TDP-43 pathology: from noxious assembly to therapeutic removal

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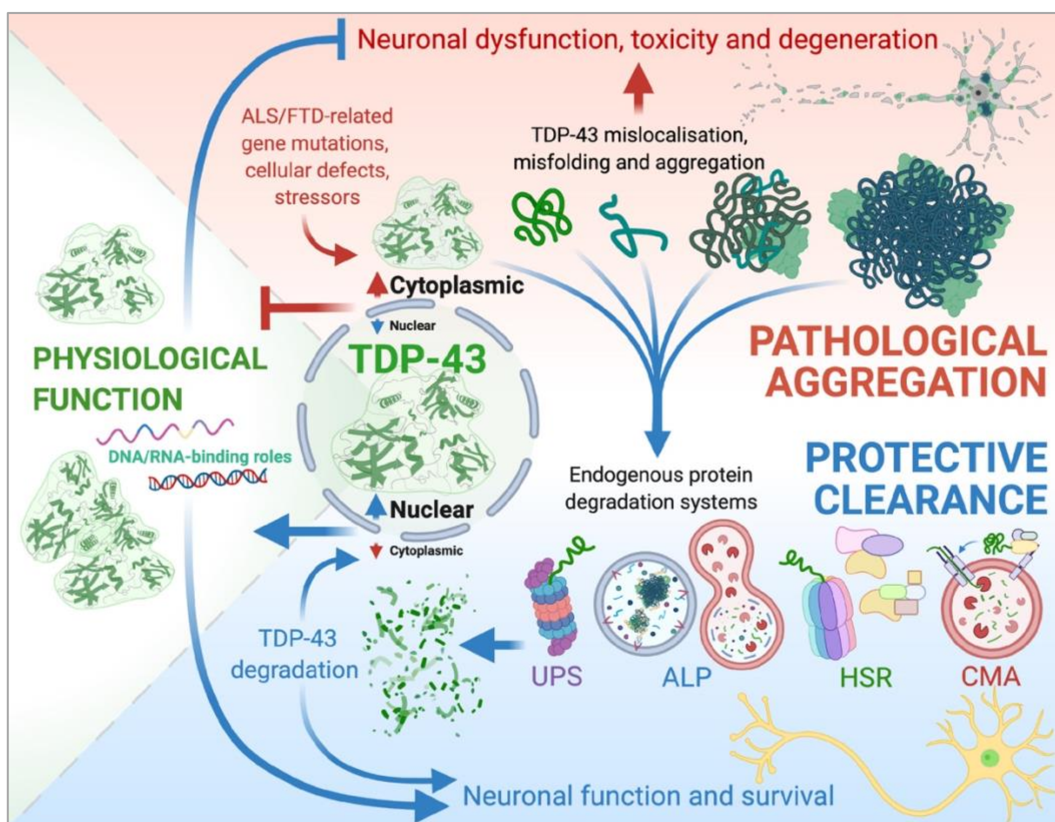
Abstract

Our understanding of amyotrophic lateral sclerosis and frontotemporal dementia has advanced dramatically since the discovery of cytoplasmic TAR DNA-binding protein 43 (TDP-43) inclusions as the hallmark pathology of these neurodegenerative diseases. Recent studies have provided insights into the physiological function of TDP-43 as an essential DNA-/RNA-modulating protein, and the triggers and consequences of TDP-43 dysfunction and aggregation. The formation of TDP-43 pathology is a progressive process, involving the generation of multiple distinct protein species, each with varying biophysical properties and roles in neurodegeneration. Here, we explore how the pathogenic changes to TDP-43, including *mislocalisation*, *misfolding*, *aberrant liquid-liquid phase separation*, *stress granule assembly*, *oligomerisation*, and *post-translational modification*, drive disease-association aggregation in TDP-43 proteinopathies. We highlight how pathological TDP-43 species are formed and contribute to cellular dysfunction and toxicity, via both loss-of-function and gain-of-function mechanisms. We also review the role of protein homeostasis mechanisms, namely the *ubiquitin proteasome system*, *autophagy-lysosome pathway*, *heat-shock response*, and *chaperone-mediated autophagy,* in combating TDP-43 aggregation and discuss how their dysfunction likely promotes disease pathogenesis and progression. Finally, we evaluate pre-clinical studies aimed at enhancing TDP-43 protein clearance via these mechanisms and provide insight on promising strategies for future therapeutic advances. Harnessing the mechanisms that protect against or ameliorate TDP-43 pathology presents promising opportunities for developing disease-modifying treatments for these neurodegenerative diseases.



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Highlights:

- *Physiological RNA-binding, self-assembly, and LLPS influence TDP-43 aggregation*
- *Distinct pathological TDP-43 species contribute differentially to cellular dysfunction and toxicity*
- *Neuronal proteostasis failure facilitates TDP-43 aggregation in ALS, FTD, and other neurodegenerative diseases*
- *TDP-43 aggregation impairs protein degradation systems to potentiate disease*
- *Modulating TDP-43 clearance holds promise for effective disease-modifying treatment*

Key words:
TDP-43 proteinopathy, protein degradation, proteostasis, neurodegeneration, motor neuron disease, ALS, FTD, LLPS

List of abbreviations:
ALP, Autophagy-lysosome pathway; ALS, Amyotrophic lateral sclerosis; C9ORF72, Chromosome 9 open reading frame 72; CMA, Chaperone-mediated autophagy; CNS, Central nervous system; CTD, C-terminal domain; CTF, C-terminal fragment; DPR, Dipeptide repeat; FTD, Frontotemporal dementia; FTLD-TDP, Frontotemporal lobar degeneration with TDP-43 proteinopathy; GWAS, Genome-wide association studies; HSF1, Heat-shock factor 1; HSP, Heat-shock protein; HSR, Heat-shock response; iPSC, Induced pluripotent stem cell; LAMP2A, Lysosome-associated membrane protein type 2A; LATE, Limbic-predominant age-related TDP-43 encephalopathy; LCD, Low-complexity domain; LLPS, Liquid-liquid phase separation; mTOR, Mammalian target of rapamycin; NLS, Nuclear localisation signal; Nups, Nucleoporins; Proteostasis, Protein homeostasis; PROTAC, proteolysis targeting chimera; PTM, Post-translational modification; RRM, RNA-recognition motif; SG, Stress granule; siRNA, Small-interfering ribonucleic acid; SOD1, Superoxide dismutase 1; TDP-43, TAR DNA-binding protein 43; UBQLN2, Ubiquilin 2; UPS, Ubiquitin proteasome system.

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1 Introduction

Dysfunction of TAR DNA-binding protein 43 (TDP-43) is the key unifying feature of most cases of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (Arai et al., 2006; Neumann et al., 2006). Despite their distinct clinical presentations, these diseases share a common neuropathology of cytoplasmic TDP-43 protein inclusions within neurons, often accompanied by a loss of normal nuclear TDP-43, which correlates strongly with neurodegeneration in the affected regions (Arai et al., 2006; Brettschneider et al., 2013; Neumann et al., 2006). ALS is characterised by the loss of upper and lower motor neurons in the motor cortex and spinal cord, leading to muscle denervation followed by progressive muscle weakening, atrophy, paralysis, and respiratory distress (Burrell et al., 2016; Grad et al., 2017). FTD involves degeneration of von Economo neurons and fork cells within the frontoinsular and anterior cingulate cortices (Kim et al., 2012; Nana et al., 2019), which commonly manifests as profound alterations in personality, emotional behaviour, social conduct, and/or language (Bang et al., 2015; Burrell et al., 2016). Together, almost 97% of ALS and 50% of FTD cases exhibit primary TDP-43 pathology, the latter being classified pathologically as frontotemporal lobar degeneration with TDP-43 (FTLD-TDP) (Ling et al., 2013). The clinical and pathological heterogeneity of these TDP-43 proteinopathies can be at least partly explained by the diverse morphologies and distributions that characterise distinct TDP-43 assemblies throughout the central nervous system (CNS) and distinguish individual ALS or FTLD-TDP subtypes (Kawakami et al., 2019; Laferriere et al., 2019; Takeuchi et al., 2016). However, there is a notable clinical overlap between these diseases, with evidence of impaired behaviour and cognitive or executive function in up to 50% of ALS patients, and in ALS animal models (Beeldman et al., 2020; Chiò et al., 2019; Gao

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et al., 2021; Kasper et al., 2015), as well as motor deficits in some FTD patients (Burrell et al., 2016). Importantly, TDP-43 pathology has also been observed in other neurodegenerative diseases, including a proportion of Alzheimer's disease (AD), Parkinson's disease, and Huntington's disease cases (de Boer et al., 2021). More recently, limbic-predominant age-related TDP-43 encephalopathy (LATE) has also been described as a primary TDP-43 proteinopathy (Nelson et al., 2019). No effective disease-modifying treatments are currently available, likely because previous therapeutic approaches have failed to target the underlying neurodegenerative pathology. However, the widespread involvement of TDP-43 suggests that therapeutic approaches that ameliorate pathological species of TDP-43 may be beneficial in treating ALS, FTLD-TDP, LATE, and other diseases with TDP-43 pathology.

Here, we examine the physiological functions of TDP-43, triggers of TDP-43 dysfunction, assembly of aggregating species, and subsequent cellular consequences. Although TDP-43 pathology is a consistent feature of ALS and FTLD-TDP, fewer than 1% of cases of either disease are caused by *TARDBP* gene mutations (Kim et al., 2020b; McCann et al., 2017). Accumulating evidence suggests that intrinsic structural characteristics of the TDP-43 protein, disease-causing mutations in other genes, extrinsic stressors, and defective protein homeostasis (proteostasis) pathways can all drive the pathological transformation of wild-type TDP-43. Four essential endogenous proteostasis systems – the ubiquitin-proteasome system, autophagy-lysosome pathway, heat shock response, and chaperone-mediated autophagy – differentially contribute to the detection, sequestration, refolding, or degradation of misfolded and aggregated proteins that may be toxic or impair cell functionality (Boland et al., 2018; Klaips et al., 2018; Nixon, 2013). While these systems normally maintain TDP-43 quality control, the persistence of TDP-43 inclusions in post-mortem patient tissue and cell and animal disease models indicates likely impairment of these protective neuronal protein clearance pathways, which are increasingly implicated in disease pathogenesis (Shahheydari et al., 2017). Understanding the mechanisms by which endogenous proteostasis systems orchestrate the clearance of different pathological TDP-43 protein species in neurons will reveal crucial protective pathways that could be exploited in therapeutic interventions aimed at combatting TDP-43 aggregation.

2 TDP-43: structure, function and mechanisms that mediate noxious assembly of pathological species

Although TDP-43 pathology is the common hallmark of sporadic and inherited cases of ALS and FTLD-TDP, the rarity of *TARDBP* mutations means that it is most often the 'wild-type' form of TDP-43 that becomes dysfunctional and undergoes aggregation. The native structure and functional interactions of TDP-43 contribute to an intrinsic aggregation propensity that links the physiological roles of this protein with the pathological changes that lead to neurodegeneration.

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1 TDP-43 is a ubiquitously expressed, highly conserved 414-amino-acid protein that includes a nuclear
2 localisation signal (NLS), two RNA-recognition motifs (RRM1 and RRM2), and an intrinsically
3 disordered C-terminal domain (CTD) comprising glutamine-/asparagine-rich and glycine-rich regions
4 (Buratti and Baralle, 2009). These functional domains mediate interactions with DNA, RNA, and other
5 proteins to regulate mRNA stability, splicing, and translation, mRNA and ribonucleoprotein transport,
6 and stress granule (SG) assembly (Afroz et al., 2017; Ayala et al., 2011b; Ayala et al., 2008). Some
7 regions also participate in binding other TDP-43 proteins for physiological homo-oligomerisation,
8 liquid-liquid phase separation (LLPS), and SG assembly (Chen and Cohen, 2019; Fang et al., 2019;
9 Gasset-Rosa et al., 2019; Guenther et al., 2018; Mann et al., 2019). The functions of these structural
10 domains therefore modulate the cellular distribution, dynamics, self-assembly, and solubility of TDP-
11 43 (Ayala et al., 2008). Specifically, the detection of the RRMs and CTD within truncated fragments
12 of TDP-43 isolated from patient tissues indicates that these domains may be involved in aggregation
13 and toxicity (Mackness et al., 2014; Wang et al., 2013b; Yang et al., 2011). In disease, TDP-43
14 pathology may therefore develop when this intrinsic aggregation propensity is exacerbated beyond a
15 critical point by extrinsic perturbations to structure, function, localisation, and solubility, to initiate or
16 accelerate the noxious assembly of pathological TDP-43 species.
17
18 The most well-characterised modifiers of ALS and FTLD-TDP pathogenesis are the disease-linked
19 mutations that account for approximately 10-15% and 15-30%, respectively, of all (sporadic and
20 inherited) cases (Pottier et al., 2016; Renton et al., 2014). Within the subset of cases with a known
21 genetic cause, mutations in the *TARDBP* gene that encodes TDP-43 account for only 5-10% (Kabashi
22 et al., 2008; Pesiridis et al., 2009; Sreedharan et al., 2008), with the remaining 90-95% related to
23 mutations in other genes such as *C9ORF72, SOD1, FUS* and *UBQLN2* (Kim et al., 2020b). *TARDBP*
24 mutations account for even fewer cases of genetic FTLD-TDP, which are largely attributable to
25 *C9ORF72* and *GRN* mitations (Pottier et al., 2016). While rare, mutations in *TARDBP* exacerbate
26 TDP-43 aggregation and can cause ALS and FTD, providing strong evidence that TDP-43 loss-of-
27 function and gain-of-toxicity drives pathogenesis (Alami et al., 2014; Budini et al., 2012; Budini et al.,
28 2015; Nonaka et al., 2009). Importantly, the pathogenic changes to TDP-43 protein (including
29 mislocalisation, aggregation, and post-translational modification) are also evident in almost all other
30 sporadic or inherited ALS and FTLD-TDP cases without *TARDBP* mutations (de Boer et al., 2021).
31
32 Cytoplasmic TDP-43 aggregation likely occurs via a progressive process, involving the formation of
33 sequential disordered intermediate species including misfolded and oligomeric TDP-43, which likely
34 precede the terminal deposition of large, insoluble TDP-43 inclusions. These distinct pathological
35 species have been reported in human disease tissues and variably recapitulated in cellular and animal
36 models. It is likely that they are each generated via different pathological mechanisms, possess
37 varying biochemical properties, and exert independent effects on neuronal function and viability (Fang
38 et al., 2014; French et al., 2019; Scotter et al., 2014). The various molecular changes that underlie

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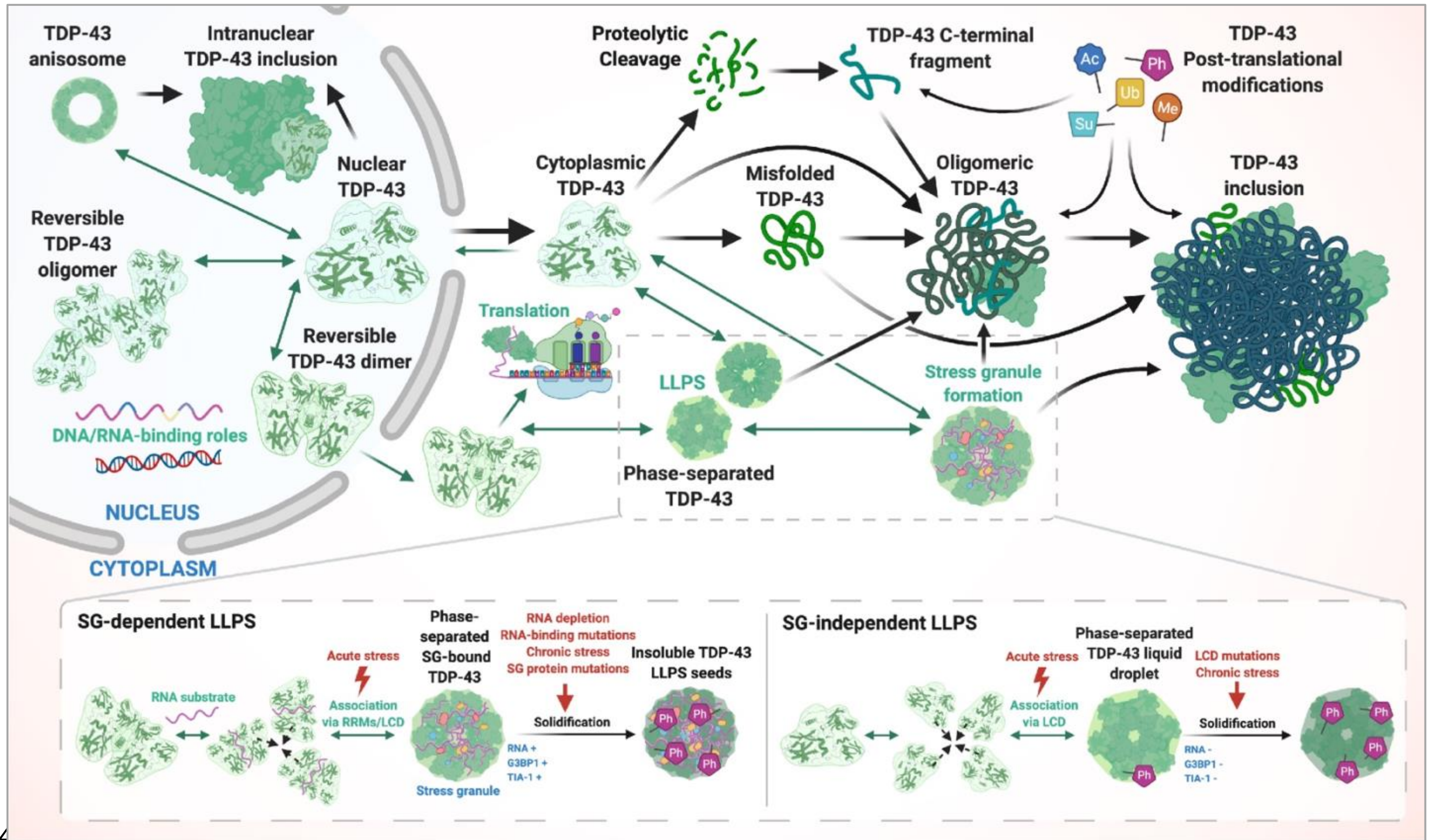
this progression of TDP-43 pathology reveal a complex network of interactions between the various pathological TDP-43 species, and their relative contributions to neurodegeneration (***Figure 1***).



<u>Figure 1:</u> The noxious assembly of distinct pathological TDP-43 species in TDP-43 proteinopathies. Schematic 'working model' for the TDP-43 aggregation process, illustrating the crucial molecular changes by which physiological TDP-43 becomes pathological (black arrows), from *mislocalisation* and *misfolding*, to *aberrant liquid-liquid phase separation* and *stress granule incorporation*, *C-terminal fragmentation* and *oligomerisation*, *post-translational modification*, and finally the deposition of insoluble *inclusions*. Multiple factors contribute to the generation of each species, including intrinsic structural characteristics/functions of TDP-43, disease-associated genetic mutations, extrinsic stressors, and defective homeostatic pathways. Green arrows represent physiological changes in localisation, structure and interactions that are required for the normal functions of TDP-43 but also have the potential to drive the formation of pathological species. These distinct cytoplasmic pathological TDP-43 species likely exist together in degenerating neurons. Schematic figure created with *BioRender*. TDP-43 tertiary structure adapted from *AlphaFold Protein Structure Database*: https://alphafold.ebi.ac.uk/entry/Q13148.

Therefore, understanding how disease-associated genetic mutations, as well as how intrinsic characteristics of the TDP-43 protein, extrinsic stressors, and defective proteostasis pathways all contribute to the dysfunction and pathological aggregation of TDP-43, should reveal crucial disease mechanisms and therapeutic targets driving ALS and FTD pathogenesis and progression, to identify important therapeutic targets.

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1 ## 2.1 RNA binding regulates TDP-43 stability and aggregation
2 Research into TDP-43 self-assembly has primarily focused on the C-terminal glycine-rich region,
3 which contains a low complexity domain (LCD) in which most ALS-/FTD-associated TDP-43 mutations
4 occur. However, new findings have revealed that the RNA-binding capacity of TDP-43 can influence
5 its aggregation propensity. Mutation analyses show that both the RRM1 and RRM2 domains are
6 required for nucleic acid binding, with high specificity towards UG-rich RNA sequences and long
7 mRNAs (Buratti et al., 2001; Kuo et al., 2014; Kuo et al., 2009; Lagier-Tourenne et al., 2010;
8 Polymenidou et al., 2011). The N-terminal domain and RRMs of TDP-43 are themselves intrinsically
9 aggregation-prone (Vivoli-Vega et al., 2020; Zacco et al., 2018), and regular interactions between
10 TDP-43 and DNA or RNA influence its native folding state and phase transitions (Huang et al., 2013;
11 Mann et al., 2019). Therefore, TDP-43 RNA-binding capacity or RNA substrate availability likely
12 contribute to disease pathology.
13
14 Mutations in the RRM domains of TDP-43 have been identified in ALS patients exhibiting TDP-43
15 proteinopathy, suggesting that aberrant RNA processing may be an important mechanism of
16 pathogenesis. Indeed, such RRM mutations abolish TDP-43-dependent RNA binding and splicing
17 (Elden et al., 2010; Flores et al., 2019; Zacco et al., 2018), impair binding with other RNA-binding
18 proteins, such as ataxin-2 (Elden et al., 2010), and prevent physiological TDP-43 dimerisation
19 (Mompeán et al., 2017). Physiological TDP-43 dimerisation and oligomerisation is likely critical for its
20 RNA binding functions, and maintains stability of TDP-43, by separating the adjoining disordered C-
21 terminal LCDs (Afroz et al., 2017). However, the RRMs may also influence TDP-43 stability
22 independently of other disordered regions, as purified TDP-43 RRMs without bound RNA have been
23 shown to undergo biophysical condensation and fibril formation, demonstrating an intrinsic propensity
24 for self-assembly (Flores et al., 2019; Yu et al., 2021; Zacco et al., 2018). Furthermore, mutations or
25 post-translational modifications within the RRMs that prevent RNA-binding lead to instability and
26 accelerated accumulation of cytoplasmic, insoluble, and hyper-phosphorylated TDP-43 species
27 (Flores et al., 2019; Yu et al., 2021; Zacco et al., 2018). These TDP-43 species can sequester wild-
28 type TDP-43 from the nucleus and resemble the pathological inclusions observed in ALS and FTD
29 (Chen et al., 2019; Cohen et al., 2015; Flores et al., 2019; Maurel et al., 2018; Zacco et al., 2018).
30 Notably, expression of RNA-binding-deficient mutant TDP-43$^{F147L/F149L}$ fails to rescue motor neuron
31 defects caused by knock-out of endogenous TDP-43, indicating that TDP-43 RNA binding is critical
32 for neuronal function and survival (Flores et al., 2019).
33
34 Impaired RNA-binding capacity of TDP-43 may not only initiate or exacerbate TDP-43 aggregation in
35 primary TDP-43 proteinopathies, but could also promote pathology formation in other
36 neurodegenerative diseases such as AD, in which characteristic tau pathology can be accompanied
37 by intracellular TDP-43 protein aggregation (Gu et al., 2017). TDP-43 normally promotes instability of
38 tau mRNA to suppress tau protein expression, but cytoplasmic TDP-43 aggregation likely disrupts this
39 function to promote the accumulation of tau and neurofibrillary tangles in the AD brain (Gu et al.,

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2017). The notion that RNA-binding deficits drive TDP-43 loss-of-function, instability and aggregation is further supported by the finding that truncated TDP-43 species lacking RRMs demonstrate high aggregation propensity and toxicity, although other truncated species that contain intact RRMs also exhibit this trend (Shenoy et al., 2020; Wei et al., 2017). Therefore, TDP-43 RNA-binding capacity likely plays an important role in the formation of pathology in ALS, FTLD-TDP, LATE, and AD, although there are also likely to be other contributing mechanisms of pathogenesis.

The abundance and stability of target RNA transcripts can also influence TDP-43 localisation, stability, and self-assembly. A deficiency of RNA substrates through global transcription inhibition accelerates the nuclear egress of TDP-43 (Ederle et al., 2018), and incubation of purified wild-type TDP-43 RRM domains induces rapid self-assembly in an RNA-free environment (Tavella et al., 2018; Zacco et al., 2018). Furthermore, the mislocalisation and aggregation of TDP-43 in *C9ORF72*-linked ALS and FTD human tissue and disease models occurs in the context of RNA foci formation (Aladesuyi Arogundade et al., 2019; Cooper-Knock et al., 2015; Donnelly et al., 2013). These RNA foci disrupt the native transcriptome and sequester RNA-binding proteins (Butti and Patten, 2019; Lee et al., 2013; McEachin et al., 2020; Simón-Sánchez et al., 2012). Indeed, both sporadic and *C9ORF72*-linked inherited ALS patient-derived induced pluripotent stem cells (iPSCs) demonstrate a subset of destabilised RNAs enriched for transcripts containing motifs for TDP-43 binding, further implicating an imbalance of TDP-43-specific RNA substrates as a key pathomechanism (Donnelly et al., 2013; Tank et al., 2018). This suggests that native TDP-43 substrate RNAs may retain TDP-43 in the nucleus, prevent its aberrant mislocalisation, and stabilise its native folding. In contrast, a deficiency of RNA substrates may create a protein-saturated environment that directly promotes cytoplasmic TDP-43 misfolding and aberrant self-assembly (Maharana et al., 2018; Mann et al., 2019). Unstable RNA-deficient TDP-43 species are usually rapidly degraded by the proteasome; however, if not efficiently cleared, they may irreversibly aggregate in neurons (Flores et al., 2019; Yu et al., 2021; Zacco et al., 2018). As RNA substrates likely play a major role in disease, novel therapies targeting abnormalities in RNA stability or abundance may be effective against neurodegeneration.

Accordingly, increasing the availability of RNA improves TDP-43 solubility. For example, the addition of RNA substrates prevents TDP-43 self-assembly in cell-free aggregation kinetics assays (Zacco et al., 2019), and decreases cytoplasmic TDP-43 inclusion formation, while increasing neuron viability in human ReNcell neurons (Mann et al., 2019). Therefore, disease mechanisms that decrease the RNA-binding efficiency of TDP-43, through RNA-binding mutations, post-translational modifications, or RNA substrate deficiency, instability or foci formation, may provide a link by which multiple forms of sporadic or inherited ALS and FTD converge on TDP-43 proteinopathy. Future research using *in vivo* models of ALS should determine how treatment with bait oligonucleotides may alter TDP-43 aggregation, neurodegeneration and disease trajectory to establish its therapeutic potential. Although changes in TDP-43 RNA-binding capacity and substrate availability may broadly influence pathology development, other interactions between TDP-43 and RNA are likely required for functional

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homodimerisation, both physiological and pathological self-assembly, co-aggregation with other RNA-binding proteins, and TDP-43 toxicity.

2.2 Physiological self-assembly of TDP-43 in liquid-liquid phase separation and stress granule formation promotes pathological nucleation

In the context of cell biology, LLPS involves the reversible self-assembly of proteins into biomolecular condensates by a process of 'de-mixing', when components within the nucleus or cytoplasm spotaneously separate into two phases of different densities, to form distinct structures with liquid-like properties (Boeynaems et al., 2018). TDP-43 undergoes LLPS, like many other proteins, to form 'membraneless organelles', which compartmentalise cellular functions and components. In particular, TDP-43 LLPS likely facilitates the spatiotemporal organisation of RNA processing (Conicella et al., 2020; Li et al., 2018b), and can occur in the nucleus under physiological conditions or be induced in the cytoplasm as a response to cellular stress (Gasset-Rosa et al., 2019; Mann et al., 2019; McGurk et al., 2018; Yu et al., 2021). As an essential RNA-binding protein, this homodimeric condensation of TDP-43 is thought to be important for neuronal functioning and controls the stability and translation of mRNA (Gao et al., 2021), including through the assembly of SGs in mouse and human neuronal models (Alami et al., 2014; Gasset-Rosa et al., 2019). Cytoplasmic SGs are 'membraneless organelles' comprising ribonucleoprotein complexes that develop under conditions of cellular stress to sequester mRNAs from disassembled polyribosomes, limiting non-essential translation (Lindquist, 1981; Nover et al., 1989). Importantly, the LLPS-mediated regulation of TDP-43-associated transcripts also includes autoregulation of TDP-43 mRNA (Hallegger et al., 2021). However, TDP-43 is able to undergo physiological LLPS independently of RNA and SG formation, suggesting that other factors also modulate TDP-43 self-assembly. Therefore, two distinct pathways for physiological LLPS of TDP-43 exist: SG-dependent and SG-independent. Although LLPS is a reversible process, TDP-43 liquid droplets can transition into a solid state, promoting spontaneous aggregation, suggesting that LLPS could play a role in TDP-43 pathology formation and neurodegeneration (Gasset-Rosa et al., 2019; Mann et al., 2019; McGurk et al., 2018; Otte et al., 2020; Pakravan et al., 2021; Ratti et al., 2020; Yu et al., 2021; Zhang et al., 2019a). Indeed, abnormalities in either the SG-dependent or SG-independent LLPS pathways may impair remodelling of RNA networks in disease (Hallegger et al., 2021) and have been related to TDP-43 inclusion formation (Asakawa et al., 2020; Chew et al., 2019).

SG-dependent LLPS

The SG-dependent LLPS pathway involves the formation of SGs under acute stress conditions, which depends on both the RRM1 and CTD of TDP-43 (Asakawa et al., 2020; Bentmann et al., 2012; Liu-Yesucevitz et al., 2010; McGurk et al., 2018; Parker et al., 2012b; Zhang et al., 2019a). The extent of TDP-43 involvement in SGs appears to vary according to the cellular stressor and cellular model. In some immortalised cell lines (such as simian COS-7 cells), ALS patient fibroblasts, and iPSC-derived motor neurons, acute exposure to arsenite-induced oxidative stress causes rapid formation of TDP-43 foci comprising the classical SG markers, G3BP1 and TIA-1 (McGurk et al., 2018). Purified protein

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assays *in vitro* have demonstrated that RNA saturation dissolves TDP-43 droplets, whereas small amounts of RNA promote TDP-43 LLPS (Ederle et al., 2018). Therefore, differences in SG-dependent TDP-43 LLPS dynamics between the nucleus and cytoplasm could be explained by differing RNA concentrations of these compartments in cellular models (Gasset-Rosa et al., 2019). The RNA-concentrated environment of the nucleus may therefore suppress this form of TDP-43 condensation (Ederle et al., 2018), while discrete cytoplasmic clusters of highly structured RNAs, such as those which form SGs, may act as scaffolds that bind TDP-43 and other ribonucleoproteins to promote SG-dependent LLPS to a greater extent (Gasset-Rosa et al., 2019; Maharana et al., 2018).

Chronic SG assembly has been reported to promote the formation of pathological inclusions (Asakawa et al., 2020; McGurk et al., 2018; Parker et al., 2012a; Ratti et al., 2020; Zhang et al., 2019a). For example, prolonged exposure to arsenite causes SG-related TDP-43 liquid droplets to transition to a 'solid' state, becoming G3BP1-negative and phospho-TDP-43-positve (McGurk et al., 2018; Ratti et al., 2020). Recently, non-pharmacological methods for mimicking chronic endogenous SG formation, by light-inducible LLPS-like multimerisation of opto-G3BP1, demonstrated recruitment of phosphorylated TDP-43, p62, and ubiquitin to 'opto-granules' in iPSC-derived cortical neurons (Zhang et al., 2019a). Whether TDP-43 within chronic SGs is RNA-bound remains inconclusive; however, these structures recapitulate hallmarks of ALS and FTLD-TDP proteinopathy and decrease neuronal viability (Zhang et al., 2019a). ALS-associated mutations in the TDP-43 RRM1 disrupt LLPS (Wang et al., 2020), but the effect on RNA binding is unclear (Dang and Song, 2020). Such RRM1 mutations promote aberrant SG assembly and dynamics (Wang et al., 2020), as do disease-linked mutations in the TDP-43 LCD (Gordon et al., 2019), or mutations in the RNA-binding protein TIA-1 (Mackenzie et al., 2017). In each case, these mutations cause excessive cytoplasmic translocation of TDP-43 to form SGs, which become phosphorylated TDP-43 inclusions upon prolonged stress (Gordon et al., 2019; Mackenzie et al., 2017; Wang et al., 2020; Watanabe et al., 2020). Together these findings demonstrate that chronic assembly or impaired dynamics of SGs provide one LLPS pathway by which TDP-43 aggregation may be nucleated. However, canonical SG assembly is not necessary for TDP-43 inclusion formation after cell stress. In zebrafish spinal cord neurons, direct stimulation of TDP-43 oligomerisation leads to the formation of cytoplasmic granules, and subsequent irreversible inclusions, that were not always immunoreactive for SG markers such as G3BP1 or TIA-1 (Asakawa et al., 2020). Likewise, cytoplasmic inclusions of acetylated TDP-43 exhibit little or no colocalisation with SG markers in cell models and human ALS spinal cord, although SGs may indirectly affect the clearance of such inclusions (Chen and Cohen, 2019). This suggests that TDP-43 phase-separated liquid droplets are heterogenous protein assemblies, and that alternative pathways in addition to chronic SG assembly are involved in both physiological and aberrant LLPS leading to pathological TDP-43 aggregation.

SG-independent LLPS

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1 TDP-43 may also undergo an alternative pathway of self-assembly via SG-independent LLPS,
2 involving the formation of RNA-deficient complexes that relies on the intrinsically disordered C-
3 terminal LCD of TDP-43, rather than functional interactions of the RRMs (Conicella et al., 2016;
4 Schmidt et al., 2019). Arsenite-mediated stress in cells over-expressing TDP-43$^{\Delta NLS}$-GFP (a mutant
5 with a defective NLS) induced two subsets of TDP-43 foci: i) small, dynamic, RNA-containing G3BP1-
6 positive SGs, and ii) large, static, G3BP1- and RNA-negative foci, in human HEK293 (Mann et al.,
7 2019) and U2OS cells (Gasset-Rosa et al., 2019), coinciding with a loss of nuclear TDP-43. The RNA-
8 negative foci contained phosphorylated TDP-43 (Gasset-Rosa et al., 2019) and p62 (Mann et al.,
9 2019), reminiscent of pathology-associated TDP-43 inclusions. Recently, optogenetic methods
10 allowing light-inducible multimerisation of wild-type TDP-43 have been developed to study SG-
11 independent LLPS, wherein a C-terminal Cry2olig tag leads to self-assembly of droplet-like
12 cytoplasmic 'opto-TDP-43' condensates that do not recruit SG components (Mann et al., 2019; Otte
13 et al., 2020; Zhang et al., 2019a). Notably, such chronic light-induced cytoplasmic TDP-43
14 multimerisation generated irreversible detergent-insoluble RNA-deficient TDP-43 inclusions. Opto-
15 TDP-43 inclusions were also hyperphosphorylated, immunopositive for p62, and accelerated the
16 death of HEK293 cells (Mann et al., 2019), and caused progressive motor dysfunction in *Drosophila*
17 (Otte et al., 2020). Although SG-independent LLPS does not require RNA binding for droplet
18 formation, the concentration of RNA in the environment may alter the dynamics of TDP-43 LLPS via
19 the LCD and also alter the toxicity of such aberrant phase transitions. It has been shown that addition
20 of low amounts of RNA to purified TDP-43 LCD results in more numerous, but smaller, TDP-43 liquid
21 droplets which was associated with a greater rate of nucleation and aggregation; however, high RNA
22 concentrations reversed this trend (Pakravan et al., 2021). Another study found that treatment with
23 RNA bait oligonucleotides prevented aberrant LLPS of G3BP1-negative TDP-43 droplets and
24 abrogated downstream toxicity and inclusion formation (Mann et al., 2019). The antagonising effects
25 of RNA in this case further differentiate the two separate LLPS pathways for TDP-43 liquid droplet
26 formation. Therefore, abnormalities in SG-independent LLPS may induce TDP-43 aggregation and
27 neurotoxicity, however further studies are required to understand the physiological significance of this
28 process and how it occurs in native systems.
29
30 A crucial determinant of SG-independent LLPS appears to be the structure of the TDP-43 C-terminal
31 LCD. The majority of the *TARDBP* mutations that cause inherited ALS lie within the LCD (Buratti,
32 2015), and likely impair dynamic LLPS and droplet formation or disassembly (Guenther et al., 2018).
33 In cell-free assays, spontaneous LLPS of purified TDP-43 LCD was prevented by disease-associated
34 A321G or Q331K mutations, while the M337V mutation led to a 50% decrease in LLPS (Conicella et
35 al., 2016). However, the A321V mutation, which was proposed to increase hydrophobicity, increased
36 TDP-43 LCD LLPS and impaired disassembly (Conicella et al., 2016). Contrasting results were
37 observed under light-inducible phase separation of the opto-LCD TDP-43 fragment in HEK293 cells,
38 whereby the A321T, Q331K, or M337V mutations increased the number, size, and stability of
39 persistent, irreversible opto-LCD droplets compared to the wild-type opto-LCD TDP-43, and

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exacerbated their maturation into TDP-43 inclusions (Mann et al., 2019). This disparity may be because the LCD mutations that ordinarily alter the binding between TDP-43 molecules do not impede the 'artificial' self-association mediated via the Cry2olig interface (Conicella et al., 2016). However, these light-inducible methods demonstrate that the LCD mutations can also affect the stability and aggregation propensity of phase-separated TDP-43 droplets once they have formed.

For wild-type TDP-43, SG-independent LLPS promotes aberrant TDP-43 amyloid fibril formation and self-assembly via the LCD, which can facilitate spontaneous aggregation of TDP-43 from within mature liquid droplets (Babinchak et al., 2019; Pakravan et al., 2021). However, like the SG-dependent pathway, wild-type TDP-43 can also be forced into pathological SG-independent LLPS by chronic stimulation and sequester endogenous nuclear TDP-43 (Asakawa et al., 2020; Mann et al., 2019). Together, these aberrant phase transitions could alter contacts with functional granules and impair TDP-43 turnover, thereby driving dysregulation of RNA processes and irreversible protein aggregation (Conicella et al., 2016; Li et al., 2018a; Mann et al., 2019).

Recent work has identified a novel mechanism of TDP-43 LLPS in the nucleus, involving TDP-43 de-mixing into anisotropic structures with spherical shells and liquid cores, termed 'anisosomes' (Yu et al., 2021). TDP-43 anisosomes form due to RNA-binding deficiency most remarkably caused by acetylation-mimicking mutations, and require the recruitment of chaperones, such as the heat-shock protein (HSP), HSP70, which form an internal phase-separated liquid core, to retain solubility (Gu et al., 2021; Yu et al., 2021). However, a decrease in ATP levels or chaperone ATPase activity, along with disease-causing mutations and post-translational modifications, may convert anisosomes into intranuclear protein aggregates, resembling pathology found in patient brain tissues (Yu et al., 2021). It remains to be seen whether the process of LLPS for generating TDP-43 anisosomes is connected to the other mechanisms for physiological self-assembly of TDP-43 discussed here, and how anisosomes are related to other nuclear forms of TDP-43 or the various pathological species, although similar mechanisms of chaperone-mediated TDP-43 LLPS have also been demonstrated in the cytoplasm (Lu et al., 2021).

The potential for phase-separated protein droplets to serve as intermediates towards irreversible aggregation has been similarly proposed in other neurodegenerative diseases, for example the formation of tau neurofibrillary tangles in AD (Wegmann et al., 2018). Soluble phosphorylated or aggregation-prone mutant recombinant tau protein, as well as high molecular weight phospho-tau isolated from human AD patient brains, can undergo aberrant LLPS that is capable of spontaneously seeding intracellular tau aggregation (Wegmann et al., 2018). Thus, the mechanisms of TDP-43 and tau aggregation could be related, supported by findings of FTLD-TDP cases exhibiting concurrent tau pathology (Koga et al., 2021); however, it is unclear whether these proteins interact, wherein a conserved LLPS mechanism is involved, or if they resemble two independent disease processes. Further research is warranted to better understand the triggers and mechanisms of TDP-43 LLPS in

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human disease or physiologically relevant *in vivo* animal models, to reveal strategies for potentially manipulating the aberrant biophysical condensation of TDP-43.

2.3 TDP-43 mislocalisation is essential for loss-of-function and toxicity, and potentiates further pathological modifications

In the absence of disease, TDP-43 is predominantly located in the nucleus, but shuttles between the nucleus and cytoplasm to complete its RNA-related functions (Ayala et al., 2008; Freibaum et al., 2010; Ling et al., 2013). Under physiological conditions, TDP-43 is imported into the nucleus by binding of the NLS to one of multiple importins in the cytoplasm and then translocated by karyopherin-β1 through the nuclear pore complex, which comprises ~30 different nucleoporin proteins (Nups) (Kim and Taylor, 2017; Nishimura et al., 2010). In contrast, the nuclear export of TDP-43 is primarily passive, despite the presence of a likely redundant nuclear export sequence (Archbold et al., 2018; Ederle et al., 2018; Pinarbasi et al., 2018). In most TDP-43 proteinopathies, TDP-43 accumulates in the cytoplasm of neurons and is depleted from the nucleus, a process which strongly correlates with neurodegeneration (Brettschneider et al., 2014; Brettschneider et al., 2013; Cathcart et al., 2021; Ditsworth et al., 2017; Lee et al., 2017; Nelson et al., 2019; Tomé et al., 2020).

Defective nuclear pore trafficking of TDP-43 has been implicated as a key process in driving TDP-43 mislocalisation. The most common genetic cause of ALS/FTD, *C9ORF72* hexanucleotide repeat expansions, involves a toxic gain-of-function mechanism whereby dipeptide repeats (DPRs) interact with and cause mislocalisation of key regulators of nucleocytoplasmic transport, thereby inducing TDP-43 mislocalisation and aggregation (Hayes et al., 2020; Zhang et al., 2015). For example, the Ran GTPase activator, RanGAP1, and the transport cargo-binding importin, karyopherin-β1, demonstrate decreased expression, mislocalisation, or co-precipitation with DPRs in *C9ORF72* patient iPSC-derived neurons and brain tissue (Coyne et al., 2020), *Drosophila* (Zhang et al., 2015), mouse neurons, and HeLa cells (Hayes et al., 2020). Several important Nups, together with karyopherin-β1, karyopherin-α2, and CAS, which recycles karyopherins to the cytoplasm, also show irregular abundance or mislocalisation in post-mortem sporadic ALS spinal motor neurons with TDP-43 pathology (Aizawa et al., 2019), and FTLD-TDP patient cortex lysates (Nishimura et al., 2010). Knock-down of these factors by small interfering RNA (siRNA) in mouse Neuro-2a cells or primary cortical neurons induces the cytoplasmic accumulation and aggregation of TDP-43, which supports the upstream role of nucleocytoplasmic transport deficits in initiating TDP-43 mislocalisation (Nishimura et al., 2010). The activity of another TDP-43-associated importin, karyopherin-α3, is regulated by cytosolic calcium through interactions with calpain-A, a pathway that may also be compromised in disease (Park et al., 2020). In ALS and FTD, these disruptions likely interfere with nuclear pore complex function, cargo loading, and nuclear import, culminating in the cytoplasmic accumulation of TDP-43 (Aizawa et al., 2019; Coyne et al., 2020; Hayes et al., 2020; Nishimura et al., 2010; Zhang et al., 2015).

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1 TDP-43 mislocalisation and cytoplasmic accumulation can be exacerbated when nuclear pore
2 complex function is in turn disrupted by pathological TDP-43. The aggregation of disease-linked
3 mutant TDP-43^{Q331K} or truncated TDP-43$^{CTF(208-414)}$ (Chou et al., 2018), the condensation of
4 cytoplasmic TDP-43 droplets by CTD-mediated LLPS (Gasset-Rosa et al., 2019), and the assembly
5 of TDP-43-containing SGs (Zhang et al., 2018), result in the mislocalisation and/or recruitment of
6 importins, multiple Nups, Ran, and RanGAP1. Furthermore, post-mortem studies of the motor cortex
7 of sporadic and mutant *TARDBP* ALS cases have shown Nup205 colocalisation with phosphorylated
8 TDP-43 inclusions (Chou et al., 2018). Therefore, pathological TDP-43 species may inhibit nuclear
9 pore complex function to further promote the nuclear depletion and cytoplasmic accumulation of TDP-
10 43 itself, together with other proteins and RNAs (Aizawa et al., 2019; Chou et al., 2018; Gasset-Rosa
11 et al., 2019; Zhang et al., 2018).
12
13 Extrinsic and intrinsic cellular stressors can also promote TDP-43 mislocalisation. Endoplasmic
14 reticulum stress and chronic excitotoxicity induce cytoplasmic accumulation of both wild-type and
15 disease-associated mutant TDP-43, promoting phosphorylation and aggregation in human
16 neuroblastoma cells, mouse Neuro-2a cells, and neuronal organotypic culture models (Ayala et al.,
17 2011a; Walker et al., 2013). Furthermore, acute oxidative stress and proteome stress, due to aberrant
18 expression of autophagy components or proteasomal inhibition, can stimulate TDP-43 mislocalisation
19 in cellular models (Ayala et al., 2011a; Foster et al., 2021; Zuo et al., 2021). TDP-43 may also
20 accumulate in the cytoplasm due to ALS- and FTD-associated *TARDBP* mutations that alter protein
21 stability. For example, mutant TDP-43^{M337V} ALS patient iPSC-derived motor neurons demonstrate
22 higher levels of both detergent-soluble and -insoluble TDP-43 compared to controls, and an increase
23 in the abundance of small cytoplasmic TDP-43 puncta (Bilican et al., 2012), which was also observed
24 in neurons expressing mutant TDP-43^{A315T} (Barmada et al., 2010). This may be due to the increased
25 stability of ALS-associated mutant TDP-43 proteins compared to wild-type TDP-43 (Austin et al.,
26 2014), further demonstrated by a longer half-life of mutant TDP-43^{M337V}, TDP-43^{G298S}, and TDP-43^{Q331K}
27 measured in isogenic cell lines (Ling et al., 2010). However, these studies did not determine the
28 mechanism by which mutant TDP-43 clearance is impaired (Ling et al., 2010). Together, these findings
29 indicate multiple routes to increased levels of cytoplasmic TDP-43 in disease.
30
31 The relative contributions of TDP-43 nuclear depletion and cytoplasmic accumulation to the toxic
32 consequences of TDP-43 mislocalisation have previously been difficult to clarify due to their
33 concomitant occurrence in ALS, FTD, and other TDP-43 proteinopathies. However, recent research
34 has worked to separate these roles in neuronal model systems and through neuropathological
35 analysis of unique TDP-43 mislocalisation patterns in patient tissues.
36
37 **TDP-43 nuclear depletion largely mediates ALS- and FTD-related loss-of-function**
38 Nuclear depletion of TDP-43 likely precedes cytoplasmic accumulation, leading to a critical loss of
39 function that contributes to neurodegeneration independently to gain of toxicity. This is supported by

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the identification of specific susceptible neuronal populations in sporadic ALS patient tissue that demonstrate complete loss of nuclear TDP-43 even in the absence of cytoplasmic TDP-43 inclusions, including layer Vb Betz cells of the primary motor cortex, and large pyramidal cells and von Economo neurons in deep layers IIIc-Vb of the anterior cingulate cortex (Braak and Del Tredici, 2018; Braak et al., 2017; Nana et al., 2019). The nuclear depletion of TDP-43 may inhibit interactions with its physiological binding partners, leading to loss of function and neurodegeneration as seen in mouse models (Igaz et al., 2011). Normally, nuclear TDP-43 binds and regulates mRNA stability, degradation, cryptic exon splicing, alternate splicing, and alternate polyadenylation (Ling et al., 2015; Melamed et al., 2019; Tollervey et al., 2011). Transcriptomics of diseased neuronal nuclei have demonstrated that loss of nuclear TDP-43 greatly alters the neuronal transcriptome in post-mortem ALS and FTD human brains, patient-derived iPSC neurons, and ALS mouse models (Liu et al., 2019). In particular, loss of nuclear TDP-43 correlates with widespread increases in alternative splicing events, cryptic exon retention, and alternate polyadenylated sequences (Amlie-Wolf et al., 2015; Polymenidou et al., 2011; Wu et al., 2019). This loss of TDP-43 RNA processing function related to pathology development is also associated with dysregulation of nucleocytoplasmic transport, histone processing, mitochondrial function, axonal and neuromuscular junction maintenance, neuronal plasticity, DNA damage repair, and many other neuronal functions (Liu et al., 2019). Faulty mRNA splicing and retention of cryptic exons leads to aberrant truncation of transcripts that encode proteins involved in these essential processes, correlating with reduced neuronal viability (Koza et al., 2019). Wild-type TDP-43 also functions in genomic DNA repair, particularly of double-stranded breaks, and its mislocalisation and loss-of-function in disease results in the accumulation of DNA damage (Wood et al., 2020), which is observed post-mortem in ALS cases and correlates with cell death (Guerrero et al., 2019; Kim et al., 2020a; Mitra et al., 2019). Indeed, interactome analyses have shown that TDP-43 associates with numerous components of both replication-dependent and -independent pathways of DNA repair, with knock-down of TDP-43 resulting in increased instability of chromosomal DNA (Kawaguchi et al., 2020). These findings indicate that loss of TDP-43 function can mediate neurodegeneration, and are supported by partial or conditional TDP-43 knock-down models that demonstrate progressive motor phenotypes, development of TDP-43 proteinopathy, and motor neuron defects (Yang et al., 2014).

Cytoplasmic accumulation of TDP-43 elicits toxic gain-of-function, sensitises cells to stress, disrupts physiological pathways and impairs neuronal function

Accumulation of cytoplasmic TDP-43, through over-expression of exogenous wild-type TDP-43, recapitulates ALS-like motor phenotypes and neurodegeneration in mice, suggesting that gain-of-toxic-function mechanisms are involved in disease pathogenesis (Walker et al., 2015). While exogenous expression of cytoplasmic TDP-43 decreases endogenous nuclear TDP-43 via autoregulation and sequestration (Ayala et al., 2011b; Igaz et al., 2011), the presence of TDP-43 in the cytoplasm also presents opportunities for pathological interactions with other cytoplasmic proteins, organelles, and signalling pathways. For example, accumulation of cytoplasmic TDP-43 may sensitise cells to stress. Pharmacological induction of acute oxidative stress after TDP-43 mislocalisation

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triggers toxic PARP cleavage, and results in abnormal recruitment of cytoplasmic TDP-43 to polyribosomes to alter protein translation in various models, including stressed human neuroblastoma cells and FTD brain lysates (Charif et al., 2020; Lee et al., 2021; Russo et al., 2017). TDP-43 mislocalisation may also cause mitochondrial impairment and damage, through sequestration or aberrant interactions with mitochondrial proteins and mRNAs, leading to a global mitochondrial imbalance that augments oxidative stress (Wang et al., 2016; Zuo et al., 2021), and triggers the release of mitochondrial DNA to initiate a strong inflammatory response (Yu et al., 2020). Inhibiting the mitochondrial localisation of wild-type and mutant TDP-43 has been shown to negate TDP-43-induced mitochondrial dysfunction and neuronal loss, thereby improving the motor phenotype in a transgenic mutant TDP-43 mouse model (Wang et al., 2016). Furthermore, the expression of cytoplasmically-directed TDP-43$^{\Delta NLS}$, but not wild-type TDP-43, causes intrinsic hyperexcitability and aberrant synaptic function in layer V excitatory neurons of the motor cortex (Dyer et al., 2021), stimulates reactive gliosis and the development of neurological abnormalities, and activates caspase-3 to drive cell death pathways (Sasaguri et al., 2016). Overall, increased cytoplasmic TDP-43 and/or nuclear depletion of TDP-43 can impair neuronal physiology and induce dysfunction of cellular pathways which promote neuronal toxicity and degeneration, independently of robust inclusion formation (Barmada et al., 2010; Igaz et al., 2011). Importantly, cytoplasmic mislocalisation of TDP-43 may also result in aberrant interactions that increase the tendency for TDP-43 to become misfolded and aggregate (Igaz et al., 2011), thus promoting further development of pathological TDP-43 species which could mediate other deleterious downstream effects.

2.4 Misfolding, self-association and aggregation of cytoplasmic TDP-43 generates pathological species that cause variable cellular dysfunction and toxicity

Although native RNA-binding and protein-protein interactions regulate the conformational integrity, stability, solubility, and self-assembly of TDP-43 under physiological conditions and have the potential to influence pathological transitions of TDP-43 (Afroz et al., 2017; Yu et al., 2021), multiple other factors promote the development of additional pathological TDP-43 species, namely misfolded TDP-43, oligomers, and inclusions.

Misfolded TDP-43

TDP-43 misfolding involves a loss of native tertiary protein structure and transition to abnormal conformation, which may occur spontaneously in cells over time and likely promotes aberrant protein dynamics and functional interactions. Levels of aggregation-prone proteins, such as TDP-43, are more likely to be ‘super-saturated’ within cells, which likely predisposes these proteins to misfolding and aggregation (Ciryam et al., 2017). The accumulation of misfolded proteins in the CNS is commonly associated with ageing-related failure of protective folding mechanisms (Santra et al., 2019), but DNA damage, cellular senescence, oxidative stress, and proteostasis impairment may also contribute (Spires-Jones et al., 2017). Misfolding of TDP-43 could then lead to a loss of normal TDP-43 function

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by masking residues necessary for physiological function or by exposing residues that mediate aberrant DNA-, RNA-, and protein-protein interactions.

Misfolded protein intermediates progress quickly into aggregated states in cell and animal models, making studies of the toxic properties of each respective intermediate in isolation extremely challenging (Johnson et al., 2008). Broadly, the accumulation of neurodegenerative disease-associated misfolded proteins is associated with pathogenic processes including excitotoxicity, neuroinflammation, defective proteostasis, oligodendrocyte dysfunction, mitochondrial impairment, triggering of aberrant signal transduction pathways, and disruption of RNA homeostasis (Soto and Pritzkow, 2018). For example, misfolded mutant TDP-43 protein exhibits a reduced ability to transport RNA appropriately (Alami et al., 2014), and may bind mitochondria to cause damage, dysfunction, and abnormal mitochondrial accumulation, leading to progressive degeneration in neurons (Wang et al., 2013a; Xu et al., 2010). Misfolded TDP-43 could also elicit intra- and inter-cellular propagation of TDP-43 pathology. This is based on the characterisation of the TDP-43 LCD as a 'prion-like' domain (King et al., 2012), yet has been hotly debated in recent years (Jo et al., 2020; McAlary et al., 2019). Thus, misfolded TDP-43 proteins may act as templates for aggregation, as this prion-like domain may induce the misfolding and self-association of native TDP-43 monomers, and be propagated as seeds to nearby cells (Jo et al., 2020). Indeed, intracellular seeding of human sporadic ALS or FTLD-TDP patient brain samples has been shown to induce pathological conformations and aggregation of wild-type TDP-43, culminating in the formation of pathologically relevant inclusions in cellular and mouse models (Laferriere et al., 2019; Nonaka et al., 2013; Porta et al., 2018). It has been shown that misfolded TDP-43 is capable of cell-to-cell transmission in cell line cultures (Cashman et al., 2020), and between neurons via axon terminals to facilitate template-directed misfolding (Feiler et al., 2015). However, it is yet to be established whether the transmissibility produced in these experimental settings replicates genuine human TDP-43 pathology. In future research, we may be able to better understand TDP-43 misfolding by investigating these species across time using live-cell imaging, or by identifying proteins, genetic manipulations, or drugs that directly modify the folding state of TDP-43.

TDP-43 oligomers

Pathological TDP-43 oligomers resemble an intermediate aggregation state between monomeric species and inclusions, in which multiple misfolded or natively-folded proteins associate in a complex that may provide a 'scaffold' for further aggregation. It is important to note that TDP-43 can undergo a form of physiological oligomerisation, adopting dynamic, 'solenoid-like' structures linked head-to-tail, that may facilitate its RNA-binding functions and antagonise pathological aggregation by spatially separating the C-terminal LCDs (Afroz et al., 2017). However, aberrant disordered TDP-43 oligomers have been shown to persist in ALS, FTD and AD brain tissue, and induce toxicity when applied in various mouse and neuronal models (Fang et al., 2014; Laferriere et al., 2019; Montalbano et al., 2020; Smethurst et al., 2020). For example, as with toxic amyloid oligomers, treatment of human

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neuroblastoma and primary mouse cortical neurons with purified oligomeric TDP-43 protein causes neurite degeneration and reduced cell viability (Fang et al., 2014). This effect was replicated *in vivo*, whereby injection of TDP-43 into the mouse hippocampus led to substantial neuronal loss in the CA1 layer (Fang et al., 2014). Furthermore, seeding of human iPSC-derived motor neurons with TDP-43 oligomers purified from sporadic ALS spinal cord extracts caused cell-type-specific toxicity (Smethurst et al., 2020). Changes in the biophysical and biochemical properties of TDP-43 due to oligomerisation may also interfere with its normal RNA-/DNA-binding capacity, either through a decrease in binding affinity or masking of binding regions, causing loss of function (Fang et al., 2014).

Like misfolded TDP-43, oligomeric TDP-43 derived from ALS brain lysates can recruit native endogenous TDP-43 and has the potential for seeding and intercellular transmission via exosomes, thereby accelerating the development of proteinopathy if not effectively targeted by protein-clearing mechanisms (Feiler et al., 2015). Interestingly, TDP-43 oligomers have been found to specifically interact with amyloid-β in AD patient brains, and the injection of TDP-43 oligomers into the brains of APP/PS1ΔE9 mice increased inflammation and exacerbated amyloid-β pathology, neuronal death, and cognitive phenotypes (Shih et al., 2020). In addition, TDP-43 oligomers may also promote cross-seeding to trigger tau aggregation *in vitro* (Montalbano et al., 2020). Thus, TDP-43 oligomers may be an important intermediate between mislocalised or misfolded species and terminal TDP-43 inclusion formation, inducing both loss of wild-type function and gain of toxic function through aberrant protein-protein interactions. However, the involvement of physiological dimerisation and oligomerisation in normal functions of TDP-43, particularly within the nucleus, suggests that therapies selectively targeting oligomeric species will likely not be specific for pathology.

Cytoplasmic TDP-43 inclusions

TDP-43 inclusions are high-order aggregates that can comprise multiple TDP-43 species associating within large, static insoluble structures. TDP-43 inclusions are a prominent feature of degenerating neurons in the affected regions of the nervous system in TDP-43 proteinopathies, suggesting that they may represent the end-stage of the aggregation process. Indeed, TDP-43 inclusions may exert toxic properties, as transfection of pre-formed human TDP-43 inclusions has been shown to induce reactive oxygen species production and apoptotic caspase-3 activation in murine neuroblastoma cells, implicating abnormal calcium homeostasis and mitochondria, without affecting endogenous nuclear TDP-43 (Cascella et al., 2019). Furthermore, injection of human FTLD-TDP-derived insoluble TDP-43 extracts induces phosphorylated TDP-43 pathology that spreads throughout the CNS in mice (Porta et al., 2021; Porta et al., 2018). Likewise, cells transfected with wild-type TDP-43 and then treated with insoluble fractions from ALS or FTLD-TDP brains show an increased rate of cell death toxicity (Nonaka et al., 2013). These results suggest that the increased death of cells affected by TDP-43 pathology correlates with the extent of TDP-43 inclusion deposition. However it must be noted that insoluble TDP-43 fractions could contain a mix of pathological species that contribute to the observed toxicity (Nonaka et al., 2013). Therefore, the overall aggregate burden may correspond with the

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burden of other pathological forms of TDP-43 that have not been accounted for, which likely also contribute to neurodegeneration.

Some findings have indicated that TDP-43 toxicity may be more dependent on cytoplasmic mislocalisation than inclusion formation *per se* (Barmada et al., 2010). This is supported by the fact that some surviving neurons in human ALS and FTD tissue and animal models harbour insoluble TDP-43 inclusions and that the overall rate of neuron loss exceeds that of inclusion deposition (Walker et al., 2015), indicating that large inclusions may actually be protective, or simply represent inert terminal storage of misfolded proteins. Indeed, the role of protein inclusions in neurodegeneration has been long debated (Ross and Poirier, 2005).

In ALS and FTD, TDP-43 inclusions may elicit cellular dysfunction by sequestering or forcing the co-aggregation of natively folded TDP-43 or other cellular proteins, thereby depleting their functional pool to impair diverse physiological pathways (Chou et al., 2018; Laferriere et al., 2019). For example, end-stage ALS brains and cellular models of TDP-43 inclusion formation demonstrate large, insoluble TDP-43 inclusions incorporating the autophagy cargo receptor, p62/SQSTM1 (Cicardi et al., 2018; Hiji et al., 2008; Mizuno et al., 2006; Wang et al., 2017) or nuclear pore complex proteins (Chou et al., 2018). Notably, TDP-43 inclusions have also been shown to colocalise with tau protein in human post-mortem AD tissue (Davis et al., 2017; Higashi et al., 2007; Kadokura et al., 2009; Tomé et al., 2021; Zhang et al., 2019b). Transgenic mice over-expressing wild-type TDP-43 show accumulation of phosphorylated tau with some evidence of potential colocalisation with cytoplasmic TDP-43 inclusions (Davis et al., 2017), while mice over-expressing disease-linked TDP-43^{M337V} exhibit increased tau expression (Gu et al., 2017). The increase in tau aggregation may be related to TDP-43-induced changes in tau expression and mRNA processing or aberrant protein-protein interactions that alter tau solubility and assembly (Davis et al., 2017; Gu et al., 2017). These studies suggest intimate links between TDP-43 proteinopathy and misfolding of other neurodegeneration-associated proteins.

Intranuclear TDP-43 inclusions

Neuronal intranuclear inclusions that may deplete the nuclear functional pool of TDP-43 can also occur in disease, being particularly predominant in the rare FTLD-TDP type D (Neumann et al., 2007). Mutations in the *VCP* gene cause FTLD-TDP type D, rare cases of multisystem proteinopathy (also refered to as inclusion body myopathy with Paget's disease of bone and FTD), or ALS (Johnson et al., 2010; Schröder et al., 2005; Watts et al., 2004). However, intranuclear inclusion pathology related to *VCP* mutations has not been thoroughly studied due to the rarity of cases (Tan et al., 2013), so the molecular mechanisms remain largely unclear. Neuronal intranuclear inclusions containing ubiquitin and VCP protein have been reported in the brain of at least one case of mutant *VCP*-associated FTD (Schröder et al., 2005), and cases of multisystem proteinopathy also demonstrate alterations in VCP localisation and both nuclear and cytoplasmic VCP inclusions in muscle (Shi et al., 2012; Watts et al., 2004). However, colocalisation of TDP-43 with intranuclear neuronal mutant VCP inclusions has not

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been clearly shown. Indeed, intranuclear TDP-43 inclusions found in FTLD-TDP type D have been described as being more abundant than inclusions labelled for VCP (Neumann et al., 2007), suggesting that nuclear TDP-43 pathology likely arises in these cases due to loss of normal VCP function rather than necessarily co-aggregation. Knock-out of VCP compromises the delivery of proteasomal and autophagic substrates for degradation, leading to the accumulation of polyubiquitinated proteins and TDP-43 aggregation, further suggesting mechanistic links between VCP function and TDP-43 (Kustermann et al., 2018; Wani et al., 2021). Further research is warranted to understand the mechanisms mediating the formation of intranuclear TDP-43 inclusions in disease.

As relatively little is known about the biophysical properties, functional interactions, and mechanisms that mediate the assembly of intranuclear inclusions, their toxic effects and role in neurodegeneration are yet to be established. It is also unclear whether intranuclear TDP-43 inclusions are released to the cytoplasm. However, it has been found that TDP-43 aggregation can also occur within other distinct but adjacent compartments called micronuclei, small nuclear fragments, in ALS patient tissues, which may provide a link between intranuclear and cytoplasmic TDP-43 pathology (Droppelmann et al., 2019). Future research investigating the incidence, molecular triggers, and consequences of intranuclear TDP-43 aggregation are needed.

2.5 Post-translational modifications that mark end-stage TDP-43 inclusions modulate pathological changes to TDP-43 structure and function

Many post-translational modifications (PTMs) to TDP-43 have been identified in ALS and FTLD-TDP tissues, including C-terminal fragmentation, phosphorylation, ubiquitination, acetylation, sumoylation, nitrosylation, and methylation, which have been extensively reviewed elsewhere (Aikio et al., 2021; Berning and Walker, 2019; Buratti, 2018; Eck et al., 2021; François-Moutal et al., 2019; Wood et al., 2021). Although TDP-43 PTMs have mostly been studied in isolation, pathological TDP-43 species likely acquire multiple different modifications throughout disease development.

It is important to note that the different TDP-43 proteinopathies and disease subtypes can be pathologically differentiated based on patterns of TDP-43 PTMs (Lee et al., 2017; Mackenzie et al., 2011). Unique PTM 'signatures' may occur at particular TDP-43 residues, decorate distinct TDP-43 species, or characterise particular regions throughout the CNS. For example, phosphorylation of TDP-43 is a consistent feature of multiple distinct TDP-43 species, observed throughout the brain and spinal cord across the entire spectrum of sporadic and familial forms of ALS and FTD (Gu et al., 2019; Guedes et al., 2017; Neumann et al., 2021; Neumann et al., 2009), while acetylation is largely a feature of TDP-43 pathology in the spinal cord (Cohen et al., 2015). One overarching feature of TDP-43 PTMs is their identification primarily on cytoplasmic or aggregated forms of TDP-43, rather than physiological nuclear TDP-43. This suggests that disease-associated mislocalisation either i) exposes TDP-43 to a modification-prone environment, ii) induces structural changes in TDP-43 that increase the abundance of TDP-43 PTMs, iii) is promoted by TDP-43 PTMs, or iv) occurs in TDP-43 species

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1 that are also prone to modification. Understanding how TDP-43 PTMs occur, and how they relate to
2 TDP-43 dysfunction and pathology formation, will reveal their role in neurodegeneration.
3
4 **TDP-43 C-terminal fragmentation**

5 Of all known TDP-43 modifications, C-terminal fragmentation was amongst the first identified
6 (Neumann et al., 2006). TDP-43 C-terminal fragments (CTFs) are formed primarily by proteolytic
7 cleavage, which can be stimulated by disease-associated TDP-43 mutations and various cellular
8 stressors (Rutherford et al., 2008; Sreedharan et al., 2008). CTFs form different truncation site-specific
9 molecular conformations which characterise distinct assemblies in TDP-43 proteinopathies,
10 prominently including 25 and 35kDa fragments (Feneberg et al., 2021; Shenoy et al., 2020). The
11 specific perturbations that contribute to the generation, and downstream cellular consequences of,
12 TDP-43 CTFs have been thoroughly reviewed elsewhere (Berning and Walker, 2019). Importantly,
13 TDP-43 CTFs are consistently observed in human ALS and FTD brain tissues and in some disease-
14 relevant animal models, although they are unlikely to be a primary driver of pathogenesis (Berning
15 and Walker, 2019).
16
17 **TDP-43 phosphorylation**

18 Phosphorylation of TDP-43 is another key pathological hallmark of TDP-43 proteinopathies (Neumann
19 et al., 2006), as has been thoroughly reviewed recently (Eck et al., 2021). Multiple TDP-43 species
20 have been shown to be phosphorylated most notably at serines 369, 379, 403, 404, 409, and 410 (Gu
21 et al., 2019; Neumann et al., 2021; Neumann et al., 2009), which is mediated by enzymes including
22 CDC7 (Liachko et al., 2013), TTBK1 (Taylor et al., 2018), casein kinase 1ε (Choksi et al., 2014), and
23 casein kinase 1σ (Nonaka et al., 2016). Furthermore, different patterns of phosphorylated TDP-43
24 pathology may be unique to particular TDP-43 proteinopathies, their subtypes, or affected regions of
25 the CNS. For example, phosphorylated TDP-43 CTFs accumulate in the cortex in ALS and FTLD-
26 TDP, but not in the spinal cord, where phosphorylated full-length TDP-43 has been observed (Igaz et
27 al., 2008). Regarding common phosphorylated residues, some AD cases exhibit TDP-43 pathology
28 specifically enriched in CTFs phosphorylated at serine residues 409/410, while other cases show
29 multiple pathological species, including full-length TDP-43, phosphorylated at serines 409/410 and
30 403/404, which are also seen in FTLD-TDP (Tomé et al., 2020). Another study observed differences
31 among FTLD-TDP subtypes, identifying phosphorylation of TDP-43 at serine 369 in ALS and FTLD-
32 TDP types B and C but not type A (Neumann et al., 2020). LATE cases exhibit phosphorylated TDP-
33 43 409/410 in the hippocampus, dentate gyrus, and amygdala (Nelson et al., 2019; Robinson et al.,
34 2020), however the full phosphorylation profile of TDP-43 is yet to be characterised in LATE. In
35 addition, recently a distinct FTLD-TDP subtype E was reported based on distinct features including
36 widespread distribution of phosphorylated TDP-43 inclusions with the appearance of fine, small
37 puncta throughout the cortical layers (Lee et al., 2017). This suggests that aggregating TDP-43
38 species in different diseases have varying tendencies for phosphorylation, distinct phosphorylation

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patterns and regional distributions throughout the CNS, which may relate to relative differences in neurodegeneration.

Phosphorylation has been shown to differentially affect the function, solubility, aggregation propensity, and toxicity of pathological TDP-43 species. Over-expression of kinases that mediate TDP-43 phosphorylation has been implicated in exacerbating pathological TDP-43 deposition, and enhancing toxicity (Choksi et al., 2014; Liachko et al., 2013; Nonaka et al., 2016; Taylor et al., 2018). While the amount of phosphorylation has been shown to correlate with the level of aggregation (Brady et al., 2011), there is also evidence challenging the hypothesis that TDP-43 phosphorylation negatively contributes to TDP-43 aggregation and disease progression. Mutations that mimic disease-associated hyperphosphorylation decrease the overall levels of phosphorylated CTFs and number of HEK293 or Neuro2a cells with TDP-43 inclusions (Brady et al., 2011). In addition, similar phospho-mimetic mutations inhibit aberrant LLPS-mediated accumulation of TDP-43 in membraneless organelles, decrease polymerisation, and enhance solubility in neurons, suggesting that phosphorylation may actually suppress the aggregation propensity of TDP-43 (da Silva et al., 2021; Wang et al., 2018). One study found that this phospho-mimic did not alter nuclear trafficking or RNA processing (da Silva et al., 2021), whereas another found a significant reduction in TDP-43 splicing regulatory activity (Wang et al., 2018). Therefore, the effects of phosphorylation on TDP-43 function and neuronal viability remain inconclusive. Phosphorylated TDP-43 is not present under normal conditions, and is usually exclusively found in the detergent-insoluble fraction of TDP-43 disease models, including cells (Cohen et al., 2015), and mouse cortex and spinal cord tissues (Walker et al., 2015), similar to human post-mortem ALS and FTD brains and spinal cords (Neumann et al., 2006). Rather than being detrimental, it remains possible that TDP-43 phosphorylation is an endogenous mechanism that enhances the solubility of cytoplasmic TDP-43, which could be protective for neurons in disease.

TDP-43 acetylation

The acetylation of select TDP-43 lysine residues is associated with alterations to RNA processing and protein solubility, likely through the electrostatic neutralisation of positively charged lysine residues as has been shown for acetylation of other unrelated proteins (Arbely et al., 2011; Ren et al., 2016). Indeed, acetylation of TDP-43 impairs the binding of negatively charged RNA, and promotes accumulation of insoluble TDP-43 species (Cohen et al., 2015). Multiple acetylation sites on TDP-43 have been identified in ALS autopsy tissues, including at lysine residues 145 and 192 located within the RRM1 and RRM2 domains, respectively (Cohen et al., 2015), as well as lysine 82 located within the NLS (Kametani et al., 2016). Expression of TDP-43 with a defective NLS (TDP-43$^{\Delta NLS}$) increases TDP-43 acetylation, suggesting that exposure to the cytoplasmic environment mediates this PTM (Cohen et al., 2015). Cells expressing acetylation-mimicking mutants of TDP-43, TDP-43$^{K145Q/K192Q}$ and TDP-43$^{\Delta NLS/K145Q/K192Q}$ form nuclear speckles and cytoplasmic inclusions, respectively (Cohen et al., 2015). These mutants also showed impaired RNA binding and were more aggregation-prone than wild-type, with increased detergent-insolubility and phosphorylation (Cohen et al., 2015). The

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cytoplasmic acetylation-mimicking mutant of TDP-43 has been shown to be occasionally recruited into SGs, but also forms SG-independent large amorphous structures that were similar in morphology and biochemical composition to inclusions found in ALS lumbar spinal cord (Chen and Cohen, 2019). Together, these findings indicate a clear link between acetylation and pathological aggregation-prone TDP-43.

While multiple PTMs have been linked with increased aggregation propensity and persistent TDP-43 inclusions in disease, it is ultimately unclear whether PTMs are causative, or a byproduct of, accumulated dysfunctional proteins. Future research should determine how changes in TDP-43 structure, function, and localisation throughout disease affect its tendency to acquire PTMs. Furthermore, the significance of different PTM profiles between TDP-43 proteinopathies, disease subtypes, and affected regions of the CNS, together with the precise molecular triggers and consequences of PTMs, must be understood to determine how PTMs impact TDP-43 pathology formation and neurodegeneration, and whether targeting PTMs could be a viable therapeutic strategy. Despite extensive and continuing study, there remain many unknowns as to the role of PTMs in disease.

2.6 Conclusions on the noxious assembly of pathological TDP-43

Within the 'life' of pathological TDP-43, the early triggers of mislocalisation and misfolding are likely the key mediators of TDP-43 loss of function, rendering the protein unable to interact with its physiological targets either spatially or allosterically. The mislocalisation and misfolding of TDP-43 are likely also necessary for subsequent cytoplasmic interactions and modifications that underlie TDP-43 toxic gain of toxic function and the development of additional pathological TDP-43 species. TDP-43 oligomerisation and inclusion deposition may not greatly affect the already compromised function of initiating pathological TDP-43 species, but are likely deleterious by sequestering other cellular proteins or natively folded TDP-43 and preventing their physiological interactions. TDP-43 PTMs are generally viewed as important markers for pathological classification; however, the contributions of PTMs to neurodegeneration and their timing within this TDP-43 pathology cascade remain unclear. Notably, observations of hallmark TDP-43 inclusions within remaining neurons at end-stage disease suggests that inclusions are not inherently toxic. Rather, the progressive noxious assembly of pathological TDP-43 species likely drives disease, and early processes of TDP-43 dysfunction and aggregation may occur well before robust inclusion pathology is established, but remain undetected within degenerating inclusion-bearing neurons.

Therefore, preventing the development of, or removing, noxious pathological TDP-43 species represents a common and crucial target for disease-modifying therapies. However, as TDP-43 is essential for survival, the ablation of endogenous TDP-43 is not a viable strategy. Understanding how endogenous protective mechanisms normally maintain TDP-43 solubility and function, prevent aggregation and refold or remove pathological species may reveal means to mitigate their toxic

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complications while restoring native-TDP-43. This will be critical to prevent further neuronal death and promote functional recovery from TDP-43 pathology-associated deficits. In the third part of this review, we explore the role of the neuronal proteostasis network in TDP-43 aggregation, and the effects on that network when TDP-43 does become aggregated, dissecting the involvement of multiple protein degradation pathways that play different roles in health and disease.

3 Therapeutic removal: clearance of TDP-43 pathology by endogenous neuronal protein degradation systems

Under physiological conditions, proteostasis is maintained by endogenous systems that refold or degrade unstable, misfolded or aggregated proteins that could be toxic or impair cell functionality (Boland et al., 2018; Klaips et al., 2018; Nixon, 2013). Mechanisms of proteostasis primarily include the ubiquitin-proteasome system (UPS), autophagy-lysosome pathway (ALP), heat-shock response (HSR), and chaperone-mediated autophagy (CMA). These systems can operate constitutively in 'housekeeping' roles or become activated under conditions of cellular stress, where each feature different regulatory mechanisms, machineries and capacities for removing distinct pathological TDP-43 species (***Figure 2***). Three key pathomechanisms relate neuronal proteostasis dysfunction with pathological TDP-43 aggregation and neurodegeneration: (i) proteostasis dysfunction has been shown to occur with ageing, and neurons are post-mitotic, long-lived, large cells, which have high demands for protein quality control, making them vulnerable to toxic protein aggregation and neurodegeneration (Aman et al., 2021; Tsuiji et al., 2017; Yerbury et al., 2020), ii) several disease-linked mutations occur in proteostasis genes, likely upstream of TDP-43 pathology formation (Medinas et al., 2017; Ramesh and Pandey, 2017), and iii) TDP-43 itself likely influences proteostasis capacity. Indeed, TDP-43 regulates the expression of important proteostasis components, a function that is likely disrupted by TDP-43 aggregation (Bose et al., 2011; Leibiger et al., 2018). Likewise, aggregating TDP-43 species may also form aberrant protein-protein interactions with proteostasis components throughout disease, further disrupting proteostasis function (Leibiger et al., 2018; Ormeño et al., 2020a; Torres et al., 2018; Xia et al., 2016).

However, it has been shown that cytoplasmic TDP-43 pathology can be effectively removed by endogenous protein clearance systems, even after disease onset, restoring the native nuclear TDP-43 pool and preventing neuronal death to facilitate phenotypic recovery in ALS mice (Walker et al., 2015). Understanding the endogenous proteostasis response to TDP-43 aggregation, how proteostasis becomes dysfunctional in disease, and which individual proteins and pathways are most effective in clearing TDP-43, may highlight critical targets to be further explored for effective disease-modifying treatments across the TDP-43 proteinopathies, including ALS, FTLD-TDP, LATE, and AD.

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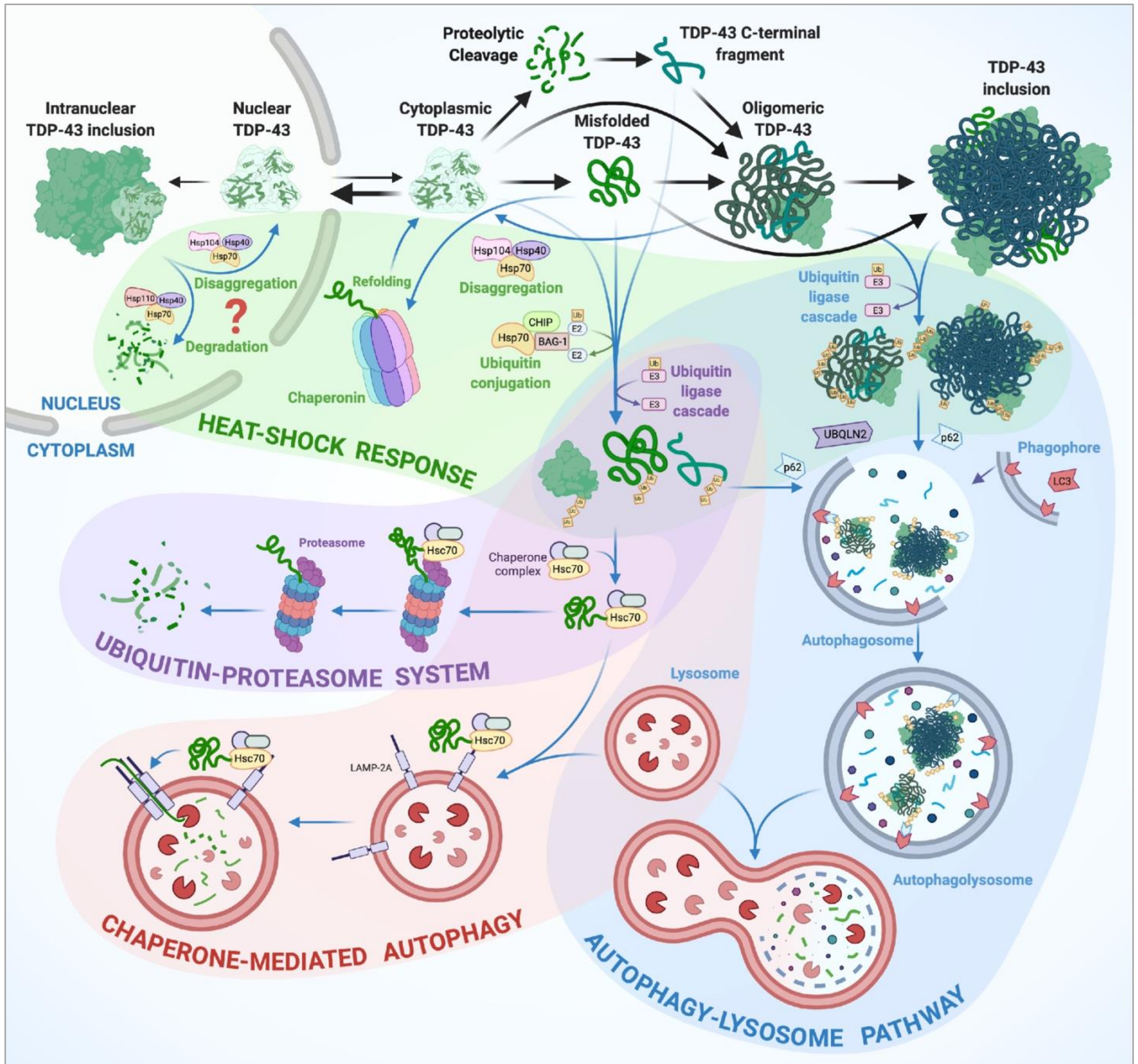

2 ***Figure 2:* Endogenous protein degradation systems are capable of removing different**
3 ***pathological TDP-43 species in neurons.*** Detection, sequestration and degradation of TDP-43
4 species occur through multiple proteostasis systems, including the ubiquitin-proteasome system,
5 autophagy-lysosome pathway, heat-shock response and chaperone-mediated autophagy. Black
6 arrows indicate the molecular changes by which native TDP-43 becomes pathological. Blue arrows
7 indicate the pathways to pathological protein clearance. Misfolded and oligomeric TDP-43 species
8 can be re-folded into functional TDP-43 monomers by the activities of the heat shock response.
9 Monomeric, misfolded and C-terminal fragments of TDP-43 can be ubiquitinated and targeted for
10 degradation by either the ubiquitin proteasome system or chaperone-mediated autophagy. Oligomers
11 and mature inclusions formed from TDP-43 can be ubiquitinated and targeted for clearance by the
12 autophagy-lysosome pathway. The pathway of intranuclear TDP-43 disassembly or clearance is
13 speculative and remains to be established. Schematic figure created with *BioRender*.
14
15

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3.1 The role of the ubiquitin-proteasome system in TDP-43 pathology

The UPS employs a network of proteins that ubiquitinate and shuttle damaged or misfolded proteins for degradation by the proteasome, predominantly degrading monomeric, unfolded polypeptides (Pohl and Dikic, 2019). Ubiquitination of persistent neurotoxic protein aggregates was one of the earliest recognised pathological hallmarks of neurodegenerative disease, and ubiquitin-binding proteins are commonly sequestered within ubiquitinated protein inclusions (Schmidt et al., 2021). ALS and FTD tissues and patient-derived fibroblasts exhibit signs of UPS dysfunction associated with TDP-43 pathology (Kabashi et al., 2012; Lee et al., 2019). For example, decreased proteasome levels, reduced catalysis of proteasomal substrates, and alterations in constitutive proteasome machinery composition have been found in lower motor neurons and spinal cord homogenates from sporadic ALS patients (Kabashi et al., 2012). Indeed, proteasomal inhibition with MG132 treatment increases disease-associated cytoplasmic accumulation of TDP-43 in cultured neurons and neuronal cell lines (Ishii et al., 2017; Scotter et al., 2014; van Eersel et al., 2011). UPS inhibition also significantly increases the formation of cytoplasmic inclusions containing endogenous and exogenous 35- and 25-kDa TDP-43 CTF in HEK293 cells (Wang et al., 2010), NSC-34 motor neuron-like cells (Cicardi et al., 2018), Neuro-2a cells (Huang et al., 2014; Walker et al., 2013), and rat primary neurons (Uchida et al., 2016). Conditional motor neuron-specific knock-out of Rtp3, a protein that is essential for 26S proteasome formation, also leads to TDP-43 aggregation, motor neuron death, and ALS-like motor deficits in mice (Tashiro et al., 2012). These findings suggest that the UPS is normally involved in the degradation of mislocalised, misfolded, or truncated TDP-43 and that disruption of the UPS can initiate and exacerbate TDP-43 pathology.

Rare inherited ALS- and FTLD-TDP-causing mutations have been identified in genes associated with the UPS, most notably including *UBQLN2* and *SQSTM1* (encoding p62), which are also involved in the ALP (Deng et al., 2011; Teyssou et al., 2013; van der Zee et al., 2014). UBQLN2 is a polyubiquitinated cargo receptor which docks target proteins to UPS or ALP adaptor proteins (Zhang et al., 2014). Wild-type UBQLN2 binds to the C-terminal region of TDP-43 with high affinity and may increase the clearance of full-length TDP-43 and CTFs (Cassel and Reitz, 2013; Watanabe et al., 2020). However, disease-association mutations in UBQLN2 may cause a loss of function that impedes protein clearance via the UPS and ALP (Wu et al., 2020). However, the precise mechanism and overall impact of such *UBQLN2* mutations alone on neurodegeneration is unclear, as complete *UBQLN2* knock-out in rats has no effect on neuronal function or behavioural phenotype (Huang et al., 2016; Wu et al., 2015), but did cause age-dependent motor decline in mice (Hjerpe et al., 2016). This *UBQLN2* knock-out phenotype in mice was more severe than the mild phenotype caused by knock-in of a disease mutation to the endogenous mouse *UBQLN2* locus (Hjerpe et al., 2016), but less severe than the exogenous over-expression of mutant UBQLN2, which resulted in dramatic and rapid motor decline (Le et al., 2016). In addition to a loss of its function, in some ALS and FTD cases caused by *UBQLN2* or *C9ORF72* mutations, the UBQLN2 protein actually forms cytoplasmic inclusions which likely contribute to neuronal dysfunction or death, through variable mechanisms which may or may

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not involve TDP-43, manifesting as different cognitive or motor phenotypes (Brettschneider et al., 2013; Deng et al., 2011; Osaka et al., 2016). *In vivo*, some studied have shown that expression of ALS- and FTD-linked mutant UBQLN2 in rats and mice leads to profound cytoplasmic UBQLN2 inclusion formation in neurons, which coincides with increased TDP-43 mislocalisation or aggregation, cognitive deficits, and motor phenotypes (Le et al., 2016; Osaka et al., 2016; Picher-Martel et al., 2019; Sharkey et al., 2018). In contrast, the expression of both human and rat wild-type or mutant UBQLN2^{P497H} in rats or in mice, has been shown to lead to development of neuronal UBQLN2-, ubiquitin-, and p62-positive inclusions without TDP-43 immunoreactivity, particularly in the hippocampus, with synaptic dysfunction, neuron death and cognitive deficits but no motor phenotype (Gorrie et al., 2014; Huang et al., 2016; Wu et al., 2015). Finally, some ALS- and FTD-linked mutant UBQLN2^{P506T} transgenic mice do not demonstrate pathological TDP-43 aggregation, or evidence of neurodegeneration or behavioural changes (Sharkey et al., 2020). It is likely that UBQLN2 aggregation further compromises ubiquitin-dependent proteostasis function, by sequestering free ubiquitin, proteasome subunits and autophagy proteins (Huang et al., 2016; Wu et al., 2015). Both this gain of aggregation propensity and loss of UPS regulation by UBQLN2 may contribute to TDP-43 proteinopathy.

Mutations in p62/SQSTM1, another UPS/ALP cargo receptor protein, have also been identified in rare cases of ALS and FTD, and likely impair binding of ubiquitinated substrates or its association with degradation machineries, leading to TDP-43 pathology (Fecto et al., 2011; Le Ber et al., 2013; Rubino et al., 2012; van der Zee et al., 2014). In particular, *SQSTM1* mutations may also increase the propensity for p62 aggregation to further impair its functional roles. Indeed, neuropathological analysis of mutant *SQSTM1* ALS and FTD cases identified large p62-positive TDP-43 inclusions in motor neurons and increased p62 and phosphorylated-TDP-43 protein levels in the spinal cord (Teyssou et al., 2013), temporal cortex, and dentate gyrus (van der Zee et al., 2014). p62-positive TDP-43 inclusions are also observed in many sporadic cases without *SQSTM1* mutations, however this may result from TDP-43-dependent sequestration (Cicardi et al., 2018; Wang et al., 2017). Therefore, ALS- and FTD-linked mutations in UPS components may hinder the binding and delivery of polyubiquitinated substrates, such as TDP-43, to the proteasome, resulting in TDP-43 inclusion formation.

3.1.1 TDP-43 pathology induces UPS impairment

TDP-43 pathology may impair UPS function by binding and sequestering important machinery components and affecting ubiquitin availability. For example, sporadic, familial, and *TARDBP*-linked ALS and sporadic ALS/dementia post-mortem spinal cord tissues show colocalisation of UBQLN2 with TDP-43 inclusions (Deng et al., 2011). Other UPS machineries including p62, as well as entire proteasomes, individual subunits and assembly proteins, have been shown to interact and become sequestered with cytoplasmic TDP-43 inclusions in primary rat neurons and NSC-34 cells expressing 25-kDa TDP-43 CTFs or an acetylation-mimicking mutant of TDP-43 (Cicardi et al., 2018;

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Riemenschneider et al., 2021; Wang et al., 2017). These inclusion-bound proteasomes exhibit a stalled conformation, suggesting a loss of proteolytic activity (Riemenschneider et al., 2021). TDP-43-induced impairment of proteasome activity is further supported by the accumulation of UPS substrate reporters such as ubiquitin^{G76V}-AcGFP (Watanabe et al., 2013) and abnormal accumulation of insoluble polyubiquitinated proteins in Neuro2a cells expressing mutant TDP-43 (Lee et al., 2020). Furthermore, internalised full-length TDP-43 inclusions in NSC-34 cells evade detection and proteasomal degradation and do not alter the activity of the UPS co-chaperones BAG1 or BAG3, suggesting impairment of substrate recruitment processes within the UPS (Cascella et al., 2017). Excessive polyubiquitination of persistent TDP-43 pathological species facilitates evasion of degradation, but also depletes the free ubiquitin pool, thereby disrupting global ubiquitin homeostasis in ALS and FTD (Farrawell et al., 2020).

3.1.2 Pharmacological activation of the UPS

Relatively few studies have investigated the effect of pharmacological enhancement of UPS function on TDP-43 pathology (***Table 1***), despite strong evidence that i) UPS impairment results in TDP-43 inclusion formation, and ii) misfolded TDP-43 impairs the UPS in a positive feed-back loop. Indeed, it has been demonstrated that the restoration of UPS function in cells leads to the clearance of TDP-43-positive and p62-positive inclusions that were generated following UPS inhibition (Scotter et al., 2014). IU1, an inhibitor of the deubiquitinating enzyme USP14, has been shown to accelerate ubiquitination and degradation of over-expressed TDP-43 in mouse embryonic fibroblasts (Lee et al., 2010), although genetic inhibition of USP14 via expression of a catalytically-inactive variant had no effect on TDP-43 levels in HEK293 cells (Ortuno et al., 2016). Additionally, forskolin treatment activated cAMP-dependent protein kinase A, leading to phosphorylation of 26S proteasomes and decreased levels of mutant TDP-43^{M337V} via the UPS (Lokireddy et al., 2015). Promisingly, small molecule proteolysis targeting chimera (PROTAC) approaches have recently been shown to induce UPS-dependent degradation of tau protein in cells, as well as in brains of non-transgenic mice and tau-related neurodegenerative disease model mice (Wang et al., 2021). This study and other advances in PROTAC technology supports promising new approaches in UPS drug discovery that could be applied to TDP-43 proteinopathies in the future (Farrell and Jarome, 2021).

Table 1. Studies of pharmacological enhancement of TDP-43 protein clearance in cell and animal models of TDP-43 proteinopathy. Summary of target selection and treatment conditions for the application of proteostasis-enhancing compounds in TDP-43 disease models, with an evaluation of therapeutic efficacy and validation for target engagement and pathway activation. Abbreviations: 1°, primary; CTF, C-terminal fragment; ER, endoplasmic reticulum; FL, full-length; FTLD, frontotemporal lobar degeneration; HSP, heat-shock protein; KD, knock-down; MDC, monodansylcadaverine; MEFs, mouse embryonic fibroblasts; NFκB, nuclear Factor kappa-light-chain-enhancer of activated B cells; mTORC1, mammalian target of rapamycin complex 1; scFv, Single-chain variable fragment; TARDBP, transactive response DNA-binding protein; TRiC, TCP-1 ring complex; VCP, valosin containing protein; WT, wild-type.

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	Target	Model	Treatment	Pathway validation	Effect	Ref.
Ubiquitin proteasome system						
IU1	USP14 inhibition	MEFs overexpr. TDP-43-FLAG	IU1 50μM, 6h	↓ free ubiquitin, no change in proteasome synthesis/subunit composition, ↑ ubiquitin conjugation to TDP-43	↑ ubiquitination and proteasomal degradation of TDP-43	(Lee et al., 2010)
Forskolin	PKA activation via cAMP	HEK293A cells expr. TDP-43^{M337V}	Forskolin 50μM, 5h in presence of cycloheximide	↑ cAMP, ↑ doubly-capped PKAα-associated proteasomes. Blocking proteasome function prevented decrease in TDP-43	↓ soluble and insoluble levels of mutant TDP-43	(Lokireddy et al., 2015)
Ubiquitin proteasome system/chaperone-mediated autophagy						
3B12A scFv-CMA (Misfolding-specific intrabody with proteolytic signals)	TDP-43 intrabody	Mouse embryonic brains expr. WT or mutant TDP-43$^{\Delta NLS, C173S/C175S}$	In utero electroporation with 3B12A scFv intrabody, 48h	No change in cell-wide autophagy or proteasome activity. However autophagy/proteasome inhibition demonstrated ALP-/UPS-dependent degradation of TDP-43	↓ size, intensity and # cytoplasmic mutant TDP-43 inclusions, ↓ neuron loss up to P21	(Tamaki et al., 2018)
Autophagy-lysosome pathway						
Rapamycin	mTORC1 inhibition	Neuro2A, SH-SY5Y cells transfected TDP-43^{C199} CTF	Rapamycin 0.5μg/mL, 24h	LC3-II/-I ratio, ↓ p70S6K (downstream target of mTOR activity)	↓ # cells with cytoplasmic TDP-43, ↓ TDP-35 and -25kDa CTFs, rescued TDP-43 mislocalisation and neurofilament instability, turnover of full-length TDP-43 unaffected	(Caccamo et al., 2009)
		FTLD-U-TDP mice	2mo mice, Rapamycin 10mg/kg, 3x/week, 4 months	↓ p70S6K (brain), ↑ LC3+ puncta (IF) and LC3-II/-I ratio (WB), no quantification of autolysosome fusion or flux	↓ # cells with cytoplasmic TDP-43 inclusions, ↓ insoluble TDP-43 and CTFs,↑ cognition, ↑ motor function, ↓ # apoptotic cells, ↓ astrogliosis	(Wang et al., 2012)
		dTDP Drosophila	Rapamycin 400μM daily for 10d prior to dTDP expr., then every 1-2 days for 14d	↓ p62 levels in control and transgenic flies (dose dependent)	↓ # neurons with TDP-43 aggregates, no effect on soluble dTDP levels, partially rescue lifespan and locomotive deficits. However was harmful to control flies	(Cheng et al., 2015)
		Glaucoma mutant OPTN (E50K) mice	5 weeks	↓ LC3-II and p62 abundance in OPTN transgenic mice, but no change in autophagy proteins in control mice.	↓ TDP-43 levels, ↑ survival of retinal ganglion cells, ↑ visual function	(Zhang et al., 2021)
Valproate	?	SH-SY5Y cells expr. TDP-25 CTF	Up to 2mM Valproate, 48h	↑ LC3-II/-I ratio, Beclin-1 levels, MDC and LysoTracker intensity	↑ autophagy, ↓ ER-stress-mediated apoptosis, ↓ TDP-25-induced neuronal toxicity	(Wang et al., 2015)
IMS-088 (analog of Withaferin-A)	NF-κB essential modulator antagonist	hTDP-43^{A315T} mice; hTDP-43^{G348C} mice	1yo mice, IMS-088 30mg/kg by oral gavage, 2x daily, 8 weeks	↓ NF-κB activity (↓NF-κB P65-luciferase reporter signal in response to TNFα+IMS-088)	↓ TDP-43 levels, cytoplasmic:nuclear ratio, and number of inclusions per cell (brain, spinal cord),↑ cognition, ↓ gliosis	(Kumar et al., 2021)
Fluphenazine (FPZ) Methrotrimeprazine (MTM) N-chlorophenoxazine (NCP)	?	1° rat neurons expr. TDP-43$^{A315T/M337V}$ and hiPSC-derived neurons	5μM each compound, 24h	↓ LC3-Dendra2 half-life, ↑ LC3-II/-I ratio, ↑ LC3-II levels, ↑ LC3+ puncta in presence of NH_4Cl (FPZ most potent)	All 3 = ↓ TDP-43^{A315T}-EGFP levels, only FPZ and MTM ↓ inclusion # by 48h, restored TDP-43 localisation, ↑ neuron/astrocyte survival	(Barmada et al., 2014)
Rilmenidine	mTOR-independent	TDP-43^{WTxQ331K} mice, P21	Rilmenidine 4x/week, 1mg/kg until late disease then 0.5mg/kg until end	↑ LC3-II and -I levels (brain, SC). Brain: no change LC3-II/-I ratio, ↓ p62/SQSTM1. SC: ↑ LC3-II/-I ratio, no change p62	Exacerbated phenotype of TDP-43^{WTxQ331K} mice (↓ lifespan), attributed to excessive mitophagy	(Perera et al., 2021)
Tubastatin-A	HDAC6 inhibition	Mutant TARDBP iPSC-derived motor neurons	Unknown concentration Tubastatin-A, 12h	Restored axonal transport deficit (contributes to autophagy deficit in these models?), no quantification of autophagy activity	↓ mutant TDP-43 mislocalisation, ↓ pTDP-43 levels, ↓ insoluble TDP-43 levels (35kDa/25kDa CTFs), no effect on endog. TDP-43 in controls	(Fazal et al., 2021)
Heat-shock response						
HSF-1A (activator)	Inhibition of TRiC complex	HEK293 cells, expr. TDP-43$^{\Delta NLS-K145Q}$	HSF-1A 10μM, 24h	No validation of HSF-1A target activation or downstream HSP gene expression changes.	↓ # TDP-43 inclusions.	(Wang et al., 2017)
17-AAG	Inhibition of HSP90 ATPase activity	HEK293 cells, expr. TDP-43$^{\Delta NLS-K145Q}$	17-AAG 5μM, 24h	No validation of changes in HSP90 activity.	↑ insoluble mutant TDP-43? ↓ soluble endogenous TDP-43 compared to vehicle control. However no quantitative densitometry analysis	(Wang et al., 2017)
		HeLa cells expr. FL TDP-43 or CTF	17-AAG 3μM, 24h	Limited validation of drug target engagement, as above.	No effect on FL and CTF TDP-43 levels	(Jinwal et al., 2012)
Celastrol	Inhibition of HSP90/CDC37 interface	HeLa cells expr. FL TDP-43 or CTF	Celastrol 3μM, 24h	Limited validation. KD and overexpr. CDC37 demonstrated Celastrol-mediated effect dependent on HSP90/CDC37 interaction.	↓ FL and CTF TDP-43 levels	(Jinwal et al., 2012)
Arimoclomol	HSF-1 and HSP gene activation	Mutant VCP mice, 4 months old	Arimoclomol 120mg/kg daily in drinking water, 10 months	↑ HSP70 expression, ↓ubiquitin levels. Proteasomal activity unchanged, ↓ LC3-II (but lysosomal inhibitor not applied)	↓ inclusion pathology, ↓ cytoplasmic TDP-43 levels, ↑ muscle force	(Ahmed et al., 2016)
Colchicine or doxorubicin	HSPB8 activation	SH-SY5Y cells expr FL TDP-43 or CTFs	Colchicine or Doxorubicin (100nM, 500nM or 1μM)	↑ hHSPB8 mRNA and protein, ↑ heat-shock-induced HSF-1,, ↑ TFEB, ↑ p62/SQSTM1, ↑ LC3.	Both ↓ # and size GFP-TDP-25 inclusions. Colchicine ↓ insoluble GFP-TDP-43 and -25 levels. Doxorubicin ↓ GFP-TDP-25 levels only	(Crippa et al., 2016)

3.1.3 Genetic enhancement of the UPS

Alternative therapeutic approaches involving direct genetic manipulation of essential UPS components that may be dysfunctional or depleted in ALS and FTD have been shown to efficiently enhance proteasomal TDP-43 degradation (***Table 2***). In human neuroglioma cells expressing full-length or CTF TDP-43, co-transfection with wild-type UBQLN2 decreased the abundance of both TDP-43 species, although changes in proteasomal activity were not detected (Cassel and Reitz, 2013). In addition, over-expression of ubiquitin in mutant UBQLN2^{P497H}-/TDP-43^{G348C}-expressing Neuro-2a cells, which demonstrate UPS impairment due to ubiquitin sequestration, has been shown to restore the free ubiquitin pool to enhance UPS-mediated degradation of cytoplasmic TDP-43 inclusions (Picher-Martel et al., 2019). TDP-43 accumulation in the mouse brain is also prevented by the expression of the E3 ubiquitin ligase Znf179, which stimulates TDP-43 polyubiquitination and 26S proteasome activity by modulating 19S/20S subunit levels, whereas knock-out of Znf179 increases insoluble TDP-43 cytoplasmic inclusion formation (Lee et al., 2018). Over-expression of another E3 ubiquitin ligase, Praja-1, also decreases cytoplasmic TDP-43 CTFs and inclusion formation in cells and mouse motor neurons (Watabe et al., 2020). Praja-1 likely facilitates greater association between pathological TDP-43 and the ubiquitin-conjugating enzyme, UBE2E3 (Watabe et al., 2020), to increase TDP-43 ubiquitination and proteasomal targeting (Hans et al., 2014). These studies indicate that strategies to increase the association of pathological TDP-43 with UPS components to facilitate specific polyubiquitination and proteasomal targeting may be more effective than stimulating increased abundance of UPS cargo receptors or proteasomal assembly.

This recognition and recruitment of substrates is one of the major rate-limiting steps to efficient protein degradation; however, emerging therapeutic agents such as synthetic antibodies may overcome these

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hurdles. For example, single-chain intracellular antibodies (intrabodies) have been generated specifically against misfolded TDP-43 and its functional RRMs, which contain proteolytic signals that direct TDP-43 degradation towards the proteasome or ALP (Pozzi et al., 2020; Pozzi et al., 2019; Tamaki et al., 2018). Viral-mediated delivery of TDP-43-intrabodies to mutant TDP-43 transgenic mice ameliorated cognitive impairment, motor defects and TDP-43 proteinopathy, which was attributed to an increased association of pathological TDP-43 with proteasomes and autophagosomes, with subsequent UPS- or ALP-dependent degradation (Pozzi et al., 2020; Pozzi et al., 2019; Tamaki et al., 2018). Testing these strategies in more complex *in vitro* neuronal systems and other *in vivo* models is required to validate whether such approaches to stimulate UPS-mediated TDP-43 degradation are clinically feasible.

Table 2. Studies of genetic enhancement of TDP-43 protein clearance in cell and animal models of TDP-43 proteinopathy. Summary of target selection and treatment conditions for the application of proteostasis-enhancing gene therapies in TDP-43 disease models, with an evaluation of therapeutic efficacy and validation for target engagement and pathway activation. Abbreviations: 1°, primary; AAV, adeno-associated virus; Baf-A1, bafilomycin-A1; CTF, C-terminal fragment; FL, full-length; HSP, heat-shock protein; KD, knock-down; KO, knock-out; NFκB, nuclear Factor kappa-light-chain-enhancer of activated B cells; NMJ, neuromuscular junction; NSC, neural stem cell; siRNA, small interfering ribonucleic acid; WT, wild-type.

Gene	Target	Model	Treatment	Pathway interaction	Effect	Ref.
Ubiquitin-proteasome system						
Ubiquitin	Overexpr. of ubiquitin	Neuro2A cells expr. TDP-43^{G348C} or UBQLN2^{P497H}	Co-transfection with pCMV-ubiquitin	↑ Ub pool,↑UPS function (chymotrypsin-like assay)	↓ % cells with cytoplasmic mutant TDP-43 inclusions	(Picher-Martel et al., 2019)
Ubiquilin-2	Overexpr. WT UBQLN2	H4 neuroglioma cells, expr. FL TDP-43 or CTF	Co-transfection with UBQLN2 for 24h	No analysis of UPS activity with UBQLN2 overexpression	↓ FL TDP-43 and CTF levels	(Cassel and Reitz, 2013)
Znf179 E3 ubiquitin ligase	Stable expr. Znf179	Neuro2A cells expr. myc-hTDP-43, 48h	Stable expression of GFP-mZnf179	↑ 26S proteasome activity, ↑ expr. 19S and 20S subunits, ↑ TDP-43 polyubiquitination	↓ insoluble TDP-43, ↓ aggregate formation, ↓ half-life of endog. TDP-43 and exog. hTDP-43.	(Lee et al., 2018)
Praja 1 RING-finger E3 ubiquitin ligase	Overexpr.. PJA1	Rat NSC-derived neuron and mouse facial neurons in vivo expr. WT TDP-43 and CTFs	AAV transduction with PJA1, 24h	↑ association of PJA1 with TDP-43 CTFs and E2 ubiquitin conjugating enzyme, UBE2E3. Showed increased ubiquitination of TDP-43 CTFs, but no analysis of global UPS activity.	↓ insoluble TDP-43 WT and CTFs, ↓ TDP-43 phosphorylation and aggregate formation. No change in neuronal survival	(Watabe et al., 2020)
USP14	USP14 inactivation	HEK293 cells expr. WT TDP-43	Co-transfection inactive mutant USP14, 48h	No validation of proteasomal activity reported.	No change in total TDP-43 levels.	(Ortuno et al., 2016)
scAAV2/9 VH7Vk9 scFv (Single-chain antibody against RRM1 of TDP-43)	TDP-43 antibody	TDP-43^{G348C} mice; TDP-43^{A315T} mice	^{G348C}intracortical or ^{A315T}intra-thecal injection AAV encoding VH7Vk9, for 2 or 4 months	Antibodies partially colocalised with ubiquitin, LC3 in HEK293. Stimulated K48 or K63 polyubiquitination of TDP-43. Autophagy blocker (BafA1) or proteasome inhibitor (MG132) showed ALP- and UPS-dependent degradation.	Both: ,↓ insoluble & total cyto TDP-43 levels,↑ nuclear:cyto ratio TDP-43. TDP-43^{G348C}:↓ cognitive decline. TDP-43^{A315T}:↓ motor decline,↓NMJ denervation, no change neuron survival,↑TDP-43 splicing function	(Pozzi et al., 2019)
Autophagy-lysosome pathway						
IκB-SR (super-repressor)	Repression of NF-κB	TDP-43^{A315T} or TDP-43^{G348C} mice	Neuron-specific expression of IκB-SR	↓ binding of Beclin-1 by Bcl-2 to induce autophagy: ↑ LC3b, ↑ Atg5 (spinal cord)	↓ TDP-43 cytoplasmic:nuclear ratio, ↓ insoluble TDP-43^{A315T} but not TDP-43^{G348C}. ↑ soluble TDP-43, ↑ neuron survival, ↑ motor performance	(Dutta et al., 2020)
C9ORF72	Overexpr. WT C9ORF72	Primary mouse cortical neurons expr. TDP-43^{D169G}	Transfection with HA-C9ORF72 plasmid	No validation of autophagic flux enhancement – but C9ORF72 siRNA impaired ↓ basal and Torin-enhanced autophagic flux	↓ % cells with TDP-43 inclusions by ~80%	(Sellier et al., 2016)
HDAC6	Overexpr. WT HDAC6	Neuro2A cells expr. WT or mutant TDP-43^{Q331K}-GFP. Drosophila expr. hTDP-43/ATXN2^{32Q}	Neuro2A: Stable HDAC6 expression. Drosophila: constitutive HDAC6 expr. (UAS-HDAC6/-TDP-43/-ATNX2 strains.	↑ LC3-II and -I accumulation, but no lysosomal degradation blocker applied. Later, Baf-A1 treatment confirmed HDAC6-mediated degradation dependent on ALP.	Neuro2A: ↓ insoluble endog. TDP-43 levels, ↓ exog. WT TDP-43-GFP Drosophila: ↓ polyubiquitylated aggregates in brain, ↑ climbing ability, ↑ lifespan.	(Lee et al., 2020)
Heat shock response						
HSF-1	Overexpr. WT or active HSF-1$^{\Delta221-315\ L395E}$	NSC34 cells expr. mutant TDP-43$^{\Delta NLS-K145Q}$, 24h	Transfected WT or active HSF-1 variant, 48h	↑ HSP40 and HSP27. Recruitment of HSP40 to TDP-43 aggregates	↓ insoluble phos. mutant TDP-43 levels, ↓ TDP-43 inclusions, prevented by KD HSP40 or HSP27 or proteasomal/autophagy blockage.	(Wang et al., 2017)
	Stable expr. WT HSF-1	SH-SY5Y cells treated 10μM MG132 to induce insoluble TDP-43	Stable expression WT HSF-1	↑ HSP70 and HSP90 levels, without increasing proteasomal activity, yet ↓ autophagy	↓ TDP-43 insolubility and fragmentation, ↓ TDP-43 CTF-associated toxicity. Effect prevented by HSK-1 KO or HSP70 inhibition	(Lin et al., 2016)
	Overexpr. WT HSF1	Rat neural stem cell-derived neuron expr. WT TDP-43 and CTFs	Adeno-associated viral transduction HSF1, 24h	Praja-1 found upregulated in HSF1 AAV genetic screen, top candidate suppressing TDP-43. No analysis proteasomal or autophagic degradation	↓ insoluble TDP-43 WT and CTFs, ↓ phos.TDP-43	(Watabe et al., 2020)
	Overexpr. WT or active HSF1	HEK293, SH-SY5Y, rat 1°neurons expr. TDP-4 WT, dNLS, M337V or Q331K	Co-transfection HSF1, 48h	No analysis proteasomal or autophagic degradation, but identified DNAJB2a (HSP40) as mediator of HSF1 effect.	HEK293/SH-SY5Y: ↓ insoluble TDP-43 levels, ↓ phos. TDP-43, ↑ cell survival. Rat neurons: ↓ TDP-43 mislocalisation, ↓ phos.	(Chen et al., 2016)
DNAJB2a	Overexpr WT DNAJB2a	Rat NSC-derived neuron expr. WT TDP-43 and CTFs	Adeno-associated viral transduction DNAJB2a, 24h	No further validation of mechanism.	↓ insoluble phos. CTFs, ↓ soluble total CTFs	(Watabe et al., 2020)
	Overexpr WT DNAJB2a	HEK293, SH-SY5Y exp WT TDP-43	Co-transfection DNAJB2a, 48h	↑ interactions with HSP70 for refolding/maintaining solubility, rather than ↑ degradation. No change proteasome activity.	↓ insoluble TDP-43 levels, no change in soluble TDP-43, ↑ cell survival, similar to HSF1 overexpr. above.	(Chen et al., 2016)
CDC37	siRNA-mediated KD CDC37	Neuronal M17 cells expr. FL or cleaved TDP-43	Co-transfection with CDC37 siRNA	↑ proteolytic clearance, ↑ destabilization of TDP-43. Effect prevented by siRNA KD Beclin 1 to inhibit autophagy.	↓ TDP-43 nuclear:cytoplasmic ratio, but ↓ total TDP-43 levels.	(Jinwal et al., 2012)
HSPB8	HSPB8 overexpr.	NSC34 expr. GFP-TDP-43 or -25	Co-transfection with human HSPB8 (transient, 3h)	No quantification other HSP expression/chaperone activity.	↓ FL TDP-43 and CTFs. Effect prevented by autophagy inhibitor 3-MA	(Crippa et al., 2016)
HSPB8	HSPB8 overexpr.	NSC34 cells, transfected with FL TDP-43 or CTF	Co-transfection with human HSPB8 (transient, 3h)	↑GFP-LC3 turnover. Autophagy blockage (Baf-A1) prevented HSPB8-induced clearance insoluble TDP-43..	↓ Soluble and insoluble TDP-43 CTFs, but not FL TDP-43.	(Crippa et al., 2010)
DNAJB1	DNAJB1 (Hsp40) overexpr.	1°rodent neurons expr. TDP-43 WT or A315T	Co-transfection with DNAJB1	No validation of target mechanism in neurons, but showed overexpr. Sis1 (DNAJB1 homologue) in yeast increased degradation of UPS reporter protein (CG*)	↓ TDP-43 toxicity, but not WT TDP-43 or mutant TDP-43^{A315T} levels	(Park et al., 2017)

3.2 The role of the autophagy-lysosome pathway in TDP-43 pathology

Autophagy is a process of 'cellular self-digestion' that involves the formation of double-membraned autophagosomes that engulf abnormal proteins or damaged organelles and fuse with degradative lysosomes containing acidic proteases that destroy the internalised cargo (Finkbeiner, 2020). While

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1 the ALP operates constitutively at basal levels for bulk catabolism of intracellular material, selective
2 autophagy may be activated under severe disruptions to proteostasis to degrade specific
3 polyubiquitinated substrates (Finkbeiner, 2020; Lim et al., 2015). Common targeting mechanisms,
4 substrate selection, and cargo receptor proteins between the UPS and ALP allow for redundancy,
5 competition, and compensation between these systems. Ubiquitinated TDP-43 inclusions bound to
6 cargo receptor proteins, p62 and UBQLN2, can be directed towards either the UPS or ALP (Zientara-
7 Rytter and Subramani, 2019). The detection of K48-linked polyubiquitin chains on cytoplasmic TDP-
8 43 inclusions suggests that a failure of degradation by the UPS and further ubiquitination with K63-
9 linked chains targets substrates towards the ALP (Lee et al., 2019; Pohl and Dikic, 2019; Scotter et
10 al., 2014).
11
12 ALP dysfunction has been identified alongside TDP-43 pathology in ALS and FTD patient tissue with
13 accumulation of stalled non-degradative autophagic vesicles (Sasaki, 2011) and co-aggregation of
14 ALP machinery components with TDP-43 inclusions (Lee et al., 2019). It is likely that TDP-43 species
15 are substrates of the ALP, as dysfunction or inhibition of this pathway causes an accumulation of
16 pathological TDP-43. Indeed, small molecule inhibition of the ALP leads to the mislocalisation and
17 accumulation of full-length TDP-43 (Caccamo et al., 2009; Wang et al., 2010), along with the
18 persistence of endogenous and exogenous 35- and 25-kDa TDP-43 CTFs (Cicardi et al., 2018; Huang
19 et al., 2014) and the formation of cytoplasmic inclusions (Kim et al., 2009). Blocking autophagy
20 prevented the final dissolution of disassembled TDP-43 inclusions, suggesting that TDP-43 oligomers
21 are also substrates for the ALP (Scotter et al., 2014). This is supported by exogenous delivery of
22 recombinant purified TDP-43 oligomers *in vitro* or optogenetic stimulation of TDP-43 oligomerisation,
23 which demonstrate selective ALP-mediated uptake and degradation (Asakawa et al., 2020).
24 Involvement of the ALP in TDP-43 oligomer clearance is supported by the finding that co-application
25 of UPS and ALP inhibitors together increases the level of insoluble high-molecular-weight TDP-43
26 species, which is not achieved by UPS inhibition alone (Scotter et al., 2014). Combined, these studies
27 show that multiple TDP-43 species (mislocalised, misfolded, oligomeric, inclusions) are substrates of
28 the ALP, and that ALP dysfunction is therefore likely to be a key pathomechanism that drives TDP-43
29 pathology in ALS and FTD.
30
31 In familial ALS and FTD, inherited mutations in genes encoding ALP components that play dual roles
32 with the UPS, including *UBQLN2*, *p62/SQSTM1, VCP, and TBK1,* or are unique to the ALP, such as
33 *OPTN, GRN,* and *TMEM106B,* directly cause ALP dysfunction, likely upstream of TDP-43 pathology.
34 ALS- and FTD-associated *UBQLN2* mutations impair the association with autophagy proteins mATG9
35 and ATG16L1, thus causing accumulation of polyubiquitinated TDP-43 and other proteins (Osaka et
36 al., 2016). Autophagic degradation capacity may also be impaired by loss of functional UBQLN2, as
37 UBQLN2 knock-out was associated with a reduction in autophagosome acidification, along with the
38 expression of ATP6v1g1, a critical subunit of the vacuolar ATPase that is required for maintaining
39 lysosome acidification and fusion with autophagosomes (Wu et al., 2020). Similar to the disruption

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caused to the UPS, disease-linked mutations in *SQSTM1*, encoding p62, also impair the binding and recruitment of ubiquitinated substrates to disrupt selective autophagy, promoting the aggregation of p62 and TDP-43 (Fecto et al., 2011; Le Ber et al., 2013; Rubino et al., 2012; van der Zee et al., 2014). Likewise, as mentioned in Section 2.4, *VCP* mutations in FTLD-TDP patients and animal models cause neuronal intranuclear TDP-43 inclusions in some cases that are intranuclear (Neumann et al., 2007; Wani et al., 2021). However, other studies in mice and iPSC-derived motor neurons expressing mutant VCP demonstrate TDP-43 mislocalisation and cytoplasmic inclusion formation (Ayaki et al., 2014; Badadani et al., 2010; Harley et al., 2021). This is concomitant with a loss of soluble nuclear TDP-43 and accumulation of non-degradative autophagosomes (Ju et al., 2009). In ALS patients, *OPTN* mutations correlate with autophagic vacuole formation and TDP-43 pathology (Kurashige et al., 2021), which is replicated in cells that demonstrate impaired autophagosome-lysosome fusion (Shen et al., 2015; Sundaramoorthy et al., 2015). Furthermore, ALS-associated *TBK1* loss-of-function mutations prevent the phosphorylation of OPTN and p62/SQSTM1, thereby impeding their activation and impairing autophagosome formation and cargo recruitment (Cirulli et al., 2015). Mutations in *PGRN* cause both sporadic and familial FTD with TDP-43 pathology (Le Ber et al., 2007), likely due to impaired lysosomal acidification and thus selective autophagic degradation, leading to the accumulation of non-degradative lysosomes (Chang et al., 2017; Elia et al., 2019; Tanaka et al., 2017). Single nucleotide polymorphisms in *TMEM106B* also increase the risk for FTD of *GRN* mutation carriers, as well as the risk of ALS (Feng et al., 2020; Werner et al., 2020). Depletion of TMEM106B exacerbates cytoplasmic TDP-43 aggregation in cells and mice, and worsens life span and motor deficits, which coincides with reduced autophagic degradation (Feng et al., 2020; Mao et al., 2021; Werner et al., 2020). Taken together, mutations in ALP genes that result in the dysfunction of cargo recognition and substrate delivery (*UBQLN2*, *p62/SQSTM1, OPTN, TBK1*), autophagosome maturation (*VCP*) and lysosome-mediated degradation (*GRN and TMEM106B*), functions which also cross-talk with the UPS, converge on TDP-43 aggregation.

3.2.1 TDP-43 pathology induces ALP impairment

TDP-43 pathological species impair or evade detection by the ALP by sequestering substrate recognition proteins into TDP-43 inclusions, as discussed for components shared with the UPS. The internalisation of exogenous full-length TDP-43 inclusions in NSC-34 cells does not alter the expression of the autophagy markers p62 or LC3, indicating that the ALP may not be effectively induced upon exposure to pre-formed TDP-43 inclusions (Cascella et al., 2017). However, high levels of 25-kDa TDP-43 CTF expression in transgenic mice may reduce autophagy induction (Caccamo et al., 2015), and the expression of 25-kDa TDP-43 in NSC-34 cells impairs overall autophagic flux, (Cicardi et al., 2018). Furthermore, the expression of TDP-43^{A315T} in SH-SY5Y neuroblastoma cells causes an accumulation of autophagosomes and lysosomes (Wang et al., 2015b). The sequestration of p62 (Hiji et al., 2008; Mizuno et al., 2006; Wang et al., 2017) and LC3 into TDP-43 inclusions (Kim et al., 2009; Liu et al., 2017; Wang et al., 2010) suggests that dysfunction might occur at the earliest stages of the ALP, including cargo recognition, substrate delivery, and phagophore formation.

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The formation of TDP-43 pathology also induces a loss of endogenous DNA-/RNA-binding function which negatively affects the splicing, stability, metabolism, transcription, and translation of thousands of mRNA targets (Bhardwaj et al., 2013), notably including the ALP genes *ATG4B, ATG7,* and *RPTOR*. RNA sequencing of ALS patient brain tissue, mouse embryonic stem cells, and HeLa cells lacking functional TDP-43 has demonstrated elevated levels of TDP-43-regulated cryptic exons in *ATG4B* mRNA (Ling et al., 2015; Torres et al., 2018). ATG4B protein is important for the cleavage and conjugation of LC3 onto the autophagosomal membrane. However, *ATG4B* cryptic exons truncate the protein upstream of important phosphorylation sites for autophagy activation, leading to impaired autophagosome formation which correlates with more severe ALS phenotypes (Torres et al., 2018). Similarly, knock-down of TDP-43 in Neuro-2a, NSC-34, and NIH3T3 cells decreases the levels of both the mRNA and protein of ATG7, essential for autophagosome biogenesis, and increases the abundance of polyubiquitinated proteins (Bose et al., 2011). TDP-43 also regulates the expression of raptor, a component of the mammalian target of rapamycin complex-1 (mTORC1) that inhibits the nuclear translocation of transcription factor EB (TFEB), a master ALP transcription factor (Xia et al., 2016). Knock-down of TDP-43 in neuronal cells has been shown to promote raptor-deficiency-mediated mTORC1 dysfunction, thereby stimulating autophagosome and lysosome biogenesis, but simultaneously downregulating dynactin-1, which impairs maturation, transport, and fusion to stall autophagic flux (Xia et al., 2016). These findings indicate that TDP-43 is not only a substrate of ALP protein clearance, but also regulates the expression of important ALP proteins and autophagic flux in cells. In disease, TDP-43 loss of function is likely initiated from early processes of aggregation, through its nuclear depletion and the generation of pathological species by cytoplasmic mislocalisation and misfolding. Therefore, the formation and persistence of pathological TDP-43 species may further impair protein clearance capacity of the ALP, which could result in a vicious cycle that exacerbates protein aggregation and proteostasis dysfunction, to drive neurodegeneration. Future research should investigate whether other proteostasis components are susceptible to TDP-43 loss of function, and how other pathological species may interfere with these pathways.

3.2.2 Pharmacological activation of the ALP

Pharmacological activation of the ALP is a promising strategy for the clearance of pathological TDP-43 species (***Table 1***). One approach to enhance the ALP involves using small molecule antagonists of mTOR, a master regulator of the ALP that phosphorylates transcription factors and components of the core machinery to inhibit autophagic flux (Dossou and Basu, 2019). The indirect mTOR inhibitor, rapamycin, is an established autophagy enhancer that is currently in clinical trials for ALS (Mandrioli et al., 2018). In preclinical studies, rapamycin reduced cytoplasmic TDP-43 aggregation and TDP-43-related neurodegeneration in cell, fly, zebrafish and mouse models of ALS and FTD (Caccamo et al., 2009; Cheng et al., 2015; Lattante et al., 2015; Wang et al., 2012), as well as a glaucoma-related mutant *OPTN* model with TDP-43 pathology (Zhang et al., 2021). One conflicting study in *SOD1*-ALS mice suggested that rapamycin may have no beneficial effect on SOD-1 pathology, and instead

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exacerbate neurodegeneration (Zhang et al., 2011). mTOR-independent autophagy enhancers such as trehalose, lithium (Scotter et al., 2014), fluphenazine, metratrimeprazine, N-chlorophenoxazine (Barmada et al., 2014), withaferin-A (Kumar et al., 2021), valproate (Wang et al., 2015a) and tubastatin-A (Fazal et al., 2021) have also shown success in combatting pathology in TDP-43 models to ameliorate neurotoxicity. However, the therapeutic efficacy of mTOR-independent mechanisms remains controversial – stimulating autophagy with rilmenidine increased the clearance of disease-associated aggregate proteins but did not slow disease progression in mutant SOD1^{G93A} or TDP-43^{Q331K} mice, an outcome which was attributed to excessive stimulation of mitophagy (Perera et al., 2018; Perera et al., 2021).

Overall, pharmacological activation of autophagy to increase TDP-43 protein degradation is a promising therapeutic strategy. However, some concerns in relation to target selection, deleterious outcomes in rodent ALS models, and multiple caveats in the studies performed have hindered its clinical application. mTOR is a broad upstream regulator of multiple metabolic processes, so mTOR inhibition could activate signalling cascades that trigger diverse off-target effects (Dossou and Basu, 2019). Of these, mTOR inhibition is thought to generally stimulate ALP gene expression, which may promote early autophagosome formation or lysosome biogenesis, but the effect on the entire pathway is unclear (Dossou and Basu, 2019). Achieving efficient 'autophagic flux', which reflects the rate of flow through the entire pathway from substrate recruitment to degradation, relies not only on biogenesis but also transport, maturation/fusion, and degradation of autophagic vesicles (Loos et al., 2014). Therefore, enhancement of the autophagy initiation steps by rapamycin may increase autophagosome formation but reach a "bottleneck" at which the rate-limiting steps of maturation and degradation result in a toxic accumulation of autophagic vesicles and cargo (Button et al., 2017). This could explain the lack of therapeutic efficacy or deleterious effects of these autophagy-enhancing drugs and indicates that more targeted approaches are needed to specifically upregulate rate-limiting steps in the ALP. High throughout screening approaches, using libraries of small molecule autophagy inducers to identify compounds that decrease toxcity caused by TDP-43 (Safren et al., 2021), hold promise for revealing better candidates for further pre-clinical and clinical development for TDP-43 proteinopathies.

3.2.3 Genetic enhancement of the ALP

Genetic approaches to enhance autophagic flux involving over-expression of ALP components have been applied in cell culture and *in vivo* models of TDP-43-related ALS and FTD (***Table 2***). In two mutant TDP-43 mouse models (A315T and G348C), neuron-specific expression of IκB-SR, a super-repressor of nuclear factor-κB (NFκB) signalling, activated autophagy and decreased insoluble mutant TDP-43 protein in spinal cord tissue by almost 50% (Dutta et al., 2020). In a TDP-43/ATXN2^{32Q} *Drosophila* model of human TDP-43 proteinopathy, over-expression of HDAC6 decreased polyubiquitinated aggregates in brain tissue, rescued climbing deficits, and increased lifespan from 15 to 21 days (Lee et al., 2020). This effect was blocked by the application of the lysosomal ATPase

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inhibitor, bafilomycin-A1, suggesting that the HDAC6-mediated reduction of TDP-43 was dependent on the ALP (Lee et al., 2020). Alternatively, TDP-43 degradation can be promoted by restoring or enhancing the function of ALP proteins impaired by ALS- and FTD-causing mutations. For example, it has been reported that siRNA-mediated depletion of endogenous *C9ORF72*, which is thought to be involved in the autophagy initation complex (Ho et al., 2019), impairs basal and Torin-induced autophagic flux, leading to the aggregation of TDP-43 and p62 proteins in primary neuronal cultures, which was significantly rescued by subsequent over-expression of wild-type *C9ORF72* (Sellier et al., 2016).

To avoid the pitfalls of previous autophagy-modulating therapeutic approaches, further research is warranted to investigate compounds or genes that individually modulate rate-limiting processes within the ALP, especially downstream of autophagosome formation. Recent research has shown that specifically modulating the activity of specialised cargo adaptors and autophagosome tethering molecules facilitates greater internalisation and subcellular shuttling of autophagy cargoes as well as lysosomal fusion (Loos et al., 2019; Wetzel et al., 2020; Zellner et al., 2021). For example, artificial tethering of LC3 and p62 targeted to the endoplasmic reticulum or Golgi apparatus successfully recruited endogenous LC3 and p62 to those organelles (Loos et al., 2019), a strategy which could be adapted to direct subcellular targeted TDP-43 degradation via the ALP. Additionally, over-expression of the ATG5-interacting TECPR1 promotes ubiquitinated aggregate clearance in neural cells by direct recruitment of LC3C autophagosomes to lysosomes (Wetzel et al., 2020). Previous work has shown that TDP-43 inclusions in ALS and FTD correlate with significant down-regulation of TECPR1 in neurons, indicating that stimulation of TECPR1 may influence TDP-43 degradation (Wu et al., 2019). It may also be beneficial to focus on enhancing the specific interaction between substrate TDP-43 and the ALP machinery, for example with a TDP-43-targeted intrabody approach (Pozzi et al., 2019; Tamaki et al., 2018). Modulating each of these steps with a combinatorial approach may lead to the development of ‘polytherapies’ to provide a synergistic effect on ALP flux and the removal of pathological TDP-43 species. Multiple criticial junctures within the ALP that determine autophagic degradation capacity may be dysfunctional throughout disease, presenting opportunities for therapeutic intervention (***Figure 3***). Indeed, treatments that restore or enhance compromised ALP processes may lead to the beneficial removal of pathological TDP-43 species to effectively combat neurodegeneration.

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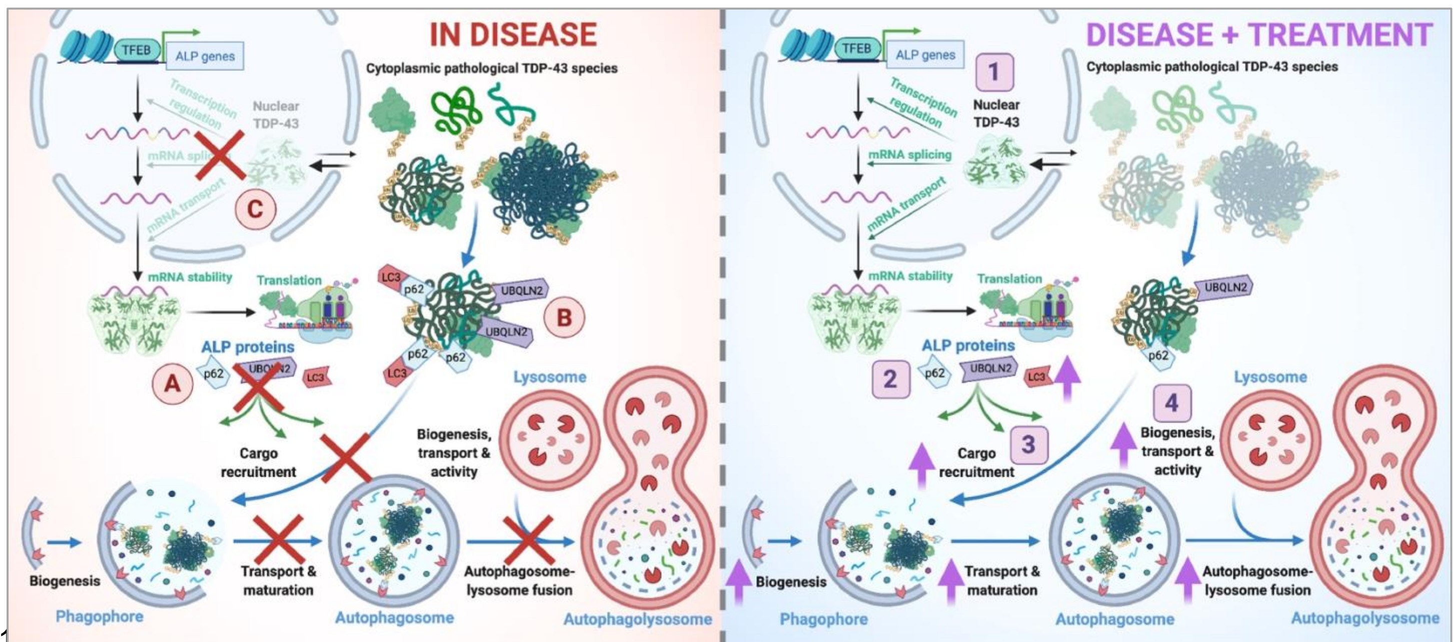

Figure 3. A model of dysfunction of the autophagy-lysosome pathway in TDP-43 proteinopathies, and effects of treatment strategies to increase overall autophagic degradation capacity. In **DISEASE**, dysfunction of the ALP likely facilitates the formation and persistence of multiple pathological TDP-43 species. The ALP may be impaired due to **(A)** disease-associated mutations in ALP genes, **(B)** sequestration of functional ALP components by insoluble TDP-43 inclusions, and **(C)** dysfunction of TDP-43-dependent ALP gene expression. Effective **TREATMENT** strategies could restore and enhance ALP-mediated degradation of TDP-43 by **(1)** increasing the overall expression of essential ALP genes, **(2)** replenishing functional components of the pathway that harbour disease-associated mutations or are depleted by TDP-43 pathology formation, **(3)** facilitating delivery of ubiquitinated protein substrates, and/or **(4)** enhancing the biogenesis, transport and activity of degradation machineries. Schematic figure created with *BioRender*.

3.3 The role of the heat-shock response in TDP-43 pathology

The HSR induces rapid expression of molecular chaperones known as HSPs in response to a wide variety of physical or environmental stressors, such as disruption of proteostasis via aberrant protein aggregation. The primary mediator of HSP transcription, heat-shock factor 1 (HSF1), integrates these proteotoxic stress signals following trimerisation to become transcriptionally active, and binds heat shock elements in promoter regions of target genes for HSR gene transcription (Mathew et al., 2001). Protein misfolding can be directly inhibited or reversed by HSPs, and HSPs can shuttle misfolded proteins to other proteostasis pathways, such as the UPS or ALP (San Gil et al., 2017).

Evidence of HSP dysregulation in ALS cases with TDP-43 pathology suggests the involvement of this pathway in disease pathogenesis. For example, HSPB1, HSP70, and HSP40 are decreased in sporadic ALS spinal cord tissue, despite no change in HSF1 levels (Chen et al., 2016; Gorter et al., 2019). Furthermore, the colocalisation of HSP40 with pathological TDP-43 inclusions at end-stage suggests that chaperones are recruited directly as a clearance mechanism and are possibly sequestered into the inclusion (Wang et al., 2017). However, as there are ~100 human HSPs, a comprehensive understanding of HSP expression levels in ALS and FTD tissues is lacking. Some work has suggested that motor neurons have a relatively high threshold for HSR induction compared

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to surrounding non-neuronal cell types. For example, neurons do not up-regulate HSP70 after heat-shock due to an inability to activate HSF1 (Batulan et al., 2003). Alternatively, neurodegenerative disease-associated pathogenic proteins may impair or evade detection by the HSR in neurons (San Gil et al., 2020; Yamashita et al., 2007).

In rare cases, mutations in HSPs are causative of familial ALS and may directly impact proteostasis. Multiple protein truncation variants of the HSP gene *DNAJC7* confer increased risk of ALS and decrease DNAJC7 protein levels in brain tissue of DNAJC7-ALS cases (Farhan et al., 2019). The *DNAJC7* mutations have been hypothesised to exacerbate proteostasis imbalance (Farhan et al., 2019), although this remains to be experimentally validated and the relationship with TDP-43 pathology remains unclear.

Inhibiting the HSR recapitulates TDP-43 pathology, providing further evidence for a role of the HSR in clearing misfolded TDP-43 species and CTFs, and preventing the accumulation of oligomers or large inclusions. For example, treatment of HEK293 cells with an HSF1 inhibitor, KRIBB11, stimulates the formation of TDP-43 aggregates following oxidative stress or heat-shock (Chang et al., 2013). Pharmacological inhibition of HSP90 and CRISPR knock-out of *STI1* in Neuro-2a and neuronal SN56 cells also increases TDP-43 insolubility, misfolding, cytoplasmic puncta, and toxicity (Lin et al., 2020). Knock-down of *HSP70* or *HSP90* also leads to the accumulation of TDP-43 CTFs (Zhang et al., 2010). Further, inhibition of the HSR by expressing a dominant-negative mutant HSF1 increases insoluble TDP-43, phospho-TDP-43, and the number of inclusions formed in cells (Chen et al., 2016; Wang et al., 2017). DNAJB2 (HSJ1a) has been identified as a potent anti-aggregation chaperone for TDP-43, which is upregulated by HSF1 expression (Chen et al., 2016; Coyne et al., 2017). Furthermore, the nucleation and oligomerisation of TDP-43 induced by casein kinase II-dependent phosphorylation triggers the HSR and requires HSP90 to maintain misfolded TDP-43 in the soluble state for later clearance, disassembly, or refolding (Carlomagno et al., 2014). Together, these studies demonstrate that the HSR is involved in the cellular response to misfolded TDP-43, CTFs, oligomers, and inclusions.

3.3.1 TDP-43 pathology induces HSR impairment

TDP-43 pathology itself may impair HSR function directly, for example by co-aggregation with HSP70 (Wang et al., 2017). In addition, aggregation of TDP-43 could also lead to sequestration of mRNA and inhibition of the translation of the synaptic HSC70-4 (HSPA8) chaperone complex, thereby disrupting HSP activity at the neuromuscular junction (Coyne et al., 2017). These studies therefore suggest multiple avenues for direct and indirect impairment of the HSR in TDP-43 proteinopathies.

3.3.2 Pharmacological activation of the HSR

The effect of pharmacological activation of the HSR on TDP-43-related neurodegeneration is currently under investigation (***Table 1***), primarily through two classes of therapeutics: 1) activators of HSF1 and

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downstream products of the HSR, and 2) antagonists of the HSF1 inhibitory complex. Enhancing HSF1 activity by arimoclomol treatment in mutant VCP mice has been shown to increase HSP70 expression and subsequently inhibit the accumulation of ubiquitin and cytoplasmic TDP-43, preventing motor decline (Ahmed et al., 2016). In recent human phase 2/3 clinical trials in rapidly progressive *SOD1*-ALS, arimoclomol did not show therapeutic efficacy, but promising preclinical studies in TDP-43 models indicate that this approach could be effective in TDP-43 proteinopathies (NCT00706147) (Benatar et al., 2018). Downstream of HSF1, stimulation of endogenous HSPB8 expression by colchicine and doxorubicin in human neuroblastoma SH-SY5Y cells expressing full-length TDP-43 or 25-kDa CTFs almost completely eliminated cytoplasmic TDP-43 aggregation (Crippa et al., 2016). It was noted, however, that both drugs activated the autophagy master regulator gene, TFEB, thereby inducing p62/SQSTM1 and LC3, which may actually mediate autophagic degradation independent of HSR chaperone activity (Crippa et al., 2016).

Other downstream approaches have targeted HSP90, which forms part of a multi-chaperone complex that stabilises and protects protein substrates from cleavage when bound to CDC37, but promotes degradation upon depletion or dissociation of CDC37 (Siligardi et al., 2002). Allosteric inhibition of the HSP90/CDC37 chaperone complex with celastrol (Jinwal et al., 2012), but not HSP90 ATPase inhibition with 17-AAG (Jinwal et al., 2012; Wang et al., 2017), promoted the HSR-mediated degradation of both full-length TDP-43 and its CTFs via autophagy in transfected HeLa and HEK293 cells. Another study also found that 17-AAG treatment failed to suppress TDP-43 aggregation, whereas treatment with an HSF1 activator, which antagonises the HSF1 inhibitory complex, was effective against TDP-43 (Wang et al., 2017). Treatment of HEK293 cells expressing acetylation-mimic mutant TDP-43$^{\Delta NLS\text{-}K145Q}$ with an HSF1 activator also significantly decreased TDP-43 inclusion formation. This suggests that the activation of endogenous HSF1 is sufficient to disaggregate TDP-43, although further validation of downstream HSP expression to confirm the precise mechanism of action is needed (Wang et al., 2017).

3.3.3 Genetic enhancement of the HSR

Genetic approaches that directly stimulate HSF1 represent a potentially effective strategy to combat TDP-43 aggregation, given that the HSR draws on the HSF1-mediated expression of a broad range of HSPs for protein refolding and degradation (***Table 2***). The over-expression of constitutively active variants of HSF1 has been shown to effectively decrease insoluble phosphorylated aggregation-prone and disease-associated mutant TDP-43 levels and/or inclusions in NSC34 (Wang et al., 2017), HEK293, and SH-SY5Y cells, and primary rat cortical neurons (Chen et al., 2016). In addition, stable expression or adeno-associated viral transduction of wild-type HSF1 also decreased insoluble levels of phosphorylated TDP-43 and CTFs in SH-SY5Y (Lin et al., 2016), and rat neural stem cell-derived neurons (Watabe et al., 2020), respectively.

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Genetic approaches that upregulate expression of specific HSPs that interact with TDP-43 and directly refold or facilitate its degradation are also promising. For example, expression of HSP70, which is known to bind endogenous TDP-43, has been shown to prevent the accumulation of 35- and 25-kDa TDP-43 CTFs by facilitating their rapid presentation to the proteasome or autophagosome (Kitamura et al., 2018; Lin et al., 2016). Similar to the pharmacological approaches described above, over-expression of HSPB8 (Crippa et al., 2016; Crippa et al., 2010) or siRNA-mediated depletion of CDC37 (Jinwal et al., 2012) in TDP-43-expressing NSC-34 or neuronal M17 cells, respectively, promoted the clearance of full-length TDP-43 and CTFs. Over-expression of DNAJB2a also provides a decrease in insoluble total TDP-43 levels in HEK293 and SH-SY5Y cells (Chen et al., 2016), and a reduction of insoluble phosphorylated CTFs in rat neural stem cell-derived neurons (Watabe et al., 2020), with a beneficial effect on cell survival. Over-expression of DNAJB1 also reduces TDP-43 toxicity, however, without providing any change in wild-type or disease-associated mutant TDP-43 levels in primary rodent cortical neurons (Park et al., 2017).

Notably, over-expression of engineered HSP disaggregases, such as HSP104, with modifications to their nucleotide-binding- and middle-domains can overcome the restraints of endogenous HSR regulation, providing a therapeutic gain of function to specifically target and antagonise TDP-43 misfolding and aggregation (Jackrel et al., 2014; Jackrel et al., 2015; March et al., 2020; Tariq et al., 2019; Tariq et al., 2018). HSP104, interacting with HSP70 and HSP40, may mediate selective unfolding of toxic protein conformations but leave benign aggregates structures intact, thereby restoring some function of the protein upon refolding to the native state, rather than stimulating protein degradation (Jackrel et al., 2014). Co-expression of potentiated prokaryotic HSP104 variants with TDP-43 effectively disassembled pre-formed TDP-43 fibrils to suppress TDP-43 aggregation and toxicity in yeast (Jackrel et al., 2014; Tariq et al., 2019; Tariq et al., 2018). This toxicity-reducing effect of potentiated HSP104 has been replicated with its eukaryotic homologs in yeast (March et al., 2020); however, further research is warranted to assess their therapeutic potential in disease-relevant *in vitro* neuronal systems and *in vivo* models.

Overall, the therapeutic strategy of activating HSF1 or overexpressing HSPs in the CNS neurodegenerative disease is promising. However, the regulation of HSR induction *in vivo* is complex and induction varies in a cell-type- and stress-type-dependent manner. Future research into the mechanisms that repress HSR induction in the CNS, and identifying the HSPs that are most efficacious in targeting pathological forms of TDP-43 and extending neuronal survival will be important for therapeutic targeting of the HSR in ALS and FTD.

3.4 The role of chaperone-mediated autophagy in TDP-43 pathology

In contrast to the ALP or conventional 'macroautophagy', CMA involves the chaperone-dependent selection and targeting of soluble cytosolic proteins directly to lysosomes. This type of autophagy is

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unique in that specific proteins are targeted for degradation, but vesicle formation for cargo uptake and transport is not required, since substrates are directly translocated across the lysosome membrane for acidic protease degradation (Cuervo and Wong, 2014; Kaushik and Cuervo, 2018). The chaperone heat-shock cognate protein 70 (HSC70) acts in the cytoplasm to bind substrate proteins and facilitate their translocation to lysosome-associated membrane protein type 2A (LAMP2A) proteins on the lysosomal membrane. This interaction stimulates the multimeric assembly of LAMP2A proteins to generate the translocation complex, through which substrate proteins begin unfolding and enter the lysosome. Early studies emphasised the requirement for CMA substrates to contain a specific amino acid sequence, the pentapeptide KFERQ motif, or protein damage, for detection and degradation (Kaushik and Cuervo, 2018). However, recent work has shown that the KFERQ-like motif is not necessarily required for HSC70 or LAMP2A binding to substrates (Kaushik and Cuervo, 2018). Further, most KFERQ-bearing proteins can also undergo CMA degradation during starvation, indicating that protein damage is not essential for CMA and that CMA may play a broader role in protein degradation than previously acknowledged (Kaushik and Cuervo, 2018).

CMA was unexpectedly found to play a role in clearing pathological TDP-43 following the detection of an alternative non-canonical CMA-recognition motif, QVKKD, in the RRM1 domain of TDP-43 (Huang et al., 2014). Remarkably, ubiquitinated wild-type TDP-43, but not a mutant lacking the QVKKD sequence, was found to co-immunoprecipitate with HSC70 (Huang et al., 2014). Recently, it was shown that HSC70 expression is reduced in sporadic ALS patients with insoluble TDP-43 pathology, and that HSC70 silencing in human neuroblastoma cells directly increases TDP-43 protein levels (Arosio et al., 2020). Both endogenous and exogenous wild-type TDP-43 that is mislocalised to the cytoplasm can be cleared by CMA, as indicated by their presence within high-CMA-activity lysosomal fractions in the rat brain, together with evidence of continued CMA-specific TDP-43 degradation upon proteasomal inhibition (Ormeño et al., 2020b). CMA may also be involved in degrading misfolded TDP-43, given that inhibition of CMA via siRNA-mediated LAMP2A knock-down increases the abundance of soluble aggregation-prone mutant TDP-43 proteins (Ormeño et al., 2020b). Furthermore, the co-expression of misfolding-specific TDP-43 intrabodies with mutant TDP-43 revealed that misfolded TDP-43 can be directed to and degraded by CMA. This was verified by the prevention of TDP-43 removal upon bafilomycin-A1-mediated inhibition of lysosomal proteolysis (Tamaki et al., 2018). TDP-43 CTFs are also likely degraded via CMA, as the siRNA-mediated knock-down of LAMP2A in transfected Neuro-2a cells increases the levels of endogenous 35- and 25-kDa TDP-43 CTFs (Huang et al., 2014). The unique requirements, regulation, and potential vulnerabilities of CMA in the context of the neuronal proteostasis network have not been well characterised. While few studies have investigated the involvement of CMA in neurodegenerative proteinopathies, and no clear role has been established for CMA dysfunction in TDP-43 pathology and ALS and FTD disease development, this is a topic of emerging attention.

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3.4.1 TDP-43 pathology induces CMA impairment

Mutant TDP-43 can interact directly with HSC70 and LAMP2A, but also co-precipitates with the autophagy proteins p62, LC3, and the HSC70 co-chaperone, BAG3 (Ormeño et al., 2020b). However, this co-precipitation was only observed for exogenous aggregation-prone mutant TDP-43 and, to a lesser extent, exogenous wild-type TDP-43, but not endogenous TDP-43 (Ormeño et al., 2020b). Interestingly, prolonged exposure of cells to TDP-43 aggregates impairs the recruitment of LAMP2A-positive lysosomes to the perinuclear region and causes lysosomal damage, resulting in dysfunction of the CMA pathway (Ormeño et al., 2020b).

3.4.2 Potential for pharmacological or genetic enhancement of CMA

Although the links to human disease are not firmly established, these studies suggest that CMA dysfunction could promote, and be influenced by, TDP-43 pathology and ALS and FTD pathogenesis. This provides a foundation for future research to investigate whether stimulating CMA can effectively combat TDP-43 aggregation. Although there is very limited research targeting TDP-43 pathology with CMA enhancement, one study showed that expression of HSPB8, which also operates through the HSR, decreased levels of truncated TDP-43 species (Crippa et al., 2016). It is important to note that the CMA pathway shares components with multiple other degradation systems (***Figure 2***), so potential treatments that upregulate CMA may broadly enhance the function of other pathways in the neuronal proteostasis network. More research is therefore warranted to identify and apply strategies for CMA activation in disease-relevant models.

3.5 Response of an integrated proteostasis network to TDP-43 aggregation: caveats and outstanding questions

The findings presented above show that TDP-43 is a substrate for key mechanisms of cellular proteostasis which work together or in isolation to degrade different pathological species involved in ALS and FTD (***Figure 2***). Notably, the various protein degradation systems share components for substrate recognition/targeting and degradation machineries, so it is likely that interactions, redundancy, compensation, or synergy between mechanisms are involved in the response to TDP-43 aggregation and toxicity (Limanaqi et al., 2020). For example, inhibition of autophagy causes the accumulation and sequestration of p62, thereby depleting the available functional pool and in-turn inhibiting p62-dependent clearance via the proteasome, leading to an accumulation of UPS substrates (Korolchuk et al., 2009). Conversely, inhibition of one system could also lead to a compensatory increase in the activity of others. For example, proteasomal inhibition by MG132, or CMA inhibition by siRNA-mediated knock-down of LAMP2A, leads to increased conversion of LC3-I to LC3-II, induction of p62 expression (Huang et al., 2014), and ULK1-/2-mediated phosphorylation (Lim et al., 2015), indicating autophagy stimulation (Li et al., 2019). Likewise, progranulin deficiency impairs lysosome function but leads to elevation of autophagy and lysosome-related genes in microglia (Elia et al., 2019; Lui et al., 2016; Zhang et al., 2020). Disruption of the CDC37/HSP90 protein complex impairs the HSR but triggers autophagic clearance of TDP-43 (Jinwal et al., 2012). Finally, when active, both the

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proteasome and autophagosome may synergistically clear polyubiquitinated TDP-43 (Urushitani et al., 2010). The application of autophagy inhibitors (such as 3-MA) or proteasome inhibitors (such as lactacystin) alone moderately prevent the the formation of cytoplasmic TDP-43 aggregates (Scotter et al., 2014; Urushitani et al., 2010). Overall, evidence degradation of polyubiquitinated wild-type and mutant TDP-43, whereas treatment with both inhibitors exacerbates for significant interactions between clearance systems demonstrates that proteostasis is executed by interconnected pathways, whereby the UPS, ALP, HSR and CMA pathways together contribute to TDP-43 accumulation and clearance. The interplay between TDP-43 aggregation and proteostasis function can be conceptually represented by a "bottleneck" model, in which the combined capacity for the clearance of TDP-43 inhibits accumulation of the various pathological species, and changes in this clearance capacity that restrict or widen this bottleneck therefore influence pathology development over time (***Figure 4***).

An important caveat to these observations on TDP-43 clearance is that most have been made using over-expression models and fluorescently tagged fusion proteins, with the roles of endogenous protein clearance systems only inferred by pharmacological or genetic modulation of specific protein degradation systems or components (Cascella et al., 2019; Cascella et al., 2017; Cicardi et al., 2018; Scotter et al., 2014; Urushitani et al., 2010; Wang et al., 2010). Exogenous over-expression and/or reporter tagging of wild-type or pathology-mimicking TDP-43 may alter its stability, abundance, and aggregation propensity. These parameters can be further impacted by varying experimental conditions and cellular or animal model used (Conicella et al., 2020; Gasset-Rosa et al., 2019; Huang et al., 2020; Nonaka et al., 2009). Experimental conditions including dosage and timing of the application of modulators can also alter endogenous protein degradation responses.

Regardless, a wealth of evidence supports a role for these essential endogenous proteostasis systems in mitigating the aggregation and persistence of TDP-43 species. Overall, many of the molecular interactions that mediate the specific and selective clearance of TDP-43, among other damaged, dysfunctional or aggregating proteins in the cell, have been determined. However, it will be necessary to further characterise the cross-talk between pathways and multiple seemingly interchangeable components of the proteostasis network in neurons, and to understand neuron-specific requirements and determinants of proteostasis, in order to develop effective and safe interventions that target proteostasis dysfunction and pathological protein aggregation in neurodegenerative disease.

Future considerations for the development of therapies aimed at removing pathological TDP-43 in disease are three-fold: i) the proteostasis network may be dysfunctional in ALS and FTD, due to ageing-related decline or disease-associated mutations which impair clearance capacity, ii) the increasing burden of cytoplasmic TDP-43 sequesters proteostasis proteins, which impair their functions, and iii) loss of TDP-43-dependent mRNA processing functions leads to further dysregulated expression of important proteostasis proteins to exacerbate deficits. Therefore, stimulation of

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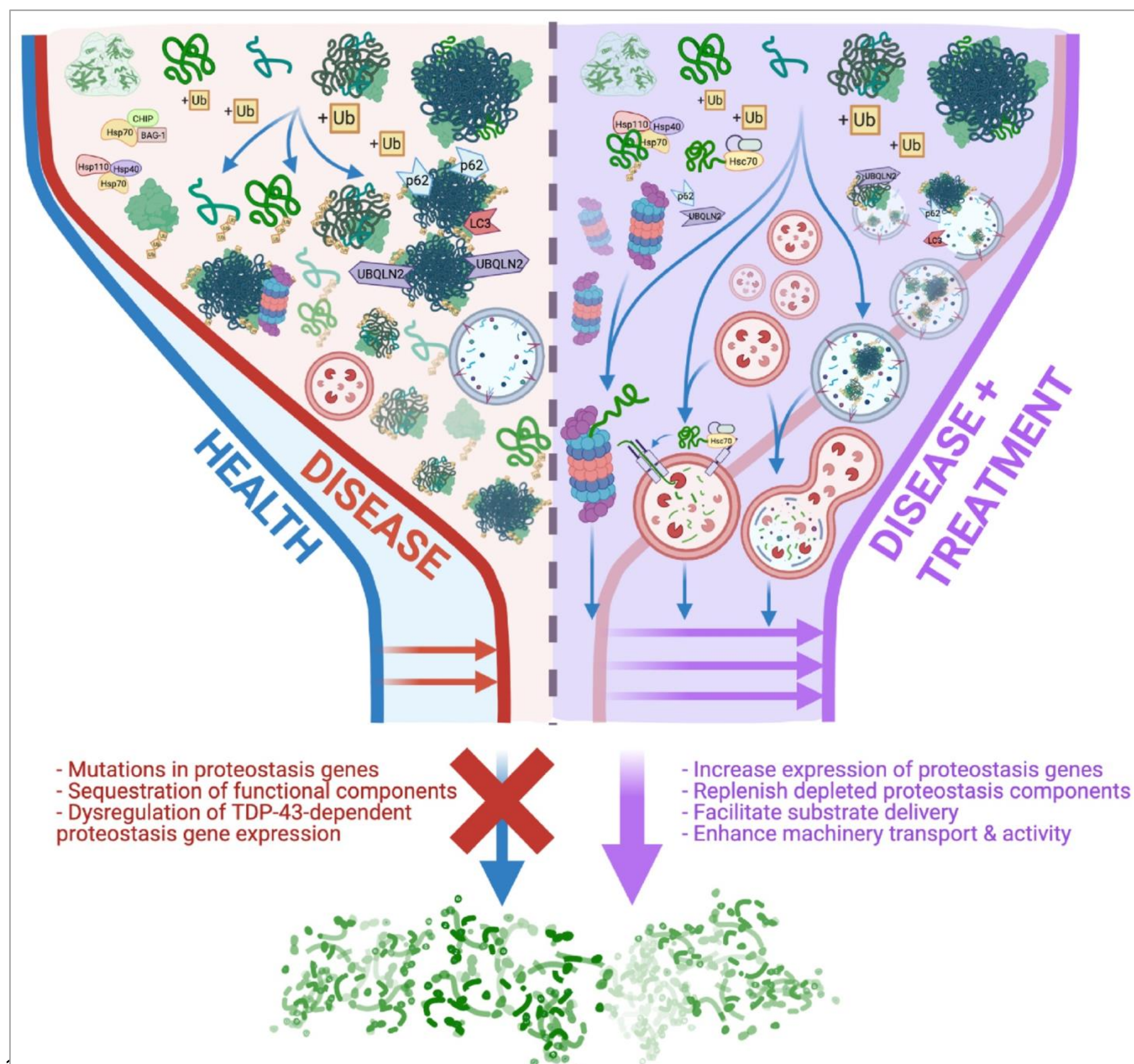
1 defective protein clearance mechanisms may be hampered by the pathological dysfunction of those
2 systems in disease. Novel therapies must therefore develop strategies for the fine regulation of
3 proteostasis components with these considerations in mind, to ensure effective restoration or
4 enhancement of neuronal protein degradation capacity in the face of TDP-43 aggregation and
5 dysfunction.
6
7 To summarise our understanding of this complex relationship between TDP-43 pathology and
8 proteostasis capacity, ***Figure 4*** illustrates the constraints of neuronal protein clearance systems in
9 relation to the accumulation of cytoplasmic pathological TDP-43 protein species, before and after
10 proposed treatments that enhance endogenous degradation capacity.



12 ***<u>Figure 4.</u> Schematic representation of the 'TDP-43 proteostasis bottleneck', depicting how***
13 ***changes in protein clearance capacity influence the accumulation and persistence of***
14 ***pathological TDP-43 species in disease, and effects of potential treatments.*** Maintaining protein

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homeostasis requires clearance of excessively abundant, misfolded or aggregated proteins. Essential protein clearance systems each feature several 'rate-limiting' steps that demand efficient coordination of the availability, localisation, transport, and activation of specialised degradation machineries. In **HEALTH**, the UPS, ALP, CMA and HSR operate together at a rate that prevents the accumulation of pathological TDP-43 proteins. In **DISEASE**, these systems may be compromised via disruptions to their components that restrict their capacity for TDP-43 degradation, leading to a 'bottleneck' effect, whereby TDP-43 aggregation exceeds degradation rate, leading to pathology formation. **TREATMENT** strategies could alleviate restrictions of these protein clearance systems and enhance their function to increase overall protein degradation capacity. This would result in "widening" the proteostasis bottleneck in disease, to facilitate greater removal of pathological TDP-43 species. Decreasing the accumulation of cytoplasmic TDP-43 will likely mitigate cellular dysfunction and toxicity, and restore the physiological abundance, localisation and function of TDP-43, to enhance neuronal function and survival. Schematic figure created with *BioRender*.

4 Advances in therapeutic approaches against TDP-43 pathology

Given that many upstream disease mechanisms converge on TDP-43 dysfunction and aggregation, therapeutics that ultimately lead to a decrease in TDP-43 pathology hold promise for ALS, FTLD-TDP, and other TDP-43 proteinopathies. However, due to the essential requirement of TDP-43 for survival, its complex autoregulatory expression mechanism, and the necessity for strict maintenance of TDP-43 levels in all cells of the body, direct depletion of TDP-43 is likely to be unviable and indirect approaches for ameliorating TDP-43 pathology will most likely be required for effective therapies. Importantly, approaches that decrease TDP-43 aggregation in the cytoplasm would be expected to result in a return of normal TDP-43 levels in the nucleus, thereby ameliorating the toxic effects from cytoplasmic accumulation of TDP-43 as well as reversing the effects of loss of normal TDP-43 function. We discuss the most promising approaches to achieving disease-modifying therapies below.

4.1 Promising human clinical trials of protein clearance/folding-modulating small molecules in ALS and FTD

Several small molecules that affect general protein clearance pathways have been tested clinically in people living with ALS. Although this approach is not specifically targeted towards TDP-43, any positive effects of such an approach are likely to be at least in part due to decreased levels of pathological TDP-43. For example, the small molecule enhancer of autophagy, bosutinib, is currently in phase 1 clinical trial for ALS (Imamura et al., 2019). Previously, another proposed small-molecule autophagy enhancer, tamoxifen, was tested in a small study of ALS patients and showed a potential for positive effects, warranting larger studies (Chen et al., 2020). Most promisingly, the dual-component AMX0035 (composed of sodium phenylbutyrate, possibly acting as a chemical chaperone, and tauroursodeoxycholic acid, possibly acting to block mitochondrial-mediated apoptosis) has shown promise in slowing functional decline in people with ALS in the CENTAUR phase 2 clinical trial (Paganoni et al., 2021; Paganoni et al., 2020), and is now progressing to phase 3 studies and therapeutic approval applications. Lithium, potentially partly acting to modulate the mTOR pathway of autophagy, has shown benefit in increasing survival specifically in ALS patients with *UNC13A* risk alleles, suggesting the potential for personalised therapies based on genetic predisposition to disease

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(van Eijk et al., 2017). Overall, these human clinical trials indicate that small molecule-mediated modulation of proteostasis may be protective against TDP-43 proteinopathies, although it remains to be determined whether any positive effects can be directly attributed to decreased levels of pathological TDP-43.

4.2 Recent gene therapy advances informing ALS and FTD treatments

Improvements in the technology for producing and delivering gene therapies now allows for safe, targeted, and efficacious modulation of disease-associated gene and protein expression. This has created a variety of promising treatment opportunities for inherited and sporadic diseases of relevance to ALS and FTD. For example, the FDA recently approved an innovative antisense oligonucleotide (ASO) gene therapy to treat spinal muscular atrophy (SMA), a rare disease affecting motor neurons, that is caused by a mutation in the survival motor neuron 1 (SMN1) gene (Finkel et al., 2017). Most children with SMA die in early childhood due to respiratory failure; however, a one-time intravenous delivery of adeno-associated virus and motor neuronal expression of fully functional SMN1 significantly improved muscle function and lifespan (Mendell et al., 2017). This provides very strong proof of concept for using targeted ASOs in people to rescue motor neuron function and survival, raising the hope for similar successes in ALS treatments. Currently, promising human ALS clinical trials are employing similar ASO approaches to target preferential knock-down of disease-associated repeat-containing transcripts in inherited forms of ALS and FTD (Klim et al., 2019a). The ASO treatment most advanced in trials for ALS is the *SOD1*-targeted Tofersen (BIIB067, NCT02623699), currently in Phase 3, which has shown decrease SOD1 concentrations within the cerebrospinal fluid at the highest concentration administered intrathecally over 12 weeks (Miller et al., 2020; Miller et al., 2013). Although Tofersen targets SOD1, an ALS pathology independent from TDP-43, it has sparked a wave of ASO development for ALS. For example, Phase 1b/2a trials have begun for an ASO targeting the hexanucleotide repeat expansion of *C9ORF72* (WVE-004, NCT04931862), in patients with *C9ORF72*-linked ALS or FTD, after showing a beneficial reduction in C9ORF72 DPRs, RNA foci, and toxicity in C9ORF72-ALS patient-derived motor neurons and mice (Chew et al., 2015; Liu et al., 2021). Reduction of C9ORF72 DPR pathology in response to targeted ASOs correlates with a decrease in concomittant TDP-43 pathology and beneficial outcomes in mice (Cook et al., 2020). Therefore, it is possible that the therapeutic benefits of correction of C9ORF72 disease are related to amelioration of TDP-43 dysfunction and aggregation. Phase 1 trials are also underway for BIIB105 (NCT04494256) in sporadic or *ATXN2*-associated ALS patients, which targets toxic intermediate-length polyglutamine repeats of *ATXN2*, and extends lifespan and decreases TDP-43 pathology in mice (Becker et al., 2017).

4.3 Genetic discoveries reveal novel modifiers of TDP-43 pathology

Given that genetic depletion of TDP-43 itself is unlikely to be a viable approach for therapy, targeting upstream modifiers of TDP-43 pathology development, or its critical downstream consequences, presents alternative approaches. Recent studies have identified gene variants that are associated

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1 with or predispose to TDP-43 pathology, which implies that these genes regulate either direct or
2 downstream cellular changes that facilitate the formation of TDP-43 pathology in neurons. Targeting
3 these genes therefore offers an opportunity for modifying pathways that cause TDP-43 dysfunction,
4 without the need to directly target TDP-43 itself. Likewise, if the critical determinants of
5 neurodegeneration downstream of TDP-43 dysfunction are identified, these could potentially be
6 harnessed for therapies. Such downstream players whose functional loss leads to neurodegeneration
7 following TDP-43 dysfunction include stathmin-2 (STMN2), the expression of which is highly
8 decreased with TDP-43 loss of function in disease, due to alterations in TDP-43-dependent *STMN2*
9 mRNA splicing (Klim et al., 2019b; Melamed et al., 2019). Loss of nuclear TDP-43 causes alterations
10 in the splicing of numerous other substrates, including *UNC13A*, leading to a loss of UNC13A protein
11 that may predispose to neurodegeneration (Brown et al., 2021; Ma et al., 2021). Indeed, genome-wide
12 association studies (GWAS) have identified alleles in the *UNC13A* gene that increase risk of ALS and
13 FTD (van Es et al., 2009; Yang et al., 2019), confirming the key role of this gene in TDP-43
14 proteinopathies. It remains to be determined whether modulation of individual downstream targets of
15 TDP-43 dysfunction will be therapeutically beneficial even in the absence of therapies that stimulate
16 clearance of pathological TDP-43, although a combination approach employing both strategies may
17 be synergistically protective.
18
19 Excitingly, the increasing powers of whole genome sequencing and GWAS are revealing greater
20 information on genetic modifiers of risk for ALS and FTLD-TDP, offering the potential to identify further
21 upstream modifiers of TDP-43 pathology amenable to therapeutic targeting. For example, variants of
22 *TMEM106B* increase the risk of FTLD-TDP (Van Deerlin et al., 2010), with TMEM106B recently shown
23 to be involved in the regulation of lysosomal transport in neuronal axons (Lüningschrör et al., 2020).
24 Such *TMEM106B* risk variants correlate with increased levels of TDP-43 pathology, while partial
25 knock-down of TMEM106B in model systems similarly increases the accumulation of aggregated
26 TDP-43 (Mao et al., 2021). Recent very large GWAS of ALS compared to other neurodegenerative
27 diseases revealed additional disease-associated risk loci (Rheenen et al., 2021), suggesting ever
28 more human-disease-relevant upstream regulators of TDP-43 pathology development. Similar
29 genome-wide analyses are also revealing differentially expressed genes associated with ALS,
30 including several likely related to ALP function, such as *ATG16L2* and *MAP1LC3A* (Saez-Atienzar et
31 al., 2021). Such genes, acting within known pathways that may directly impact TDP-43 dysfunction,
32 are promising candidates for therapeutic targeting, potentially using ASO approaches.
33
34 This progress in revolutionary genetic technologies and human clinical trials holds promise for the
35 development of effective disease-modifying therapies for ALS and FTD. In particular, the specificity
36 and adaptability of these molecular therapies will allow for rapid translation of emerging therapeutic
37 targets and biological pathways of interest. This provides hope for developing approaches to target
38 TDP-43 proteinopathy in the near future, potentially through the modulation of proteostasis capacity
39 in people living with ALS and FTD. Further developments of new biomarkers to allow for effective

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tracking of changes in TDP-43 pathology in both clinical trials and in clinical practice, will be key to harnessing these developments. This is particularly important for the identification of patients early in the disease course, as it remains unclear when treatments should be initiated for maximum benefit.

5 Conclusions

The field of TDP-43-related research is currently in a transition period, with increased investment in the development of disease-modifying therapeutics. Treatments that restore native TDP-43 function and remove pathological TDP-43 proteins hold great potential. In this review, we have evaluated key literature focused on the complexity of how TDP-43 pathology is triggered, how pathology develops over time, which endogenous protein degradation mechanisms become activated or impaired throughout disease, and how this information could be used for targeted therapeutic design. Importantly, the formation of TDP-43 pathology is a progressive process, involving the generation of disordered intermediate aggregating protein species that exhibit unique biophysical properties, acquire modifications, and play different roles in the neurodegenerative process. The intrinsic structural characteristics and functions of the TDP-43 protein, ALS- and FTD-causing genetic mutations, extrinsic stressors, and defective proteostasis pathways all likely culminate in the generation of pathological TDP-43 species. With improvements in therapeutic technologies and delivery methods, exploitation of endogenous protective protein clearance mechanisms and targeting of biological pathways that lead to TDP-43 pathology provides a promising opportunity to develop future treatments for ALS, FTD, and other neurodegenerative TDP-43 proteinopathies.

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