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RNA dysfunction and aggrephagy at the centre of an amyotrophic lateral sclerosis/frontotemporal dementia disease continuum

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Amyotrophic lateral sclerosis and frontotemporal dementia form two poles of a genetically, pathologically and clinically-related disease continuum. Analysis of the genes and proteins at the heart of this continuum highlights dysfunction of RNA processing and aggrephagy as crucial disease-associated pathways. TAR DNA binding protein and fused in sarcoma (FUS) are both RNA processing proteins whose dysfunction impacts on global cellular RNA regulation. The recent discovery that expression of repeat expansions in the *C9orf72* gene may induce RNA foci that could sequester RNA binding proteins such as TAR DNA binding protein and FUS highlights a further possibly important mechanism of RNA dysfunction in disease. Furthermore, sequestration of key RNA binding proteins may also play an important role in sporadic disease due to the association of TAR DNA binding protein and FUS with stress granules. In a further functional convergence, ubiquilin 2, p62, valosin-containing protein and optineurin are all linked to aggrephagy, a cargo-specific subtype of autophagy important for degrading ubiquitinated target proteins through the lysosome. Notably these two key pathways interact; TAR DNA binding protein and FUS bind and regulate key aggrephagy-related genes whereas dysfunction of aggrephagy leads to cytoplasmic relocalization and aggregation of TAR DNA binding protein. The convergence of amyotrophic lateral sclerosis and frontotemporal dementia linked genes into these two pathways highlights RNA dysfunction and aggrephagy as promising areas for drug discovery. In this review we discuss the importance of each of these pathways and suggest mechanisms by which they may cause both sporadic and familial disease.

Keywords: amyotrophic lateral sclerosis; frontotemporal dementia; RNA processing proteins; *C9orf72*; aggrephagy

Abbreviations: ALS = amyotrophic lateral sclerosis; FTD = frontotemporal dementia; FTLD = frontotemporal lobar degeneration; TDP-43 = TAR DNA binding protein

Introduction

Amyotrophic lateral sclerosis (ALS) is a subtype of motor neuron disease that affects upper and lower motor neurons, causing muscular paralysis and eventual death through respiratory failure in 3 to 5 years (Cleveland and Rothstein, 2001). By contrast, frontotemporal dementia (FTD) is the second most common cause of presenile dementia, and includes four clinical subgroups: semantic dementia, progressive non-fluent aphasia, behavioural variant FTD and FTD with motor neuron disease/ALS (Snowden

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et al., 2007; Josephs et al., 2011). Neuropathologically FTD, together with the atypical parkinsonian disorders progressive supranuclear palsy and corticobasal degeneration, are defined under the bracket of frontotemporal lobar degeneration (FTLD), which is characterized by atrophy of the frontal and temporal brain lobes.

An amyotrophic lateral sclerosis/frontotemporal dementia disease continuum: clinical, pathological and genetic overlaps

Clinical data have demonstrated for some time that ALS and FTD are highly related conditions, occupying two poles of a disease continuum (Lomen-Hoerth et al., 2002). Up to 50% of ALS sufferers display some degree of cognitive impairment, whereas up to 16% of patients diagnosed with FTD display a motor neuron disease phenotype, usually first recognized by the presence of fasciculations or difficulty swallowing (Lomen-Hoerth et al., 2002; Hodges et al., 2004; Ringholz et al., 2005; Kertesz et al., 2007). Patients presenting with both FTD and ALS symptoms are frequently diagnosed as having a mixed FTD-ALS syndrome (McKhann et al., 2001). Strong molecular links between the two syndromes were first found with the discovery that aggregations of ubiquitinated TAR DNA binding protein (TDP-43) or FUS, two highly related RNA processing proteins, define the vast majority of ubiquitin-positive inclusions in both ALS and FTLD (Arai et al., 2006; Neumann et al., 2006, 2009). TDP-43 pathology is present in 90% of ubiquitin positive FTLD cases and non-SOD1 ALS cases with FUS-positive inclusions accounting for the majority of remaining ubiquitin-positive TDP-43-negative inclusions (Neumann et al., 2006, 2009; Mackenzie and Rademakers, 2008). Following these seminal discoveries, cases of FTLD and ALS were renamed to reflect the underlying pathology, for example FTLD-TDP or ALS-FUS (Mackenzie et al., 2009). More recently, inclusions containing p62, ubiquilin 2 or optineurin, all linked to protein degradation pathways, have been found in cases with ALS/FTLD associated with mutations in the genes encoding the respective proteins as well as in other familial and sporadic cases (Deng et al., 2011b; Hortobágyi et al., 2011; King et al., 2011).

Multiple pathological divisions within the ALS-FTLD disease spectrum are highlighted in Table 1. SOD1 and tau define subgroups of ALS and FTLD that show little clinical overlap and have been reviewed extensively elsewhere (Kato et al., 2000; Dickson et al., 2011; Seelaar et al., 2011).

Genetic links between ALS and FTD were first noted by the presence of several cases of familial ALS-FTD with, in some cases, even a change of phenotype from FTD to ALS between generations (Hudson, 1981; Gunnarsson et al., 1991). Multiple specific genetic links between ALS and FTD have now been described—the genes underlying these links are listed together with their functions, clinical phenotypes and frequencies, inheritance patterns and associated neuropathology in Table 2.

Mutations in *TARDBP*, which encodes TDP-43, are responsible for 4–6% of cases with non-SOD1 familial ALS and ~1% of apparently sporadic ALS (Andersen and Al-Chalabi, 2011). Furthermore, rare mutations in *TARDBP* are also causative for FTD (Borroni et al., 2009; Kovacs et al., 2009; Lagier-Tourenne et al., 2010). Mutations in *FUS*, again encoding a pathological feature of both diseases, are causative of ~1 and 4% of apparent sporadic and familial ALS respectively, but are yet to be shown definitively to be causal for FTD—only a single case of FTD with *FUS* mutations has been putatively assigned (Kwiatkowski et al., 2009; Vance et al., 2009; Van Langenhove et al., 2010; Chio et al., 2011; Lai et al., 2011).

In a significant recent discovery, expanded GGGGCC hexanucleotide repeats in the first intron of the *C9orf72* gene have been shown to segregate in cases with FTD, ALS and FTD-ALS (Dejesus-Hernandez et al., 2011; Renton et al., 2011). *C9orf72* encodes a protein of unknown function, however, the prevalence

Table 1 TDP-43 and FUS at the centre of the ALS/FTD disease spectrum

Pathological disease divisions	Causative genes	Protein species found in inclusions
ALS-SOD1	*SOD1*, sporadic	**SOD1**, p62, ubiquitin, ubiquilin 2 (Deng et al., 2011a, b; Hortobágyi et al., 2011; Kato et al., 2000)
ALS-TDP	*TARDBP*, *C9ORF72* *OPTN*/ *UBQLN2* Sporadic	**TDP-43**, p62, ubiquitin, ubiquilin, ubiquilin 2, optineurin (Arai et al., 2006; Brettschneider et al., 2012; Deng et al., 2011a, b; King et al., 2011; Williams et al., 2012)
ALS-FUS	*FUS* *UBQLN2* Sporadic	**FUS**, p62, ubiquitin, ubiquilin 2 optineurin (Deng et al., 2010; Deng et al., 2011a, b; Williams et al., 2012)
FTLD-FUS	Unknown, sporadic	**FUS**, p62, ubiquitin (Neumann et al., 2009)
FTLD-TDP	*GRN*/*VCP*/ *TDP-43*/ *C9ORF72* Sporadic	**TDP-43**, p62, ubiquitin, ubiquilin, ubiquilin 2, optineurin (Neumann et al., 2007; Deng et al., 2011b; King et al., 2011; Brettschneider et al., 2012)
FTLD-*MAPT*	*MAPT* Sporadic	**Tau**, p62, ubiquitin (Dickson et al., 2011; Hortobágyi et al., 2011)

Major pathological disease subtypes along the ALS/FTD spectrum are shown from ALS-SOD1 (dark blue), through shared TDP-43 or FUS pathology (light blue/red) to FTLD-tau (dark red) at the opposite pole. Associated causative mutations and characteristic inclusion constituents are shown for each pathological subtype. FUS and TDP-43 define large subtypes of both ALS and FTLD whereas SOD1 and tau pathology define distinct pathological subtypes at each end of the continuum (shown in darker blue/red). Notably, the presence of p62 and ubiquitin is shared between all inclusion types. Optineurin pathology has to date only been described in cases defined by TDP-43 or FUS suggesting it may be a more specific additional feature of TDP-43/FUS proteinopathies.

Table 2 Mutations associated with both ALS and FTD typically occur in genes encoding RNA processing proteins or components of the protein degradation machinery

Mutated Gene	Function	Clinical phenotype	Mode of Inheritance	Neuropathology in mutation cases
C9orf72	Unknown	ALS (+ + +), FTD (+ + +), FTD-ALS (+ + +) (Dejesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011; Majounie *et al.*, 2012)	Dominant	ALS/FTLD-TDP (Simón-Sánchez *et al.*, 2012)
TARDBP	RNA processing protein (Lagier-Tourenne *et al.*, 2010)	ALS (+ +), FTD (+), FTD-ALS (+) (Kabashi *et al.*, 2008; Sreedharan *et al.*, 2008; Benajiba *et al.*, 2009; Borroni *et al.*, 2009)	Dominant, recessive	ALS-TDP (Van Deerlin *et al.*, 2008)
VCP	Protein turnover via UPS and autophagy (Dai and Li, 2001)	ALS (+ + / +), FTD (+*), FTD-ALS (+) (Guyant-Marechal *et al.*, 2006; Gitcho *et al.*, 2009; Johnson *et al.*, 2010)	Dominant	ALS/FTLD-TDP (Gitcho *et al.*, 2009)
FUS	RNA processing protein (Lagier-Tourenne *et al.*, 2010)	ALS (+ +), rare FTD (Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009; Blair *et al.*, 2010; Van Langenhove *et al.*, 2010)	Dominant, recessive	ALS-FUS (Mackenzie *et al.*, 2011)
UBQLN2	Protein turnover via UPS and autophagy (Ko *et al.*, 2004)	ALS (+), FTD-ALS (+) (Deng *et al.*, 2011*b*)	Dominant (X-linked) in ALS	ALS-TDP/ALS-FUS (Deng et al., 2011*b*)
SQSTM1	Autophagy, inflammation and apoptosis (Moscat and Diaz-Meco, 2009)	ALS (+), FTD (+) (Fecto *et al.*, 2011; Rubino *et al.*, 2012)	Unclear, segregation yet to be shown	Unclear

The normal cellular function, associated clinical phenotype, frequency in disease, mode of inheritance and associated neuropathology are shown for genes linked to both ALS and FTD. Notably segregation analysis has yet to be performed for *SQSTM1* associated cases meaning these mutations may act as risk factors rather than causative mutations.
+ Mutations are usually a rare cause of the disorder (> 2% of familial cases).
+ + Mutations in the gene are generally causative for familial forms at a reasonable frequency (2–8% of familial cases).
+ + + Mutations are often a common cause of familial disease (> 8%).
*VCP mutations are more often described as part of the mixed disorder inclusion body myopathy associated with Paget's disease of the bone and frontotemporal dementia (IBMPFD). UPS = ubiquitin proteasome system.

of repeat expansions within both ALS and FTD make *C9orf72* expansions extremely interesting within the scope of this review. More than 30 GGGGCC repeats within *C9orf72* are classified as pathological, with most disease-associated expansions estimated at between 700 and 1600 repeats (Dejesus-Hernandez *et al.*, 2011). However, technical difficulties using repeat primed PCR mean the number of repeats required for disease is still unclear (Renton *et al.*, 2011; Xi *et al.*, 2012).

Estimates for the prevalence of expanded *C9orf72* repeats in ALS and FTD have consistently shown that the locus represents, in at least some populations, the single greatest genetic cause of ALS, FTD and ALS-FTD (Majounie *et al.*, 2012; Smith *et al.*, 2013). Studies in European, Northern American and Australian populations have suggested an overall average frequency of ~33% in familial ALS and 8% in sporadic ALS, with prevalence rising as high as 83% and 73% in Belgian and Swedish cohorts, respectively (Dejesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011; Dobson-Stone *et al.*, 2012; Gijselinck *et al.*, 2012; Majounie *et al.*, 2012; Ratti *et al.*, 2012; Smith *et al.*, 2013; Garcia-Redondo *et al.*, 2013). By comparison, the frequency of expanded *C9orf72* repeats in Japanese and Chinese ALS populations appears to be much lower (<5%), consistent with recent suggestions of an initial founding effect due to the repeat expansion arising within Northern Europe (Ogaki *et al.*, 2012; Ratti *et al.*, 2012; Smith *et al.*, 2013; Garcia-Redondo *et al.*, 2013). Fewer studies of the prevalence of expanded *C9orf72* repeats in FTD cohorts have been published but prevalence again seems to be high with an average of ~20% and 6% suggested for familial and sporadic European populations, respectively (Dejesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011; Gijselinck *et al.*, 2012; Majounie *et al.*, 2012). Furthermore, an exceedingly prominent clinical overlap between ALS and FTD has also been noted within *C9orf72* disease cohorts (Dejesus-Hernandez *et al.*, 2011). Clearly, understanding the pathogenesis of *C9orf72* mutations must be a priority and it should include the functional analysis of the previously uncharacterized C9orf72 protein, which may potentially have a cellular role similar to other ALS/FTD related proteins.

At lower frequencies, mutations in the valosin-containing protein (*VCP*) gene lead to both ALS and FTD (Gitcho *et al.*, 2009; Johnson *et al.*, 2010; Mackenzie *et al.*, 2010). Similarly mutations in *SQSTM1*, encoding the p62 protein, have been described in both ALS and FTD cases, although segregation analysis has yet to be performed in either ALS or FTD families meaning *SQSTM1* mutations may function as risk factors rather than being directly pathogenic (Fecto *et al.*, 2011; Rubino *et al.*, 2012). Furthermore *UBQLN2*, encoding ubiquilin 2, has recently been linked to ALS, ALS-FTD and FTD at relatively low frequencies (Maruyama *et al.*, 2010; Deng *et al.*, 2011*b*; Synofzik *et al.*, 2012).

The genes listed here reflect those shared between ALS and FTD, however, many further genes have been linked to ALS or FTD individually. The full genetic basis of these diseases has been reviewed extensively, and will not be listed here (Andersen and Al-Chalabi, 2011; Seelaar *et al.*, 2011).

Notably, as discussed in more detail below, these ALS and FTD linked genes segregate into two major functional groups; those associated with RNA processing and those involved in protein degradation pathways. The convergence of ALS and FTD genes into

these pathways highlights RNA processing and cargo-specific autophagy as central to the pathogenesis within the ALS/FTD continuum. The importance of these pathways in ALS and FTD, and how they might interact in both familial and sporadic disease will be the focus of this review.

Shared cellular pathways in amyotrophic lateral sclerosis and frontotemporal dementia

RNA processing and dysregulation

Genetic and pathological analysis has therefore demonstrated that *TARDBP*, *FUS* and *C9orf72* are at the centre of the ALS-FTD spectrum. Notably all three genes may share a common link to cellular RNA dynamics.

The involvement of TDP-43 and FUS in RNA-related pathways is strong: both are RNA processing proteins with roles in multiple steps of RNA regulation including: RNA transcription, splicing, transport, translation and microRNA production (Lagier-Tourenne *et al.*, 2010). Both proteins directly interact with the heterogeneous nuclear ribonucleoprotein complex, which regulates RNA splicing and transport, suggesting that they may have similar roles in the cell (Calvio *et al.*, 1995; D'Ambrogio *et al.*, 2009). Indeed dual knockdown experiments in zebrafish suggest that TDP-43 and FUS operate within the same pathway, with FUS acting downstream of TDP-43 (Kabashi *et al.*, 2011).

The role of TDP-43 and FUS in RNA processing is mediated through direct interaction with RNA, both TDP-43 and FUS bind RNA through two RNA recognition motif (RRM) protein domains (Hoell *et al.*, 2011; Tollervey *et al.*, 2011). TDP-43 binding sites are found in the RNA encoding TDP-43, FUS and other RNA processing proteins such as poly(A)-binding protein cytoplasmic 1 (PABPC1) suggesting TDP-43 and FUS may participate in a large co-regulatory network (Sephton *et al.*, 2011). Downregulation of TDP-43 in the mouse brain has been shown to reduce levels of FUS by 60% suggesting that feedback and crosstalk mechanisms are required to maintain precise expression levels across this network (Polymenidou *et al.*, 2011; Sephton *et al.*, 2011; Tollervey *et al.*, 2011). Notably, TDP-43 RNA targets include genes important for synaptic function, neurotransmitter release and the neurodegeneration-related genes progranulin (*GRN*), α-synuclein (*SNCA*), tau (*MAPT*) and ataxin 1 and 2 (*ATXN1/2*) (Polymenidou *et al.*, 2011; Sephton *et al.*, 2011). Dysfunction in this complex network of RNA binding proteins is therefore likely to have severe downstream consequences. It is, however, important to note that TDP-43 and FUS have many thousands of targets within the genome; TDP-43, for example, has binding sites in ~30% of transcribed mouse genes (Polymenidou *et al.*, 2011). Individual studies have highlighted different sets of genes targeted by these RNA binding proteins making the physiological importance of single reported interactions difficult to understand without further molecular insights (Polymenidou *et al.*, 2011; Sephton *et al.*, 2011; Tollervey *et al.*, 2011). A recent study mapping both TDP-43 and FUS binding to RNA has, however, cast some light on transcripts regulated by both TDP-43 and FUS, and hence likely to be central to understanding the downstream effects of TDP-43/FUS dysfunction that lead to ALS/FTD. Whilst TDP-43 and FUS have largely distinct binding patterns—only 86 shared gene regulation events were highlighted in the study—genes that are regulated by both TDP-43 and FUS are enriched for the presence of very long introns (Lagier-Tourenne *et al.*, 2012). Notably the co-regulated genes in this study were also enriched for neuronal functionality, suggesting a conserved role for TDP-43 and FUS in maintaining levels of neuronal proteins whose pre-RNA feature elongated introns (Lagier-Tourenne *et al.*, 2012). Aside from affecting messenger RNA translation, TDP-43 and FUS also have clear roles in alternative splicing with, for example, knockdown of TDP-43 in SH-SY5Y cells leading to 228 splicing changes amongst genes containing alternative isoforms (Tollervey *et al.*, 2011). Interestingly, TDP-43 activity is required for inclusion of exon 18 of *SORT1*. *SORT1* encodes a receptor for progranulin, although not the receptor mediating the effects of progranulin on neurite outgrowth, and regulates progranulin levels, providing a possible link between TDP-43 dysfunction and disease (Carrasquillo *et al.*, 2010; Hu *et al.*, 2010; Polymenidou *et al.*, 2011; Gass *et al.*, 2012). Similarly, FUS has been shown to bind RNA at splice acceptor sites and associates with transcriptional machinery such as RNA polymerase II and the TFIID complex consistent with a role in splicing and transcriptional regulation (Lagier-Tourenne and Cleveland, 2009; Hoell *et al.*, 2011).

The key role of TDP-43 and FUS at different stages of RNA processing is clear, but how do mutations in these genes cause disease? In the neurons of all patients with ALS or FTLD with either TDP-43 or FUS pathology, the defining protein (TDP-43 or FUS) relocates from the nucleus to the cytoplasm and forms aggregates (Arai *et al.*, 2006; Neumann *et al.*, 2009; Deng *et al.*, 2010). Three possible causes of cytotoxicity in mutant and/or cytoplasmically localized TDP-43 and FUS can be proposed: (i) loss of normal nuclear function leading to dysregulation of nuclear RNA processing; (ii) gain of extraneous cytoplasmic RNA binding activity; or (iii) aggregation-dependent toxicity.

The finding that the majority of FUS mutations cluster within a nuclear localization sequence and directly lead to a loss of normal nuclear localization makes a loss of function an attractive idea for FUS toxicity (Dormann *et al.*, 2010). FUS toxicity in yeast has been shown to be suppressed by over-expression of RNA processing proteins such as the human or yeast RNA helicases *UPF1* and *ECM32*, which function in RNA quality control and appear to compensate for loss of FUS activity (Ju *et al.*, 2011). A loss-of-function mechanism is also supported by an apparent correlation between the degree of mutation-induced relocalization and phenotypic severity of associated disease (Dormann *et al.*, 2010; Mackenzie *et al.*, 2011). However, these findings do not necessarily show that FUS mutations act through a loss of function mechanism—a toxic role in the cytoplasm could give similar data. With regard to a toxic gain-of-function it is notable that human wild-type and mutant FUS is equally toxic when expressed in yeast due to the lack of nuclear localization sequence conservation

across species (Ju *et al.*, 2011). Addition of a yeast nuclear localization sequence abrogates toxicity, suggesting that toxicity is directly related to cytoplasmic accumulation (Ju *et al.*, 2011). Analysis of RNA binding by wild-type or mutant FUS shows an altered, rather than simply reduced, set of binding targets in cytoplasmically localized mutant FUS (Hoell *et al.*, 2011). Furthermore, use of serially deleted *FUS* expression constructs in a yeast model demonstrated that both N and C terminal regions, including RNA binding domains, are required for toxicity, suggestive of aberrant functionality in mislocalized FUS (Ju *et al.*, 2011; Sun *et al.*, 2011). A further argument for a gain-of-function effect is seen in the weak clearance of FUS from the nuclei of many affected neurons—arguing against complete loss of nuclear action (Neumann *et al.*, 2009). The evidence for direct toxicity of FUS aggregates remains unclear; one study using expression of a series of deletion constructs of FUS in yeast demonstrated that aggregation was only weakly correlated with toxicity (certain constructs that formed aggregations did not show toxicity) whereas a further contradictory yeast study has demonstrated that FUS aggregation is correlated with toxicity and highly dependent on expression level (Ju *et al.*, 2011; Sun *et al.*, 2011). Notably these toxicity-dependent aggregates appear to be stress granules—aggregations of RNA and RNA binding proteins thought to function in a protective manner during periods of cellular stress by protecting untranslated messenger RNA from destruction or modification in the cytoplasm (Sun *et al.*, 2011). This finding infers that FUS must localize to stress granules to mediate toxicity and is somewhat surprising—stress granule sequestration of FUS is likely to ameliorate any aberrant RNA binding functionality in the cytoplasm—unless stress granules, or their possible ubiquitinated derivatives are actively toxic. Furthermore, screens in yeast for suppressors of FUS toxicity highlighted various stress granule components including the yeast homolog of PABP1, a protein involved in stress granule assembly inferring that stress granules may be key to FUS mediated toxicity (Ju *et al.*, 2011). It is also notable that the requirement of RNA binding activity for toxicity may reflect binding to stress granules rather than aberrant cytoplasmic processing targets.

As such the mechanism by which FUS mutations lead to disease seem to be intrinsically linked to loss of nuclear localization but may proceed through both loss and gain-of-function. Further experiments to define the importance of aggregation and stress granule association on FUS toxicity in further model systems would be instructive.

Like FUS, pathological TDP-43 is associated with nuclear clearance and cytoplasmic aggregation (Arai *et al.*, 2006). However, unlike FUS, TDP-43 mutations do not cluster around a nuclear localization sequence, meaning a direct relocalization appears not to be the primary toxic feature of mutations. Indeed, mutations in genes other than *TARDBP*, such as *VCP*, can lead to cytoplasmic TDP-43 accumulation (Gitcho *et al.*, 2009). Furthermore, TDP-43 pathology has also been seen in other seemingly unrelated disorders such as Alzheimer's disease, suggesting that it may be an indirect downstream effect of mutations that leads to cytoplasmic clearance of TDP-43 (Nakashima-Yasuda *et al.*, 2007; Wilson *et al.*, 2011). Within model systems relocalization of mutant TDP-43 is often only seen with the addition of further stress, and concomitant formation of cytoplasmic stress granules, although a small degree of relocalization in the absence of exogenous stress has been reported (Barmada *et al.*, 2010; Liu-Yesucevitz *et al.*, 2010). It is therefore possible that *TARDBP* mutations confer toxicity through increased aggregation or stress granule association, leading indirectly to a loss of nuclear TDP-43 due to cytoplasmic sequestration. In support of this hypothesis, ALS associated *TARDBP* mutations, unlike mutations in *FUS*, have been shown to increase TDP-43 aggregation propensity (Johnson *et al.*, 2009). While loss of nuclear RNA processing activity is again likely to explain aspects of TDP-43 toxicity due to the important role of TDP-43 in the nucleus, other factors seem to be involved. Although 93% of TDP-43–RNA interactions (with the exception of 3′ untranslated region binding) occur in the nucleus, TDP-43 does regulate the translation of RNAs in the cytoplasm and interacts with cytoplasmic proteins (Freibaum *et al.*, 2010; Tollervey *et al.*, 2011). Furthermore, within multiple model systems, overexpression of wild-type and mutant TDP-43 has been shown to be toxic in a dose dependent manner, arguing for a gain of toxicity (Wegorzewska *et al.*, 2009; Barmada *et al.*, 2010). Together with the requirement for RNA binding for TDP-43 to mediate toxicity in several disease models, it appears that pathogenic TDP-43 has a cytoplasmic gain-of-function due to aberrant processing of cytoplasmic RNAs as well as possible loss of normal nuclear function (Voigt *et al.*, 2010). The major difference between the two proteins appears to be that loss of nuclear relocalization is a primary feature of *FUS* mutations whilst, by contrast, increased aggregation propensity may be the major feature of *TARDBP* mutations. The most powerful evidence for the impact of *TARDBP* and *FUS* mutations is the importance of RNA binding to toxicity; both proteins require RNA binding domains to mediate toxicity whilst FUS toxicity has been shown—in two separate yeast models—to be suppressed by overexpression of similar RNA binding proteins (Voigt *et al.*, 2010; Ju *et al.*, 2011; Sun *et al.*, 2011).

As alluded to above, a possible explanation for the propensity of TDP-43 and FUS to deposit in the cytoplasm in cases without clear disruption of nuclear import lies in their known association with stress granules. Mutant TDP-43 and FUS have been shown to localize to stress granules under conditions of cytoplasmic stress, such as heat shock or induction of reactive oxidative species through arsenite exposure (Colombrita *et al.*, 2009; Bosco *et al.*, 2010). It is therefore possible that periods of extended cellular stress, even in the absence of disease associated mutations, may lead to a cytoplasmic relocalization and sequestration of key RNA binding proteins within stress granules. In support of this idea, in mouse models of neural injury (axotomy), cytoplasmic TDP-43 levels have been shown to increase in the post-injury period, with TDP-43 interacting with components of RNA granules (Moisse *et al.*, 2009). Furthermore, in SH-SY5Y cells exposed to oxidative stress, *FUS* messenger RNA levels have been shown to be decreased by 40%, consistent with either direct *FUS* messenger RNA sequestration in stress granules or downstream sequestration of *FUS* regulating proteins such as TDP-43 (Blechingberg *et al.*, 2012). As such, cellular stress could provide a mechanism for sporadic disease in which stress granule mediated sequestration, rather than specific mutations, leads to dysfunction of key RNA binding

proteins such as TDP-43 and FUS. Recent evidence has also suggested that stress granules may transition, over time, into the larger ubiquitinated aggregates seen in post-mortem disease tissue; both TDP-43 and FUS positive aggregates in post-mortem tissue colocalize with key stress granule proteins such as TIA1, PABP1 and eIF3 (Dormann *et al.*, 2010; Liu-Yesucevitz *et al.*, 2010). Furthermore, TDP-43 containing stress granules have been shown to survive as cytoplasmic aggregates once cellular stress is removed—a finding not replicated for non-TDP-43 stress granules, and to be less likely to disassemble in the presence of chemical inhibitors (Parker *et al.*, 2012). These data suggest that TDP-43 and FUS containing stress granules may transition to disease associated aggregates, perhaps through the formation of overly stable stress granules. As such, stress granules may provide a mechanism through which cellular stress leads to the sequestration of RNA processing proteins causing a loss of function in these proteins, or alternatively may promote the formation of toxic aggregations of TDP-43 or FUS. The importance of stress granules in disease is further highlighted by their association with other neurodegeneration associated proteins including survival of motor neuron, huntingtin and ataxin 2 (Hua and Zhou, 2004; Elden *et al.*, 2010; Ratovitski *et al.*, 2012).

Interestingly ataxin 2, associated with an increased risk of ALS when carrying an intermediate number of polyglutamine repeats, has been shown to interact within a common complex with TDP-43 and localize to stress granules (Elden *et al.*, 2010). Ataxin 2 is a modifier of TDP-43 toxicity in yeast and *Drosophila* where increased levels of ataxin 2 enhance TDP-43-mediated toxicity (Elden *et al.*, 2010). Furthermore, ataxin 2 affects stress granule formation in a concentration-dependent manner (Nonhoff *et al.*, 2007; Elden *et al.*, 2010). Notably, as mentioned above, TDP-43 binds *ATXN2* messenger RNA suggesting a possible co-regulatory interaction (Sephton *et al.*, 2011). As intermediate polyglutamine repeats have been suggested to increase the effective cellular concentration of ataxin 2 through increased protein stabilization, it is possible that these expansions lead to greater formation of stress granules, and hence a greater chance of stress granule-mediated sequestration of TDP-43 or FUS (Elden *et al.*, 2010). By contrast, more recent reports suggest that whilst TDP-43 C terminal fragments and FUS recruit ataxin 2 to stress granules, overexpression of *ATXN2* reduces the association of TDP-43 and FUS with stress granules while increasing their cytoplasmic levels—arguing that stress granule sequestration of TDP-43 may be protective in some cases (Nihei *et al.*, 2012). Notably, *ATXN2* repeat expansions seem to be associated only with ALS and not FTD, suggesting that ataxin 2 contributes to an ALS-specific pathway of disease rather than one common to the ALS/FTD continuum, although *FUS* is also almost exclusively genetically linked to ALS but still has a clear role in FTD (Van Langenhove *et al*. 2012; Vance *et al.*, 2009). Similarly, senataxin (*SETX*) and angiogenin (*ANG*), two genes linked exclusively to ALS, are RNA interacting proteins, whereas the survival of motor neuron (SMN) protein associated with spinal muscular atrophy is also an RNA-binding protein that localizes to stress granules (Hua and Zhou, 2004; Wu *et al.*, 2007; Hirano *et al.*, 2011). Notably angiogenin has been shown to promote the formation of arsenite-induced stress granules through cleavage of transfer RNA to form transfer RNA-derived stress-induced RNAs (tiRNAs), which inhibit protein translation in an eIF2 (eukaryotic initiation factor 2) independent manner—leading to stress granule assembly (Emara *et al.*, 2010). A number of ALS-associated *ANG* mutations have been ascribed to a loss of function effect, implicating impaired stress granule formation in disease (Wu *et al.*, 2007). As such, four ALS and FTD genes, plus SMN in a related disorder, seem to either localize to, or influence the formation of stress granules. If stress granules lead either to sequestration of RNA binding proteins, direct aggregate toxicity or to remove toxic TDP-43/FUS then this will be an exciting disease associated pathway to investigate. Given the contradictory reports as to the effect of stress granule formation on toxicity, it will be important to investigate further the impact of stress granule-mediated sequestration of TDP-43/FUS in a variety of disease models.

Further to the clear role of TDP43 and FUS in RNA pathways, the recent discovery of the *C9orf72* hexanucleotide expansion in ALS and FTD has provided additional evidence that impairment of RNA processing could be a general mechanism of disease in ALS and FTD. Abnormal intranuclear RNA foci containing the expanded RNA transcript have been described in cases of FTLD with *C9orf72* mutations (Dejesus-Hernandez *et al.*, 2011). The formation of RNA foci has been suggested to sequester RNA binding proteins impairing their function (Miller *et al.*, 2000; Simón-Sánchez *et al.*, 2012). Indeed the hexanucleotide motif of *C9orf72* has been predicted *in silico* to interact with the A2/B1 regions of the heterogeneous ribonucleoprotein particle complex which contains FUS and directly interacts with TDP-43 (Iko *et al.*, 2004; Buratti *et al.*, 2005; Dejesus-Hernandez *et al.*, 2011). However, although rare nuclear RNA foci were found in a subset of cases, it is not yet clear how the sequestering of RNA-binding proteins in the nucleus could lead to the more widespread cytoplasmic aggregates of TDP-43 found in cases with the *C9orf72* mutation (Dejesus-Hernandez *et al.*, 2011; Hsiung *et al.*, 2012). Furthermore, other studies have failed to find *C9orf72*-derived RNA foci using different *in situ* hybridization probes and TDP-43/FUS have, to date, not been shown to localize to C9orf72-derived foci (Simón-Sánchez *et al.*, 2012). Expanded RNA repeats have, however, been described as sequestering RNA-binding proteins in various other neurological disorders. In myotonic dystrophy, the most common adult onset muscular dystrophy, expression of RNA containing either expanded CUG or CCUG repeats leads to the presence of nuclear RNA foci and the sequestering of RNA binding proteins such as musclebind-like splicing regulator 1 (MBNL1) (Mahadevan *et al.*, 1992; Philips *et al.*, 1998; Miller *et al.*, 2000; Liquori *et al.*, 2001; Higashi *et al.*, 2007). As a direct result of MBNL1 sequestration, downstream genes such as *BIN1* have been shown to be misspliced, with these alterations in *BIN1* splicing shown to lead to muscle weakness and T tubule alterations in mouse models (Fugier *et al.*, 2011). Furthermore, in another neurodegenerative disease, Fragile X-associated tremor ataxia syndrome (FXTAS), medium length (55–200) expanded CGG repeats also lead to the sequestering of RNA binding proteins and resultant splicing alterations in patients (Tassone *et al.*, 2004; Iwahashi *et al.*, 2006; Sellier *et al.*, 2010). The parallels between these cases and the GGGGCC

expansion in *C9orf72* are clear; expression of *C9orf72* expanded repeats could lead to sequestration and aberrant function of RNA binding proteins, consistent with the emerging concept of RNA dysregulation as a central theme within the ALS/FTD continuum. The parallels between stress granule mediated aggregation of RNA binding proteins and sequestration through aberrant binding to expanded RNA repeats suggest accumulation of TDP-43, FUS or other RNA binding proteins in either nuclear or cytoplasmic foci could be of great importance. It should, however, be noted that alternative mechanisms for *C9orf72*-derived disease are possible; the presence of repeat expansions has been suggested to reduce expression of the *C9orf72* gene leading to disease through haploinsufficiency (Renton *et al.*, 2011). Early reports have demonstrated reduced C9orf72 levels within post-mortem brain tissue, although this finding has not been reported by all groups (Dejesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011; Gijselinck *et al.*, 2012). Manipulation of *C9orf72* expression in model systems or functional analysis of the C9orf72 protein will be required in order to investigate whether it is a reduction of *C9orf72* expression that leads to disease (Gijselinck *et al.*, 2012).

Additional links between RNA processing and neurodegeneration were recently provided by the discovery of mutations in the *EXOSC3* gene, which encodes a component of the RNA exosome complex, in pontocerebellar hypoplasia and spinal motor neuron degeneration (Wan *et al.*, 2012). Given the current rate of discovery of mutations in RNA processing protein genes in neurodegenerative disease, dysfunction of RNA processing is clearly evolving into a central theme within neurodegeneration. This association appears to be especially common in conditions affecting motor neurons, with *TARDBP*, *FUS*, *C9orf72* and *EXOSC3* adding to information previously gained from *SMN* within the motor neuron condition spinal muscular atrophy (Lefebvre *et al.*, 1995; Wan *et al.*, 2012). Within the ALS/FTD continuum overall, deregulation of RNA processing through the expansion at the *C9orf72* locus, formation of stress granules and mutations in the *FUS* and *TARDBP* genes appear to be of great interest. In particular, defining the interactions between wild-type and mutant forms of TDP-43, FUS and C9orf72, together with elucidating the effect of TDP-43 and FUS stress granule localization on toxicity should be extremely instructive. It will be interesting to investigate whether stress granule localization of TDP-43 and FUS is also seen in *C9orf72*-associated disease cases.

Protein degradation pathways

The protein degradation machinery of the cell has long been demonstrated to be of critical importance in dealing with the misfolded and aggregated proteins that define many neurodegenerative disorders (Rubinsztein, 2006). Two major pathways for protein recycling are seen in the cell; the ubiquitin proteasome system, where proteins are specifically targeted for destruction within the proteasome by the addition of poly-ubiquitin residues, and macroautophagy, where long-lived proteins and organelles are sequestered within autophagosomes which then fuse with lysosomes leading to the degradation of vesicle cargo. Knockout of the key autophagy gene *Atg7* in a mouse model led to severe neurodegeneration and the accumulation of polyubiquitinated aggregates, demonstrating both the importance of autophagy within long-living non-dividing neuronal cells, and its relevance to neurodegenerative disease (Komatsu *et al.*, 2006). Furthermore, several neurodegeneration-linked genes, for example *GBA* and *LRRK2* in Parkinson's disease and *OPTN* and *SQSTM1* in ALS/FTD have been linked to autophagy (Bjørkøy *et al.*, 2006; Alegre-Abarrategui *et al.*, 2009; Velayati *et al.*, 2010; Wild *et al.*, 2011). The possible involvement of the ubiquitin proteasome system in neurodegeneration is highlighted by the ubiquitination of aggregates in multiple disorders, and through—as discussed later—the presence of mutations in *UBQLN2* and *VCP* in ALS and FTD. Although clear evidence of a causal role of ubiquitin proteasome system defects in neurodegeneration has been elusive, various pieces of evidence have linked protein aggregate toxicity to ubiquitin proteasome system defects and have been reviewed in detail elsewhere (Dennissen *et al.*, 2012). Importantly, TDP-43 aggregations appear to be degraded through both autophagy and the ubiquitin proteasome system, meaning both pathways could be of relevance to ALS/FTD pathogenesis (Brady *et al.*, 2011).

Four genes, *UBQLN2*, *SQSTM1*, *OPTN* and *VCP* linked to ALS and/or FTD have strong links to protein degradation pathways highlighting this important pathway as central to pathogenesis within the ALS/FTD continuum.

Ubiquilin 2 is a member of the four-strong ubiquilin family of proteins that regulate the destruction of ubiquitinated proteins through the ubiquitin proteasome system or autophagy. Ubiquilin family proteins all contain a ubiquitin-like and a ubiquitin-associated domain (UBL/UBA) (Ko *et al.*, 2004). The ubiquitin-like domain is responsible for binding proteasome subunits, whereas the ubiquitin-associated domain functions in binding poly-ubiquitin chains, suggesting that ubiquilin proteins function in the recognition and transport of ubiquitnated proteins to the proteasome for destruction (Ko *et al.*, 2004). Furthermore, ubiquilin also appears to function in autophagy through binding the autophagosomal protein LC3, to transport certain ubiquitinated cargoes or aggregates to the autophagosome for degradation (Rothenberg *et al.*, 2010). Rare mutations in *UBQLN2* have been linked to ALS and ALS/FTD and have been suggested to lead to an impairment of protein degradation by the ubiquitin proteasome system, perhaps reducing clearance of aggregated proteins (Deng *et al.*, 2011*b*). Pathologically, ubiquilin 2 co-localizes with TDP-43 and FUS, suggesting that ubiquilin 2 acts within the pathway required for degradation of TDP-43 and FUS aggregations and remains trapped in aggregates that are not degraded (Deng *et al.*, 2011*b*; Williams *et al.*, 2012). Notably, *UBQLN1*, encoding a further member of the ubiquilin family, has strong links to neurodegenerative conditions (Mah *et al.*, 2000). Ubiquilin pathology has recently been suggested to be present, and act as a marker in cases with ALS and FTLD-TDP with the *C9orf72* mutation (Brettschneider *et al.*, 2012). Within the ALS/FTD continuum, ubiquilin 1 has been shown to bind polyubiqitinated TDP-43 aggregates *in vitro* with overexpression of *UBQLN1* leading to TDP-43 being recruited to aggregates containing the autophagosomal marker LC3, suggesting a role for ubiquilin 1 in the destruction of TDP-43 containing aggregates by autophagy (Kim *et al.*, 2008). Within a *Drosophila* model of TDP-43 proteinopathy,

co-expression of ubiquilin leads to a reduction in both soluble and insoluble TDP-43 levels and, perhaps somewhat surprisingly, an increase in TDP-43 mediated toxicity, even though cytosolic TDP-43 aggregates were not seen (Hanson *et al.*, 2010). One case of atypical motor neuron disease has been associated with *UBQLN1* mutations, but a recent screening of ~100 cases of both familial and sporadic ALS failed to highlight any association, although this does not rule out a possible rare association and should not discourage further screens (González-Pérez *et al.*, 2012). These data do, however, suggest a role for ubiquilin 1 and 2 in the destruction of ubiquitinated ALS and FTD aggregations by either the ubiquitin proteasome system or autophagy.

Notably, p62, another protein involved in protein degradation pathways and linked to ALS and FTD, has also been shown to bind polyubiquitin chains. Unlike ubiquilin 2, p62 appears to function in autophagy only, acting as a cargo receptor recruiting large polyubiquinated aggregates to autophagosomes (Bjørkøy *et al.*, 2005, 2006). *In vivo*, p62 coats TDP-43 inclusions, and p62 overexpression has been reported to reduce the formation of TDP-43 aggregates (Brady *et al.*, 2011). As such depletion of p62 might be expected to lead to the formation of intracellular aggregates. However, in an apparent contrast p62 also appears to have a role in aggregate formation, autophagy mediated degradation of p62 is required to prevent the build-up of ubiquitinated p62-containing aggregates (Komatsu *et al.*, 2007). Furthermore p62/*SQSTM1* knockdown in autophagy deficient mice suppresses the formation of ubiquitinated protein aggregates within neurons (Komatsu *et al.*, 2007). As such, maintaining 'homeostatic levels of p62' may be important in both the formation, marking for autophagic destruction and subsequent fusion of aggregates with autophagosomes (Komatsu *et al.*, 2007). In keeping with this idea, p62 overexpression has been shown to enhance the aggregation of mutant SOD1 protein (which defines another pathological subtype of ALS), but that these aggregates do not affect cell viability (Gal *et al.*, 2007). p62, alongside another autophagy cargo-receptor, NBR1, has been suggested to structurally maintain larger ubiquitinated aggregates with smaller aggregates not requiring p62 to form, consistent with p62 being a ubiquitin binding protein (Yamamoto and Simonsen, 2011). Whether the effect of p62 on aggregate formation is beneficial to the cell depends, of course, on whether the build-up of specific ubiquitinated aggregates is toxic or beneficial.

By contrast, excess p62 accumulation in the liver, due to inhibition of autophagy, has been demonstrated to lead to liver damage by deleteriously high induction of oxidative stress response genes through activation of the stress response factor Nrf2 (Komatsu *et al.*, 2010). Loss of p62 suppresses liver dysfunction in autophagy deficient mice; however, the same result is not seen in the brain (Komatsu *et al.*, 2007). Although this finding argues against a toxic stress response induction of p62 in neurodegeneration, the lower levels of basal autophagy (and hence smaller impact on p62 levels) in the brain coupled with the long timescales associated with neurodegenerative disease mean this feature of p62 is still worthy of investigation within ALS/FTD, especially if stress granules are seen on neuronal Nrf2 activation (Komatsu *et al.*, 2007).

As such two contrasting ideas for the role of p62 in disease can be suggested; first, p62 may be a crucial component in the selective formation of large ubiquinated aggregates and the subsequent fusion of these aggregates with autophagosomes. Second, and somewhat paradoxically, accumulation of p62 due to deficits in autophagy may lead to aberrant induction of oxidative stress response genes.

Remarkably, in a manner similar to p62 and ubiquilin 2, optineurin seems to act as an 'autophagy receptor', binding ubiquitin or ubiquitinated aggregations to direct them to autophagosomes (Wagner *et al.*, 2008; Wild *et al.*, 2011). Optineurin, like p62, contains a LC3 interacting motif allowing direct binding of LC3 at autophagosomal membranes (Wild *et al.*, 2011). ALS associated mutations in *OPTN* appear to affect the ubiquitin binding motifs of optineurin, suggesting that loss of ubiquitin binding activity is the pathogenic feature of *OPTN* mutations in ALS (Maruyama *et al.*, 2010). It would therefore appear that ubiquilin 2, p62 and optineurin all function in a selective type of autophagy referred to as aggrephagy due to its role in the specific elimination of ubiquitinated protein aggregates through the lysosome (Yamamoto and Simonsen, 2011). Dysfunction in any of these three proteins would be expected to lead to an inability of aggregates to be removed, consistent with the neuropathology of ALS and FTD.

VCP (also known as p97), which is a member of the diverse AAA-ATPase protein super family, has a role in protein turnover by the ubiquitin proteasome system (Dai and Li, 2001). VCP complexes bind to ubiquitinated target proteins and structurally remodel them through an ATP-dependent unfolding process to allow targeting to the proteasome (Meyer *et al.*, 2012). Expression of dominant-negative mutant VCP leads to accumulation of ubiquitinated proteins, suggesting defects in VCP may impair recruitment of proteins to the proteasome (Dalal *et al.*, 2004). In the context of ALS and FTLD-associated inclusions it is tempting to speculate that the unfolding activity of VCP may be required to separate individual aggregated proteins from within large inclusions for destruction by the proteasome. Furthermore, in a notable convergence, VCP—like ubiquilin 2, p62 and optineurin—appears to also play a role in autophagy. In fact, VCP mutations cause inclusion body myopathy associated with Paget's disease of the bone and frontotemporal dementia (IBMPFD), which is characterized by the accumulation of non-functional autophagosomes together with p62 and LC3 due to defects in vacuole maturation (Ju *et al.*, 2009). Specifically, VCP seems to play a role in the selective maturation of ubiquitin containing autophagosomes to autolysosomes, suggesting that defects in this pathway may be involved in both FTD and IBMPFD (Tresse *et al.*, 2010). Indeed IBMPFD-associated VCP mutations lead to an impairment in the specific fusion of ubiquitin-containing autophagosomes with lysosomes (Tresse *et al.*, 2010). As such, like ubiquilin 2, OPTN and p62, VCP may also function at the interface of the ubiquitin proteasome system and autophagy, selectively coupling target protein ubiquitination to autophagy. Although acting at a different stage of the pathway, VCP provides further evidence that aggrephagy may well be at the heart of the ALS/FTD disease spectrum.

VCP, ubiquilin 2, optineurin and ubiquilin 2 all then act in coupling ubiquitinated target proteins to autophagy, or more specifically, aggrephagy. The clustering of ALS/FTD associated proteins within the aggrephagy pathway suggests that it is primarily defects within cargo-specific autophagy, rather than the system classically associated with the clearance of ubiquitinated proteins—the ubiquitin proteasome system—that is impaired within certain cases of ALS and FTD. Further genes encoding proteins acting within the aggrephagy pathway, especially those coupling ubiquitin to LC3, such as NBR1, would make excellent candidate genes for ALS and FTD. Given the suggested involvement of the ubiquitin proteasome system in ALS and FTD it is also noteworthy that cargo-specific autophagy can take over in situations where the ubiquitin proteasome system is not working to full capacity; indeed it appears that the two systems are interconnected and impairment of one is likely to affect the other (Korolchuk *et al.*, 2010). As such it is possible that aggrephagy is largely used when the ubiquitin proteasome system is overwhelmed by the production of protein aggregates, a possible outcome in TDP-43 and FUSopathies. Therefore defects in the ubiquitin proteasome system are still of interest within the ALS/FTD continuum and should be investigated further.

Convergence of themes: RNA processing proteins and protein degradation pathways interact

Defects in autophagy lead to accumulation of cytoplasmic RNA-processing proteins

Alterations in protein degradation and RNA processing pathways therefore seem important in ALS and FTD, but could these pathways be interrelated? One possibility is that the impairment of protein degradation pathways in neurons affected in ALS and FTD results in the abnormal function of RNA-binding proteins such as TDP-43 or FUS, perhaps through protein aggregation (Fig. 1). In support of this hypothesis, the pathology in cases harbouring mutations in the *VCP*, *OPTN* and *UBQLN2* genes is dominated by abnormal cytoplasmic levels and aggregations of TDP-43 (Neumann *et al.*, 2007; Gitcho *et al.*, 2009; Maruyama *et al.*, 2010; Ritson *et al.*, 2010; Deng *et al.*, 2011*a*, *b*). Cases with *SQSTM1* mutations await pathological characterization, but it would be no surprise to find TDP-43 pathology. In the case of FUS, however, despite some reports of FUS accumulation in cases with *UBQLN2* mutations, *in vitro* mislocalization of FUS in response to autophagy/ubiquitin proteasome system defects has not been shown in the same manner as TDP-43, suggesting that the link between protein degradation and RNA dysfunction may go through TDP-43 solely, with relocalization of FUS occurring through a more primary defect. It is notable that in cases of ALS and FTD with mutations in protein degradation genes, pathology is specific to TDP-43 (and perhaps FUS), suggesting a direct link between impaired protein degradation and accumulation of RNA processing proteins as opposed to general accumulation of aggregation prone proteins such as SOD1 or tau.

Additional support for this hypothesis comes from studies in primary hippocampal cortical neurons and motor neuron lines in which the direct manipulation of protein degradation pathways by the addition of proteasome inhibitors, or expression of mutant VCP, results in TDP-43 relocalization (Ritson *et al.*, 2010; van Eersel *et al.*, 2011). Once cytoplasmically localized (due to either *TARDBP* or *VCP* mutations) TDP-43 and VCP appear to interact and enhance neurotoxicity and aggregation (Ritson *et al.*, 2010). Within the cytoplasm, it is possible that accumulation of TDP-43 and FUS over a threshold level leads them to aggregate. Alternatively, small aggregates could spontaneously form even under normal conditions, but are usually degraded by cellular recycling pathways (Fig. 1). In either case, both TDP-43 and FUS have been shown to be intrinsically aggregation prone, with an initial seeding reaction important for wild-type and mutant TDP-43 aggregation (Johnson *et al.*, 2009; Furukawa *et al.*, 2011; Sun *et al.*, 2011). Therefore, rapid recognition and destruction of small aggregates could be of crucial importance, before a threshold of aggregated, cytosolic, TDP-43 is reached. In support of this idea it is notable that three of the four autophagy/ubiquitin proteasome system proteins linked to ALS-TDP function in coupling ubiquitinated protein material to the proteasome or autophagosome rather than at later degradation steps.

A further mechanism in which defects in protein degradation could lead to accumulation of TDP-43/FUS is through stress granules. Stress granules, as highlighted above, may be the first stage in the formation of large ubiquitinated aggregates and sequester RNA binding proteins such as TDP-43 and FUS. Notably, inhibition of the ubiquitin proteasome system has been demonstrated to lead to the formation of stress granules in a cell culture model, and hence possibly increased TDP-43 or FUS cytoplasmic sequestration or aggregation dependent toxicity (Mazroui *et al.*, 2007).

This ubiquitin proteasome system-dependent induction of stress granules is mediated by increased phosphorylation of eIF2α (Mazroui *et al.*, 2007). Notably, phosphorylation of eIF2α, a translation initiation factor, is required not only for stress granule assembly but also for starvation-induced autophagy (Kedersha *et al.*, 2002; Talloczy *et al.*, 2002). Furthermore, induction of specific oxidative stress has been demonstrated to induce autophagy (Chen *et al.*, 2008). As such, both stress granule formation and autophagy induction seem to be regulated through the same oxidative stress response-based pathway that leads to eIF2α phosphorylation. Additionally, basal autophagy is also required to prevent the build-up of reactive oxygen species, one of the conditions required to induce the formation of TDP-43 or FUS-containing stress granules (arsenite exposure leads to accumulation of reactive oxygen species) (Mathew *et al.*, 2009). Defects in autophagy could therefore lead to a build-up of reactive oxygen species, and hence stress granule mediated sequestration of TDP-43 and FUS. In this context, the ability of p62 to cause liver toxicity through upregulation of the stress response gene *Nrf2* is notable due to the role of Nrf2 in reactive

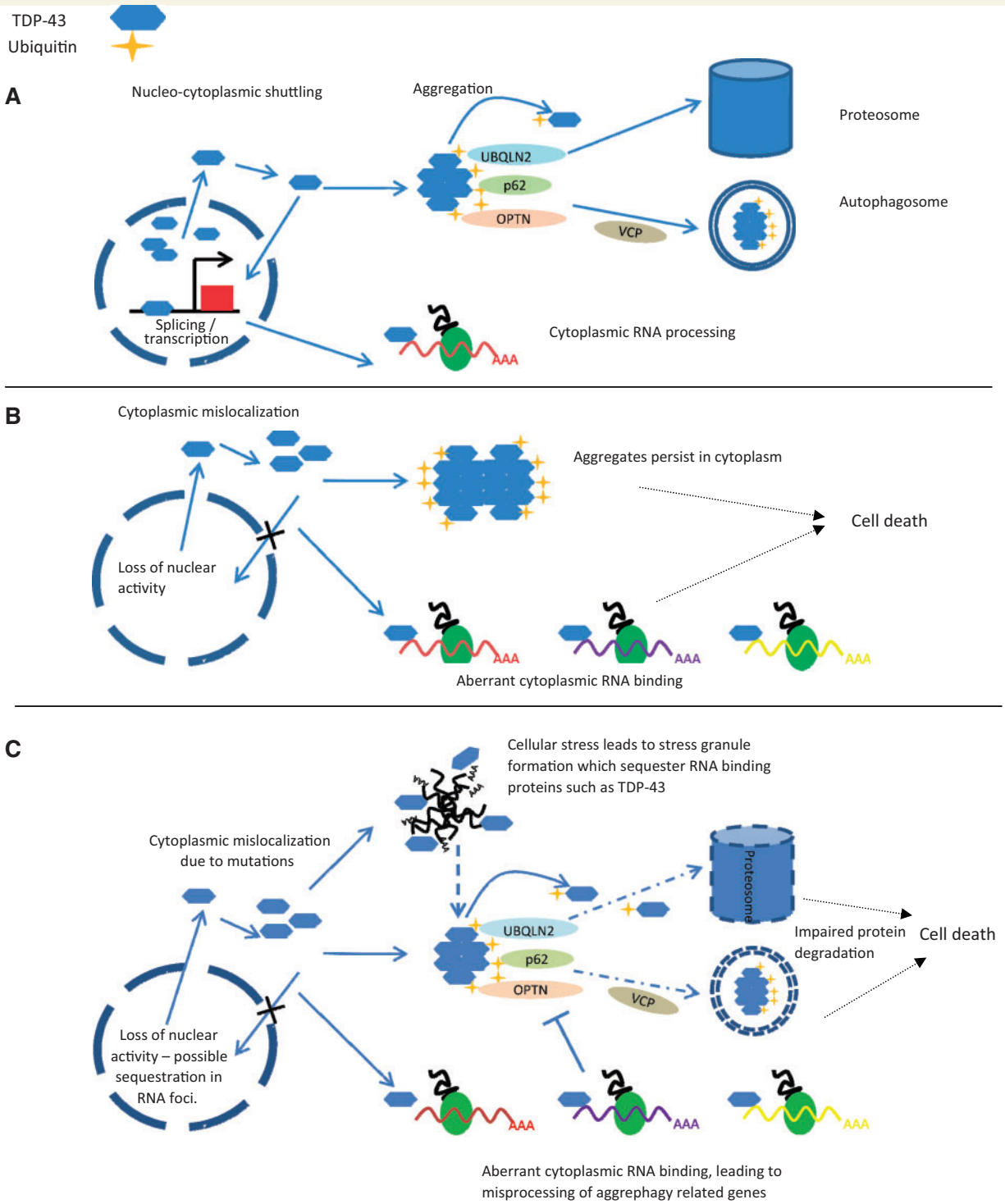

Figure 1 Pathogenesis pathways in sporadic and familial disease. Possible disease associated pathways are shown for TDP-43. FUS is likely to operate in highly similar pathways, but key details of its involvement in several steps are still to be elucidated and only TDP-43 is shown for clarity. (**A**) Normal cellular functions of TDP-43. TDP-43 shuttles between the nucleus, where it regulates splicing and transcription, and the cytoplasm, where further RNA targets are bound. Any stochastically forming aggregates are degraded by the ubiquitin proteasome system or autophagy. (**B**) Defects in protein degradation lead to a loss of nuclear TDP-43, either by directly affecting nuclear import/export or by failed aggregate destruction. Relocalization of TDP-43/FUS causes a loss of nuclear and concomitant gain of cytoplasmic RNA processing. (**C**) Loss of TDP-43 function due either to direct mutations in *TARDBP* or through sequestration in cytoplamsic stress granules or nuclear RNA foci, causes dysfunctional RNA processing which may in turn lead to defects in aggregate clearance.

oxygen species dependent signalling. Nfr2 has been shown, not only to be activated by oxidative stress but also to be required for the formation of ubiquitinated aggregates in autophagy-deficient mouse models, suggesting that oxidative stress-dependent signalling could lead to the formation of ubiquitinated aggregates, perhaps by way of stress granules (Riley *et al.*, 2010). As such, defects in protein degradation could lead to the formation of stress granules, through either ubiquitin proteasome system or autophagy inhibition, leading to the sequestration of RNA binding proteins and perhaps formation of TDP-43/FUS ubiquitinated aggregates.

Defects in RNA processing proteins may lead to dysregulation of protein degradation pathways

On the other hand, it is possible that in other cases, such as those with mutations in TDP-43 or FUS, a primary alteration in RNA processing leads to a secondary impairment in protein degradation (Fig. 1). In support of this hypothesis, depletion of TDP-43 has been shown to reduce the level of expression of the important autophagy-related protein Atg7, leading to an inhibition of autophagy (Bose *et al.*, 2011). Similarly, small interfering RNA knockdowns of TDP-43 in primary cortical neurons causes an increased vulnerability of cells to proteasome inhibition (van Eersel *et al.*, 2011). TDP-43 also appears to bind and regulate the stress response gene *Nrf2*, which is linked to the formation of ubiquitinated aggregates and is regulated by the autophagy related protein p62 (Colombrita *et al.*, 2012). Furthermore, knockdown of TDP-43 has been shown to produce downregulation of histone deacetylase 6 (HDAC6), a protein with diverse links to neurodegenerative diseases (Pandey *et al.*, 2007; Fiesel *et al.*, 2010; Cook *et al.*, 2012). In a remarkable convergence, HDAC6, a ubiquitin binding protein, appears to function within the aggrephagy pathway with a suggested function similar to that of VCP—maturation of ubiquitin specific autophagosomes to lysosomes (Lee *et al.*, 2010). As such, loss of TDP-43 function can be linked to the ubiquitin-specific autophagy pathways that have been strongly highlighted by mutations in *SQSTM1 VCP*, *OPTN* and *UBQLN2*. A further point arising from this convergence is whether HDAC6, like VCP, could play a genetic role in neurodegeneration—HDAC6 has already been shown to rescue neurodegeneration caused by ubiquitin proteasome system defects through compensatory cargo-specific autophagy (Pandey *et al.*, 2007). Notably HDAC6 has also been implicated in Alzheimer's disease through involvement in the regulation of microtubule transport dynamics and in the regulation of tau levels through acetylation of the molecular chaperone heat shock protein 90 (HSP90) (Ding *et al.*, 2008; Cook *et al.*, 2012). Acetylation status affects the propensity of HSP90 to direct misfolded proteins such as tau to a refolding or degradation-based pathway, suggesting another manner in which HDAC6 levels could affect protein degradation pathways in ALS-FTD (Cook *et al.*, 2012).

Meanwhile, analysis of RNA binding targets of FUS using RIP-chip (RNA immunoprecipitation and microarray analysis) in NSC-34 cells highlighted ubiquitin dependent proteolysis as a functional gene category enriched for FUS binding (Colombrita *et al.*, 2012). FUS binding was mapped to five separate members of the Cullin family of proteins that make up part of the cullin-RING E3 ubiquitin ligases, placing FUS as an important regulator of protein ubiquitination genes (Colombrita *et al.*, 2012). Furthermore, wild-type and mutant FUS binding has been mapped to the transcripts of *UBQLN1*, *UBQLN2*, *SQSTM1* and *VCP* (Hoell *et al.*, 2011). In the experiments by (Hoell *et al.*, 2011), it is transcripts that are uniquely bound by mutant FUS that show an overrepresentation of ubiquitin-associated proteolysis functions, providing a clear link between defective RNA processing proteins and protein degradation (Hoell *et al.*, 2011). FUS binding has also been mapped to *OPTN* messenger RNA, although this result was not found in a second, UV-CLIP, experiment (Colombrita *et al.*, 2012). FUS also appears to bind the messenger RNA of components of the eukaryotic translation initiation factor 2 required for induction of starvation-dependent autophagy as well as stress granule formation (Hoell *et al.*, 2011; Colombrita *et al.*, 2012).

Clearly, data from various studies have highlighted the fact that TDP-43 and FUS can bind to, and likely regulate, the messenger RNA of many autophagy/ubiquitin proteasome system associated genes. Impaired function of TDP-43 and FUS due to pathogenic mutations could therefore drive defects in either ubiquitin-specific or general protein clearance pathways in the cell through dysregulation of RNA processing. It is, however, necessary to note that many thousands of TDP-43 and FUS binding sites have been mapped within the transcriptome meaning the biological relevance of these interactions needs further investigation before individual interactions can be ascribed important disease-associated functions.

Another possible mechanism by which RNA binding proteins could lead to defects in autophagy or the ubiquitin proteasome system is through simple overloading of these pathways through their aberrant accumulation. The fact that ubiquilin 1 and 2, OPTN and p62 have are all found in TDP-43 and/or FUS aggregates in post-mortem disease lends support to this hypothesis. The presence of TDP-43 and FUS in stress granules seems to be key to their aggregation and pathology and may mean that large numbers of aggregations can arise quickly in the cell where they may trap key autophagy/ubiquitin proteasome system-related proteins. This could be especially important if something intrinsic to these aggregates, which could possibly derive from stress granules, makes them hard to degrade. It is also notable that in cases with *C9orf72*-associated ALS and FTLD, ubiquilin and p62 positive, TDP-43 negative aggregates have been described (Brettschneider *et al.*, 2012; Troakes *et al.*, 2012). As such a gene with a putative RNA-mediated mechanism of toxicity may also be leading to the aggregation and hence impairment of proteins required for normal cellular protein degradation pathways.

Various data has therefore shown that defects in RNA processing proteins could well have downstream effects on protein degradation pathways, either through improper regulation of key ubiquitin proteasome system or autophagy-related genes or through their tendency to form stress granule-associated aggregations, which may overwhelm cellular clearance mechanisms.

Conclusions

Much progress has been made in explaining the continuum existing between ALS and FTD based on an ever-expanding set of shared clinical, pathological and genetic data. Pathologically, TDP-43 and FUS proteinopathies provide much of this overlap, suggesting that events leading to the cytoplasmic relocalization of these two similar RNA processing proteins are key for the development of ALS/FTD, with SOD1 and tau pathology being distinct pathological entities located at the very ends of the spectrum.

Functional analysis of the genes along this ALS/FTD continuum suggests that RNA processing and protein degradation pathways, especially aggrephagy, are central pathogenic mechanisms. Dysfunctional RNA processing is linked strongly to each side of the ALS/FTD continuum, either genetically or pathologically, by FUS, TDP-43 and C9orf72. The association of both TDP-43 and FUS with stress granules and the possible formation of RNA foci due to *C9orf72* repeat expansions specifically highlight cytoplasmic sequestration of key RNA processing proteins in disease. Both dysfunction of RNA processing leading to impairments of key downstream targets, and the formation of toxic, possibly stress granule-derived, aggregates are implicated in disease progression.

Protein degradation is linked to both ALS and FTD pathologically and genetically, though it is notable that, currently at least, genetic links to protein degradation are stronger for ALS than FTD. Although *VCP* and *SQSTM1* have been associated with both ALS and FTD, to date no *OPTN* mutations have been found in FTD, despite a screen of 371 cases (Rollinson *et al.*, 2012). Furthermore, only a single *UBQLN2* mutation of unconfirmed pathogenicity has been linked to a case of pure FTD, although the number of cases screened was low ($n = 45$) (Synofzik *et al.*, 2012). Whilst this may reflect a greater sensitivity of motor neurons to protein degradation pathways it is also possible that further screening will lead to the discovery of causative *UBQLN2* and *OPTN* mutations in FTD as well as ALS. The association of *UBQLN2*, *VCP*, *OPTN* and *SQSTM1* with the ALS/FTD continuum specifically links ubiquitin-specific autophagy, or aggrephagy, to disease. This genetic inference fits with the pathological findings of both ALS and FTD in which end-stage disease shows the presence of ubiquitinated aggregates within affected neurons.

Furthermore, it is clear that dysfunction in either RNA processing or aggrephagy may impact upon the other pathway; both may play interrelated roles in the pathogenesis of ALS and FTD. Within sporadic disease, the close relationship of both stress granule-mediated sequestration of RNA binding proteins and autophagy with oxidative stress is notable and should be explored further.

Given the possible relationship between key autophagy/ubiquitin proteasome system proteins and those involved in RNA processing, it will be interesting to look at the relationship between aggregation and toxicity in wild-type and mutant TDP-43, and whether this relationship is modified by defects in ubiquitin-specific autophagy or the ubiquitin proteasome system. Interactions between mutant and wild-type TDP-43, FUS and VCP, ubiquitin 2, optineurin and p62 should also been investigated to define mutation-specific effects on the interplay of these interlinked proteins.

Within cases with sporadic ALS and FTD it would be interesting to investigate whether general impairments in protein degradation or RNA processing are seen. In fact, while we have argued that mutations in several genes can lead to a primary alteration in either RNA processing or protein degradation pathways with a secondary impairment in the other pathway, the question remains whether defects in these same mechanisms are also causing sporadic disease. Although some evidence suggests that proteasome activity is decreased with age or in cases with Alzheimer's or Parkinson's disease, this observation may not hold true in cases with sporadic ALS and FTD (Keller *et al.*, 2000; McNaught *et al.*, 2001). Regarding a primary alteration in RNA pathways in sporadic ALS and FTD, it is also possible that sequestering of RNA processing proteins is mediated by aberrant, stochastically forming, RNA foci or that prolonged cellular stress due to a variety of sporadic factors could lead to sequestration of TDP-43 or FUS in stress granules causing general RNA dysfunction.

In order to further study the pathology of ALS and FTD, more relevant models of the disease are likely to be required; current ALS and FTD transgenic models are often not fully relevant to the ALS/FTD continuum pathways, with, for example, much of ALS research based on SOD1 models, which may show an entirely separate model of pathology to that of ALS-FTD. Attention should therefore be focused on creating both *in vivo* and *in vitro* models to study TDP-43, FUS, C9orf72, p62/SQSTM1, OPTN, VCP and UBQTN1/2 and the interactions between wild-type and mutant forms of each protein. Finally, construction of disease-associated pathways should allow putative therapeutic targets to be considered. Although not yet fully characterized at a molecular level, the pathways constructed here highlight aberrant RNA processing and defects in aggrephagy as possible targets for therapeutic action in ALS and FTD. Modulation of aggrephagy through chemical or genetic means to inhibit or enhance the levels or activity of key proteins such as VCP, p62, OPTN, UBQLN2 and HDAC6 could all hold promise in the reduction of protein aggregation in ALS/FTD. However, as the finding that excess p62 levels lead to liver damage demonstrates, simply increasing the activity of autophagy-related proteins could lead to undesired side-effects (Komatsu *et al.*, 2010). How one might therapeutically combat the loss of nuclear TDP-43 or FUS is less clear, given the ubiquity of nuclear import and export processes and the global roles of TDP-43 and FUS within the transcriptome and beyond. The recent discovery that arginine methylation is a potent modifier of FUS nuclear import does however highlight that novel mechanisms to achieve this aim may be possible as our understanding of these central proteins and pathways increases (Dormann *et al.*, 2012).

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