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Phenotypic diversity in ALS and the role of poly-conformational protein misfolding

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Abstract

In many types of familial amyotrophic lateral sclerosis (fALS), mutations cause proteins to gain toxic properties that mediate neurodegenerative processes. It is becoming increasingly clear that the proteins involved in ALS, and those responsible for a host of other neurodegenerative diseases, share many characteristics with a growing number of prion diseases. ALS is a heterogenous disease in which the majority of cases are sporadic in their etiology. Studies investigating the inherited forms of the disease are now beginning to provide evidence that some of this heterogeneity may be due to the existence of distinct conformations that ALS-linked proteins can adopt to produce the equivalent of prion strains. In this review, we discuss the *in vitro* and *in vivo* evidence that has been generated to better understand the characteristics of these proteins and how their tertiary structure may impact the disease phenotype.

Keywords

Amyotrophic Lateral Sclerosis; Superoxide dismutase-1; TDP-43; prion; strains

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that leads to paralysis and ultimately death of those afflicted with the condition. Although weakness can first appear anywhere in the body, including the respiratory system, most cases manifest in limb or bulbar muscles as a focal weakness before spreading throughout the motor axis to weaken respiratory muscles. This muscle loss is caused by the degeneration of both upper and motor neurons throughout the CNS, and, unfortunately, there are no treatments that slow disease progression or significantly lengthen survival. The prevalence of ALS is about 5

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cases per 100,000 with an incidence of 1-2 individuals diagnosed per 100,000 each year, revealing how quickly the disease progresses. The majority of cases are fatal within 2-4 years after diagnosis, but factors such as the site of onset, duration from first symptom to diagnosis, presence of cognitive impairment, and genotype can increase survival to more than 10 years after onset [1, 29]. The motor dysfunction in ALS has also been demonstrated to overlap with frontotemporal dementia (FTD), a disease characterized by the degeneration of the frontal and temporal lobes. Up to 50% of patients with ALS develop frontal lobe dysfunction, whereas approximately 15% of patients with FTD develop motor neuron loss.

Although clinically indistinguishable, ~90% of ALS cases are sporadic and have no known origin (sALS) while ~10% are familial (fALS) and are typically passed down with inheritance. There are now more than 50 genes that have been implicated in ALS pathogenesis, yet many of these have yet to be completely validated. Interestingly, the proteins encoded by these ALS-causing genes have a diverse set of functions, highlighting the complex nature of this disease and suggesting a range of mechanisms that ultimately lead to the degeneration of motor neurons. The first of these mutations to be discovered, and most widely studied, was found in the gene encoding the Cu/Zn superoxide dismutase 1 (*SOD1*) gene [112], of which there are now more than 180 clinically relevant mutations identified (http://alsod.iop.kcl.ac.uk/). However, mutations in SOD1 account for only 10-20% of all fALS diagnoses. Other genes associated with a significant portion of fALS cases include *C9ORF72* and those encoding the 43 kDa transactive response (TAR) DNA-binding protein (TDP-43) and the fused in sarcoma/translocated in liposarcoma (FUS/TLS) protein (http://alsod.iop.kcl.ac.uk/). Together, the identification and characterization of these disease-causing mutations and their proteins have greatly enhanced our understanding of ALS.

Aside from the key neuropathological feature of upper and lower motor neuron loss in cases of ALS, molecular neuropathology has demonstrated the appearance of ubiquitinated skein-like, or dense and round, inclusions in the cytoplasm of motor neurons [83, 85]. In the majority of ALS cases (~97%), including disease caused by repeat expansions in C90RF72, these protein inclusions are immunoreactive for TDP-43. In ALS cases caused by mutations in the genes encoding for SOD1 and FUS, protein inclusions are composed of those gene-products, and, generally, TDP-43 pathology is lacking [94]. Studies of mutant SOD1, TDP43, and FUS in cell and animal models have revealed that they harbor many of the properties of the infectious PrP prion protein (PrP^{Sc}) and of other proteins associated with neurodegeneration such as synuclein, tau, and amyloid-β (Aβ). These properties include the protein's ability to aggregate into non-native structures, template its misfolded conformation, spread from cell-to-cell in culture, spread along neuroanatomically connected regions *in vivo*, and, in some cases, adopt distinct conformations or "strains" [8, 30, 74, 101, 115, 118, 137]. With the recent advances in imaging, such as cryo-electron microscopy (cryo-EM), along with better *in vitro* and *in vivo* tools, we now have the ability directly assess the existence and consequences of conformationally distinct strains of misfolded proteins in neurodegenerative diseases.

In this review, we will discuss the existing evidence linking ALS to prion diseases and emphasize those studies investigating the conformational heterogeneity of SOD1 and other proteins implicated in ALS.

PrP prions and strains

The classical PrP prion diseases are a group of infectious neurodegenerative diseases affecting humans and a wide range of other mammals with the most notable being domesticated cattle and sheep that are consumed by humans. The major neuropathological hallmarks in the central nervous system (CNS) include neuronal loss, spongiform degeneration, gliosis, and the accumulation of amyloid plaques or aggregates consisting of the abnormal, protease-resistant form of the host-encoded prion protein (PrP) [36, 37, 107]. The identification and nature of the causative agent of these diseases was a highly contentious topic that resulted in the recognition of a novel infectious pathogen: a prion – a small proteinaceous infectious particle [18, 106]. Prior to its identification, and based upon the central dogma of molecular biology, the infectious agent was believed to be due to an “unconventional” or “slow” virus [52]. Ultimately, it was the transmission of the scrapie agent to hamsters and its subsequent purification and biochemical characterization that led to the identification of the infectious prion protein (PrP^{Sc}) [76, 87, 105].

Though the prion hypothesis was supported by the data, many characteristics of PrP^{Sc} existed that perplexed researchers; the most puzzling being the finding that transmission studies with different PrP^{Sc} inocula resulted in disease with distinct incubation periods and neuropathological features [77]. At the time, the only explanation for these findings was the existence of a strain-specific nucleic acid as part of the infectious agent. It was a theorized mechanism by J.S. Griffith in which he explained the potential for a pathogenic protein that enciphers its own replication without the need for nucleic acid that led researchers to examine and hypothesize that the structural heterogeneity of PrP^{Sc} could account for the disparate symptoms observed in experimental animals [61]. To address this hypothesis, researchers utilized two hamster-adapted strains of the transmissible mink encephalopathy (TME) agent that had been isolated following multiple passages in Syrian golden hamsters [16]. These two isolated strains had vastly different incubation periods and neuropathology and were designated hyper (HY) and drowsy (DY) due to their differing symptoms at the clinical stage of disease [16]. Biochemical studies of HY and DY PrP^{Sc} revealed differences in their migration by western blot analysis, their sensitivity to digestion with proteinase K, and their sedimentation in *N*-lauroylsarcosine [15]. It was also demonstrated that protease-resistant fragments of PrP that were unique to the two strains were maintained upon *in vitro* conversion of radiolabeled recombinant hamster PrP^{c} (the normally folded cellular version of the protein) to either labeled HY or DY PrP^{Sc} [14]. Further studies investigating both human and animal prion diseases using a variety of biochemical and histological techniques also provided persuasive evidence to the origins of PrP prion strains [31, 126]. It is now well-established that strain-specific information is encoded in the tertiary structure of PrP^{Sc} and that the protein can adopt a wide variety of conformations [116].

Prion strains are operationally defined by the distinct phenotypic characteristics they display upon infection when certain variables are held constant, such as the dose of the agent, the

route of infection, and the genotype of the host [98]. The differences commonly observed when such variables are accounted for include differences among clinical symptoms, incubation periods, the location and severity of neuropathology, and biochemical properties of abnormal PrP^{Sc}. Although differences in biochemical properties such as a protein's sensitivity to protease digestion or denaturation can be explained based on the tertiary structure and the solvent accessibility of its amino acids, it is not well understood how the conformation of a protein can account for variations in clinical symptoms and neuropathology [9].

As mentioned previously, it is now beginning to be understood that proteins involved in other neurodegenerative disorders share many of the same features and characteristics of the PrP protein. This includes the existence of distinct protein conformers or strains. For instance, it has been hypothesized that the various clinical phenotypes among tauopathies, such as Alzheimer's disease (AD), progressive supranuclear palsy (PSP), and corticobasal degeneration (CBD), were due to different tau strains; however, it wasn't until recently with the advent of new technologies like cryo-EM, that researchers were able to directly analyze the structure of protein fibrils at the atomic scale. Using this technology, it was directly shown that the tau fibrils isolated from the brains of patients who succumbed to these different diseases had distinct fibril conformations [43, 47, 56, 140]. These studies are just now starting to shed light on the structural heterogeneity that can exist for a given misfolded, pathogenic protein, and it will be quite interesting to explore whether this same phenomenon holds true for other neurodegenerative diseases, such as ALS.

General properties of SOD1

SOD1 is an antioxidant protein whose normal function is to catalyze the dismutation of highly reactive superoxide anions to hydrogen peroxide and dioxygen. It is a ubiquitously expressed and abundant protein mainly localized in the cytosol [33]. Some localization to the mitochondrial intermembrane space and nuclear compartments has also been reported [46, 54]. In its natively folded state, SOD1 exists as an extremely stable 32-kDa homodimer, in which each 153-amino-acid subunit contains one copper and one zinc ion along with an intra-subunit disulfide bond. Post-translational maturation and proper folding of SOD1 to a mature, highly stable dimer first involves the non-chaperone mediated incorporation of zinc (Zn) [51]. The Zn-bound form of SOD1 is then recognized by the copper chaperone for SOD1 (CCS), forming a heterodimer that facilitates copper (Cu) delivery and disulfide bond formation [11, 117]. The disulfide-oxidized holo version of human SOD1 is an extremely stable enzyme that melts at 92°C and resists proteolytic digestion at concentrations up to 1 mg/ml for 30 minutes at 37°C [108, 129]. However, even in the absence of Cu, the incorporation of Zn has shown to significantly alter its secondary structure and stabilize SOD1, so much so that this binding alone is sufficient to allow the formation of its normal quaternary structure [119].

SOD1 was the first dominantly inherited gene described to have a role in ALS pathogenesis [112] and now accounts for ~10-20% of all fALS cases. To date, there are now more than 180 different mutations that have been identified that span the entire length of the gene. These are predominantly single amino-acid residue substitutions; however, deletions,

insertions, and C-terminal truncations have also been described [120, 129]. It was initially hypothesized that mutations in SOD1 resulted in the loss of enzymatic activity and toxicity, though many SOD1 variants display normal enzymatic activity. In studies with transgenic mice overexpressing the G93A variant, animals developed disease despite increased levels of enzymatic activity [19, 63, 66]. Additionally, mice lacking endogenous SOD1 do not exhibit ALS-like phenotypes [109]. Ultimately, studies in transgenic models expressing various fALS-linked SOD1 mutants demonstrated that aggregation of the mutant protein was an invariant pathologic feature of mice that developed an ALS-like paralytic disease [23, 72, 134, 135]. Together, these data implicated a gain-of-toxic mechanism for SOD 1-mediated pathogenesis and led researchers to focus on the misfolding of SOD1 as a critical event in disease pathogenesis.

Familial ALS point mutations in SOD1 protein folding may act primarily by slowing the protein's rate of maturation rather than completely preventing the acquisition of its native conformation. Studies in which the residues involved in Cu binding (H46 and H48) were mutated did not prevent, but slowed the rate of dimerization and intramolecular disulfide bond formation [24]. This was also the case for mutations in the cysteine residues that produce the intramolecular disulfide bond (C57 and C146); even though these mutations prevented the formation of the disulfide bond, they allowed ~50% of the C146R-SOD1 to acquire resistance to proteinase [24]. This data suggests that although post-translational modifications of SOD1 have impacts on the rate of maturation, the inherent properties of the SOD1 sequence determine whether the protein will achieve its native conformation.

Aggregation and conformational templating of SOD1

Although not all of the more than 180 fALS-SOD1 associated mutations have been characterized, those that have were found to result in an increased propensity for SOD1 to adopt misfolded conformations and acquire detergent insolubility [23, 72, 103]. These variants have been primarily investigated by transient overexpression in cell models [103], but in transgenic mouse models overexpressing SOD1 mutants, detergent-insoluble aggregates of SOD1 are also observed [23, 133]. Importantly, similar proteinaceous inclusions immunoreactive for SOD1 are also detected in the CNS of fALS-SOD1 patients, leading to the widely accepted hypothesis that SOD1 aggregation is associated with the etiology of SOD1-linked fALS. Protein aggregation is associated with the pathogenesis in many other neurodegenerative diseases, such as AD, Parkinson's disease, and Huntington's disease, in which intracellular inclusions and/or extracellular amyloid fibrils are the main pathological hallmarks. Like SOD1, mutations within the proteins implicated in the pathogenesis of these other diseases result in their misfolding and aggregation. However, unlike SOD1, patients that succumb to the sporadic form of these diseases accumulate proteinaceous inclusions comprised of the wild-type (WT) versions of their associated toxic protein. Although conformational antibodies specific for "misfolded" SOD1 have revealed sparse immunopositive inclusions within the CNS of postmortem sALS patients, the role for WT SOD1 in the pathogenesis of sALS remains controversial [5, 20, 21, 35, 60, 99, 113, 127].

As previously discussed, WT SOD1 is an extremely stable protein due to its post-translational modifications, and preventing these modifications from occurring affects the kinetics of maturation and destabilizes the protein. The apo (metal free) version of SOD1 has been demonstrated to form detergent-insoluble amyloid fibrils under destabilizing conditions such as high temperature, low pH, or in the presence of organic solvents [50, 123]. Moreover, under mild denaturing conditions at 37°C and with constant shaking, it was demonstrated that both WT and mutant SOD1 spontaneously form fibrils [27, 28]. Similar cell-free assays were initially used in the prion field to study the kinetics of PrP^{C} to PrP^{Sc} conversion and supported the hypothesis that the PrP prion protein undergoes aggregation via a multistep process referred to as template-dependent polymerization [4, 78]. It was observed that fibrillization initiates through a rate-limiting nucleation step in which PrP^{Sc} "seeds" are produced that can then go on to convert the PrP^{C} substrate into additional PrP^{Sc} fibrils. More recently, through the optimization of techniques such as protein misfolding cyclic amplification (PMCA) and real-time quaking-induced conversion (RT-QuIC), it has been demonstrated that fibrillization can be greatly enhanced by adding a pre-formed seed, such as PrP^{Sc}-containing homogenates, and also by applying sonication or shaking to break up the continually growing fibrils, thereby producing more seeds [4, 114]. The kinetics of SOD1 fibrillization in the cell-free assays closely mirror those involving PrP, indicating a similar mechanism of fibril assembly. Chia et al. demonstrated the ability to induce fibrillization of both recombinant WT and mutant SOD1 under mild denaturing conditions following the addition of homogenates prepared from the spinal cords of transgenic mice overexpressing mutant SOD1 [28]. This study was paramount in demonstrating the ability of SOD1 to act as a seed for fibrillization and was one of the first studies to highlight the similarities of PrP and SOD1.

These cell-free studies then led researchers to investigate the transmission properties of SOD1 in living cells. Using neuroblastoma cell lines, Munch et al. were the first to demonstrate that exogenously added recombinant mutant SOD1 fibrils were able to be internalized via lipid-raft dependent micropinocytosis [91]. Once inside the cells, these SOD1 species were capable of inducing the aggregation of the overexpressed cytosolic mutant SOD1, causing it to adopt a detergent-insoluble conformation [91]. The authors observed that over time, as the cells continued to divide and the internalized exogenous SOD1 aggregates disappeared, the induced aggregates remained abundant for over a month. This finding revealed that SOD1 aggregation in this cell system was a heritable phenotype and/or the induced aggregates were capable of cell-to-cell transmission [91]. Although the induction of SOD1 aggregation in this assay was dependent on the overexpression of the SOD1 substrate, another study revealed the ability for endogenously expressed WT SOD1 to misfold and aggregate upon the transient overexpression of mutant SOD1 [59]. The WT SOD1 protein upon misfolding also acquired detergent insolubility and the ability to infect neighboring cells, either through the association with exosomes or from being released by dying cells [60, 121].

The *in vivo* demonstration of mutant SOD1 transmission was dependent on identifying transgenic animal lines that were permissive for transmission. Early work in our laboratory examined whether the onset of motor neuron disease (MND) in mice expressing the G93A or G37R fALS variants of SOD1 could be accelerated by injecting tissue homogenates from

paralyzed mice of the same genotype into the spinal cords of newborn transgenic mice. In our experience, using small numbers of mice initially, mice expressing these mutants were not permissive; however, we subsequently observed that the onset of MND could be accelerated in mice expressing the G85R fALS variant of SOD1 fused to yellow fluorescent protein (YFP) [7]. When bred to homozygosity, the G85R-SOD1:YFP transgenic mouse lines develop an ALS-like phenotype comprising of paralysis and muscle loss at ~16 months of age [132]. Mice that are hemizygous for the transgene rarely develop disease over their lifespan [7]. It was previously demonstrated for PrP transmission that utilizing a mouse model that expresses the mutant transgene below the threshold level required to spontaneously develop neurodegenerative disease enabled induction of pathology and disease by injecting PrP^{Sc}-containing homogenates [67]. Similar to this paradigm, our group prepared spinal cord homogenates from paralyzed mice that overexpressed different SOD1 mutants and injected them into the spinal cords of neonatal (P0) hemizygous G85R-SOD1:YFP mice to determine whether MND could be transmitted. The homogenates were prepared from diseased transgenic mice overexpressing the G93A or G37R SOD1 proteins. Surprisingly, both preparations resulted in the induction of G85R-SOD1:YFP misfolding and aggregation throughout the CNS and the onset of paralysis. Homogenates prepared from mice that did not contain SOD1 aggregates were unable to induce pathology or disease following injection. Similar outcomes were reported by another group that used a line of mice expressing the G85R variant (no tag) at levels that produce disease between 12-16 months. Intraspinal injection of tissue homogenates from paralyzed G85R mice into young adult animals led to a markedly accelerated age to paralysis [17]. Together, these studies demonstrated that mice expressing the G85R variant of SOD1 were permissive for mutant SOD1 prion-like seeding. Subsequent studies in our laboratories demonstrated that mice expressing truncation mutants of SOD1 are also permissive to mutant SOD1 seeding [6].

In other work, we also demonstrated that injection of tissue homogenates unilaterally into the sciatic nerve of G85R-SOD1:YFP mice produces a unilateral paralysis in the ipsilateral hind limb that spreads to the contralateral hind limb; reminiscent of the spreading paralytic features observed in ALS patients [8]. A longitudinal study of these sciatic-nerve-injected mice revealed that the induced G85R-SOD1:YFP aggregates accumulated in the CNS in a predictable manner; first in the ipsilateral spinal cord before spreading to neuroanatomically connected regions of the CNS.

Importantly, recombinant SOD1 fibrils and homogenates prepared from the spinal cords of fALS-SOD1 patients were also capable of transmitting the disease [6]. An equally important negative finding from these transmission studies was observed when homogenates prepared from the spinal cords of transgenic mice used to model other neurodegenerative diseases, such as those for synucleinopathies and tauopathies, were used as inoculum in the G85R-SOD1:YFP injection paradigm [6]. These lines (expressing mutant synuclein or mutant tau) also develop progressive paralytic phenotypes, have extensive spinal cord pathology, and have a massive glial response within the CNS that many believe could have a role in the disease process. However, upon injecting these homogenates, none of the G85R-SOD1:YFP mice developed MND nor accumulated SOD1 inclusions, indicating the specificity of SOD1 prions to induce MND transmission [6].

SOD1 strains

As mentioned previously for PrP prions, the variability observed among prion diseases in regard to clinical symptoms, incubation period, and neuropathology has been attributed to the existence of prion strains, or alternative conformations of the PrP^{Sc} protein. Immunohistological studies of mutant SOD1 pathology in transgenic mouse models provided evidence that different fALS mutants of SOD1 produced alternative conformations of misfolded SOD1 [13]. Whether such conformational differences underlie aspects of human disease remains unclear. In both sporadic ALS and SOD1-ALS, muscle loss and paralysis can begin in the arm(s), leg(s), or bulbar region, the degree of upper vs. lower motor neuron involvement can vary, the age of onset is observed anywhere from 25 to 70 years of age, and the course of disease can last from one to more than 20 years [104]. A similar phenotypic variability is also observed in patients harboring mutations within the *PRNP* prion gene. In PrP prion diseases, particular polymorphisms within *PRNP* dictate whether the individual develops Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker disease (GSS), or fatal familial insomnia (FFI) – three diseases that have distinct pathological manifestations [57, 79]. These disparate phenotypes are believed to arise due to different tertiary conformations that the mutant PrP protein adopts, which create distinct strains of prions. Similar data are being observed for tauopathies, in which it is now beginning to be understood, due in large part to the advances in cryo-EM, that distinct tauopathies like AD, PSP, and CBD are caused by distinct conformations that tau fibrils can adopt [41, 42, 47, 140]. Based on these observations for PrP and tau prions, it seems possible that mutations in SOD1 could lead to distinct strains of misfolded protein and account for some of the heterogeneity observed among fALS-SOD1 cases (Fig. 2).

A characteristic of PrP prion strains that has been implicated to impact the course of disease is PrP's rate of replication and aggregation kinetics [9]. To better understand the aggregation propensity of SOD1 and its relationship to fALS-SOD1 disease features, a number of clinically relevant SOD1 mutants were transiently transfected into human embryonic kidney (HEK293) cells [104]. It was found that the inherent aggregation propensity varied considerably among the more than 30 SOD1 mutants tested and that this property did not correlate with properties such as enzyme activity, protein thermostability, mutation position, or degree of change in protein charge the mutation imparts [104]. Interestingly, though the variability in disease duration among fALS-SOD1 patients could not be explained by the SOD1 mutant's aggregation propensity, it was observed that those mutations associated with shorter disease durations were more prone to aggregate [104]. Although the differences in the biochemical and biophysical properties of the SOD1 mutants did not correlate within many of the clinical features, these studies demonstrated inherent differences in the characteristics of SOD1 that are enciphered within the amino acid sequence or the tertiary structure it adopts.

The conformational variability among strains of PrP prions has also been demonstrated via immunohistochemistry, using a panel of antibodies that spans the entire length of the protein. This technique is referred to as "epitope mapping" and was used extensively to distinguish experimental and natural sources of scrapie and bovine spongiform encephalopathy (BSE) in sheep and goats [58, 69]. A similar approach has been used to

study the different conformational states that misfolded SOD1 can adopt. Using a panel of eight antibodies that span the length of SOD1, Bergh et al. revealed that the detergent-insoluble SOD1 aggregates from various lines of SOD1 transgenic mice differed in their immunoreactivity; WT, G85R, and G93A overexpressing mice accumulated one immunohistochemical profile (Strain A), whereas D90A overexpressing mice accumulated two profiles (Strain A and Strain B) [13]. Interestingly, these structural variants also displayed differences in molecular properties and growth kinetics, and both Strain A and Strain B were different from recombinant SOD1 fibrils generated *in vitro* under a variety of conditions. The authors also determined whether the Strain A and Strain B structural features would be inherited when passaged in G85R-overexpressing transgenic mice [17]. Spinal cord homogenates from diseased mice expressing G85R (Strain A) or D90A (Strain B) were injected into the spinal cords of G85R overexpressing mice, and following disease onset, epitope mapping was performed on their spinal cords. Supporting the notion of conformation-dependent templated propagation, the induced aggregates had the same immunohistochemical profile as the injected SOD1 prions [17]. Together, this data further demonstrates the structural heterogeneity that misfolded SOD1 can adopt and the consequences of these variations on the course of disease, strongly implicating the existence and similarity of SOD1 strains to PrP strains.

Studies performed in our lab demonstrating the transmissible properties of SOD1, discussed earlier, also provided findings that implicated the existence of SOD1 strains. When recombinant WT SOD1 fibrils or homogenates prepared from paralyzed SOD1-overexpressing mice were injected into the permissive G85R-SOD1:YFP mouse line, distinct pathologies were observed [6, 7]. Mice that succumbed to disease following injection with homogenates prepared from paralyzed G93A-SOD1 mice developed round punctate G85R-SOD1:YFP inclusions, whereas mice injected with recombinant WT SOD1 fibrils developed distinct intracellular skein-like inclusions (Fig. 1). More importantly, when spinal cord homogenates prepared from these pathologically distinct mice were passaged a second time within naïve G85R-SOD1:YFP mice, the pathologic inclusions in the recipient mice retained the characteristics of the original donor mice (Fig. 1). Similarly, we have shown that fibrilized recombinant SOD1 can induce motor neuron disease in G85-SOD1:YFP mice and that mutation of the primary sequence of recombinant SOD1 in these fibrils can influence the morphology of inclusions that develop in paralyzed G85R-SOD1:YFP mice [34]. Therefore, the most plausible explanation for these finding is that the unique SOD1 conformations within these preparations are capable of templating these conformations to naïve G85R-SOD1: YFP protein, manifesting as distinct pathologies. This phenomenon is also observed for PrP prions in which heritable phenotypes are observed upon successive passages and occurs as a result of conformation-dependent templated propagation [16, 126].

These transmission studies also revealed other prion-like characteristics of the misfolded SOD1, including shorter incubation periods upon second passage in recipient G85R-SOD1:YFP mice and increased penetrance [6]. For PrP prion strains, this phenomenon is termed “host adaptation” and is thought to occur due to the greater compatibility between the inocula “seed” and the host substrate when the sequences of both are identical [22]. Notably, in the PO injection paradigm, the second passage of inocula derived from G93A

mice produced paralysis at ~3 months of age, whereas inocula derived from G37R mice produced paralysis at ~6 months of age [6]. This difference did not appear to be due to the titer of the SOD1 seeding species, as the seeding dose for both inocula appeared similar when tested in a G85R-SOD1:YFP organotypic spinal cord slice culture assay [6]. Interestingly, fALS-SOD1 patients carrying the G37R-SOD1 mutation have one of the longest disease durations (18.7 ± 11.4 years) among all other fALS-SOD1 mutations, whereas the G93A variant has one of the shortest (2.4 ± 1.4 years). Notably, in the mouse study, we observed a modulation of onset by sequence variant, whereas in human fALS the sequence variation appears to dictate disease duration. Understanding whether different fALS variants of SOD1 produce sequence-specific strain attributes that influence the evolution of disease requires further study with a greater number of SOD1 variants associated with long and short disease durations.

General properties of TDP-43

As mentioned previously, abnormal localization of the 43 kDa transactive response (TAR) DNA-binding protein (TDP-43) is observed in ~97% of all ALS cases, making it the most prevalent pathological hallmark in ALS; however, TDP-43 pathology is not limited to ALS. It is also observed in other neurodegenerative disorders, including frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U) [94], AD [73], chronic traumatic encephalopathy (CTE) [89], and cerebral age-related TDP-43 [92]. This 414 amino-acid protein is encoded by the highly conserved *TARDBP* gene, and is ubiquitously expressed and localized primarily within the nucleus where it has multiple roles in RNA metabolism, including splicing regulation, trafficking, and degradation [25, 71]. Although mainly localized to the nucleus, the protein contains both a nuclear localization signal (NLS) and a nuclear export signal (NES). It also contains two tandem RNA-recognition motifs (RRM) and a C-terminal low complexity domain (LCD), or prion-like domain (PrLD), that is glycine-rich and appears to be of key importance to the intrinsic aggregation propensity of the protein.

Although little is still known about the aggregation pathway for TDP-43, its N-terminal region has been observed to play a regulatory role in the formation of different types of aggregates while the LCD, under certain experimental conditions, undergoes liquid-liquid phase separation that has been hypothesized to be the precursor to fibril formation [10, 32, 88, 124]. TDP-43 inclusions are hyperphosphorylated, ubiquitinated, and accumulate intracellularly in both upper and lower motor neurons and in some glial cells [94]. In those neurons containing TDP-43 inclusions, the nuclear TDP-43 is depleted, indicating that the loss of its normal function in RNA metabolism may contribute to toxicity [55, 94]. Endogenous nuclear TDP-43 is known to be tightly regulated and appears to be critical for survival. Overexpression in yeast is toxic, and overexpression in the CNS of mice and rats causes neuronal degeneration [70, 125, 139], whereas knockout of *TARDBP* in mice led to embryonic lethality [80]. However, similar to SOD1, mutations in TARDBP that have been associated with ALS are dominantly inherited, implicating a gain-of-toxic property. TARDBP mutations have been found in ~5% of fALS and ~1% sALS cases, where there is no known family history. There are now more than 50 mutations that have been identified, and they are primarily localized to the C-terminal LCD (http://alsod.iop.kcl.ac.uk/). These

findings highlight the uncertainty that still exists as to whether TDP-43 associated toxicity is caused by a gain-of-toxic function, loss-of-toxic function, a combination of the two, or an unknown mechanism.

It is becoming increasingly evident that the C-terminal portion of the protein plays a critical role in the aggregation and toxicity of TDP-43. The PrLD or LCD is found in a number of other proteins, many of which are also RNA/DNA binding proteins and implicated in the pathogenesis of various neurodegenerative diseases [64]. In yeast proteins containing a PrLD, it has been demonstrated that this region can switch from a disordered conformation to a self-templating, cross-β-sheet-rich amyloid-like conformation, and similarly, the PrLD in TDP-43 was found to be intrinsically disordered and crucial for aberrant protein aggregation *in vitro* and *in vivo* [3, 71, 84]. Furthermore, toxicity associated with TDP-43 was found to be eliminated when the PrLD was deleted; however, this was observed when the RNA-binding ability of TDP-43 was eliminated as well [3, 70, 131]. Biochemical characterization of the insoluble fractions of ALS patient tissue has also demonstrated that C-terminal fragments of TDP-43 are produced ranging in size from ~25-35 kDa and that these fragments contain a primarily β-sheet-rich conformation and are highly amyloidogenic and cytotoxic [62, 94, 141, 142].

TDP-43 aggregation and conformational templating

Given that TDP43 exhibits biochemical properties that overlap with prions, studies began to elucidate its ability to template misfolding of the normally folded TDP-43 protein. The first demonstration of this utilized recombinant WT and sarkosyl-insoluble aggregates of TDP-43, which were observed to be taken up by cultured cells and also induce the aggregation of endogenous, intracellular TDP-43 [49]. Nonaka et al. then isolated sarkosyl-insoluble TDP-43 from ALS and FTLD-TDP brains and demonstrated that when added to a neuronal cell line overexpressing TDP-43, the cells accumulated phosphorylated, ubiquitinated, and fragmented cytoplasmic inclusions of TDP-43, recapitulating the features of pathological TDP-43 inclusions in the brains of patients [97]. The induced TDP-43 inclusions were also capable of inducing subsequent pathology when re-administered back onto naïve TDP-43 overexpressing neurons. Smethurst et al. also demonstrated the ability for TDP-43 isolates to induce TDP-43 aggregation *in vitro* through the addition of brain and spinal cord extracts from ALS patients to cells overexpressing TDP-43 [122]. Although modest, they also demonstrated the spread of TDP-43 aggregates to neighboring cells. This spread was also investigated in primary neurons cultured in microfluidic devices and shown to undergo both anterograde and retrograde transport [44]. Together, this data implicates TDP-43 as an additional prion protein capable of inducing misfolding and aggregation via template-dependent polymerization.

Successful studies demonstrating the *in vivo* transmission of TDP-43 pathology is limited to one recent study by Porta et al. [102]. As discussed previously, the successful demonstration of *in vivo* transmissibility of prions is very dependent on the animal models available, and, unfortunately, due to the tight regulation of endogenous TDP-43, the majority of TDP-43 transgenic models that overexpress the protein have led to an aggressive toxic phenotype [128]. To circumvent this issue, Porta et al. utilized a doxycycline-regulatable transgenic

mouse model expressing a cytoplasmic NLS mutant of human TDP-43 that had been previously described and develops little to no TDP-43 pathology throughout its lifespan [2, 68]. The authors induced transgene expression one week prior to intracerebrally injecting the mice with TDP-43 enriched samples prepared from FTD patients and found the induction of *de novo* TDP-43 pathology at just one-month post-induction [102]. In addition, the localization of TDP-43 pathology over time indicated the spread of the infectious agent via connected neuroanatomical regions of the brain, similar to propagative spread observed for SOD1 and other prions. Interestingly, injection of these same samples into non-transgenic mice induced aggregation of endogenous mouse TDP-43, albeit at a much lower level.

TDP-43 strains

As mentioned, TDP-43 pathology is observed in the majority of ALS cases along with many other neurodegenerative disorders, including FTD. Cases of FTD that contain TDP-43 inclusions are referred to as FTLD-TDP and account for approximately half of all FTD cases. These cases can also present with different cognitive deficits such as behavioral or language impairments. Because these cases indicate the potential presence of strains, the variation in the distribution and morphology of TDP-43 species has been stratified into at least four subtypes (types A-E) [82, 86]. Type A neuropathology is primarily seen in FTLD-TDP patients that have mutations in the gene encoding progranulin (*PGRN*) and is defined by abundant dystrophic neurites and crescent or oval shaped neuronal cytoplasmic inclusions (NCIs) primarily in cortical layer 2. Type B is observed most commonly in patients with ALS and FTD and presents with mild levels of NCIs in all cortical layers and displays few short dystrophic neurites. Type C neuropathology is associated with sporadic disease and is observed most often in the semantic dementia variant of FTLD-TDP. TDP-43 pathology in type C is characterized by numerous long dystrophic neurites that are primarily found in cortical layer 2. Type D is observed in patients carrying mutations in the gene for the valosin-containing protein (VCP), which gives rise to inclusion body myopathy with early onset Paget's disease and FTD (IBMPFTD). The pathology within these patients consists of short dystrophic neurites, lentiform neuronal intranuclear inclusions, and few NCIs within all layers of the cortex. Lastly, type E pathology is associated with a rapidly progressive subtype termed behavioral variant FTD (bvFTD) that has no known genetic cause. TDP-43 pathology in these cases has a wide neuroanatomic distribution that consists of ubiquitin-negative granulofilamentous neuronal inclusions, fine grey matter grains, and oligodendroglial inclusions. In addition to the variation in clinical and pathological phenotypes, hyperphosphorylated, sarkosyl-insoluble TDP-43 fractions isolated from brain homogenates of each of these subtypes reveal distinct western blot banding patterns of the C-terminal fragments, suggesting that different conformations of TDP-43 exist in the brains of these patients [65]. Together, this data demonstrates the variability that exists among ALS and FTLD-TDP patients in respect to their clinical symptoms, TDP-43 pathology, and western blot banding patterns, yet it also reveals the correlation that is observed among five distinct subtypes. This strongly implicates the existence of TDP-43 strains as a cause for some of the variability observed.

Although only a handful of *in vitro* studies have investigated TDP-43's prion-like characteristics, one of these studies describes results that strongly support the existence of

TDP-43 strains. By adding extracts from ALS patients to HEK293 cells overexpressing WT TDP-43, Smethurst et al. demonstrated that a range of morphologically distinct TDP-43 aggregates were observed following immunostaining [122]. These aggregates displayed morphologies reminiscent of those observed in ALS patients – including skein, round, dot-like, and granular inclusions. Although the authors go on to reveal that extracts from these cells could then induce naïve cells through serial passages, they do not describe the resultant TDP-43 morphologies and whether they resemble those from the primary passage, perhaps due to the heterogeneity of the morphologies within a given sample.

Prion properties of other ALS-linked proteins

There is mounting evidence that other proteins implicated to have a role in ALS also have properties akin to prion proteins, though they have not been studied to the extent of SOD1 and TDP-43. These include FUS and the dipeptide repeat (DPR) proteins produced in response to the repeat expansion in *C90RF72*. FUS was discovered shortly after the identification of TDP-43, and mutations within the gene account for ~4% of fALS patients [81, 130]. Similar to TDP-43, FUS is an RNA binding protein that plays a critical role in transcriptional regulation, RNA metabolism, and, potentially, DNA repair [12, 48, 136]. It also has many of the same domains as TDP-43 including a NES, NLS, and a disordered PrLD; however, unlike TDP-43, the bulk of the identified mutations (predominantly frameshift deletions) have not been observed in the PrLD but rather in an arginine-glycine-glycine (RGG) rich region (http://alsod.iop.kcl.ac.uk/). In patients carrying mutants of FUS, the protein forms cytoplasmic inclusions in neurons and glial cells within the brain and spinal cord whereas the nuclear localization is either partially or completely abrogated [93, 130]. In regard to the characteristics and mechanisms of FUS aggregation and transmissibility, there have only been a couple of studies to date. Nomura et al. demonstrated the increased propensity for purified recombinant FUS containing the G156E mutation, but not WT, to spontaneously form fibrils and revealed the ability for these fibrils to induce the aggregation of the WT FUS protein [96]. In addition, they demonstrated that when co-transfected into rat hippocampal primary neurons, the G156E mutant FUS was capable of recruiting and inducing the aggregation of WT FUS [96]. Another study utilized *Drosophila* primary neuronal cultures to reveal the ability of mutant forms of FUS to transfer between neurons, revealing another prion-like feature of the protein [45]. There are many additional studies investigating the ability of FUS to form liquid-liquid phase separation and how this contributes to its gain and/or loss of function, including its role in the formation of pathological cytoplasmic inclusions [75, 95, 100]. While these findings have begun to attribute prion properties to FUS, further studies will need to be completed to better understand its characteristics and whether misfolded FUS is capable of acquiring multiple conformations.

An intronic hexanucleotide GGGGCC (G_4C_2) repeat expansion within the first intron of the *C9ORF72* gene is one of the more recently found genes to have a role in ALS and FTD and is now considered to be the most frequent genetic cause of ALS with ~40% of fALS and 5-20% of sALS patients carrying the expansion [38, 110, 111]. In addition to the toxic mechanisms that may arise due to the production of these DPRs [90, 143], there is also the theory that toxicity comes about from the loss of normal C9orf72 function [38] or a gain-of-

toxic function caused by the formation of expanded toxic RNA species [39, 53]. Though there is data that has revealed the toxicity associated with five different DPRs produced from translation of the repeat RNA (poly-GR, poly-GA, poly-GP, poly-PA, and poly-PR), there are limited studies investigating their prion-like properties. One such study demonstrated that all of these DPRs, with the exception of poly-PR, was capable of undergoing cell-to-cell transmission via exosome-dependent and exosome-independent pathways *in vitro* [138].

Conclusion

With an ever-increasing amount of data, it is now becoming obvious that the proteins responsible for many neurodegenerative diseases share numerous properties with PrP, leading some to classify them as prions (Table 1). The ALS-linked proteins discussed here are no exception. Though studies have implicated distinct conformers of these toxic proteins as the basis for disease heterogeneity, the recent advances in technologies like cryo-EM now give researchers the ability to assess the structures of protein fibrils isolated from the brains of patients at the atomic scale. These techniques, as they are now doing for tauopathies, will help to reveal the range of misfolded conformations SOD1 and TDP-43, among others, can adopt and provide insight on the complexity of these diseases. These will be important studies for therapeutic strategies for ALS as they will begin to elucidate how stratified the patient population is and whether targeted therapies to the misfolded protein will be useful among a range of protein conformers.

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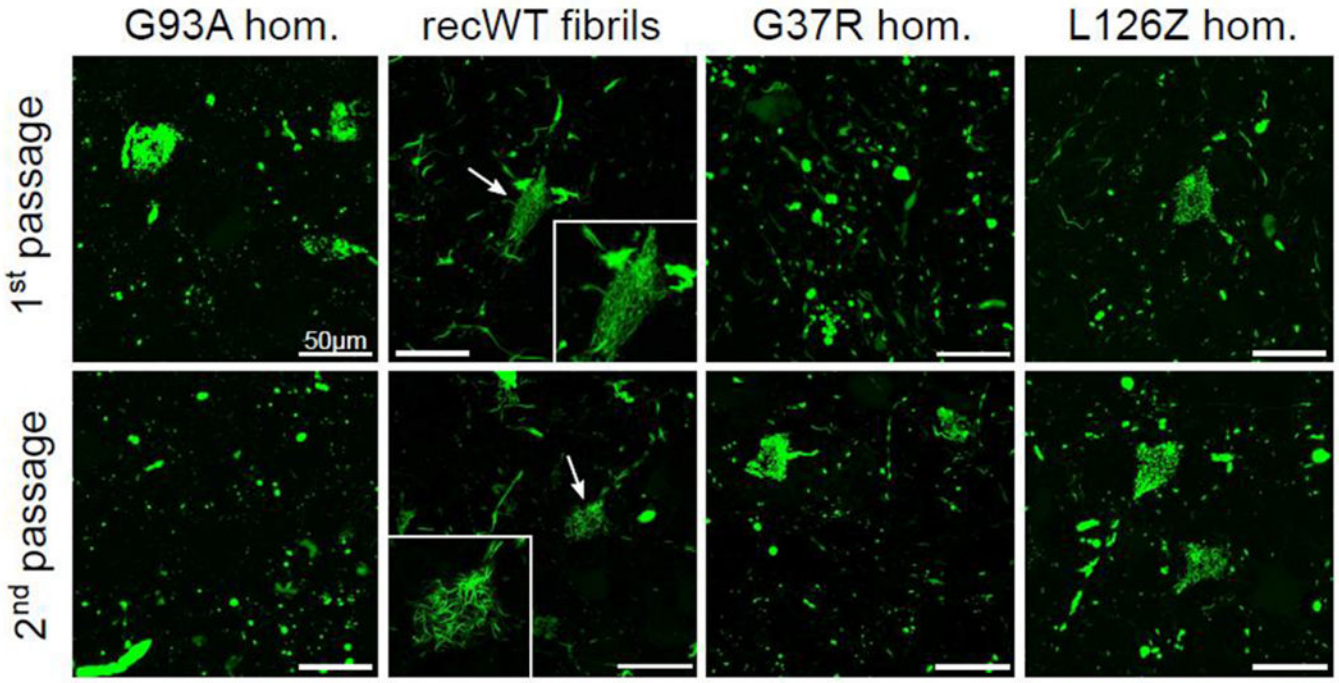

Fig. 1.

Distinct pathologies induced in G85R-SOD1:YFP inoculated mice. Images of spinal cords from diseased G85R-SOD1:YFP mice injected within their spinal cords at P0 with the first or second passages of the indicated homogenates. Significant differences were observed among those G85R-SOD1:YFP inclusions induced by homogenates from diseased G93A expressing mice versus those induced by recWT fibrils. These differences were retained among secondary passaging and is a strong indication of SOD1 conformational templating. Reprinted by permission from **Springer Nature Customer Service Centre GmbH**: [**Springer**] [**Acta Neuropathologica**] (Distinct conformers of transmissible misfolded SOD1 distinguish human SOD1-FALS from other forms of familial and sporadic ALS, Ayers, J.I., et al., 2016 [6]

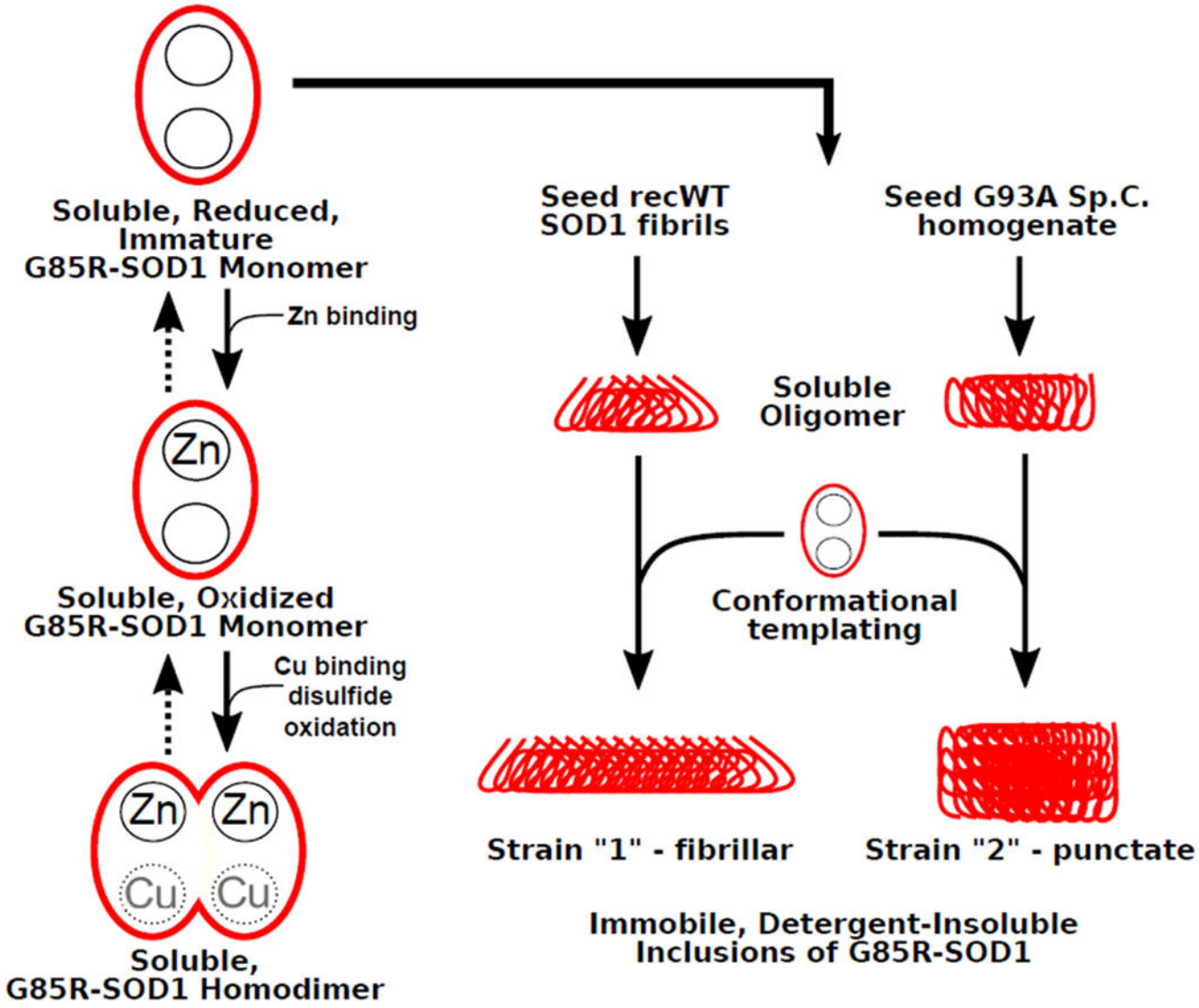

Fig. 2.

Alternative folding pathways for G85R-SOD1 fALS missense point mutant of SOD1 exposed to misfolded SOD1 seeds. Human G85R-SOD1 exhibits defects in the binding of Zn and Cu (indicated by grayed out Cu) that destabilize native structure [26]. A subset of G85R-SOD1 subunits can acquire Zn and Cu and achieve near native conformation. Immature mutant SOD1 exposed to different SOD1 seed aggregates is susceptible to conformational templating, leading to alternative conformations of misfolded protein. In the example here, seeding with fibrils of recombinant (recWT) WT SOD1 produce a strain that forms fibrillar inclusion pathology while seeding with spinal cord homogenates from paralyzed G93A mice induces punctate pathology [6]. A similar type of diagram could be drawn for G85R SOD1 seeded with tissue homogenates from paralyzed G85R-SOD1 mice (Strain 1) and spinal homogenates from paralyzed D90A-SOD1 mice (Strain 2) [17].

Table 1.

Prion-like properties of ALS-linked proteins

Protein/ Gene	Seeded Aggregation			Spread		Inducible MND in mice			Strain properties
	Cell-free	Cell culture	*In vivo*	Cell-culture	*In vivo*	Recombinant protein	Murine tissue lysate	Human tissue lysate	
SOD1	[27, 28, 50]	[59, 60, 91]	[7]	[59, 60, 91]	[8]	[6]	[7, 17]	[6, 40]	[6, 17]
TDP-43	[49, 71]	[44, 49, 97, 122]	[102]	[44, 97, 122]	[102]	n.d.[1]	n.d.	[102]	[65, 102, 122]
FUS/TLS	[96]	[96]	n.d.	[45]	n.d.	n.d.	n.d.	n.d.	n.d.
C9orf72	n.d.	n.d.	n.d.	[138]	n.d.	n.d.	n.d.	n.d.	n.d.

[1] not done