNAs, which disrupts their translation and promotes nonsense-mediated decay[63]. Further analysis of RNA-sequencing data derived from HeLa cells treated with TDP43 small interfering RNA identified 1274 genes within which TDP43-dependent cryptic splicing repression is present, whereas analysis of *Tardbp* knockout mouse embryonic stem cells identified 1,550 genes, the

◀ Fig. 1 | **TDP43 protein structure. a** | Schematic of the structure of TAR DNA-binding protein 43 (TDP43), which is characterized by two RNA recognition motifs (RRM1 and RRM2) involved in RNA binding, a nuclear localization signal (NLS) recognized by karyopherins during nuclear import and a nuclear export signal (NES); however, it is important to note that recent experiments have questioned the existence of a bona fide NES[94,100–103]. Several mutations associated with amyotrophic lateral sclerosis and/or frontotemporal dementia are indicated in blue boxes. The majority of these mutations reside in the glycine-rich low-complexity domain (also known as the prion-like domain (PrLD)) in the carboxy terminus[29,30], which is prone to intermolecular interactions and is involved in phase separation[34,36,38,129] and the formation of pathological aggregates. This region contains three heavily phosphorylated sites at amino acid residues S379, S403/S404 and S409/S410 (REFS[138,139]). An additional three sites within this domain have been shown to be important for phase separation: residues W334, W385 and W412 (REF.[129]). **b** | Solution structure of the human TDP43 amyloidogenic core region of the low-complexity domain (residues 311–360), showing an α-helical structure[31]. **c** | Disorder score (numeric prediction of the lack of an ordered 3D structure, based on X-ray and/or spectroscopic data) of the human TDP43 low-complexity domain; shown are two intrinsically disordered regions (IDR1 and IDR2), interspersed by a highly conserved region (CR)[32]. **d** | Hydrophobic residues (indicated in orange) are positioned at conserved intervals in the intrinsically disordered regions of mammalian and bird TDP43 homologues. Each grey lane represents a different homologue[32]. **e** | Secondary structure prediction scores (indicating the likelihood of amino acid residues to adopt an orderly 3D structure) for residues 290–370 of human TDP43, a region that can form an α-helix and/or β-strand inside the TDP43 low-complexity domain. Grey area indicates the hotspot within which gene mutations have the strongest effects on protein function[36]. Horizontal brackets indicate the position of the low-complexity aromatic-rich kinked segments (LARKS; residues 312–317) and an α-helix (residues 321–330), respectively[36]. **f** | TDP43 low-complexity domain segments can form steric zippers, stretches of very short peptide sequences that fold into self-complementary β-sheets. These are shown for residues 328–333 of TDP43, which can adopt anti-parallel packing (as shown by the alternating orientation of arrows representing the direction of strands). Top view shows the structure along the fibril axis, whereas lateral view shows the structure perpendicular to the fibril axis. Colour-coding represents the relative conformation of the β-sheets: red sheets are kinked, blue sheets are straight. Cyan droplets represent water molecules found in the structure[38]. Part **b** adapted with permission from PDB ID 2N3X. Sehnal, D., Rose, A. S., Kovca, J., Burley, S. K. & Velankar, S. (2018) Mol*: Towards a common library and tools for web molecular graphics. *MolVA/EuroVis Proceedings* https://doi.org/10.2312/molva.20181103, RCSB PDB. Parts **c** and **d** adapted from REF.[32], CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/). Part **e** adapted from REF.[36], CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/). Part **f** adapted from REF.[38], Springer Nature Limited.

Phase separation
The creation of two different phases from a homogeneous mixture, which can occur by liquid demixing of soluble monomeric proteins and includes their dispersion into liquid droplets and accumulation into irreversible cytoplasmic aggregates.

Polymorphs
Proteins whose sequences enable them to adopt different structural conformations.

Cellular stress
A consequence of environmental or chemical stressors, such as extreme temperatures, alterations in pH or exposure to toxins, that threatens the internal state and, ultimately, the survival of a cell.

Cryptic exons
Coding sequences within a precursor mRNA that are normally not included in the mature RNA but can occur in an alternative form of the mRNA.

Nonsense-mediated decay
A surveillance and degradation mechanism in eukaryotes, mainly eliminating mRNAs containing premature stop codons.

Alternative polyadenylation
A process of gene regulation leading to the production of mRNA isoforms in which the formation of poly(A) tails is initiated at different positions in the precursor mRNA.

RNA exosome
A ring-structured multi-protein complex found in the cytoplasm, nucleus or nucleolus, responsible for degrading different kinds of RNA molecules.

majority of which overlapped with the RNA-sequencing data set derived from HeLa cells[64]. Among the identified genes were two known ALS-related genes, *HNRNPA2/B1* and *HNRNPH1*, as well as several nucleoporin genes (*NUP43*, *NUP50-AS1*, *NUP85* and *NUP2010*) and genes involved in nucleocytoplasmic transport (*RANBP1*, *RANBP17*, *RABGAP1* and *TNPO1*)[64].

Alternative polyadenylation has also been identified as a mechanism of TDP43-mediated RNA processing regulation[65,66]. Using cell and mouse models, as well as patient-derived induced pluripotent stem cells and post-mortem tissue from patients with ALS or FTD, three independent studies showed that lowered levels of, or mutations in the gene encoding TDP43 suppress expression of the microtubule-associated protein stathmin 2 (STMN2), through a process involving alternative polyadenylation[66–68]. Depletion of nuclear TDP43 led to the inclusion of a region within intron 1 of *STMN2* that introduced a premature stop codon and polyadenylation site. This resulted in the production of a truncated form of STMN2 that was further shown to be associated with TDP43 pathology in the brains of patients with FTD[68]. Similarly, mutations affecting the C terminus of TDP43 have been shown to cause a gain of splicing function and to drive ALS-related phenotypes in mice[62,69], whereas deletion of its PrLD reduces TDP43 toxicity, as well as misfolding and aggregation[70].

As noted above, initial findings in patients with ALS or FTD revealed increased levels of TDP43 in the cytoplasm, accompanied by nuclear depletion of the protein[3,4]. Subsequent experimental evidence in fruit flies[71,72], zebrafish[73], mice[74–76], rats[77] and non-human primates[78] established that either insufficient nuclear levels or enhanced cytoplasmic levels of TDP43 are directly related to neuronal dysfunction and cytotoxicity, suggesting a causal nexus between deregulated, mislocalized TDP43 and disease[6]. As a consequence, TDP43 levels and localization must be tightly regulated to maintain cellular homeostasis and shield neurons and glial cells from TDP43-mediated toxicity.

Autoregulated TDP43

It has been shown that the regulation of TDP43 levels and localization is achieved in part through autoregulation[22,79,80]. TDP43 can adjust or mitigate its own expression levels via a negative feedback loop in which it targets a TDP43 binding region (TDPBR) in the 3′ UTR of its own transcript[22,24,79]. Two mechanisms have been proposed to explain how this might be achieved (FIG. 2). According to the first model, elevated levels of TDP43 protein recognize and bind the TDPBR[22,24,79], which triggers alternative splicing of its own transcripts and leads to the removal of two alternative introns (6 and 7) within the last exon of *TARDBP* pre-mRNA[22]. The resulting mRNA is rather unstable and thought to be removed by nonsense-mediated decay, thereby leading to reduced levels of TDP43 protein[22,79,81]. In the second model, TDP43 binding to the TDPBR mediates intron 7 splicing, which also removes pA1 (one of the three polyadenylation sites located in the 3′ UTR of *TARDBP*)[22]. This results in an alternative *TARDBP* transcript that has a cryptic exon 7 in the 3′ UTR (with the donor splice site residing in exon 6) and only contains the remaining polyadenylation sites pA2 and pA4 (REFS[22,79,80]). This 3′ UTR splicing event results in decreased levels of *TARDBP* mRNA by altering the nucleocytoplasmic distribution of these mRNA transcripts, leading to nuclear retention and their ultimate degradation by the RNA exosome[79,80,82]. Evidence for the first and second mechanisms has been obtained by studies in transgenic mice and human embryonic kidney cells. The net result of both mechanisms remains the same: TDP43 autoregulation reduces *TARDBP* mRNA template availability and, as a consequence, regulates the level of expressed protein (FIG. 2).

As has been shown in *Drosophila melanogaster*, this autoregulatory feedback loop can be mimicked by integrating the TDPBR found in the 3′ UTR of human *TARDBP* pre-mRNA into the fly genome, where it recapitulates key features of TDP43 autoregulation — including the induction of alternative splicing events, the differential usage of polyadenylation sites and the regulation of steady-state levels of mRNA — previously described in mammalian and cellular model systems[22,79–82]. In the *Drosophila* model system, TDP43 autoregulation could be modified by splicing factor 2 (SF2), RNA-binding protein 1 (Rbp1) and splicing factor 3b subunit 1 (Sf3b1), which act as genetic modulators of TDP43 production[83]. It remains to be shown, however, whether these genetic modulators also play a similar role in mammals/humans.

Of note, altered TDP43 autoregulation has been directly related to disease. Experiments with human cell cultures and transgenic mice revealed that mouse *Tardbp* RNA undergoes complex alternative splicing, the dysregulation of which can lead to isoforms that accumulate in the cytoplasm and cause impaired autoregulation, although their impact on cytotoxicity was not determined[84]. In mice, the inhibition of splicing through the introduction of antisense oligonucleotides targeting intron 6 resulted in increased levels of total TDP43 protein and of insoluble fragments of TDP43, both of which were associated with a loss of spinal motor neurons in ageing mice[85]. RNA sequencing data obtained from HEK293 cell lines overexpressing TDP43 (REF.[86]) identified alternatively spliced, short *TARDBP* isoforms from which exon 6 was almost entirely removed[87]. Finally, analysis in rodent primary cortical neurons transfected with EGFP-tagged isoforms of TDP43 revealed cytoplasmic accumulation of a short isoform (sTDP43) that was also impaired in its capacity to induce splicing and autoregulation[87]. These data suggest a direct link between dysfunctional autoregulation and the cellular mislocalization of TDP43.

In addition to these findings, a recently developed mouse model revealed a direct link between an ALS-related *TARDBP* mutation and altered autoregulation of TDP43 (REF.[69]). In this model, a point mutation equivalent to the ALS-related human TDP43 Q331K mutation was introduced in the endogenous mouse *Tardbp* gene, thereby retaining the endogenous gene structure (including promoter and autoregulatory 3′ UTR) and maintaining ubiquitous expression of TDP43 during development and in the adult[69]. The resulting $TDP43^{Q331K/Q331K}$ knock-in mice showed an

approximately 80% increase in retention of *Tardbp* intron 7, a 14% increase in *Tardbp* expression and an approximately 45% increase in nuclear TDP43, when compared with wild-type mice. This suggests that the Q331K mutation perturbs TDP43 autoregulation by causing a gain-of-function defect; however, the mechanism through which it has this effect is unknown[69].

These experimental data are in line with the effects of mutations found in familial and sporadic cases of ALS and FTD. For example, it has been reported that patients with FTD with mutations in the 3′ UTR of *TARDBP* show increased levels of TDP43 expression[88]. Elevated levels of TDP43 protein were also observed in the post-mortem frontal cortex and hippocampus of both, patients with sporadic ALS or FTD and those in whom the disease is driven by mutations in *C9ORF72* (REF.[89]), the most common genetic form of ALS and FTD, which is characterized by cytoplasmic mislocalization and nuclear depletion of TDP43. Altogether, these findings indicate that the key pathological features of TDP43 proteinopathy might be linked, at least in part, to defective autoregulation of its own expression levels, leading to accumulating TDP43 protein and resultant mislocalization.

Localization of TDP43

Autoregulation of TDP43 is dependent on its localization to the nucleus, where it recognizes the TDPBR in the *TARDBP* transcript. Nuclear localization of TDP43 is achieved by active transport (FIG. 3a) during which TDP43 is carried as cargo across the nuclear envelope by karyopherin proteins, which function as importins that shuttle macromolecules from the cytoplasm into the nucleus[90]. Karyopherin-α (KPNA) recognizes the nuclear

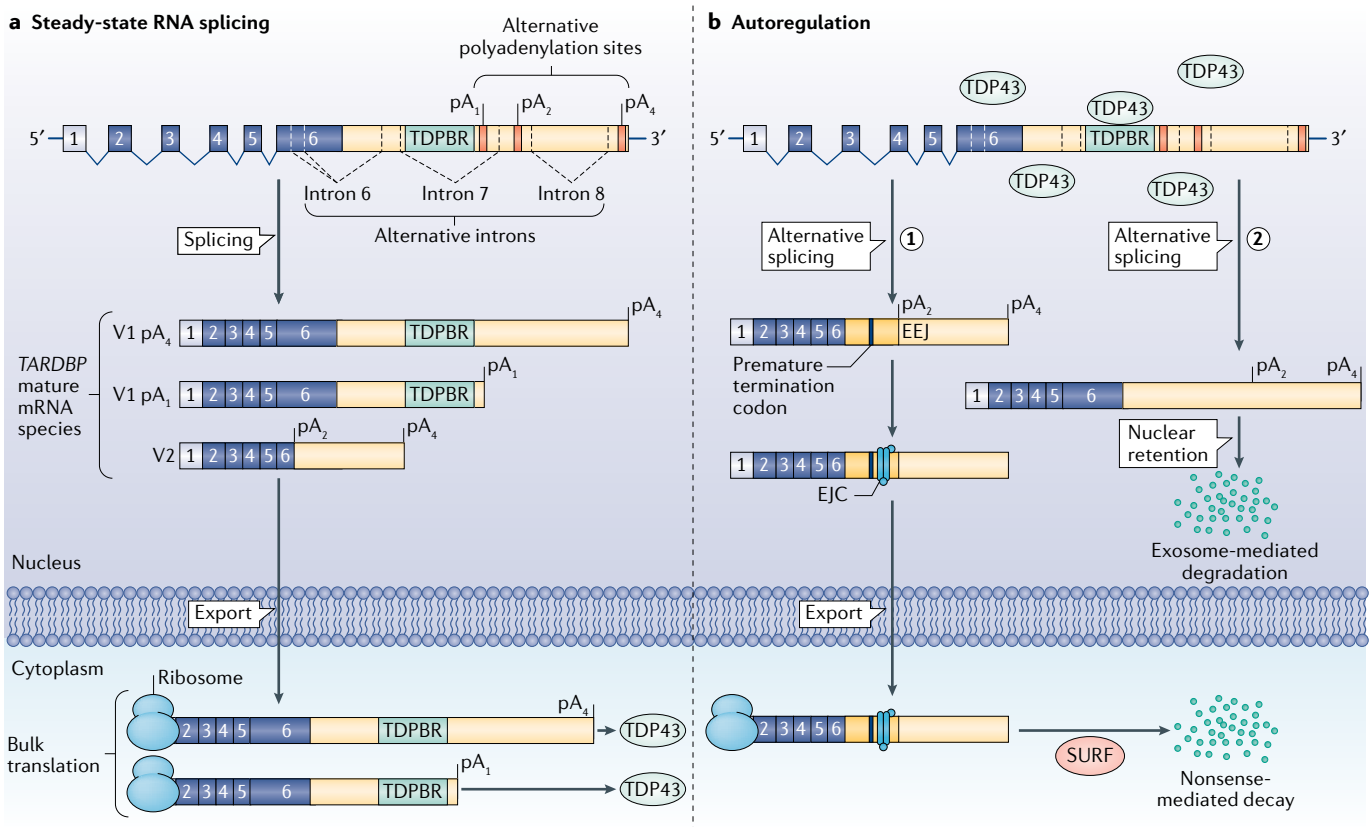


Fig. 2 | **Mechanisms of TDP43 autoregulation.** *TARDBP* pre-mRNA splicing within the nucleus leads to the formation of mature mRNA species that contain different polyadenylation sites (pA_1, pA_2 and pA_4). **a** | During steady state, in which cellular homeostasis is maintained, pA_1 is the major polyadenylation site used by mature *TARDBP* mRNA, with pA_4 utilized at a much lower rate[80,82]; note that a shorter mRNA species, V2, has been reported as well[22,79]. These mature RNAs are exported to the cytoplasm, where bulk TAR DNA-binding protein 43 (TDP43) translation takes place. **b** | TDP43 autoregulation is dependent on the TDP43 binding region (TDPBR) located in the extended 3′ untranslated region of its own pre-mRNA, a region that also contains three alternative introns (introns 6, 7 and 8)[79–82]. An increase in the levels of TDP43 results in its increased binding of the TDPBR, which leads to increased processing of alternative introns and alternative splicing. Two hypotheses have been proposed to explain how TDP43-mediated autoregulation can lead to unstable mRNA transcripts that disrupt the regulation of its endogenous protein levels through nonsense-mediated mRNA decay[22,81] in the cytoplasm (proposed mechanism 1) or nuclear retention and exosome-mediated degradation of the mRNA (proposed mechanism 2)[79,80]. In proposed mechanism 1, TDP43 binding to the TDPBR causes splicing and removal of alternative introns 6 and 7, leading to the formation of a premature termination codon and exon–exon junctions (EEJs) that are used for the assembly of exon–junction complexes (EJCs). During translation in the cytoplasm, ribosomes usually displace EJCs and EEJs; however, in the presence of a nearby premature termination codon, translation is stalled and interaction with the SMG1–UPF1–eRF1/2 (SURF) complex induces nonsense-mediated decay of the mRNA[22,79,81]. In proposed mechanism 2, TDP43 binding to the TDPBR mediates intron 7 splicing that also removes the polyadenylation site pA1, leading to a transcript that includes a 'cryptic exon 7' (not shown) and the polyadenylation sites pA_2 and pA_4. The resulting highly unstable mRNA is retained in the nucleus, where it undergoes exosome-mediated degradation. Adapted with permission from REF.[81], Oxford University Press.

localization signal (NLS) of TDP43 and together with karyopherin-β1 (KPNB1) forms a trimeric complex, through which TDP43 is imported into the nucleus[91,92]. Once in the nucleus, Ras-related nuclear protein guanosine triphosphate (RanGTP) binds the trimeric complex and dissociates it by inducing a conformational change, resulting in TDP43 release. As a result, two complexes arise: KPNB1–RanGTP and KPNA–RanGTP–CAS, with cellular apoptosis susceptibility protein (CAS) acting as a nuclear export factor in the latter. These complexes are shuttled back to the cytosol, where RanGTP is hydrolysed to RanGDP, and KPNA and KPNB1 are dissociated from RanGDP and RanGDP–CAS, respectively. As a consequence, these proteins become available again to enable more TDP43 transport into the nucleus. In the other direction, the nuclear export of TDP43 can be mediated by exportins that recognize its nuclear export signal (NES)[91,93]. TDP43 can also be exported by interacting with the mRNA export complex[94] (FIG. 3b), with the RNA helicase DDX19 and its cofactor GLE1 (REF.[95]) thought to provide directionality by preventing the backsliding of messenger ribonucleoprotein complexes into the nuclear pore[96–99].

Recent data suggest that a significant amount of TDP43 is exported to the cytoplasm via passive diffusion (FIG. 3c). It was reported that the nuclear egress of TDP43, and also of FUS, does not depend on exportin 1 (XPO1; also known as CRM1) and deletion of the TDP43 NES did not impede nuclear export of TDP43 in HeLa cells. Indeed, export occurred even when the mRNA export factor ALYREF was silenced[94]. These findings are in contrast to those of another study carried out in *D. melanogaster,* which showed that downregulation of the ALYREF orthologue *Ref1* mitigated TDP43-mediated neurodegeneration in the fly eye[100]. Furthermore, artificial enlargement of TDP43 significantly impaired its movement in HeLa cells from the nucleus to the cytoplasm, indicating that diffusion was size-dependent[94]. These findings are corroborated by a study that showed that the NES is not required for nuclear egress but, when mutated, affects both the solubility and the splicing function of TDP43 in HeLa cells and rat hippocampal neurons[101]. Similarly, pharmacological application of selective inhibitor of nuclear export (SINE) compounds, which target XPO1, had no effect on TDP43 localization in rodent primary cortical neurons[102]. Together, these data question the functional significance of the NES of TDP43 and indicate that nuclear egress occurs via passive diffusion[94] and/or might be the result of its interaction with the RNA export machinery[103].

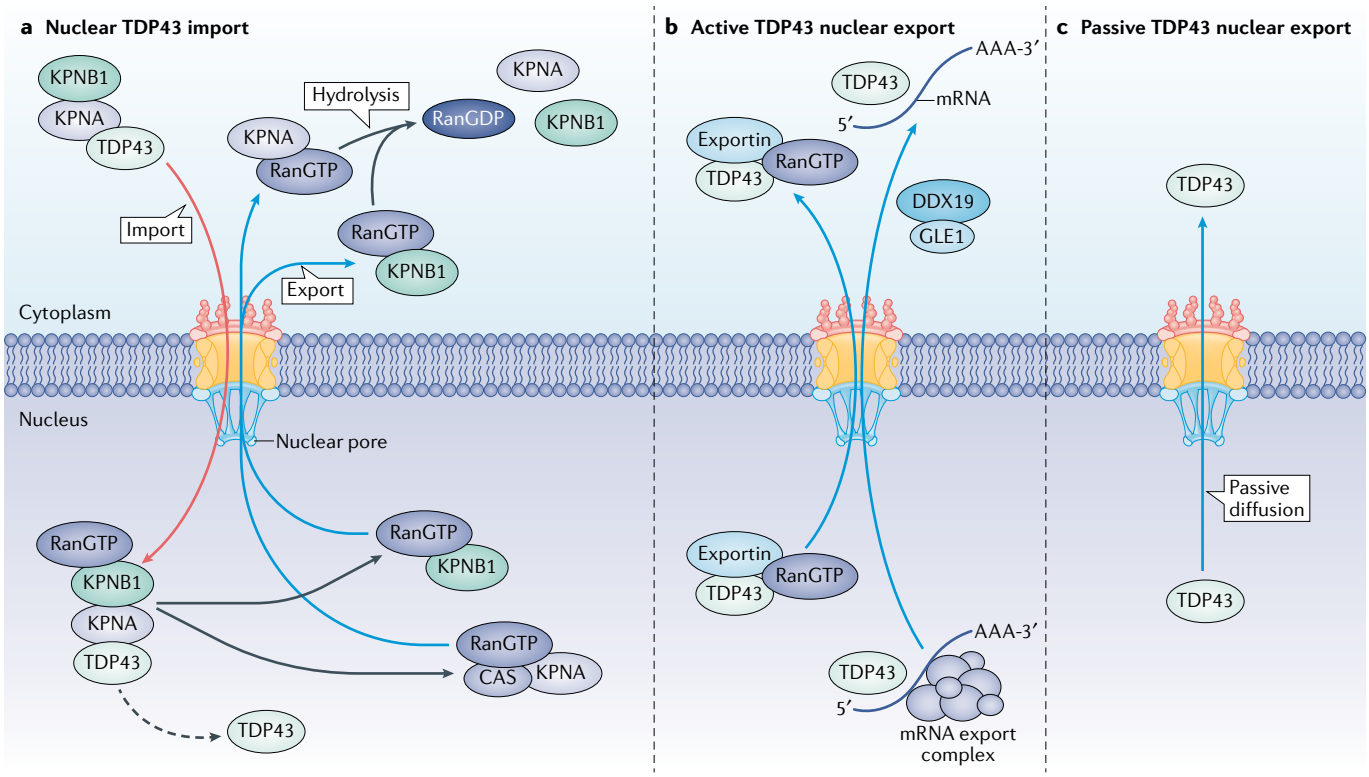


Fig. 3 | **Nucleocytoplasmic transport and diffusion of TDP43. a** | TAR DNA-binding protein 43 (TDP43) is imported into the nucleus with the help of karyopherin-α proteins (KPNAs) and karyopherin-β1 (KPNB1)[92,94,101]. There, Ras-related nuclear protein guanosine triphosphate (RanGTP) dissociates the complex and releases TDP43 in the nucleus. KPNB1 associates with RanGTP and KPNA associates with RanGTP and cellular apoptosis susceptibility protein (CAS), a nuclear export factor. These two complexes exit from the nucleus and, after RanGTP undergoes hydrolysis, the karyopherins are free in the cytoplasm to start another round of import. **b** | Two suggested mechanisms of active TDP43 export. The mechanism on the left depicts TDP43 forming a complex with exportin and RanGTP, which is continuously produced by guanine nucleotide exchange factor (GEF). The mechanism on the right shows mRNA export, in which TDP43 is exported through an interaction with mRNA or with the mRNA–export complex[96]. On the cytoplasmic side of the nuclear pore complex, DDX19 RNA helicase and its cofactor GLE1 ensure the success of the mRNA transport. **c** | Passive diffusion has also been suggested as a means of TDP43 nuclear export[94,100,101].

Molecular chaperones
Proteins that assist in the correct and timely folding or unfolding of other proteins or the assembly and disassembly of macromolecular complexes.

The significance of nucleocytoplasmic shuttling of TDP43 for its proper function is further illustrated by experiments in which its localization signals have been artificially deleted. Expression of human TDP43 with a defective NLS in transgenic mice led to a dramatic decrease in endogenous mouse TDP43 levels and caused corticospinal tract degeneration and motor spasticity in the absence of insoluble aggregates[104]. Another study that utilized mouse primary hippocampal neurons reported that expression of *TARDBP* carrying a mutation in the NLS-coding sequence results in insoluble cytoplasmic TDP43 aggregates that, in turn, sequester endogenous TDP43 protein, leading to the nuclear clearance of endogenous TDP43 (REF.[93]). The significance of these findings is further illustrated by recent studies utilizing transgenic mice with doxycycline-suppressible expression of human *TARDBP* with deletion of the TDP43 NLS. This resulted in accumulation of cytoplasmic aggregates of TDP43, the loss of endogenous nuclear mouse TDP43, dramatic motor neuron loss and progressive motor impairments leading to premature death[105]. Of note, reversion of the expression of the mutated gene after disease onset decreased TDP43 pathology, increased nuclear endogenous TDP43 to control levels and prevented further motor neuron loss[105].

Comparable phenotypes have also been observed in patients with ALS or FTD caused by a G4C2 hexanucleotide repeat expansion in *C9ORF72* (REFS[106,107]) in which neurodegeneration is always accompanied with TDP43 pathology[108]. These 'C9ALS/FTD' cases are characterized by accumulation of G4C2 mRNA and dipeptide repeat proteins (DPRs) that are generated by repeat-associated non-ATG (RAN) translation from the hexanucleotide repeat RNA[109]. Functional studies in cell and animal models identified defective nucleocytoplasmic transport as being directly related to TDP43 proteinopathy[92] and C9ALS/FTD[110–115]. Further studies showed that accumulating cytosolic TDP43 caused cytoplasmic mislocalization of KPNAs in *D. melanogaster* models of C9ALS/FTD, a phenotype that was also observed in post-mortem frontal cortex tissue of patients with C9ALS and in sporadic cases of FTD with TDP43 pathology[116,117]. Moreover, a vicious feedback cycle was identified by which G4C2-derived DPRs mediate TDP43 and subsequent KPNA pathology. These TDP43 and KPNA abnormalities in turn become independent of the initiating DPR trigger, leading to further nuclear depletion and cytoplasmic mislocalization of TDP43 that precedes degenerative cell loss[117]. Together, these data demonstrate that nucleocytoplasmic transport and karyopherin function are essential processes to maintain correct TDP43 localization, which is required for cellular homeostasis.

TDP43 phase separation

As described above, TDP43 harbours a prion-like low-complexity domain[29,30], which is an intrinsically disordered region that is devoid of charged amino acids but enriched in polar ones (glutamine, asparagine, serine and glycine), with interspersed aromatic residues (tyrosine and phenylalanine). Recent functional studies showed that PrLDs similar to that found in TDP43 make the proteins that harbour them (known collectively as intrinsically disordered proteins) vulnerable to changes in the surrounding milieu that can alter their physical properties, eventually altering their physical state to generate pathological aggregates[118,119] (FIG. 4a). These changes are similar to the phase separations that occur in some mixed solutions, which range from the liquid–liquid demixing that produces liquid droplets to phase transitions into gel-like structures (hydrogels) that, over time, can mature into irreversible, fibrillar aggregates such as amyloid-like fibres[120,121] (FIG. 4b). These transitions, especially the initiating phase separation of soluble proteins into droplets and hydrogels, are sensitive to changes in temperature, salt, pH or protein concentration, and tend to occur in membraneless compartments such as stress granules, P granules, paraspeckles and nucleoli[121].

Several studies demonstrated that, like other intrinsically disordered proteins, TDP43 undergoes liquid–liquid phase separation[34,118,122] that is modulated by karyopherins acting as molecular chaperones[123,124]. Thus, under physiological conditions, TDP43 can form membraneless liquid droplet-like structures and can undergo phase separation[125]. Phase separation of many intrinsically disordered proteins has been shown to be mediated by cation–π, π–π and hydrophobic intermolecular interactions between the charged and polar amino acids in the PrLD[121], although it remains to be determined whether similar mechanisms mediate phase separation of human TDP43. In addition, in vitro studies using cell-free and cell culture assays demonstrated that TDP43 can form spherical liquid droplet-like structures[118,122,124,126,127]. It is known that persistent cellular stress or mutations affecting the 3D structure of the low-complexity domain[32,36] can lead to droplet ageing and cause droplets to transition into hydrogels, which could fibrillate over time, as has been shown for hnRNPA1 (REF.[128]). The phase transitions of TDP43 are crucially dependent on its C-terminal domain[118], especially its α-helical structure[34] and three tryptophan residues within it: W334, W385 and W412 (REF.[129]) (FIG. 1).

These changes in TDP43 aggregation state have been directly linked to nucleocytoplasmic transport and its mediators, the karyopherins (FIG. 3). Using hydrogels, it was shown that KPNA and KPNB1 can interact with and bind to the NLS of TDP43. Upon binding of karyopherins, the aberrant phase transition of TDP43 was reversed and prevented from occurring any further[123]. These findings are consistent with the discovery that ALS-related mutations in the PrLD of TDP43 can accelerate the transition of liquid-like droplets of TDP43 to pathologically fibrillar aggregates[128], whereas overexpression of KPNA and KPNB1 resulted in the reversal and further prevention of these pathological processes[123]. These findings are also consistent with recent studies in *Drosophila*, mouse cortical neurons, transgenic mice expressing TDP43 and induced pluripotent stem cells or post-mortem material derived from patients with sporadic or familial forms of ALS or FTD, which revealed that disease-initiating cytoplasmic accumulation of TDP43 is associated with KPNA abnormalities that are accompanied by KPNB1 depletion and subsequent dysfunctional nucleocytoplasmic transport[113,116,117,124]. Of note, these studies

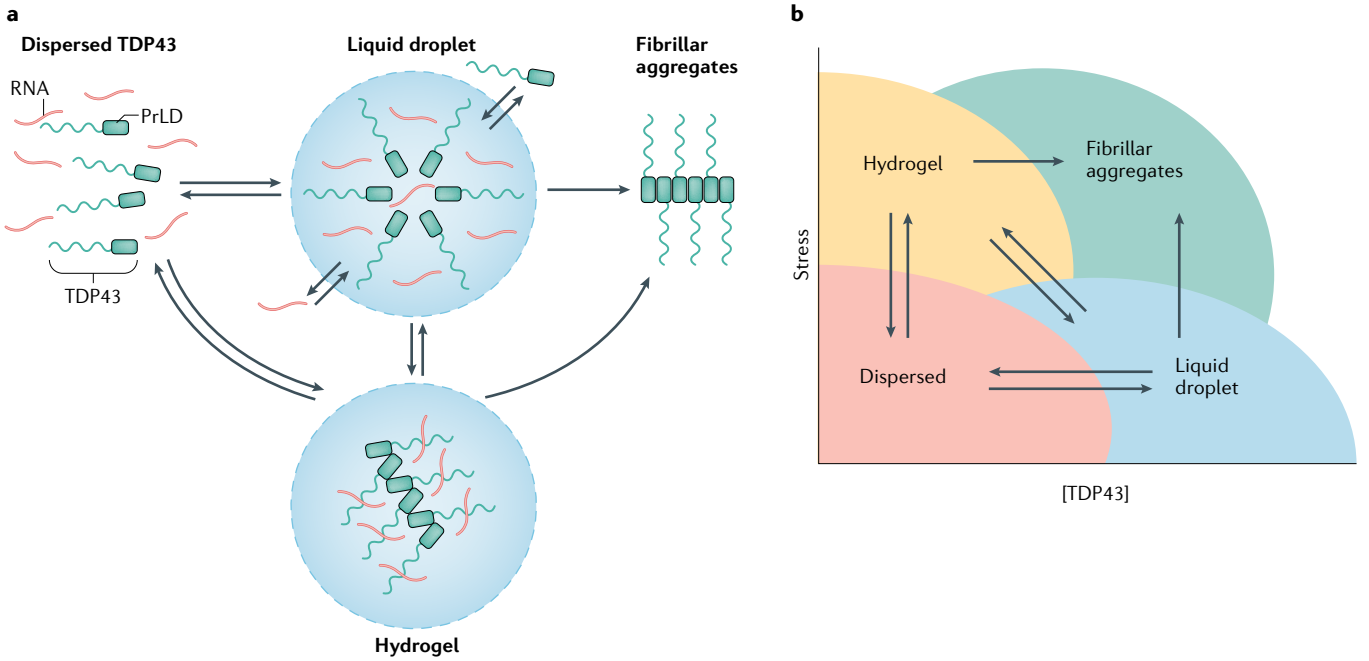


Fig. 4 | **Phase transition of TDP43. a** | Liquid–liquid phase separation of TAR DNA-binding protein 43 (TDP43). In physiological conditions, TDP43 exists in a dispersed state (left). In response to stress or other factors, such as disease-related mutations, protein accumulation or protein interactions with its low-complexity domain, TDP43 undergoes liquid demixing and converts to liquid droplets; however, a return to the dispersed state is still possible[123–129]. Interactions of the protein with RNA molecules are possible in both the dispersed and droplet states, and movement of protein or RNA from the droplet to the surrounding environment is also still possible[123,125]. Later, if the stress persists, the droplets can turn into hydrogels or directly into fibrillar aggregates[124]. Droplet and hydrogel fusion are both possible and all states can be reversed, apart from that of the fibrillary aggregates. **b** | Phase diagram depicting how stress and protein concentration [TDP43] can lead to phase separation; arrows depict reversible or irreversible phase transitions that correlate with abundance of cytoplasmic TDP43 (REFS[123–125,128]). PrLD, prion-like domain.

consistently observed a reduction in and eventual loss of endogenous nuclear TDP43 as a result of its cytoplasmic accumulation and defective nucleocytoplasmic transport. Given the essential role of nuclear TDP43 in regulating its own mRNA (and thus protein) levels[22,79], these findings suggest that there may be a detrimental feedforward cycle of cytoplasmic mislocalization, aberrant phase transition and defective autoregulation that can affect each other and interdependently trigger TDP43 pathology (FIG. 5).

TDP43 out of control

TDP43 pathology is the defining characteristic of ALS and FTD and is used to determine the severity and stages of disease progression[130–132]. The predominant features of this pathology are the cytoplasmic accumulation and aggregation of TDP43 strains, often accompanied by nuclear depletion[133]. Although it remains to be proven why both phenotypes co-occur, they have both been causally related to disease[23], emphasizing the need for tight control of the abundance and subcellular localization of TDP43. The three pathogenic mechanisms reviewed above suggest they are part of an intrinsic control system regulating the physiological levels and localization of TDP43 (FIG. 5).

Autoregulation safeguards TDP43 abundance to ensure that it remains within a healthy range, which is essential for the processes that are regulated by TDP43 in order to maintain cellular homeostasis[22,24,69,79–82,85]. Nucleocytoplasmic transport is the predominant mechanism that shuttles TDP43 across the nuclear membrane and also secures its enrichment in the nucleus[92,101], where its key functions (such as splicing regulation) take place[19]. Liquid–liquid phase separation realizes the transition between physiological aggregation states (ranging from native monomers to liquid droplets and assembly into membraneless organelles[34,118,123,124,129,134]), thereby retaining TDP43 in its functional form and devoid of irreversible aggregation. These three mechanisms thus individually regulate various aspects of TDP43 homeostasis and function. Dysregulation or malfunction of any one of them affects the other (FIG. 5), and the resulting phenotypes provide compelling evidence that the three mechanisms are connected in a delicate interplay to maintain normal TDP43 function, which otherwise results in cytotoxicity and progressive neurodegeneration.

This model is supported by recent studies using cell cultures, transgenic mice and patient-derived induced pluripotent stem cells, which reported that phase transition of TDP43 into cytosolic liquid droplets resulted in the accumulation of phosphorylated TDP43, which converted into gels upon cellular stress[124]. These gels in turn recruited karyopherins and nuclear pore proteins, which impaired nucleocytoplasmic transport, leading to clearance of nuclear TDP43 and resulting in cell death[124]. Similar clearance of nuclear TDP43 has been observed to be triggered by accumulation of G4C2-derived DPRs, resulting in cytoplasmic accumulation and subsequent nuclear depletion of TDP43 (REFS[116,117]). In line with the

idea that there is a direct nexus between karyopherin abnormalities and TDP43 pathology, several studies have shown that KPNA or KPNB1 overexpression is able to reverse and prevent the formation of fibrillar TDP43 (REFS[116,123]). These data demonstrate that reduced levels of karyopherins or disrupted karyopherin binding can enhance phase transition and irreversible aggregate formation of TDP43, a conclusion that was independently demonstrated in human cell culture experiments using cytoplasmic fragments of TDP43 (REF.[135]).

Typical ALS-related mutations in TDP43 result in an accelerated transition from liquid droplets to irreversible aggregates[128] or directly affect the autoregulation of TDP43 (REF.[69]). The resultant nuclear import deficits or enhanced phase separation impair TDP43 autoregulation. Once 'trapped' in droplets and irreversible aggregates, the protein can no longer monitor its mRNA synthesis and/or splicing[124] and fails to regulate the endogenous protein level. The resulting accumulation of TDP43 affects its phase transition[124] and nuclear import[116,117], further accelerating TDP43 pathology. In addition, cellular stress leads to TDP43 phase transition, which — over time — can transform liquid droplets into fibrillar aggregates that cannot reverse into soluble forms, and thus TDP43 becomes dysfunctional[118]. These cytoplasmic TDP43 fibrils can attract and coexist with interaction partners, such as importins[117,123]. The ensuing nucleocytoplasmic transport deficits lead to mislocalization and accumulation of TDP43 in the cytoplasm, and its nuclear clearance[116,117], which also affects autoregulation and, ultimately, causes degenerative cell death.

Conclusion and future directions

Altogether, these findings provide evidence that TDP43 autoregulation, nucleocytoplasmic transport and phase transition are interrelated and are part of an intrinsic regulatory system that maintains the physiological

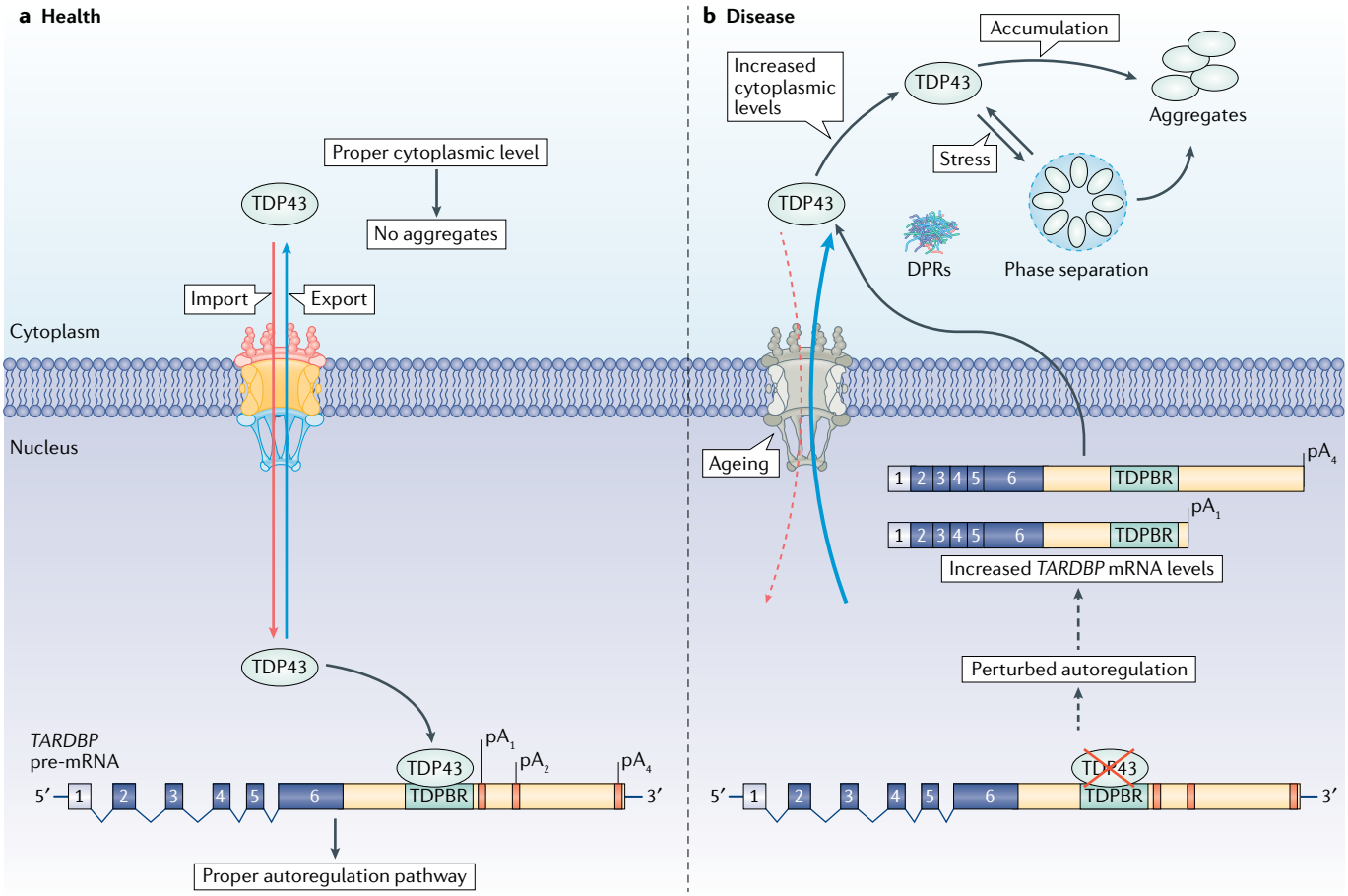


Fig. 5 | **Mechanisms of TDP43 control.** A potential vicious cycle leading to the perturbation of TAR DNA-binding protein 43 (TDP43) homeostasis. According to this model, nucleocytoplasmic transport, autoregulation and phase transition may all be part of an intricate regulatory system related to TDP43 pathology and contribute to a feedforward loop promoting disorder, cytotoxicity and cell death. **a** | In a healthy cell, TDP43 nucleocytoplasmic shuttling is normal, keeping the nuclear to cytoplasmic ratio of the protein within a healthy range. This shuttling also contributes to physiological levels of nuclear TDP43 and the maintenance of a normal level of autoregulation, with TDP43 in the nucleus binding to the 3′ untranslated TDP binding region (TDPBR) of the *TARDBP* pre-mRNA. **b** | In disease, nuclear import of TDP43 appears to be disturbed (indicated by red dashed arrow). This could be the result of multiple factors, including the accumulation of dipeptide repeat proteins (DPRs) that occurs in cases of amyotrophic lateral sclerosis or frontotemporal dementia caused by a G4C2 hexanucleotide repeat expansion in *C9ORF72*, or simply because of ageing. As a result, TDP43 cytoplasmic levels increase, which could lead to accumulation of the protein or trigger phase separation and its irreversible aggregation. Furthermore, nuclear depletion of TDP43 renders its autoregulation machinery dysfunctional, leading to accumulation of deregulated *TARDBP* mRNA levels and, eventually, to increased levels of TDP43 that accumulate in the cytoplasm. pA_1, pA_2 and pA_4, polyadenylation sites.

levels, subcellular localization and native aggregation state of TDP43. They constitute three vectorial forces of a triangular system to safeguard TDP43 function that is required for cellular homeostasis and to restrain the vortex of TDP43 pathology that otherwise will result from dysfunction of any one of them (FIG. 5). This system of TDP43 control is vulnerable to many insults, including cellular stress, gene mutations or initiating triggers (such as the DPRs derived from hexanucleotide repeats in C9ALS/FTD)[117].

Despite recent advances in understanding the underlying causes of TDP43 pathology, it remains to be shown how cytoplasmic accumulation and nuclear depletion are coordinated during the onset and progression of disease. Dissection of these mechanisms will require experimental assays in which, for example, autoregulatory sequences in the 3′ UTR of *TARDBP* pre-mRNA are ablated[81] in order to determine whether and how cytoplasmic accumulation can affect nuclear depletion despite defective autoregulation. Similarly, it will be interesting to determine whether and how mutations of the tryptophan residues that are required for liquid–liquid phase separation of TDP43 (REF.[129]) are necessary and sufficient to avoid cytoplasmic accumulation and subsequent aggregate formation, and how this may spare nuclear depletion of TDP43. Furthermore, transgenic or pharmacological targeting of nucleocytoplasmic transport could test whether and how it might be able to alleviate the detrimental effects of cytoplasmic accumulation of TDP43, whether and to what extent these interventions might reverse or slow down nuclear depletion of TDP43 and how this may delay or even rescue (at least to some extent) loss-of-function phenotypes.

The triangular system of TDP43 control may indeed represent a novel therapeutic target. Several compounds that inhibit nuclear transport of specific cargo substrates have been reported as anticancer agents, with more than 20 nuclear transport inhibitors being identified[136]. Verdinexor (KPT-335) and KPT-350, which selectively interfere with the XPO1-dependent nuclear export pathway, have been shown to alleviate motor symptoms and to inhibit inflammatory demyelination and axonal damage in a preclinical mouse model of demyelination, which resulted in attenuation of disease progression[137]. Pharmacological treatment with KPT-335 and KPT-276, another XPO1 inhibitor, were able to suppress 55–62% of the cell death caused by selective overexpression of C-terminal fragments of TDP43 or mutant TDP43^{Q331K} in mouse cortical neurons[116]. Corresponding therapeutic effects were also observed in *D. melanogaster* models of C9ALS, in which application of KPT-276 was able to ameliorate eye degeneration caused by overexpression of G4C2 hexanucleotide repeat expansions[110]. It remains to be shown how these therapeutic interventions prevent TDP43 pathology, and resolving this question may require more specific compounds that target the triangle of TDP43 control; however, a promising start has been made.

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