

Profilin 1 mutants form aggregates that induce accumulation of prion-like TDP-43

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ABSTRACT. Mutations in the profilin 1 (PFN1) gene have been identified as a cause of familial amyotrophic lateral sclerosis (ALS), and neuropathological studies indicate that TDP-43 is accumulated in brains of patients with PFN1 mutation. Here, we investigated the role of PFN1 mutations in the formation of prion-like abnormal TDP-43. Expression of PFN1 with pathogenic mutations resulted in the formation of cytoplasmic aggregates positive for p62 and ubiquitin, and these aggregates sequestered endogenous TDP-43. TDP-43 accumulation was facilitated in the presence of proteasome or lysosome inhibitor. Co-expression of mutant PFN1 and TDP-43 increased the levels of detergent-insoluble and phosphorylated TDP-43, and this increase required the C-terminal region of TDP-43. Moreover, detergent-insoluble fractions prepared from cells expressing ALS-linked mutant PFN1 induced seed-dependent accumulation of TDP-43. These findings indicate that expression of PFN1 mutants induces accumulation of TDP-43, and promotes conversion of normal TDP-43 into an abnormal form. These results provide new insight into the mechanisms of TDP-43 proteinopathies and other diseases associated with amyloid-like protein deposition.

KEYWORDS. ALS, degradation system, FTL, profilin 1, prion, TDP-43

Transactivation response DNA-binding protein 43 (TDP-43), encoded by the *TARDBP* gene, is a highly conserved, ubiquitously expressed heterogenous nuclear ribonucleoprotein (hnRNP) involved in exon splicing, gene transcription, regulation of mRNA stability and biosynthesis, and formation of nuclear bodies.¹⁻⁵ Structurally, TDP-43 is characterized by 2 RNA-recognition motifs

(RRM1 and RRM2), and the C-terminal region includes a glycine-rich domain and a glutamine/asparagine (Q/N)-rich domain that are implicated in interactions with other proteins.⁶⁻⁹ In pathological conditions, such as frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS), TDP-43 is accumulated mainly as ubiquitin-positive tau-negative inclusions in

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the cytoplasmic compartment of neuronal and glial cells in the brain and spinal cord of patients.^{10,11} Biochemical and histological studies have demonstrated that TDP-43 is deposited in a filamentous form and most of the filaments are abnormally phosphorylated.¹² Therefore, both loss of normal function of nuclear TDP-43 due to cytoplasmic mislocalization and gain of toxic function due to TDP-43 aggregation in the cytoplasm are thought to be involved in the pathogenesis of ALS and FTLD accompanied with TDP-43 inclusions (FTLD-TDP).¹³

The discovery of mutations in the *TARDBP* gene in familial and sporadic ALS indicated a pivotal role of TDP-43 in the pathogenesis of ALS and FTLD.¹⁴⁻¹⁶ These mutations are predominantly located in the C-terminal region, suggesting that conformational change of this region is closely related to the pathogenesis.¹⁷ Actually, the glycine-rich and Q/N-rich domains in this region contribute to amyloid-like fibril formation as well as aggregation propensity of TDP-43.¹⁸⁻²¹ The RRM2 domain also plays a key role in TDP-43 aggregation.²² On the other hand, we previously demonstrated that insoluble TDP-43 aggregates in brains of ALS and FTLD-TDP patients have prion-like properties.²⁰ When insoluble TDP-43 from ALS or FTLD-TDP brains was introduced as seeds into cells expressing TDP-43, seed-dependent TDP-43 accumulation was induced. Interestingly, the C-terminal fragment-banding patterns of converted host proteins resemble those of insoluble TDP-43 used as seeds, suggesting that the conversion is template-dependent. Moreover, mass spectrometric analysis of insoluble TDP-43 from brains of ALS patients suggested that RRM2, the glycine-rich domain, and a part of the Q/N-rich domain form the core region of TDP-43 aggregates.²³ These results suggest that seed-dependent accumulation of prion-like, conformationally changed TDP-43 via interaction at the C-terminal region has a pivotal role in the pathogenesis of ALS and FTLD-TDP. However, it is largely unknown how and when such abnormal TDP-43 is formed in cells.

Recently, mutations in the profilin 1 (PFN1) gene have been identified as a cause of familial ALS.²⁴ PFN1 is involved in various cellular

functions by binding to actin monomer, phosphoinositides and proline-rich proteins.²⁵⁻²⁷ Previous studies have found that loss-of-function of PFN1 resulting from mutations linked to familial ALS causes cytoskeletal disruption and altered stress granule dynamics.^{24,28} On the other hand, classical TDP-43 pathology was present in the brains of patients with autosomal dominant mutations in the PFN1 gene²⁹ and co-aggregation of PFN1 and TDP-43 was observed in cells expressing ALS-linked mutant PFN1.²⁴ These findings imply that gain-of-toxic function PFN1 mutations cause TDP-43 aggregation.

To elucidate how mutant PFN1 induces TDP-43 pathology, we transiently expressed wild-type (WT) or ALS-linked PFN1 mutants (C71G, M114T, E117G, or G118V) in SH-SY5Y neuroblastoma cells. As shown previously, PFN1 mutants that cause ALS (C71G, M114T, and G118V) are destabilized and are prone to aggregate in cells. These aggregates were also positive for ubiquitin and p62 (characteristic of inclusions detected in FTLD or ALS) and were predominantly localized in cytoplasm.³⁰ On the other hand, the E117G PFN1 mutant, a moderate risk factor of familial ALS, was diffusely localized in cells, like WT PFN1, suggesting that the propensity of PFN1 mutants to aggregate is implicated in the pathogenesis of ALS. In support of this hypothesis, another pathogenic mutant A20T PFN1 displays a high aggregation propensity.²⁹ Interestingly, in some cells expressing mutant PFN1, co-aggregates of PFN1 and endogenous TDP-43 were formed in the cytoplasm, the downstream functions of TDP-43 were disturbed, and TDP-43 disappeared from the nucleus. Further, immunoprecipitation analysis demonstrated that PFN1 interacts with TDP-43, but not with fused in sarcoma (FUS), a hnRNP implicated in FTLD and ALS.³¹ These findings indicate that TDP-43 is sequestered into PFN1 aggregates due to its affinity for PFN1. This mechanism might at least partly explain why nuclear protein TDP-43 forms inclusions in the cytoplasm, and why inclusions mainly composed of TDP-43 are formed. That is, TDP-43 localized in the cytoplasm (either produced or transported there) might be sequestered into

structures with affinity for TDP-43, and start forming inclusions in the cytoplasm.

Deficits in the protein degradation system, including the ubiquitin-proteasome and autophagy-lysosome systems, with aging are implicated in the pathogenesis of various neurodegenerative diseases.³² In the case of PFN1 mutations, inhibition of proteasome by MG132 increased PFN1 and TDP-43 aggregation in cells transfected with mutant PFN1, primarily because the total amount of mutant PFN1 and TDP-43 was increased. On the other hand, PFN1 aggregates suppressed the formation of aggresome-like structures when proteasome was inhibited. Although it is not clearly known to what extent this suppression is involved in the increased TDP-43 aggregation, formation of aggresomes is recognized as a cytoprotective response serving to sequester potentially toxic misfolded proteins and facilitate their clearance by autophagy.³³ Therefore, increased misfolded TDP-43 and decreased function of ubiquitin-proteasome systems might coordinately increase TDP-43 aggregation in cells harboring PFN1 aggregates in the presence of a proteasome inhibitor. PFN1 aggregates also suppressed the conversion of LC3-I to LC3-II, an essential event for autophagy, possibly because of sequestration of LC3 into PFN1 aggregates. It is not clear whether LC3 is directly sequestered into PFN1 aggregates or whether the process is mediated by the binding of other proteins such as p62 sequestered into PFN1 aggregates. Importantly, TDP-43 aggregates increased in the presence of the PFN1 aggregates when lysosomal function was inhibited by bafilomycin A1, a specific inhibitor of vacuolar ATPase. Bafilomycin A1 did not markedly increase PFN1 aggregates, different from MG132. Therefore, this finding may imply that prion-like conformationally changed TDP-43 is formed in the presence of PFN1 aggregates, and seed-dependent TDP-43 accumulation is facilitated by decreased lysosomal degradation of TDP-43 fibrils that function as a template for seed-dependent accumulation of TDP-43. These effects of PFN1 aggregates on the degradation system, and the increased endogenous TDP-43 accumulation when the degradation system is inhibited, might partly explain why ALS pathogenesis develops with aging. Further studies are needed in order to elucidate in detail the

mechanism through which PFN1 aggregates suppress the degradation system.

The process of seed-dependent TDP-43 accumulation progresses through the conversion of normal TDP-43 into conformationally changed (abnormal) TDP-43 that serves as a template of TDP-43 fibrils, leading to TDP-43 filament formation.^{20,34} To investigate the effect of PFN1 aggregates on seed-dependent TDP-43 accumulation, TDP-43 monomer was supplied by means of overexpression. Insoluble TDP-43 accumulation markedly increased in cells co-transfected with mutant PFN1 and TDP-43, indicating that seed-dependent TDP-43 accumulation progressed in the presence of PFN1 aggregates. Moreover, insoluble phosphorylated TDP-43, a hallmark of pathological TDP-43, was detected dependently on the amount of insoluble TDP-43 accumulation. Thus, TDP-43 might be phosphorylated after seed-dependent TDP-43 accumulation. We propose that TDP-43 sequestered into PFN1 aggregates in the cytoplasm has a prion-like property and induces seed-dependent TDP-43 accumulation. To test the idea that TDP-43 is sequestered into PFN1 aggregates in the cytoplasm, we co-expressed mutant PFN1 and TDP-43 lacking the nuclear localization signal (Δ NLS) in cells. This resulted in higher accumulation of insoluble TDP-43 and phosphorylated TDP-43 than did co-expression of mutant PFN1 and TDP-43. The results of co-expression of C71G PFN1 with GFP-tagged full-length, N-terminal, or C-terminal TDP-43 also revealed that increased TDP-43 aggregation in the presence of PFN1 aggregates requires the C-terminal region of TDP-43, which includes the prion-like domain.^{18,21} Furthermore, seed-dependent TDP-43 accumulation and insoluble TDP-43 phosphorylation were increased in cells after introduction of detergent-insoluble fraction from cells transfected with ALS-linked mutant PFN1, indicating that conformationally changed, prion-like TDP-43 was formed in the presence of mutant PFN1. Interestingly, detergent-insoluble fraction from cells expressing soluble PFN1 E117G mutant also induced seed-dependent TDP-43 accumulation. This finding indicates that conformational change of TDP-43 induced by mutant PFN1 does not necessarily require PFN1 aggregation. Possibly, TDP-43 might not be easy to aggregate under

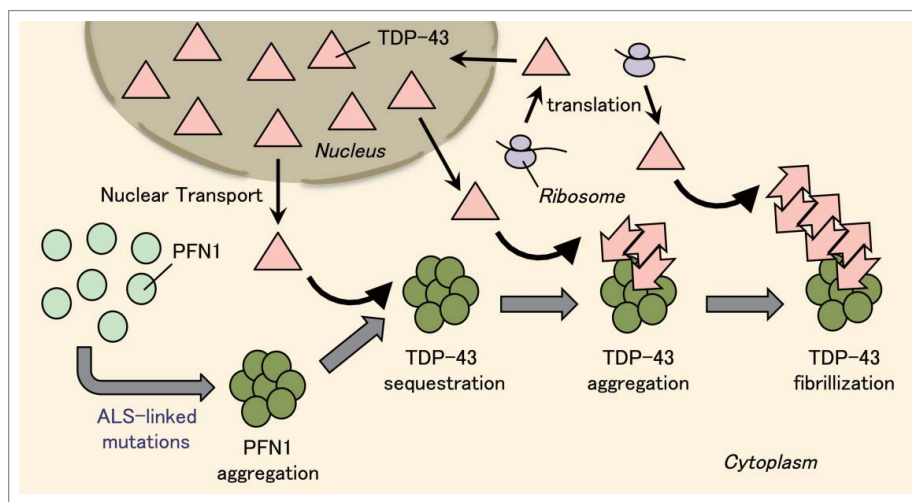
physiological conditions due to the presence of multiple interacting partners.³⁵ Although the precise mechanism remains unknown, PFN1 mutations linked to ALS might increase the affinity of PFN1 for TDP-43, and this in turn might disrupt interactions with other partners and induce conformational change of TDP-43. We propose that structures, such as PFN1 aggregates, that sequester TDP-43 might be a trigger to induce TDP-43 proteinopathy (Fig. 1).

Interestingly, as seed-dependent TDP-43 accumulation increased over time, endogenous PFN1 was increased in the detergent-insoluble fraction, indicating that TDP-43 aggregates also sequestered endogenous PFN1. In support of this idea, PFN1 is accumulated in inclusions positive for phosphorylated TDP-43 in the brain of sporadic ALS or progranulin-deficient FTLD patients. Therefore, endogenous PFN1 would be locally lost in cells harboring TDP-43 aggregates. It has been reported that phosphorylated TDP-43 aggregates sequester RNA polymerase II, and induce cell death via a non-apoptotic mechanism in SH-SY5Y cells and FTLD-TDP patients.³⁶ Considering that PFN1 is

involved in various cellular functions, localized loss of endogenous PFN1 might also mediate the toxicity of TDP-43 inclusions. Further, it is possible that WT PFN1 aggregates sequester TDP-43. In the brain of ALS or FTLD patients without PFN1 mutations, PFN1 accumulation might be simply a result of sequestration into TDP-43 aggregates. On the other hand, PFN1 accumulation might precede TDP-43 accumulation. Post-translational modifications of PFN1, such as phosphorylation or acetylation, which accumulate with aging, might induce conformational change of PFN1 and lead to its aggregation.³⁷⁻³⁹ Further studies are needed to examine whether accumulated PFN1 in ALS or FTLD patients is post-translationally modified or whether post-translational modifications of PFN1 induce conformational change of PFN1. We propose that co-aggregation of PFN1 and TDP-43 is a potential therapeutic target for TDP-43 proteinopathy.

In summary, PFN1 mutations associated with ALS induce formation of PFN1 aggregates, which sequester TDP-43 and form co-aggregates of PFN1 and TDP-43 that are positive for ubiquitin

FIGURE 1. Conversion of normal TDP-43 into prion-like species by ALS-linked PFN1 mutants. PFN1 with mutations that cause ALS forms aggregates in the cytoplasm. TDP-43 localized in the cytoplasm for its production or transportation from nucleus is sequestered into PFN1 aggregates, where it is converted into prion-like species, and forms TDP-43 aggregates. Furthermore, these TDP-43 aggregates serve as a template for conversion of normal TDP-43 into prion-like TDP-43 aggregates. As a result, TDP-43 filaments are formed. Thus, TDP-43 aggregation is facilitated firstly by sequestration of TDP-43 into PFN1 aggregates and secondly by self-template-type aggregation of prion-like TDP-43.



and p62, which is characteristic of inclusions observed in ALS and FTLT-DTP brains. Furthermore, TDP-43 sequestered into PFN1 aggregates functions as a template for seed-dependent TDP-43 accumulation, suggesting that PFN1 aggregates provide a scaffold for conformational change of TDP-43. Our findings provide a clue as to how prion-like TDP-43 may be generated, and highlight the importance of the formation of conformationally changed, prion-like TDP-43 in the pathogenesis of ALS and FTLT-DTP.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors report no potential conflicts of interest.

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