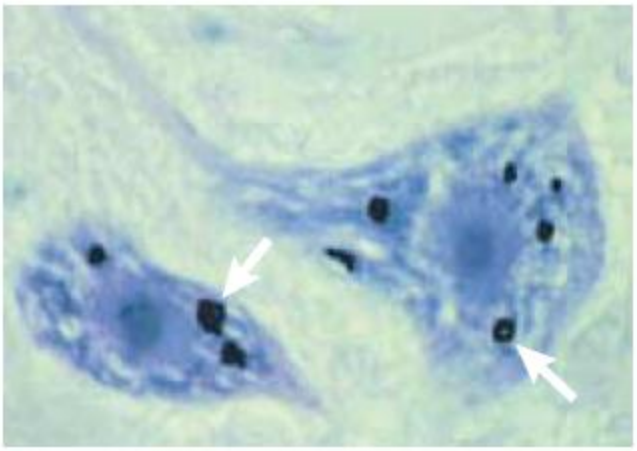
# Aggregates in Amyotrophic Lateral Sclerosis: hallmark or cause?



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**Abstract**

Amyotrophic lateral sclerosis (ALS) is a late-onset neurodegenerative disorder characterized by the loss of upper and lower motor neurons. A hallmark all ALS patients show are aggregates in these neurons. It is still unknown whether these aggregates are just hallmarks or contribute to disease progression. Mutations discovered to be a cause of ALS are among others mutations in SOD1, TDP-43 and FUS/TLS. This paper tries to establish whether aggregates caused by these mutations are an underlying cause of ALS or just a hallmark and concludes that aggregates can be considered as causative to ALS. Aggregates are toxic to cells by impairing essential cellular functions. Aggregates caused by mutant SOD1 are found in mitochondria and presumably impair mitochondrial function. Aggregates caused by mutant TDP-43 and FUS/TLS need their RNA-binding domain to be toxic and most likely disrupt RNA translation and metabolism.

**Contents**



Figure frontpage:
Soto C. (2003) Unfolding the role of protein misfolding in neurodegenerative diseases. *Nature reviews Neuroscience* 4(1): 49-60

## Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder. It is characterized by the selective loss of upper and lower motor neurons. Upper motor neurons connect the motor cortex with the spinal cord. Lower motor neurons project from the brain stem or spinal cord to the muscles. Loss of these motor neurons leads to progressive paralysis, muscle atrophy and severe disability. The onset of ALS is on average between 50 and 60 years of age and is fatal between 2-5 years after disease onset as a consequence of respiratory failure. Despite numerous attempts to understand the etiology of ALS, the underlying cause is not clear. There is currently no cure. (Bendotti et al. 2011)

In about 90% of ALS patients the etiology is unclear, this is called sporadic ALS (sALS). However, the other cases are familiar (fALS). In these cases a mutation results in disease, most of the times inherited in an autosomal dominant manner (Lanson and Pandey 2012). fALS and sALS are clinically indistinguishable. Since the cause of ALS is understood in fALS, this is the best research target to provide insight in the underlying cause and eventually develop therapy.

Mutations in the SOD1 gene were the first to be identified as a cause of ALS (Rosen et al. 1993). This mutation is now known to be a cause of fALS in about 20% of the cases. Since then, more and more mutations were discovered to be a cause of fALS of which SOD1, FUS/TLS, TDP-43 and C9ORF72 are the most prevalent. (Table 1)

Most of these genes have a single mutation and are then responsible for disease onset. Recently, a GGGGCC repeat expansion in the first intron of the C9ORF72 gene was identified to be a cause of fALS in 23,5% of an American population (Dejesus-Hernandez et al. 2011) and 46,0% of a Finnish population (Renton et al. 2011). In controls the GGGGCC hexanucleotide expansion was found to repeat 2-23 times, but in ALS patients it was

**Table 1** Mutations found to be a cause of fALS

| Prevalance in fALS | Gene | Function protein | References |
|---|---|---|---|
| ~20% | SOD1 | Antioxidant | Rosen et al. (1993) |
| Rare | ALS2/Alsin | Guanine nucleotide exchange factor | Hadano et al. (2001) |
| Rare | DCTN1 | Binding to both microtubules and dynein | Puls et al. (2003) |
| Rare | SETX | DNA/RNA helicase | Chen et al. (2004) |
| Rare | VAPB | Association with both microtubules and intracellular vesicles | Nishimura et al. (2004) |
| Rare | ANG | Ribonuclease | Greenway et al. (2006) |
| ~5% | TDP-43 | DNA/RNA binding, formation of RNA-granule | Kabashi et al. (2008) |
| ~5% | FUS/TLS | DNA/RNA binding, formation of RNA-granule | Kwiatkowski et al. (2009) |
| Rare | ELP3 | RNA-polymerase II component | Simpson et al. (2009) |
| Rare | VCP | ATP-binding, involved in vesicle transport | Johnson et al. (2010) |
| Rare | OPTN | Vesicle trafficking | Maruyama et al. (2010) |
| Rare | SPG11 | Unknown | Orlacchio et al. (2010) |
| Rare | SIGMAR-1 | ER chaperone, involved in lipid transport | Al-Saif et al. (2011) |
| ~33% | C9ORF72 | Unknown | Dejesus-Hernandez et al. (2011) Renton et al. (2011) |
| Rare | UBQLN2 | Association with both proteasome and ubiquitin ligase | Deng et al. (2011) |

discovered to repeat 177-1600 times. Since this mutation has recently been discovered, it is poorly investigated. Therefore, the mutations reviewed in this paper will be the other three most prevalent: SOD1, TDP-43 and FUS/TLS.

The mutations found to be a cause of fALS can roughly be divided in six groups based on the function of the protein they encode. 1. RNA-binding and processing: SETX, ANG, TDP-43, FUS/TLS, ELP3. 2. Transport: DCTN1, VAPB, VCP, OPTN, SIGMAR-1. 3. Degradation: UBQLN2. 4. Antioxidant: SOD1. 5. Guanine nucleotide exchange factor: ALS2/Alsin. 6. Unknown function: SPG11, C9ORF72. It could be that the proteins with comparable functions induce ALS by common pathways. This needs to be investigated.

In ALS several cellular and molecular processes are impaired. First of all mitochondrial dysfunction and abnormal mitochondrial morphology is seen in mutated SOD1 and TDP43 mouse models (Sotelo-Silveira et al. 2009; Wegorzewska and Baloh 2011). In mutant FUS/TLS motor neuron cellular model mitochondria appear to be significantly shorter (Tradewell et al. 2012). Mitochondria are, among other functions, the energy suppliers of cells and involved in cell death. Second, autophagy seems to be dysregulated. Autophagy is responsible for degradation of organelles and malformed proteins (Chen et al. 2012). Third, dysfunction of the ubiquitin-proteasome is observed. This system is responsible for clearance of misfolded and abnormal proteins (Bendotti et al. 2011). Further, axonal transport defects are observed. Axonal transport is extremely important for motor neurons which can have axons of more than one meter. Axonal transport supplies the axon with newly synthesized material and transfers old or damaged material back to the cell body (Ikenaka et al. 2012). Glutamate exicitotoxicity also seems to play a role in ALS. Riluzole, the only approved drug to slow the progression of ALS, is a presynaptic glutamate inhibitor. However, its exact mechanism in the slowing of ALS progression is unknown (Gordon 2011).

The most prominent clinical hallmark all ALS patients show are aggregates (Watanabe et al. 2001). These accumulations of insoluble proteins are found in post mortem studies. This is usually the clinical end stage of ALS. Whether these aggregates are just a hallmark or really contribute to disease onset and progression is currently still debated. Therefore the purpose of this paper is to search the literature for evidence whether aggregates caused by mutated SOD1, TDP-43 or FUS/TLS are just a hallmark or may cause disease onset. To answer this question the following issues are addressed: First, why are mSOD1, mTDP43, mFUS/TLS aggregation prone? Second, what is the evidence for toxicity of aggregates? And third, how do mSOD1, mTDP43, mFUS/TLS contribute to disease?

**SOD1**

Cu/Zn binding superoxide dismutase, also known as SOD1, is a human homodimeric metalloenzyme that catalyzes the dismutation of $O_2^-$ to $O_2$ and $H_2O_2$ (Rosen et al. 1993). $O_2^-$ is a naturally occurring, but harmful free radical. The encoded protein occurs as two isoenzymes, one is located in the cytoplasm and the other one is located in the inner membrane space of mitochondria. SOD1 is predominantly a cytoplasmic protein (Kawamata et al. 2008).

SOD1 mutations associated with ALS are gain of function mutations. SOD1 knockout mice do not develop motor neuron degeneration associated with ALS (Reaume et al. 1996). This rules out the possibility that SOD1 mutations are causing disease only by a loss of function mutation. Moreover, it rules out the possibility of gain of function by a dominant negative effect. Furthermore, animal models expressing mutant SOD1 show ALS-like phenotypes (Deng et al. 2006).

*Why are SOD1 mutations associated with ALS aggregation prone?*

Currently it is not clear what the exact aggregation mechanism of mutant SOD1 is. There are some reports addressing this question. Deng et al. propose a mechanism which links oxidative stress with increased aggregation of mutated SOD1. They suggest that mutations in SOD1 destabilize SOD1 in vivo. This prevents formation of the intramolecular disulfide bond between cysteine-57 and cysteine-146 and leads to increased amounts of reduced SOD1. The reduced monomers form insoluble dimers and multimers by oxidation-mediated intermolecular disulfide bonding via oxidation of cysteine residues. Eventually, these dimers and multimers aggregate (Deng et al. 2006). This hypothesis seems to be highly unlikely, since mutated SOD1, lacking its cysteine residues, still has high potential to aggregate (Karch et al. 2008). So disulfide cross-linking is not required for aggregation. Instead Karch et al. identify two regions that seem to promote aggregation. These regions are residues 42-50, component of the $4^{th}$ β-strand, and residues 109-123, component of the $7^{th}$ β-strand. They suggest that mutated SOD1 destabilizes the structure which leads to increased protein-protein interaction between these regions. This eventually leads to aggregation. SOD1 with mutations within these regions seem to show lower aggregation properties (Karch et al. 2010). This confirms their hypothesis that these regions are critical for aggregation. Furukawa et al. show that each mutant forms aggregates with different structures. They identify three core regions of aggregates: residues 1-30, residues 90-120 (these residues are partially overlapping with a region identified by Karch et al.) and residues 135-153. These residues are protease resistant. They take into account that aggregates formed by different mutations are forming structurally different aggregates and show that the core structure of SOD1 aggregates is dependent on the kind of mutation. They propose that mutations induce non-native folding and promote interactions between the protease resistant regions, thereby forming an aggregate core (Furukawa et al. 2010). Ding et al. hypothesize that disease associated mutations cause destabilization of SOD1, which leads to local unfolding. This partially unfolded protein exposes hydrophobic regions which are prone to aggregate (Ding et al. 2011).

In my opinion the hypothesis of Furukawa et al. is most likely. They determine three essential regions for aggregation and they explain why these specific regions are aggregation prone. These regions are in fact protease resistant and when they interact with each other due to a mutation, they seem to form a good seed of aggregation. More research should be done to determine whether this is the underlying mechanism of SOD1 aggregation.

*What is the evidence for toxicity of aggregates caused by mutated SOD1?*

Numerous studies address this question since it is not clear whether the aggregates found in ALS are an underlying cause of the disease and whether they have a harmful or protective effect. No correlation is found between mutated SOD1 aggregates and cell death in a nerve growth factor differentiated PC12 cellular model of ALS (Lee et al. 2002). It is questionable whether PC12 cells are a good model to study cell death in ALS, since in ALS a significant loss of motor neurons is seen. Motor neurons have a peculiar morphology, consequently anything not harmful to PC12 cells could be disastrous to motor neurons with their extremely long axons.

Mutant SOD1, but not wild-type SOD1 is found to co-localize with a dynein subunit, DIC, in protein aggregates in the spinal cord of transgenic mice. Dynein is a protein involved in retrograde axonal transport. This transport mechanism is important for cell function, especially for motor neurons, since these neurons have exceptionally long axons. This co-localization of mutated SOD1 and dynein is found in the pre-symptomatic mice and increases over time with disease progression (Zhang et al. 2007). This proves that aggregates are not only found in the clinical end stage of ALS, but also pre-symptomatic. Furthermore, it suggests that the aggregates are toxic by binding dynein and preventing it from performing its function. Since mutations in dynactin, a dynein binding protein, are found to be a cause of fALS (Puls et al. 2003), impaired axonal transport is thought to be a cause of motor neuron degeneration.

Mutant SOD1 is found to aggregate in mitochondria. These mitochondria show swollen and severely damaged cristae. Mitochondria without aggregates show normal phenotype (Figure 1). Aggregates associate with the outer and inner membranes (Deng et al. 2006) and are located near the cytoplasmic surface of spinal cord mitochondria (Liu et al. 2004) while normally functioning SOD1 is only found in the inner membrane space of mitochondria (Kawamata et al. 2008). Besides, mutant SOD1 is causing severe damage to mitochondria. In mutant SOD1 transgenic mice the mitochondrial respiration, electron transfer chain and ATP synthesis are severely impaired (Mattiazzi et al. 2002). Furthermore, mutant SOD1 is found to bind directly to voltage dependent anion channels of the outer membranes of mitochondria leading to a decreased ADP flux in rats (Israelson et al. 2010). So there are numerous examples of harmful effects of mutant SOD1 on mitochondria. Moreover, accumulation of mutant SOD1 with mitochondria was selective for affected tissue. Interestingly, mutant SOD1 association with mitochondria is not an intrinsic property of motor neurons but is acquired with age. At 5 weeks of age mutant SOD1 did not associate with mitochondria in mice. At 6 months of age the pre-symptomatic mice show aggregates associated with mitochondria. The amount increases 2-fold after disease onset (Liu et al. 2004). This shows that aggregates are not just a hallmark of the clinical end stage, but are formed before disease onset. Besides, the absence of mutant SOD1 association with mitochondria at young age is consistent with the late onset of ALS. The localization of mutant SOD1 in

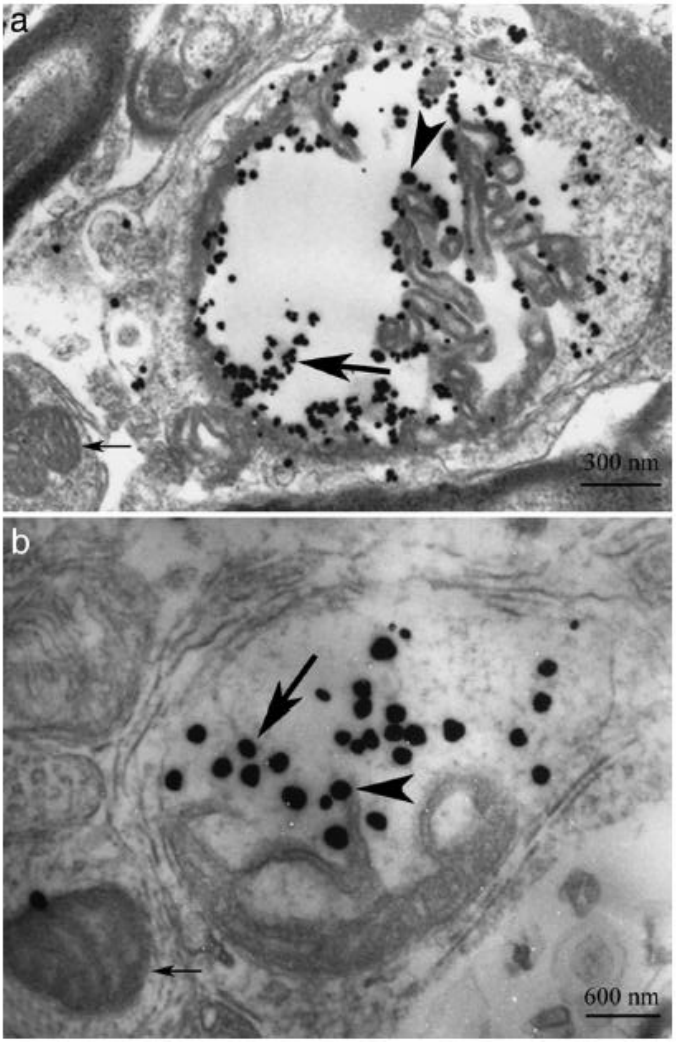


**Figure 1** ImmunoGold electron microscopy of the spinal cord sections of mutant SOD1 mice with a SOD1 antibody. The morphology of cristae was altered in aggregate dense areas. Some aggregates were closely associated with swollen cristae (arrowheads). Some cristae were severely damaged (big arrows). This damage was not observed in relatively normal mitochondria with fewer aggregates in the same mice (small arrows). (Deng et al. 2006)

mitochondria presumably has a direct role in mitochondrial damage since mutant SOD1 specifically targeted for the inter membrane space causes neuronal toxicity in motor neurons (Magrané et al. 2009). It is questionable whether this model is suitable to investigate the role of mutant SOD1 in mitochondria. SOD1 is predominantly localized in the cytoplasm and when it is specifically targeted for the mitochondria it will be present in much higher concentrations than normal. This abnormal concentration could be harmful to mitochondria rather than the mutation. Aconitase, a mitochondrial protein responsible for the transition of citrate to isocitrate in the citric acid cycle, is found to aggregate in a SOD1 mouse model. It is found in co-localization with SOD1. The accumulation was present before the onset of disease (Basso et al. 2009). This is another link between aggregate formation and mitochondria.

There is some evidence that clearance of aggregates reduces cell death. Overexpression of glutaredoxin 1 increases the solubility of mutant SOD1 in the cytosol but it does not prevent apoptosis or mitochondrial damage in neuronal cells and immortalized motor neurons. However, overexpression of glutaredoxin 2 increases the solubility of mutant SOD1 in mitochondria and inhibits apoptosis in neuronal cells and motor neurons (Ferri et al. 2010). This is a strong indication that the toxicity of SOD1 arises primarily from aggregates in the mitochondria. Furthermore, Hsp70 and Hsp40 are found to co-localize with intracytoplasmic aggregates formed by mutant SOD1 in Neuro2A cells. Overexpression of these two heat shock proteins reduced aggregate formation, improved neurite outgrowth and prevented cell death (Takeuchi et al. 2002).

All this taken into account, there is convincing evidence that the aggregation of mutant SOD1 is toxic. Aggregation prevents other proteins or structures from performing their function, this is seen with dynein and aconitase. Moreover, the aggregation of mutant SOD1 seems to be extremely harmful to mitochondria.

*How does mutant SOD1 contribute to disease?*

I think that mutant SOD1 could affect several cellular processes leading to motor neuron degeneration and ALS. Most importantly, there are numerous examples of detrimental effects of mutant SOD1 on mitochondria. In my opinion mitochondrial dysfunction is the most important process caused by mutated SOD1. Motor neurons are very active and are extremely dependent on their high energy production. Mutant SOD1 is found to aggregate in mitochondria, alters its morphology and prevents it from performing its function. This could lead to energy deficiency and ultimately to cell death. Mutant SOD1 is also found to co-aggregate with dynein and thereby impairing axonal transport. This could be detrimental for motor neurons and their long axons. Still, mitochondrial dysfunction seems to be the primary mechanism by which mutant SOD1 promotes cell death.

**TDP-43**

TAR DNA/RNA Binding Protein, also known as TDP-43, was originally identified as a modulator of HIV-1 gene expression (Ou et al. 1995). It is now known to be involved in both DNA and RNA binding (Strong et al. 2007). TDP-43 is normally a nuclear protein (Johnson et al. 2008). RNA binding proteins are involved in local RNA translation and protein synthesis. They regulate mRNA distribution by forming RNA-protein complexes (RNA granules) in which translation is repressed. Whenever needed, RNA granules are transported to the synapse where translation takes place. TDP-43 is associated with stress granules, a type of RNA granule. Stress granules function in stress conditions to promote translation of stress response proteins (Liu-Yesucevitz et al. 2011).

Most of the mutations discovered in TDP-43 are dominant missense mutations in the glycine rich C-terminal (Kabashi et al. 2008; Sreedharan et al. 2008; Pesiridis et al. 2009). Mutations in TDP-43 seem to be both a loss

and gain of function mutation. Knockdown of TDP-43 in zebrafish embryos causes developmental defects and a loss of function motor phenotype, which indicates a loss of function mutation. Human wild-type TDP-43 is able to partially rescue this phenotype. Interestingly, mutant TDP-43 fails to rescue this phenotype. Overexpression of mutated TDP-43 causes a motor neuron phenotype of shorter motor neuronal axons and premature branching. Furthermore, it causes swimming deficits.which indicates a gain of function mutation (Kabashi et al. 2009).

*Why are TDP-43 mutations associated with ALS aggregation prone?*

TDP-43 is intrinsically aggregation prone. In the absence of other components it spontaneously forms aggregates, which closely resemble the morphology of TDP-43 aggregates found in degenerating motor neurons of ALS patients (Johnson et al. 2009). The C-terminal (residues 277-414) of wild-type TDP-43 is required for spontaneous aggregation (Figure 2). Fragments without the C-terminals show exclusive nuclear localization, are not toxic and show no cytoplasmic aggregates (Johnson et al. 2008). Interestingly, this C-terminal was recently discovered to be prion-like (Cushman et al 2010). Moreover, most of the mutations discovered appear to be in the C-terminal (Pesidiris et al. 2009). Six ALS-linked mutations within this domain were tested and found to increase the number of TDP-43 aggregates and promote toxicity in yeast. Furthermore, the minimal fragment necessary for both aggregation and toxicity was determined to be the 25 kDa fragment, which includes the RRM2 (RNA-Recognition Motive 2) and the C-terminal domain (residues 188-414) (Johnson et al. 2009). This indicates that mutated TDP-43 is dependent on its ability to bind RNA to be toxic.

It is known that mutant TDP-43 forms fragments more rapidly than wild-type TDP-43 in vitro and in Chinese hamster ovary cells (Sreedharan et al. 2008). To test whether these aberrant cleavage products promote toxicity, HEK293 cells were made to express 35 kDa and 25 kDa fragments (Figure 2). These fragments correspond to the products formed by proteolytic cleavage by caspases under apoptosis in TDP-43 expressing cells (Nishimoto et al. 2010). These fragments miss the N-terminal, which contains the nuclear localization signal. They cause cellular toxicity and form cytoplasmic inclusions (Zhang et al. 2009). The 25 kDa fragment corresponds to the C-terminal and RRM2. The 35 kDa fragment corresponds to the C-terminal, RRM1 and RRM2. This is a strong indication that the fragments are toxic. It remains unknown how much mutant TDP-43 is abnormally cleaved and whether only the fragments or also full-length TDP-43 contribute to toxicity. Therefore, it is questionable whether this HEK293 model overexpressing only TDP-43 fragments is a good representation of ALS caused by mutated TDP-43.

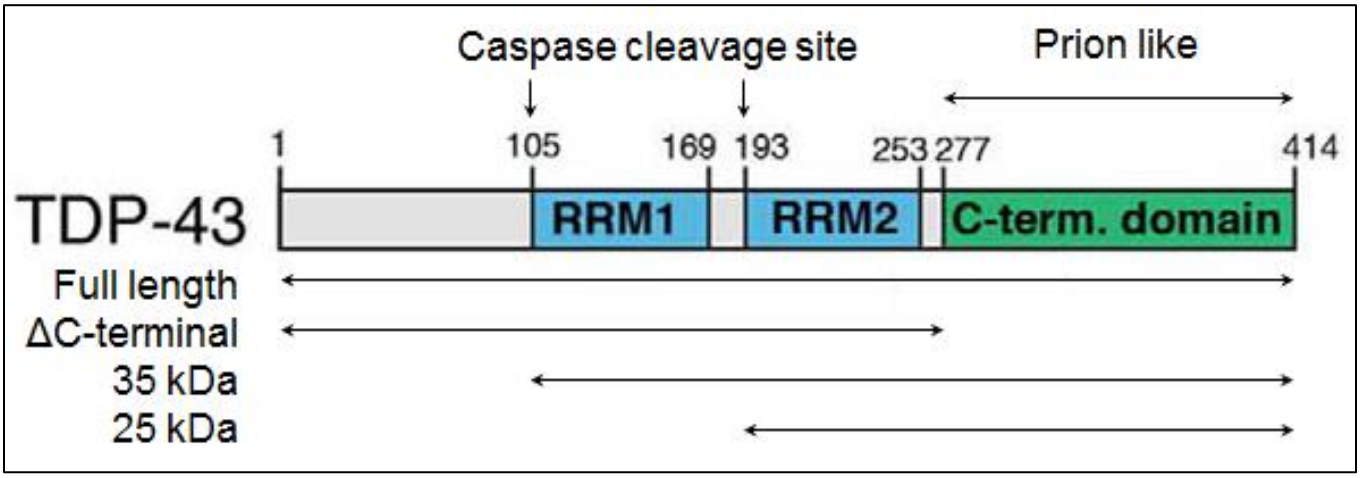


**Figure 2** Schematic of domain architecture of TDP-43 (Sun et al. 2011)

In my opinion ALS-linked mutations cause rapid fragmentation and since these fragments miss the nuclear localization signal, they mislocate to the cytoplasm. In the cytoplasm the TDP-43 fragments aggregate. This is highly likely since the aggregation properties of TDP-43 are dependent on the C-terminal and RRM2, corresponding with the 25 kDa fragment. Further research should be done to determine the amount of abnormally cleaved TDP-43 and how much impact these fragments have in causing ALS.

*What is the evidence for toxicity of aggregates caused by mutated TDP-43?*

TDP43 accumulation is toxic in yeast (Johnson et al. 2008). But whether this is a cause of ALS or a consequence of another process remains unknown in this study. The toxicity of TDP-25 and TDP-35, is not dependent on the loss of the nuclear localization signal. This is proved by comparing a TDP-43 fragment missing the N-terminal as well as RRM1 and a RNA-binding deficient TDP-43 fragment. These two different mutations of TDP-43 do not enhance motor neuron loss compared to control in a chick model and a Drosophila model. These data suggest RNA-binding activity to be necessary for neurotoxicity (Voigt et al. 2010). In neuroblastoma cells 50% of the 25 kDa TDP-43 inclusion positive cells were apoptotic. These inclusions were also positive for caspase-3. This pattern was not seen in TDP-43 wild-type transfected cells (Zhang et al. 2009). This provides evidence for the toxicity of aggregates. There is a proposed mechanism which gives an explanation why the aggregates are toxic. The study identifies a kind of mRNA that binds to TDP-43. When the levels of TDP-43 are decreased, the levels of Atg7 mRNA, encoding an autophagy component, are lower. TDP-43 could bind Atg7 through RRM1. Loss of TDP-43 leads to loss of Atg7 mRNA and ATG7 protein. This causes impairment of autophagy and facilitates accumulation of polyubiquitinated proteins (Bose et al. 2011). So it could be that TDP-43 aggregates with Atg7 mRNA resulting in the loss of ATG7 protein, which leads to autophagy dysfunction. In this scenario the aggregates are the cause of loss of other cellular functions.

Very convincing evidence for mutated TDP-43 to be toxic came from a recent study. In this study, a transgenic rat was created in which the expression of ALS-associated mutant TDP-43 was restricted to motor neurons and could be switched on and off. These rats showed ALS-like paralysis when the transgene was switched on. Progression of ALS was stopped after transgene expression was switched off on time. Accumulated ubiquitin in motor neurons was detectable only in rats with active disease. The rats in which the disease was halted showed no accumulated ubiquitin (Huang et al. 2012). TDP-43 is a major component of polyubiquitinated inclusions of pathological cellular samples of ALS (Neuman et al. 2006). This suggests that accumulated ubiquitin may be cleared after disease progression is halted. This provides strong evidence for ubiquitin positive aggregates to be at least an accompanying event of motor neuron degeneration and gives strong indication that aggregates are a cause of motor neuron degeneration.

In a Drosophila model, inhibition of aggregation of TDP-43 by pharmacological or genetic upregulation of small heat shock protein CG14207 results in partial reduction of its neurotoxic effect on motor neurons. Inhibition of aggregation of the disease associated 25 kDa fragment results in complete suppression of its toxicity and also clearance from the brain (Gregory et al. 2012). These results suggest that both TDP-43 and TDP-25 aggregates are toxic. The more promising results with TDP-25 could indicate a preference of CG14207 for TDP-25 or suggest that TDP-25, although more aggregation prone, is less toxic than full length TDP-43. This study provides strong evidence that aggregates are toxic to motor neurons.

*How does mutant TDP-43 contribute to disease?*

In my opinion mutant TDP-43 contributes to ALS, because of the RNA-binding capacity of this protein. The toxicity of TDP-43 aggregates is dependent on their RNA-binding properties. Mutant TDP-43 is found to form fragments rapidly, these fragments miss the nuclear localization signal and mislocate to the cytoplasm. TDP-43 is thus prevented to perform its function in the nucleus. In the cytoplasm TDP-43 and truncated TDP-43 aggregate and it seems highly likely that they aggregate with mRNA, thereby preventing RNA to travel down to axons and be regionally translated. This abnormal RNA processing is in my opinion a significant contributor to ALS progression.

**FUS/TLS**

Fused in sarcoma or translocated in sarcoma, from this point referred to as FUS, is originally identified as a fusion protein caused by translocation of the CHOP dominant negative transcription factor gene with FUS in malignant myxoid liposarcoma (Rabbitts et al. 1993). It is now known to function in both DNA and RNA metabolism and it is predominantly a nuclear protein (Kwiatkowski et al. 2009). FUS is an RNA-binding protein and like TDP-43, it is associated with the formation of stress granules (Liu-Yesucevitz et al. 2011).

Mutations in FUS are considered to be both loss of function and gain of function mutations. Knock-out of cabeza, the Drosophila ortholog of FUS, causes decreased adult viability, diminished locomotor speed and reduced life span which indicates a loss of function (Wang et al. 2011). Furthermore, it is shown that mutant FUS, but not wild-type FUS, causes cytoplasmic mislocalization and reduced lifespan. The mutant phenotype could not be rescued by wild-type FUS, even though wild-type FUS was not recruited to cytoplasmic mutant FUS aggregates (Murakami et al. 2012). This suggests that mutant FUS/TLS causes neuronal dysfunction by a dominant gain of function mutation, but not via a dominant negative effect of mutant FUS on wild-type FUS.

*Why are FUS mutations associated with ALS aggregation prone?*

FUS is intrinsically aggregation prone. In isolation it spontaneously forms pore-like oligomers and filamentous structures similar to FUS inclusions found in ALS patients (Sun et al. 2011). The full-length FUS protein is most aggregation prone (Figure 3). Residues 1-422 and residues 292-526 are also able to form aggregates, but their potential to form accumulations is significantly reduced compared to full length FUS in yeast (Kryndushkin et al. 2011). In FUS the N-terminal domain was recently discovered to be prion-like (Cushman et al. 2010). In TDP-43

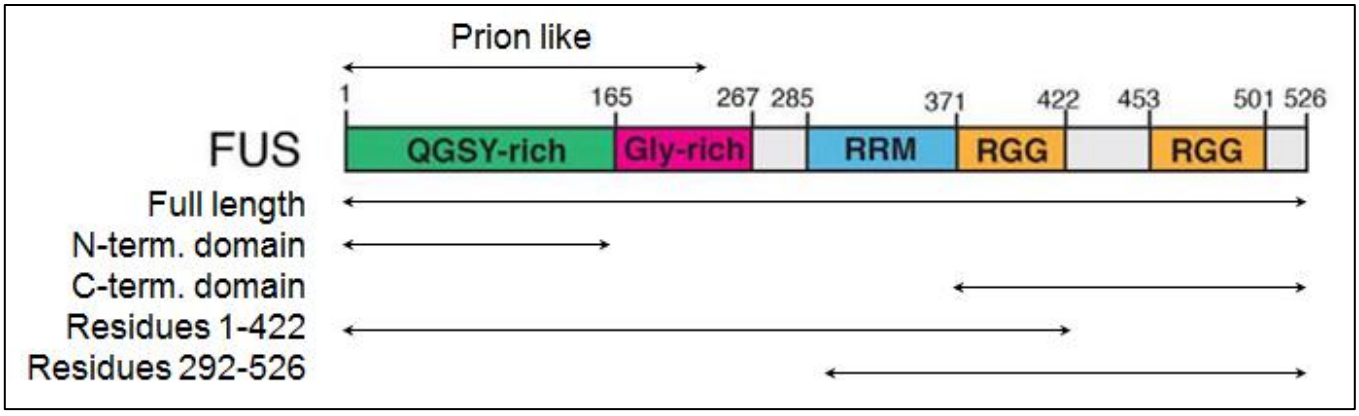


**Figure 3** Schematic of domain architecture of FUS (Sun et al. 2011)

this prion-like domain is sufficient to promote aggregation on its own. However, the N-terminal of FUS is not sufficient for aggregate formation on its own. Expressing of this N-terminal in absence of the other domains of FUS lead to entirely nuclear localization and no toxicity. Adding back the RRM, which is essential for TDP-43 toxicity, resulted again in nuclear localization and absence of toxicity. In addition the RGG domain, which is located in the C-terminal region, was required for cytoplasmic aggregation and toxicity in yeast. This means that residues 1-422 are essential to promote toxicity and aggregation (Sun et al. 2011). This arginine-glycine-glycine (RGG) tri-peptide repeat is hypothesized to perform a direct function in a-specific RNA binding (Burd and Dreyfuss 1994). This is an indication that mutant FUS, just as mutant TDP-43 needs its RNA-binding domain to be toxic. (Figure 3)

In contrast with the findings of Kryndushkin et al., Sun et al. found that deletion of the N-terminal resulted in the absence of aggregates. Interestingly, most mutations are found in the C-terminal of FUS (Lanson and Pandey 2012), which is not entirely required for aggregation. This region contains the nuclear localization signal. This indicates that changes in this region cause mislocalization to the cytoplasm. It is possible that mutant FUS is more aggregation prone in the cytoplasm than in the nucleus. Perhaps mutations in the nuclear localization signal cause mislocalization and the other regions promote aggregation.

*What is the evidence for toxicity of aggregates caused by mutated FUS?*

Toxicity of FUS is associated with greater expression and accumulation of FUS in cytoplasmic aggregates in yeast (Kryndushkin et al. 2011). This study only examines the aggregation and toxicity of wild-type FUS and not of mutations linked to ALS. In contrast it is shown that expression of wild-type FUS is barely or not toxic at all (Murakami et al. 2012). Besides, it could be debated whether yeast is a good model to investigate FUS toxicity, since yeast is poorly comparable with motor neurons. Studies that compare wild-type FUS with mutated FUS could provide better evidence for toxicity of aggregates. Ectopic expression of FUS carrying ALS-causing mutations in Drosophila leads to a mutation-dependent neurodegenerative phenotype. Expression of wild-type FUS does not lead to this phenotype. It is shown that mutated FUS localizes to both the cytoplasm and nucleus whereas wild-type FUS localized only to the nucleus. Furthermore, deletion of the nuclear export signal suppresses toxicity of mutated FUS (Lanson et al. 2011). This indicates that mutated FUS requires cytoplasmic localization to be toxic. The toxicity of cytoplasmic localized FUS was also shown in C. elegans. Mutated FUS cause FUS accumulation in cytoplasmic inclusions. These aggregates were associated with progressive motor dysfunction and reduced lifespan. Two mutations, R522G and P525L, associated with severe ALS show more cytoplasmic accumulations, significantly more impaired motor function and reduced lifespan than mutations associated with mild ALS, R514G and R521G. This suggests that cytoplasmic accumulation of mutant FUS is toxic. Moreover, wild-type FUS does not rescue mutant FUS and it is not recruited to mutant FUS aggregates (Murakami et al. 2012). This indicates a gain of function for mutant FUS and its aggregates instead of a loss of function. In rats mutant FUS R521C leads to neuronal loss accompanied by ubiquitin aggregation. This increased accumulation was not seen in transgenic rats overexpressing wild-type FUS. Interestingly, mutant FUS was not co-localized with ubiquinated inclusions, indicating that mutant FUS could escape proteosomal degradation (Huang et al. 2011). However, it remains unknown whether all FUS aggregates are ubiquitin negative or just this single mutation.

Furthermore, the aggregates were positive for the mitochondrial marker COXIV (Huang et al. 2011). Mitochondria are defect in FUS motor neuron cellular model (Tradewell et al. 2012). It is possible that these defect mitochondria are ubiquinated for degradation.

Human HSPA1L is able to reduce human FUS toxicity by decreasing the amount of insoluble forms of the protein in Drosophila. In this model, the amount of insoluble FUS correlated with neurotoxicity (Miguel et al. 2012). This indicates a relation between FUS neurotoxicity and progressive accumulation of FUS aggregates.
The RNA/DNA binding seems to be important for FUS to be toxic. In yeast overexpression of DNA/RNA binding proteins other than FUS could rescue toxicity of overexpression of human FUS. This rescue was without changing the expression level of FUS, without affecting the cytoplasmic translocation and without changing the amount of inclusions formed (Ju et al. 2011). This indicates that the mutated FUS aggregates are toxic by preventing FUS to perform its normal RNA/DNA binding function. This is further supported by the finding that expression of ALS linked FUS mutations resulted in accumulation into stress granules, a type of RNA granule (Ito et al. 2011). This supports the hypothesis that disruption of translational regulation and metabolism of RNA may be essential for FUS toxicity. The aberrant RNA processing is due to the formation of inappropriate or excessive stress granules.
It is not exactly clear whether the FUS aggregates are toxic, but in my opinion there are strong indications for a direct correlation between the amount of FUS aggregates and neurotoxicity.

*How does mutant FUS contribute to disease?*
In my opinion mutant FUS contributes to ALS because of its RNA-binding capacity. The neurotoxicity of FUS aggregates is dependent on their RNA-binding properties. Most of the mutations found to be a cause of ALS appear in the nuclear localization signal of FUS. These mutations could cause mutant FUS to mislocate to the cytoplasm, where it aggregates and forms stress granules. Thus abnormal RNA processing by mutated and aggregated FUS is in my opinion a significant contributor to ALS progression.
Furthermore mutant FUS seems to have a devastating effect on mitochondria. The mitochondrial dysfunction could lead to disease. It is unclear whether this is an early cause of ALS or a late consequence of another fatal process. This mechanism should be further investigated.

**Discussion**

The main question of this paper was whether aggregates are the cause or just a hallmark of ALS. In my opinion aggregates are both hallmark and cause of this disease. Aggregates in ALS are not performing a similar toxic function as aggregates in Alzheimer's disease and Parkinson's disease. Parkinson's disease is characterized by accumulation of α-synuclein. Aggregation of α-synuclein into oligomers with a ring-like appearance has been proposed to play a role in toxicity. A-synuclein aggregates penetrate the membrane and form pore-like structures that fully perforate the lipid bilayer, leading to increased membrane permeability which could be harmful to cells (Tsigelny et al. 2012). Alzheimer's disease is related with β-amyloid aggregation. It is proposed that the primary agents of this disease have a common molecular structure and that this structure can spontaneously integrate into the bilayers of membranes to form aqueous pores. This ability to produce permeable channels is a key element in the toxicity of β-amyloids (Singer and Dewji 2006). In these two diseases there is a direct link between aggregates and cellular toxicity. This seems to be the case in ALS as well. However, protein aggregates in ALS are not forming pores in membranes, but they are causing disease by impairing essential cellular functions. This means that protein aggregates are a direct cause of cell death. There are strong indications that aggregation of mutated SOD1 in mitochondria and near the cytoplasmic surface of mitochondria leads to mitochondrial dysfunction. Mutated SOD1 is the cause of mitochondrial damage and mitochondrial defects. This leads to neurotoxicity. There are strong indications that mutated TDP-43 and mutated FUS cause

cell death by a comparable mechanism. TDP-43 and FUS are both RNA-binding proteins and in my opinion the cytoplasmic aggregation of these proteins, perhaps with mRNA, leads to abnormal RNA-processing. Disrupting of RNA metabolism could lead to less translation of mRNA and consequently to a deficiency of essential proteins.
It is not surprising that TDP-43 and FUS function in a comparable mechanism leading to ALS, since they are both RNA-binding proteins. Furthermore TDP-43 and FUS are found to genetically interact with each other (Lanson et al. 2011). Cross-rescue analysis demonstrated that FUS acted together and downstream of TDP-43. Moreover, these proteins associate with each other in an RNA-dependent complex (Wang et al. 2011). Further investigation should focus on these two proteins together.
In my opinion aggregates are the underlying cause of ALS. Therefore, more research should focus on prevention or reversal of aggregate formation, either by molecular chaperones, inducing autophagy or stimulation of the ubiquitin proteasome system. This would provide very convincing evidence that aggregates are the cause of ALS. Furthermore, prevention of aggregates could be a promising therapeutic target for ALS. Perhaps this will eventually lead to the first cure for ALS. Lots of research should be done before this can be established.

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