FAMILIAL AMYOTROPHIC LATERAL SCLEROSIS WITH A FOCUS ON C9ORF72 HEXANUCLEOTIDE GGGGCC REPEAT EXPANSION ASSOCIATED ALS WITH FRONTOTEMPORAL DEMENTIA

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STATEMENT BY AUTHOR

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Abstract:

Amyotrophic Lateral Sclerosis (ALS) is a rare and fatal neurodegenerative disorder resulting in the loss of motor neurons from the spinal cord and frontal cortex. The patterns of neurodegeneration, affected regions, age of onset, and time course of disease progression are all highly variable between and within variants of the disease. Familial ALS (fALS), inherited versions of ALS due to genetic changes, accounts for between 5-20% of all ALS cases, while the rest are sporadic, with either no causative mutation identified or no familial history of ALS. Recently, the discovery of *C9orf72* hexanucleotide repeat expansions have been identified as one of the most common causes of familial ALS, with some patients presenting with dual phenotypes of ALS and frontotemporal dementia, leading to new hypotheses about the nature of neurodegenerative diseases. Despite the continued discovery of new ALS causative genes, little is known about the pathogenesis of the disease. While almost all variants include the presence of intracellular protein inclusions, the site of the protein plaques and involved proteins varies between genetic and phenotypic variants of this disease. Due to the lack of clear pathogenic mechanisms, several hypotheses have been developed to explain the process of neurodegeneration. Autophagy, the process of self-eating, leading to destruction of damaged or excess proteins and organelles, has been implicated as being altered in ALS. Multiple variants have demonstrated altered mitochondrial morphology and cellular energetic dynamics, which could explain previous observations that implicate the process of apoptosis in cellular death. Many of the involved proteins in ALS have functional roles for intracellular, nucleocytoplasmic, and axonal transport of various proteins or RNA. These three competing hypotheses are currently the most prominent hypotheses in the pathogenesis of ALS, and have largely been considered as separate and competing in past research. Is there a chance that the true pathogenesis leading to neuronal destruction via apoptosis involve all three hypotheses? Altered protein and RNA transport dynamics could lead to changes in cellular stress responses or overload autophagy pathways, leading to exacerbated cellular stress responses, leading to alterations in mitochondrial morphology and eventually cell death via apoptosis.

Introduction:

Amyotrophic lateral sclerosis (ALS) comprises a heterogeneous group of neurodegenerative disorders that result in the destruction of motor neurons, primarily in the ventral horn of the spinal cord. This destruction of motor neurons in the spinal cord is, in some cases, also accompanied by the destruction of neurons in the frontal and temporal lobes of the brain, resulting in a condition known as Amyotrophic Lateral Sclerosis with Frontotemporal dementia (ALS/FTD). Over 20 different subtypes of ALS have been identified, with the following being used in clinical diagnostic criteria: pattern of inheritance, pattern of neurodegeneration, affected muscles, time course of progression, involved proteins and mutations in genes, age of onset, and presence or absence of frontotemporal dementia symptoms (Yamashita and Ando 2015). Because of the range of involved muscle groups, and the potential for the presence or absence of frontotemporal dementia, ALS is commonly considered to exist as part of a spectrum with diseases of pure motor neuron degeneration at one end, and diseases of purely cortical neural degeneration (Figure 1).

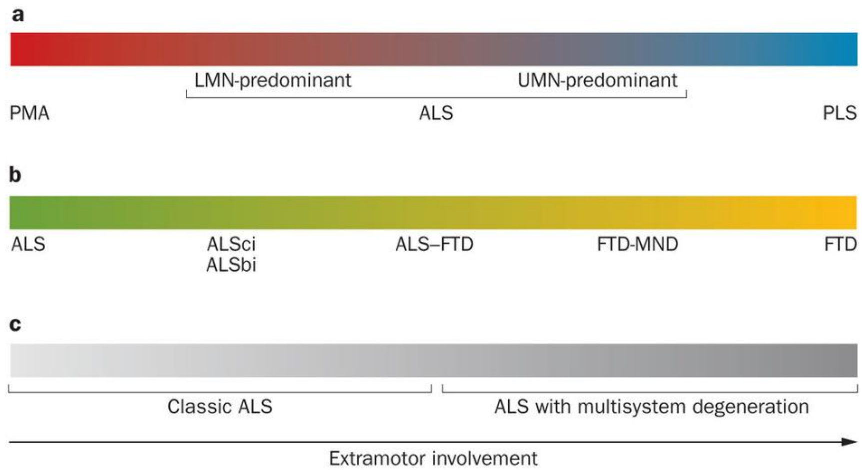


Figure 1. The spectrum of disorders encompassing ALS and associated pathologies. The current thinking about ALS has placed it in several different spectrums of other neurodegenerative disorders. The middle scale is the most important for this paper, with ALS at one end and frontotemporal dementia (FTD) at the other end of the spectrum, with intermediate disorders commonly observed in neurodegenerative diseases. With the discovery that *C9orf72* expansions can clnically correlate with both ALS and FTD, this spectrum has become very important in the thinking about ALS.
Swinnen and Roberecht 2014

Common symptoms of ALS begin with muscle weakness of the proximal muscles, most notable in the neck, upper arms, chest, and in many cases muscles of the face that aid in swallowing or speaking (bulbopharyngeal onset), ultimately progressing to severe muscle weakness, and in most cases death due to loss of control of muscles involved in inspiration, primarily the diaphragm and intercostal muscles. Because these symptoms are somewhat common in many neurodegenerative disorders, this leads to some difficulty in diagnosis of the disease, with genetic analysis being the only true way to confirm ALS as the correct diagnosis (Mancuso and Navarro 2014). With upwards of 20 different genes having been implicated as causative or contributing to ALS, even this can be somewhat of a challenge. Mutations in SOD1 were the first analyzed (Rosen et al 1993), and were previously thought to be the most common cause of familial ALS (fALS), resulting in 15-20% of all cases (Bruijn et al 1998), until the discovery of the gene locus at C9orf72, a chromosomal location corresponding to chromosome 9, which is thought to account for upwards of 40% of all fALS cases, and another 5-6% of sporadic cases (Farg et al 2014).

The most common pathological feature of ALS is the presence of protein inclusions in the cytoplasm of pyramidal neurons. These protein inclusions are markers for pathology of the disease, and do not occur normally. Protein inclusions typically consist of one of several proteins that have been ubiquitinated and commonly hyperphosphorylated: SOD1, TDP43 and FUS are the most commonly occurring proteins to be found in these insoluble inclusions. Whether the presence of protein inclusions is causative of ALS or a symptom of the disease progression is a topic that is under intense study, with little hope for a clear answer anytime soon.

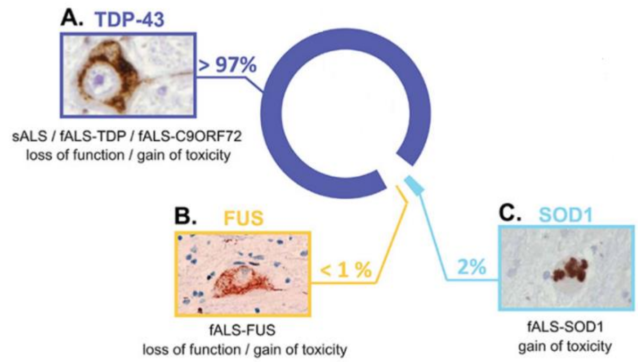


Figure 2: Examples of the protein inclusions seen in ALS, and the prevalence of specific proteins in these inclusions. A) TDP-43 is most commonly found in plaques, seen in about 97% of all ALS cases. An example of an inclusions stained using imunohistochemistyr for TDP-43 can be seen. B) Mutations in FUS are one of the more commonly seen. In FUS mutant patients, FUS containing inclusions are commonly seen, but only account for ~1% of all cases. C) SOD1 mutations are some of the most commonly seen as causative of ALS, but SOD1 is only found in ~2% of protein inclusions seen in ALS. Laferrière and Polymenidou (2015).

Clinical Picture:

The nature of ALS results in significant geneotype-phenotype heterogeneity in terms of age and location of onset, progression and further affected neuromuscular groups, time-course of disease progression, inheritance pattern in familial cases, involved gene mutations, and overall severity of the symptoms.

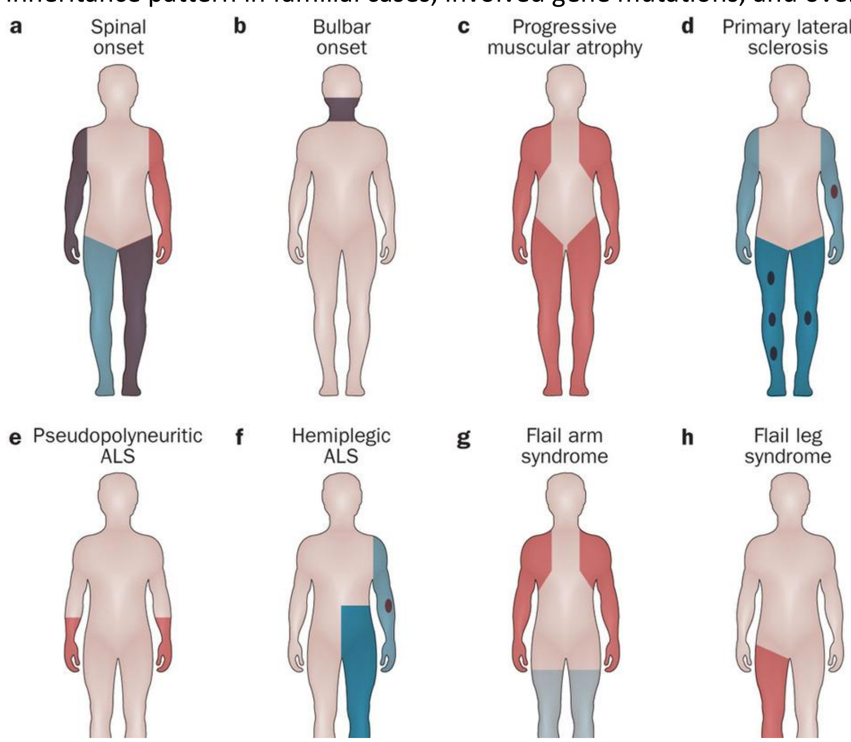


Figure 3. Common phenotypic presentations associated with variants of ALS. Swinnen and Roberecht (2014)

Figure 3, to the left, demonstrates some of the common phenotypes associated with ALS, and their respective patterns of upper and lower motor neuron involvement (Swinnen and Roberecht 2014). Upper motor neuron symptoms stem from neurodegeneration in the primary motor cortex of the brain, whereas lower motor neuron symptoms stem from neurodegeneration of motor neurons in the spinal cord, and in different variants of ALS, remarkably distinct patterns of involvement of these two regions are seen. Upper motor neuron degeneration typically results in spasticity or hyperreflexia, whereas lower motor neuron degeneration results in atrophy, loss of function, and twitching. All of these phenotypes have been observed in at least one of the many variants of ALS. Because of the vast phenotypic heterogeneity associated with ALS, there are multiple different factors by which the phenotypes of ALS can be sorted.

Status as an Orphan Disease

Based on consensus from the the FDA and NIH, ALS is currently considered an orphan disease (FDA *Orpan Products* page 2016; NIH Genetic and Rare Disease). By definition, an orphan disease is a disease in which less than 200,000 persons are affected nationwide. With an anticipated worldwide incidence of between 1:100,000 to 1:300,000, and a worldwide population of approximately 9 billion, we can expect between 30,000 and 90,000 people to be affected at any point in time worldwide. Because of the nature of orphan diseases, in trials and experimental research, sample size is not considered as important of a factor in establishing linkage to specific genes, in establishing specific morphological or pathological features of the disease, or in consideration of study design. In most studies, as many people as possible are included. However, the nature of the disease leading to progressive paralysis makes travel impossible for many of these patients, and with a limited population distributed across the globe, considerations of the sample size of the disease are less important than confirming the diagnosis of ALS in these patients via genetic and clinical analysis. Confirmation of an ALS diagnosis, as such, is the truly important factor in the design of studies focusing on ALS.

Gentoype:

In cases of sporadic and familial ALS, the underlying cause is commonly a mutation in one or more genes associated with ALS. In cases where an established mutation is discovered to be causative, patients can be sorted on the basis of the involved gene, and associated mutation. This provides limited clinical utility, as within variants of ALS there is significant heterogeneity in terms of progression, though some do show specific preponderances. For examples, *C9orf72* hexanucleotide repeat expansion carrying ALS patients typically present with bulbar involvement, and upper motor neuron dominance. On the other hand, patients with *SOD1* mutations almost never develop bulbar symptoms as the first sign of disease progression (Swinnen and Roberecht 2014).

Upper motor neuron versus lower motor neuron dominance

Upper motor neuron symptoms are the result of neurodegeneration in the primary motor cortex, and include weakness, spasticity, and increased deep tendon reflexes. Lower motor neuron symptoms are the result of neurodegeneration in the spinal cord and include fasciculations (abnormal electrical activity in the muscle fascicle layer), atrophy, and weakness.

In many cases of ALS, either upper motor neuron signs or lower motor neuron signs are seen as the predominant clinical symptoms associated with the disease. Indeed, many patients with primary lateral sclerosis only display upper motor neuron signs, failing to meet the criteria for a diagnosis of ALS, but displaying many of the symptoms. Conversely, patients with progressive muscular atrophy or flail arm syndrome tend to predominantly show lower motor neuron signs, with little to no upper motor neuron involvement. However, all of these diseases fit within a spectrum of neurodegeneration related to ALS, and these phenotypes can commonly be seen as the onset of ALS symptoms. The level of neuronal involvement in the disease has been shown to correlate to the severity of symptoms.

Location of onset, age of onset, and disease progression

Multiple different sites of origin are demonstrated within clinical ALS, and have variability relating to the specific variant of ALS the patient is affected with. As previously stated for example, *C9orf72* hexanucelotide repeat expansions tend to correlate with bulbar symptoms as the site of onset. Alternatively, causative mutations in *VAPB* tend to correlate with onset in any of the limbs (Li and Wu 2016).

Age of onset is also highly variable between and within disease variants. For example, several familial ALS variants display a juvenile progression, such as ALS associated with mutations in *SETX* and *SPG11*. This is directly in contrast to classical ALS with an adult onset in the 5^{th}-6^{th} decade of life.

Time course of disease progressions displays significant phenotypic variability as well, between and within variants. Certain mutations in *SOD1* have been seen to correlate to a rapid progression, such as the A4V variant commonly seen in North America. In contrast, other variants display a slow progression, as seen in cases associated with *VAPB* or *OPTN* mutations (Li and Wu 2016).

Diagnostic Criteria

Two different major diagnostic criteria systems have been established in the diagnosis of ALS over the last several decades. The most commonly used diagnostic criteria include the El Escorial criteria established in 1990, which was revised in 2000 to become the revised El Escorial criteria, and more recently the Awaji criteria, established in 2008.

El Escorial Criteria

Established in 1990 by a committee of neurologists in El Escorial, Spain, the El Escorial criteria were considered the essential features in patients that would lead to a diagnosis of ALS (Brooks 1994).

The necessary clinical features for a diagnosis of ALS via the El Escorial criteria include: presence of lower motor neuron signs by clinical, electrophysiological, or neuropathological examination, the presence of upper motor neuron signs by clinical examination, and progressive spread of symptoms within muscle groups or to other regions. In addition, diagnosis necessitates lack of evidence supporting other disease process that can explain the presence of electrophysiological or neuropathological findings and neurological imaging that lacks evidence of other disease processes. In the process of diagnosing ALS, an examination of medical history, physical analysis of the patient, and neurological or electrophysiological analyses to confirm lower motor neuron signs will be performed early in the process of the diagnosis. Further studies to determine ALS will include neuroimaging studies to identify patterns of degeneration, clinical laboratory examinations to rule out other disease processes, and neuropathological examinations to determine motor neuron involvement and location of involvement. If early studies suggest the presence of clinical ALS on any of the defined levels (suspected, possible, probable, and definite ALS), a follow-up examination should be performed after at least 6 months to establish progression of the disease.

In the diagnosis of ALS, upper motor neuron and lower motor neuron signs can occur in any of the 4 spinal cord regions; bulbar, cervical, thoracic, and lumbrosacral. In diagnosing the presence of lower motor neuron involvement, symptoms of weakness, atrophy, and fasciculations, irregular electrical activity leading to contractions in the fascicle. In diagnosing upper motor neuron symptoms, the presence of abnormal reflexes, repeated and rhythmic muscular contractions, and spastic tone are clinical features suggesting upper motor neuron involvement. Spread of symptoms between vertical regions of the body can be best used to establish progression, as lateral spread of the disease may not indicate the involvement of any new neuronal tissues.

Within the El Escorial criteria, 4 different diagnoses are available: suspected, possible, probable, and definite ALS. A diagnosis of suspected ALS is based on the presence of only lower motor neuron clinical symptoms in two or more affected regions. Possible ALS is the diagnosis given to patients who demonstrate upper and lower motor neuron involvement in only one region, display only upper motor neuron signs in two or more regions, or when lower motor neuron signs occur in muscle groups closer to the front of the body than the location of upper motor neuron signs. Probable ALS requires involvement of upper and lower motor neuron signs in at least 2 different affected regions. Definite ALS has the most stringent criteria, and requires presence of upper and lower motor neuron symptoms in the bulbar region and at least two other regions, or the presence of upper and lower motor neuron involvement in 3 different spinal cord regions. In addition,, diagnoses of ALS can be differentiated into familial and sporadic by the presence of familial involvement, and can also co-exist with other disorders, or occur with only motor neuron involvement, leading to diagnoses of: sporadic ALS in patients with no other comorbid disorders, coexistent sporadic ALS if seen with other neurological symptoms, ALS-linked disorders occur when disease progression leads to other abnormalities, and based on genetic and epidemiological differences into variants of ALS.

Revised El Escorial Criteria

In revising the El Escorial criteria, limited changes were made to the diagnostic process, but with the progression of diagnostic and imaging techniques, have been made slightly more stringent, while expanding the range of disease diagnoses (Brooks et al 2000).

While the initial diagnostic criteria have been unchanged in this revision, it has now been established that the presence of upper and lower motor neuron involvement must be demonstrated. In addition, a diagnosis of Clinically Probable- Laboratory Supported ALS was added for patients who demonstrate involvement of upper and lower motor neuron involvement in only one region, or patients who demonstrate upper motor neuron involvement in one region with lower motor neuron symptoms in two regions, and available laboratory and imaging studies have been performed to exclude diagnosis of related disorders. At the same time, the recognition that ALS can be associated with multiple other disorders was made, leading to the establishment of ALS-Plus diagnoses to explain patients with extrapyramidal involvement and patients with comorbid frontotemporal dementia, as examples of possible ALS-Plus disorders. ALS-Mimic syndromes were also discussed, which include diseases that match some of the clinical phenotypic characteristics of ALS and may lead to a misdiagnosis. For patients with suspected ALS and laboratory results indicating abnormalities in motor neuronal function and innervation patterns that are not distinct to ALS, a diagnosis of ALS with Laboratory Abnormalities of Uncertain Significance was established.

Awaji Criteria

The Awaji criteria for establishing clinical ALS were determined in 2006 at a consensus symposium held on Awaji Island in Japan, from which the diagnostic criteria have derived their name. During this symposium, the previously established El Escorial Criteria were reviewed point by point, and comments were generated (de Carvalho et al 2008).

The most significant change proposed during the Awaji symposium was the concept that needle electromyography (EMG) results should be accepted with the same diagnostic value as clinical examinations that demonstrate the presence of neurogenic changes. The expected result of this change is to allow for earlier detection of single limb involvement, which may allow for earlier diagnosis in clinical ALS patients. This also led to the removal of the diagnostic criteria Clinically Probably ALS Laboratory Supported, with the inclusion of needle EMG data in diagnosis this category becomes essentially non-existent.

When performing needle EMG on patients, it was also decided that presence of detectable fasciculations should hold the same value as the presence of fibrillations. However, these only hold value in cases of clinically probable ALS, as fasciculations can occur in normal muscles. In addition, the symptoms of unstable motor unit potentials which can be detected via needle EMG as changes in the firing of motor units.

In addition, this conference noted the difficulty in diagnosing the presence of upper motor neuron symptoms. While available techniques, such as central motor conduction time tests and the triple stimulation method, a variation of motor conduction tests that involves transcranial magnetic stimulation, and induces 2 impulses simultaneously to determine the presence of abnormal conductance patterns (Magistris et al 1999), have been shown to have some success in facilitating the diagnosis of upper motor neuron symptoms, the development of new techniques is needed, and further analysis of currently available techniques is suggested.

Further Steps

While the El Escorial and Awaji criteria allow for the successful diagnosis of many ALS patients, the sensitivity of the Awaji criteria is currently suggested to be around 60%, while the sensitivity of the El Escorial criteria is seen to be about 45% (Geevasinga et al 2016). While neither of these provide the necessary sensitivity to diagnose all affected patients, they do provide clinical diagnostic value for the determination of diagnoses of ALS versus other neurodegenerative disorders. With advancements in genetic techniques, genetic material isolated from patients can be analyzed to look for changes in one of multiple causative genes associated with ALS to aid in confirmation of the diagnosis. However, almost all researchers will agree that the diagnostic criteria need to be further studied, further revised, and will require the creation of new diagnostic techniques and possibly the implementation of new diagnostic criteria to allow for identification of all cases of ALS and ALS with associated disorders (Agosta et al 2015).

The necessity for a more sensitive criteria is one of the most crucial steps in further expanding the clinical spectrum of ALS and neurodegenerative diseases in this author's opinion. While criteria with a sensitivity of approximately 70% is acceptable for a rare disease such as ALS, a major goal of research should be to determine if changes to these criteria can improve the sensitivity of the test without decreasing the specificity and creating even more overlap in diagnostic criteria for neurodegenerative diseases, such that we can gain new insights by identifying new rare variants.

In addition, a major goal should be the development of clinical criteria that can allow for early diagnosis of ALS, as many studies have generated results demonstrating that earlier intervention and treatment can decrease severity of symptoms in both patients and mice (Riviere et al 1998; Li et al 2015). Thus, one of the goals of ALS research should be determining useful early clinical biomarkers in patients affected patients, or in persons who would be suspected to be affected, such as those children of affected ALS patients in familial ALS cases.

ALS Functional Rating Scale

The ALS Functional Rating Scale provides a rating of remaining motor function for patients, based on 12 factors, each assigned a rating between 0 and 4 (ACTS 1996) This rating scale is used to evaluate the decline of motor function over time, and has been shown to provide clinical predictive value in determining the progression and likely death of patients. A table of the rating system can be seen below.

In 1999, one of the research groups involved in the clinical assessment of the ALS functional rating scale released a set of revised criteria for the functional rating of ALS symptoms (Cedarbaum et al 1999). The revised rating scale included three new symptoms expanded from the "Breathing" rating component of the original scale. These three new factors can be seen in the table below as well; these have been italicized for differentiation as part of the revised scale rather than classical scale.

Factors	Rating	0	1	2	3	4
1	Speech	Loss of useful speech	Speech combined with non-vocal communication	Intelligible with repeating	Detectable speech disturbance	Normal speech processes
2	Salivation	Marked drooling; requires constant tissue or handkerchief	Marked excess of saliva with some drooling	moderately excessive saliva; may have minimal drooling	Slight but definite excess of saliva in mouth; may have nighttime drooling	Normal
3	Swallowing	NPO (exclusively parenteral or enteral feeding)	Needs supplemental tube feeding	Dietary consistency changes	Early eating problems--occasional choking	Normal eating habits
4	Handwriting	Unable to grip pen	Able to grip pen but unable to write	Not all words are legible	Slow or sloppy; all words are legible	Normal
5a	Cutting food and handling utensils (without gastronomy)	Needs to be fed	Food must be cut by someone, but can still feed slowly	Can cut most food, although clumsy and slow; some help needed	Somewhat slow and clumsy; no help needed	Normal

5b	Cutting food and handling utensils (with gastronomy)	Unable to perform any aspect of the task	Provides minimal assistance to caregiver	Some help needed with closures and fasteners	Clumsy but able to perform all manipulations independently	Normal
6	Dressing and hygiene	Total dependence	Needs attendant for self-care	Intermittent assistance or substitute methods	Independent and complete self-care with effort or decreased efficiency	Normal function
7	Turning in bed and adjusting bed clothes	Helpless	Can initiate, but not turn or adjust sheets alone	Can turn alone or adjust sheets, but with great difficulty	Somewhat slow and clumsy; no help needed	Normal
8	Walking	No purposeful leg movement	Non-ambulatory functional movement	Walks with assistance	Early ambulation difficulties	Normal
9	Climbing stairs	Cannot do	Needs assistance	Mild unsteadiness or fatigue	Slow	Normal
10	Breathing	Ventilator dependent	Intermittent ventilatory assistance required	Shortness of breath at rest	Shortness of breath with minimal exertion	Normal
R10	*Dyspnea*	*Significant difficulty, considering using mechanical respiratory support*	*Occurs at rest, difficulty breathing when either sitting or lying*	*Occurs with one or more of the following: eating, bathing, dressing*	*Occurs when walking*	*None*
R11	*Orthopnea*	*Unable to sleep*	*Can only sleep when sitting up*	*Needs extra pillows in order to sleep (more than two)*	*Some difficulty sleeping at night due to shortness of breath, does not routinely use more than two pillows*	*None*
R12	*Respiratory Insufficiency*	*Invasive mechanical ventilation by intubation or tracheotomy*	*Continuous use of BiPAP during the night and day*	*Continuous use of BiPAP during the night*	*Intermittent use of BiPAP*	*None*

Table 1: The ALSFRS (ALS Functional Rating Scale) and ALSFRS-R (Revised ALS Functional Rating Scale) criteria for the evaluation of neurodegenerative decline in ALS patients. Clinically, these scales have proven to be valuable predictors of the time course of the disease, and provide clinicians a means for

determining necessary associated care and management of symptoms, as well as allowing for determination of when to perform drastic procedures, such as full-time respiratory ventilation and end of life support.

Treatment

While ALS is an incurable disease, there have been many attempts to generate treatments that would improve the lifespan of patients, or that would alter the progression of symptoms to allow patients to exist with a more normal quality of life for extended periods of time compared to the absence of treatment.

Currently, the only approved molecular therapy for the treatment of ALS is administration of riluzole. Treatment of ALS with riluzole was approved in the United States in 1995 after a double-blind placebo controlled study demonstrated increased survival in patients treated with riluzole compared to control (Benisom et al 1994; Hugon J 1996; Petrov et al 2017). In this study, after 1 year, 74% of patients receiving riluzole were still alive versus placebo. The calculated survival advantage for riluzole treated patients was 49% versus 37% in placebo treated patients, indicating an increase in survival for patients treated with riluzole. In addition, treatment of transgenic mice with riluzole has shown similar efficacy as in that of human patients (Gurney 1997). Despite this, the efficacy of riluzole is still limited. Data from clinical trials suggested a 16% improvement in survival in ALS patients, which is limited as best. As the average disease progression ranges anywhere from 1-5 years, this amounts to an increased life expectancy of 2-10 months, which while significant, is a somewhat limited extension in lifespan for the patients. Functionally, riluzole acts as a neuroprotective compound via anti-glutamatergic effects (Doble 1996).

In the years since the approval of riluzole, over 60 compounds have been evaluated for clinical efficacy in large clinical trials. Of these, none have displayed clinical efficacy in the treatment of ALS (Petrov et al 2017).

Of the many compounds that have been developed, few have displayed clinical efficacy on the primary endpoint of the study, which in most cases is to improve the lifespan of patients. Edaravone, a compound possessing free-radical scavenging and anti-oxidative properties has displayed some success in clinical trials, though this drug once again did not demonstrate efficacy versus placebo for the full ALS population studied, but may provide efficacy in specific patient populations, specifically those who display increased levels of free radical species (Tanaka et al 2016).

Masitinib is a receptor tyrosine kinase inhibitor which in ALS SOD1 G93A mice has been demonstrated to improve life span and decrease symptoms in mice that had already reached end-stage paralysis (Trias et al 2016). Currently, a Phase 3 clinical trial is underway to evaluate the clinical efficacy of masitinib in the treatment of ALS, with preliminary analysis of results halfway through the study suggesting clinical efficacy in improving the ALSFRS-R rating in treated patients versus placebo. In addition, the inclusion criteria for the study are significantly broader than many, indicating that the study designer's suspect this treatment may be effective for a large number of affected ALS patients, as normally, inclusion criteria for ALS clinical trials tend to be limited based on genetic differences. The pending final results of this study are very interesting, as the efficacy of the drug was not expected to be seen in mice, let alone humans, and does not seem to target any of the involved or implicated proteins in the progression of ALS, which will certainly make understanding the mechanism of action of the drug in reducing symptoms of ALS an area of research importance for many years to come, particularly if the results of the Phase 3 study end in approval of the drug for treatment.

Treatment with mesenchymal stem cells has also been suggested as a possible therapy for modulation of ALS symptoms. Transplantation of human motor neurons generated from mesenchymal stem cells into the meningeal spaces of the spinal cord has shown efficacy in mice, resulting in delayed onset of symptoms and increased lifespan, both of which were statistically significant (Lee et al 2014). Early clinical trials have suggested that this form of therapy may provide beneficial results. Two Phase I clinical trials were conducted in 2012 and demonstrated safety and efficacy of mesenchymal stem cell transplant and injection into the lumbar tracts of patients, with no adverse side effects noted (Riley et al 2012; Glass et al 2012). A second study looking at the safety of intraspinal injection was also performed in 2014, with similar results documented (Feldman et al 2014). In a phase one clinical trial, 6 end stage patients were randomized, and 3 injected with mesenchymal stem cells unilaterally, while the other three were injected bilaterally in the lumbar cord tract (Mazzini et al 2015). Two of the patients in the study displayed increases in their ALSFRS-R score, with a third displaying increased motor response and coordination in the tibialis anterior. This stage I trial demonstrated some clinical efficacy in the treatment of ALS patients with mesenchymal stem cells. In a second stage I/IIa study performed in 2017, injection of 26 patients with bone marrow derived mesenchymal stem cells was performed (Sykova et al 2017). In this study, many patients reported adverse events of a severe headache following injection. However, functional improvements in several ALSFRS-R categories was noted in patients, which lasted for 6 months after improvement began (a total of 9 months from injection to functional decline).

It will be interesting to see the development of Stage II and Stage III clinical trials looking at the efficacy of mesenchymal stem cell injection, as this appears to be a potentially very useful therapeutic technique. With the death of neurons being the ultimate pathology of ALS, if these could be replaced with the use of mesenchymal stem cells, it would seem that the symptoms of the disease could be ablated. The modest effects seen in clinical trials so far are likely indicative of the continued progression of the disease, making it likely that in patients where this treatment is given, continued injections every 3-6 months would be necessary to see continued improvement in patients.

Recently, in a transgenic line of ALS mice, expressing human mutant G93A SOD1, researchers have demonstrated that administration of a PET imaging compound Cu^{II}(atsm) which results in the release of copper from tissues with damaged mitochondria has shown success in preventing the development of symptoms and extending quality and length of life. Mice expressing *SOD1* mutations tend to have SOD1 that is deficient in copper, though available research has not made this mechanism clear; while treatment with Cu^{II}(atsm) has been shown to increase the expression of mutant SOD1 (Roberts et al 2014), it also results in excess copper for SOD1 to bind which theoretically increases reactive oxygen species scavenging. In addition, if administered to mice from birth onward, survival of SOD1 G93A mice was extended to over 600 days of asymptomatic life, significantly longer than the expected 70-90 days for onset in untreated controls, and controls where treatment was stopped 21 days post-natal (Hilton et al 2017).

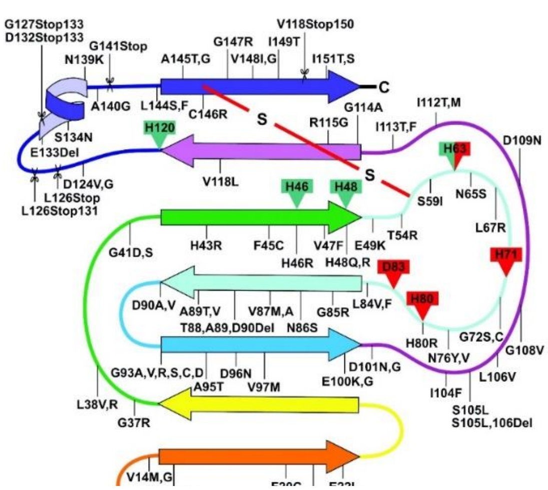
End of life care and support is particularly necessary in ALS patients. Typically, as progression of the disease continues and motor function is lost, the patient needs more and more specialized care from a team of physicians, nurses, hospice workers, and will often require in-home care. As patients decline, they will begin to need help with everyday tasks such as getting dressed, maintaining personal hygiene, feeding oneself, and even breathing. All of these can be supported with the use of in-home, in-hospital, or in-hospice care. In addition, as the patient declines, they will be more than likely need to attend additional physician visits to monitor their progression. All will necessitate the patient to acquire assistance, likely in the form of a private nurse, or in end stage disease hospice care. It may also be necessary for ventilation or intubation of patients to occur to provide mechanical support for respiration and physical support for feeding (Soriani and Desnuelle 2017).

For the treatment of *C9orf72* hexanucleotide repeat expansion associated ALS with FTD, one specific treatment options may someday be available, having shown significant efficacy in mice. Antisense oligonucleotide ablation of repeat expanded RNA in mice transgenically expressing GGGGCC repeat expanded *C9orf72* has shown significant efficacy in reducing the formation of RNA containing inclusions, commonly referred to as RNA foci, and dipeptide repeat protein containing inclusions, two of the most common histopathological features of *C9orf72* linked ALS with FTD (Jiang et al 2016). In addition, treatment ameliorated behavioral deficits, but with limited effect on motor neuropathy. This is likely due to the fact that dipeptide repeat inclusions are seen more commonly in the neurons of the cerebral cortex than spinal cord, and may play a larger role in determining FTD pathogenesis than ALS pathogenesis in the combined phenotype.

ALS1 *(SOD1):*

SOD1 encodes the protein Copper/Zinc Superoxide dismutase 1, which is involved in the conversion of the superoxide radical (O_2^-) into hydrogen peroxide. This results in the conversion of a dangerous free radical ion into a more stable molecule which can then be further metabolized by the body into water. Superoxide is formed as a byproduct of oxidative metabolism and this conversion is necessary to prevent oxidative damage from occurring to the cells. Because of this metabolic role, SOD1 is associated with the mitochondria, and has been seen to interact with anti-apoptotic proteins such as BCL-2 (Pedrini 2010).

Mutations in SOD1 were first identified as causative of ALS by analysis of DNA from 18 familial ALS cases. All cases analyzed showed mutations that could be traced back to the locus of chromosome 21q that is known to be the position of ALS, and further analysis showed all ALS patients from these 18 families



Characteristic	G93A	G85R
	Line Gur1; (high Tg copy #); B6SJL Hybrid	Line 148
Inheritance	Dominant	Dominant
Protein Aggregation Propensity	High[7]	High[7]
Enzyme Activity	Active[3]	Inactive[1,5]
Protein Stability	Stable[5]	Reduced[5]
Disease Onset	Early (3- 4 mo)[3]	Late (7.5 mo)[2]
Disease Progression	Moderate (3 wks)[3]	Fast (2 wks)[2]

Figure 4: Left) Distribution of mutations in SOD1 associated with ALS (Valentine et al 2005). Right) Commonly used SOD1 mutations in mouse models of ALS and the characteristics of the mice (Leitner et al 2009).

carried mutations in the SOD1 gene (Rosen et al 1993). Further analysis throughout the past 20 plus years has shown over 160 variations in the SOD1 gene, all causative of ALS. These mutations can be seen in the figure below (Figure 1). As can be seen, the mutations are spread throughout the entirety of the protein, with no clustering, and a variety of different mutations: nonsense and missense mostly with many partially conserved mutations. The number and distribution of mutations seen in *SOD1* result in markedly different severities of disease and progression. For example, the A4V mutation is the most common in North America, and results in an aggressive and fast progressing form of ALS that commonly leads to death within 1-3 years (Cudkowicz et al 1997). In contrast, the D90A mutation results in a mild form of ALS with a typical progression course of around 10 years (Andersen 1996). Because of the heterogeneity of disease phenotypes, it was important to determine what kind of effect mutations in SOD1 had on the wild type protein. Mutations in SOD1 appear to cause a toxic gain of function, as first elucidated by Gurney et al 1994, who expressed human G93A SOD1 in mice at high levels, and demonstrated an ALS phenotypes. This result was later reinforced when SOD1 knockout mice showed no decreased survival relative to wild type, but mice with a G85R mutation (see Figure 2) in SOD1 showed decreased survival times relative to wild type (Bruijn et al 1998). Another important observation was the presence of wild-type SOD1 in plaques composed largely of mutant SOD1, indicating a possible dominant negative gain of function for the mutant SOD1 protein. A dominant negative gain of function could lead to conscription of wild type SOD1 into plaques via interactions with mutant SOD1.

Two of the most commonly used ALS mouse models include the G93A and G85R mutants, which are transgenic mice expressing human *SOD1* mutations. The details of these two mouse lines can be seen in Figure 2. The G85R model follows the most classical ALS progression, with a long time course to disease onset, and a quick disease progression. The G93A model is one of the most commonly used due to the quick timeframe in which symptoms develop relative to other models. Both display classical ALS symptoms: muscle weakness, dysphagia (difficulty eating), and histologically show protein inclusions common to ALS. However, of these two commonly used models, the G85R SOD1 mouse is likely the more appropriate model to use, as this more closely follows the clinical features of ALS and a timeframe more similar to human disease.

Current research into SOD1 mediated ALS is looking at protein misfolding. Protein misfolding is thought to play a role in the formation of SOD1 containing inclusions, and recent research has posited the idea of using protein chaperones to ameliorate the effects of the disease. In fact, work by Novoselov et al (2013), demonstrated that overexpression of HSJ1a, a member of the Hsps40 protein family was capable of increasing neuronal survival and performance in late stage G93A ALS model mice. This result has recently been seen in the G85R mouse model as well, by overexpressing Hsp110, another protein chaperone (Nagy et al 2016). This new area of research has provided the potential for a therapeutic target; if protein misfolding is causative of SOD1 mediated ALS, then overexpression of protein chaperones should help decrease misfolding and lead to increased survival. Other areas of current research are looking at the interactions between SOD1 and the mitochondria, as well as pro and anti-apoptotic proteins, seeing if the interactions between SOD1 and these proteins influence integrity of the mitochondrial membrane and possibly lead to cell death through an incorrectly triggered apoptotic pathway (Pickles et al 2016). New mutations in SOD1 are still being discovered as well (Hayashi et al 2016). The involvement of SOD1 in the reactive gliosis seen in ALS is also currently a subject of intense study (Madji Hounoum et al 2017), and in addition, many groups are hoping to establish the functional role that different *SOD1* mutations exert on disease progression (Srinivasan et al 2017, Keskin et al 2017, Abdolvahabi et al 2017).

ALS10 *(TARDBP):*

TARDBP is the name of the gene encoding the protein TDP-43. TDP-43 was first identified in 1995 as a protein capable of binding to the TransActivator Response element (TAR) of the HIV genome (Ou et al 1995). Wild-type TDP-43 is localized to the nucleus of cells, while in ALS, TDP-43 appears to be almost exclusively cytoplasmic, indicating a clearance of TDP-43 from the nucleus. This could be caused by acquisition of a mutation in the nuclear localization or export domains of the protein. Multiple different isoforms of the protein appear as well, many without a functional domain necessary for TDP43's exon skipping function. Initial functional analysis performed by Ou et al demonstrated that TDP-43 was functionally incapable of binding to RNA; more recent studies have questioned this result. I*n vivo*, TARDBP has been shown to be capable of binding both DNA and RNA targets, and appears to play a central role in the processing of at least some RNA targets. TDP-43 has a high affinity for DNA and RNA transcripts containing multiple TG (UG) direpeats, and generally appears to interact well with RNA and DNA that have repeated elements (Buratti and Baralle 2001). One notable target of TDP-43 is the Cystic Fibrosis Transmembrane Receptor (CFTR) transcript. Binding of TDP-43 induces skipping of exon 9, which produces a non-functional CFTR protein, and this RNA processing may be a vital step in the development of CF by affected individuals (Buratti and Baralle 2001). TDP-43 may also be involved in the formation of stress granules, intracellular vesicle like structures that sequester RNA transcripts and free ribosomal components to prevent translation during periods of cellular stress, such as if levels of amino acid charged tRNA molecules for use in translation falls too low (Ling et al 2013). The functional roles of TDP-43 in the maintenance of RNA processing and transcriptional/translational dynamics can be seen in Figure 5. Even more recent research has demonstrated an interaction between TDP-43 and C9orf72, another protein where genetic alterations have been implicated as causative of ALS. Ishiguro et al (2016) demonstrated that TDP-43 is capable of binding and transporting G-quadruplex containing mRNA's from the nucleus in the cell body to the length of the dendrites where these proteins can be locally transported and provide the expected

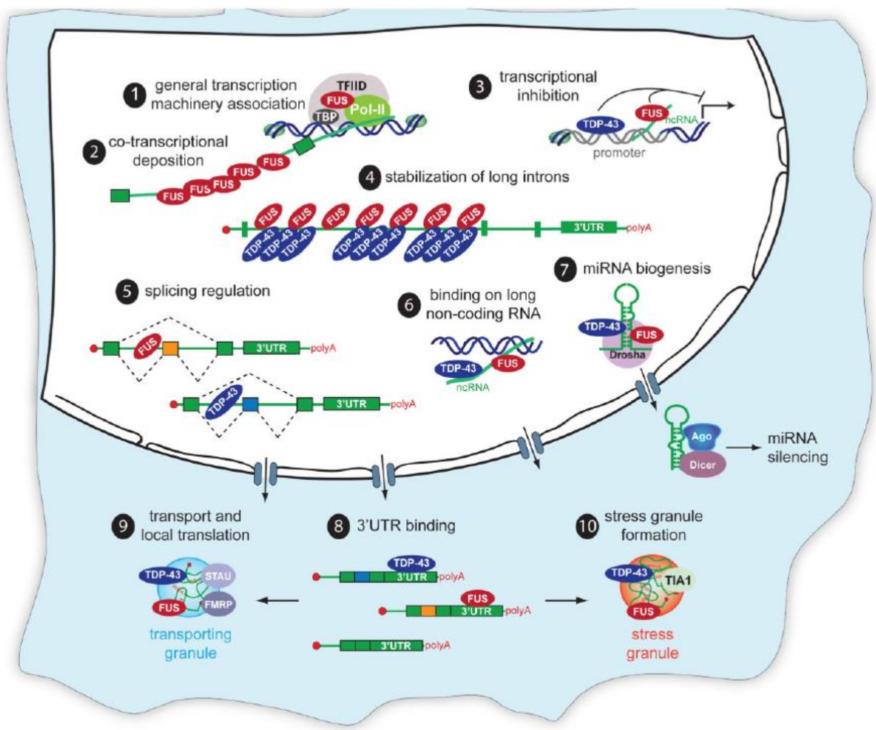


Figure 5: Many of the DNA/RNA binding roles of TDP-43 and FUS/TLS (Ling et al 2013)

function. *C9orf72* is one such G-quadruplex RNA that TDP-43 is capable of binding and transporting, and is heavily implicated in the progression of ALS with FTD. One final emerging function of TDP-43 is protection from incorporation of cryptic exons into RNA transcripts. Cryptic exons are small, intronic DNA sequences which occasionally can be improperly inserted into the RNA transcript of a protein (Zhang 2007). This function was lost when TDP-43 was knocked out in mice, and led to an increase in cryptic exons present in RNA transcripts, resulting in incorrect translation, and leading to increased nonsense mediated decay of RNA transcripts (Ling et al 2015).

TDP-43 was first identified as potentially causative of ALS in 2006 through the work of Neumann et al. This group discovered that the major component of protein inclusions in ALS is an ubiquitinated, cleaved, misfolded, and hyperphosphorylated version of TDP-43, though wild-type TDP-43 was also found to be present within these protein inclusions (Neumann et al 2006). This hyperphosphorylated ubiquitinated TDP-43 protein has since been reported to be present in protein inclusions in upwards of 97% of all sporadic ALS cases (Ling et al 2015)

TDP-43 has a unique gene structure which can be seen in Figure 6. This gene includes many expected elements for a DNA/RNA binding protein, such as the presence of a nuclear localization and export signal, and two RNA recognition motifs. However, TDP-43 also includes a glycine rich region, which is commonly referred to as a "prion-like region."

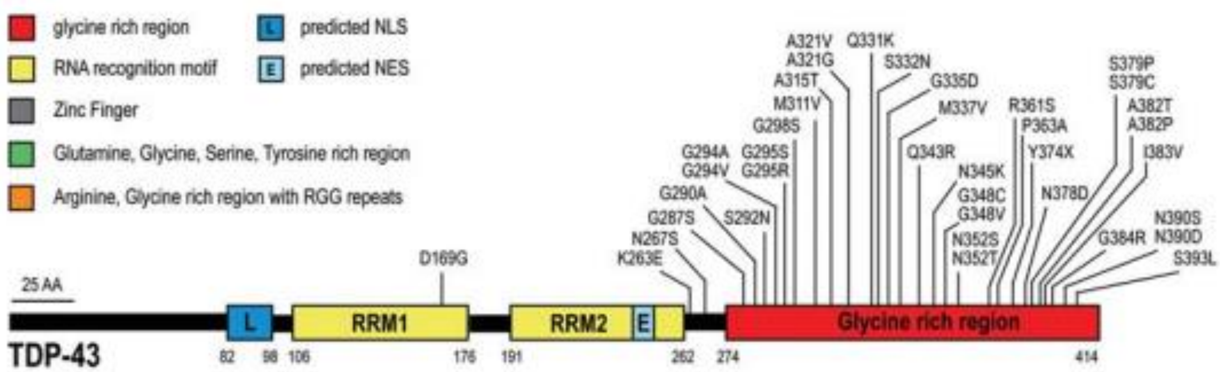


Figure 6: The gene structure of *TARDBP*. There are 5 main regions. L) Nuclear Localization. RRM1) RNA Recognition Motif 1. RRM2) RNA Recognition Motif 2. E) Nuclear Export Signal. Glycine Rich Region) Prion-like domain. (Lagier-Tourenne et al 2012)

The mutations tend to cluster in the glycine rich region (prion like), which has led to the development of the "Prion Hypothesis of ALS" (Lee and Kim 2015), though increased research and new results are leading thoughts away from this direction. The Prion Hypothesis of ALS essentially states that mutations in the prion like region of the TDP-43 protein leads the protein to act more prion like, demonstrating a propensity to bind and sequester wild type TDP-43 in insoluble plaques, while also demonstrating some ability to misfold wild type TDP-43, which allows for further disease progression. In addition, prions have been shown to propagate between cells; in patients with TDP-43 mutations causative of ALS, a similar spread of proteins between healthy cells and damaged or dying cells has been observed. Much of this thought is based on two facts: the glycine rich region of TDP-43 does indeed have many sequence and structural similarities with prion proteins, and the presence of wild-type TDP-43 in TDP-43 protein inclusions. Based on this, the Prion Hypothesis was developed to explain the presence of wild-type TDP-

43 in the protein inclusions, though many other simpler mechanisms, such as a dominant negative mechanism whereby mutant TDP-43 monomers can interact and sequester wild-type TDP-43 proteins. The prion-like region of TDP-43 indeed appears to have significant functional importance, as deletion of the Prion domain lead to a 90% reduction in CFTR exon 9 skipping in one study (Wang et al 2004), indicating that this region may itself be important in either selecting RNA for binding or in the process of binding splicing machinery.

Attempts have been made to generate TDP-43 transgenic mice for use in research, with several models having successfully mimicked human ALS, such as the one used by Wegorzewska et al (2009). This mouse model develops TDP-43 staining protein inclusions and also appears to develop symptoms similar to those seen in affected humans with ALS, including degeneration of motor neurons in both the upper and lower CNS, and can also replicate the symptoms of ALS with FTD in some cases, where some transgenic mice appear to have markedly worse maze tests than others. TDP-43 in these mice also appears to delocalize from the nucleus as is seen in ALS, making this a useful model. Other models have been unsuccessful, leading to difficulty in continuing research, largely due to the self-regulatory actions of TDP-43. The presence of elevated levels of wild type TDP-43 is enough to lead to discontinuation of TDP-43 mRNA production, which can lead to difficulty in generating the protein plaques seen in ALS. The major issue associated with the use of almost all available TDP-43 transgenic mouse models has to do with the fact that these mice overexpress TDP-43, either endogenous or human mutant, and this over-expression does not mimic the normal conditions of the disease, and while almost all cases develop associated TDP-43 pathologies including cytoplasmic inclusions, very few of the mice develop the clinical symptoms of ALS (Phillips and Rothstein 2015). Transgenic knockout of TDP-43 in mice models leads to embryonic lethality (Sephton et al 2010).

Recently, it has been demonstrated that mutant TDP-43 mislocalizes and aggregation in the mitochondria, where it exerted effects such as binding mitochondrial mRNA involved in generation of complex I, leading to complex I dysfunction, and general mitochondrial dysfunction (Wang et al 2016). By abolishing the ability of TDP-43 to enter the mitochondria, neuronal loss in TDP-43 transgenic mice was diminished, mitochondrial dysfunction was decreased, and the phenotype of the mice was significantly improved.

Current research is studying the effects of different isoforms of TDP-43 and seeing what effects these isoforms have- forms lacking the prion domain do not have the ability to cause exon skipping, but can bind RNA, meaning they likely have an effect on binding and regulating RNAs through some other mechanism, possibly exon stabilization, RNA transport, or binding to the 3'UTR of specific mRNAs (D'alton et al 2015, Caccamo et al 2015). Other research is examining the interaction between C9orf72 and TDP-43, which have recently been shown to interact, and it has been suggested that TDP-43 may transport C9orf72 the length of the axon to where it is transcribed (Ishiguro et al 2016), which would provide a link between the presence of TDP-43 in protein inclusions even in ALS cases without TARDBP mutations. Indeed, in many cases of TDP-43 proteinopathy associated with ALS, there is no mutation in TARDBP, but there may be mutations or changes in other ALS causative genes. In particular, *C9orf72* hexanucelotide expansions, which will be discussed in detail later, are commonly associated with TDP-43 proteinopathy and the presence of TDP-43 protein inclusions.

ALS6 *(FUS/TLS):*

FUS/TLS (Fused in Sarcoma/ Translocated in Liposarcoma) is a protein that belongs to a unique class of DNA/RNA binding proteins that is able to function at all stages of gene expression, from transcription through translation (Sama et al 2014). FUS/TLS was first identified as a part of fusion protein in various sarcomas and liposarcomas. In cancer, the N-terminal domain of FUS is fused with the C-terminus of an endogenous DNA binding factor, leading to excessive transcriptional activation and issues with RNA processing (Crozat et al 1993, Rabbitts et al 1993). FUS appears to have many wild type functions *in vivo*, which can be seen in Figure 5, the first and foremost of which is directly binding to DNA. By directly binding to DNA, FUS appears to play a role in DNA repair by aiding in pairing of homologous single stranded DNA (Baechtold et al 1999), as well as by aiding in D-loop formation, structures similar to telomeres which mark sites of damage in DNA (Takahama et al 2009). The N-terminal domain appears to be involved in protein dimerization and the initiation of transcription, one of the most important functions of FUS (Yang et al 2014). As a role in transcriptional processing, FUS is involved in the selection of the adenylation site of the transcript and can use alternate adenylation sites to create alternate transcripts (Masuda et al 2015). Several different groups have demonstrated the direct ability of FUS to interact with the spliceosome components, particularly small nuclear riboproteins, and mutations in FUS seen as causative of ALS appear to disrupt proper spliceosome formation (Yu et al 2015). Several studies have reported the ability of FUS to bind to the 3’UTR of many different mRNAs, and appears to be involved in affecting the stability of these mRNAs, with knockdown mostly leading to mRNA destabilization (Udagawa et al 2015). Finally, FUS appears to be involved in RNA translation and sequestering, with multiple groups reporting its involvement in transporting mRNA the length of the axon, and the presence of FUS in stress granules. Due to its involvement in RNA processing, FUS is primarily located in the nucleus. FUS proteins have been shown to bind to a specific GUGGU motif in RNA (Lagier-Tourenne et al 2012).

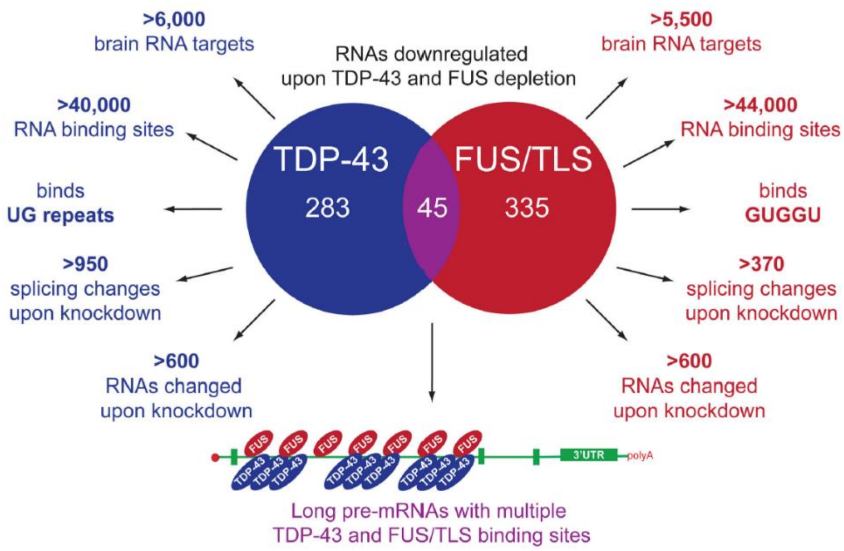


Figure 7: While TDP-43 and FUS/TLS have similar functions in the processing and maintenance of DNA and RNA, when comparing the two proteins, it is evident that they have little overlap in terms of RNA that the individual proteins are capable of processing. As can be seen, when knockdown of TDP-43 or FUS occurs, approximately 300 RNA targets are downregulated. Between these pools of RNA that are downregulated, 45 are the same between TDP-43 depletion and FUS depletion. (Ling et al 2013).

As can be seen in Figure 5, FUS and TDP-43 both have a variety of shared functions in RNA processing and transcriptional/translational control. This led researchers to determine which RNA targets are downregulated by the knockdown of either FUS or TDP-43. The results of these knockdowns can be seen in Figure 7. Ultimately, an analysis of multiple TDP-43 and FUS knockdown experiments demonstrated a knockdown of approximately 300 RNA targets when either was depleted, of which 45 of these targets were found in both knockdown pools.

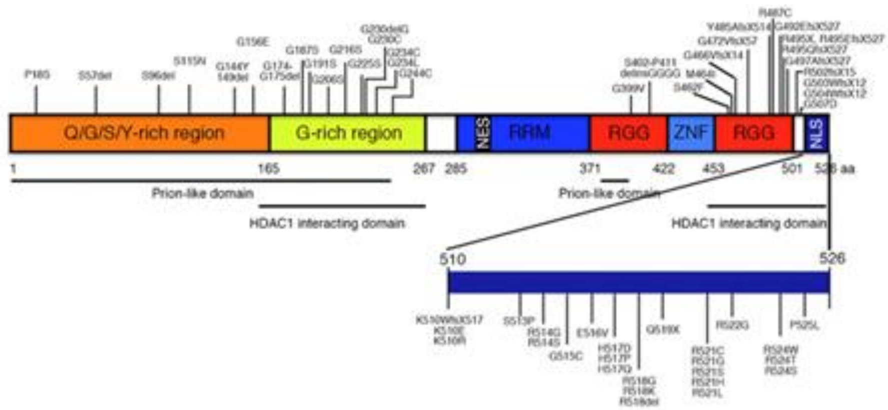


Figure 8: Gene structure of *FUS/TLS* showing the different domains and sites of common mutations causative of ALS. Q/G/S/Y Region) Prion-like. N-terminal domain. G-rich region) Glycine rich region, Prion-like. RRM) RNA Recognition Motif. RGG) Arg-Gly-Gly rich region. ZNF) Zinc Finger. NES) Nuclear Export Sequence. NLS) Nuclear Localization Sequence (Shang and Huang 2016).

While linkage to chromosome 16 was shown to be associated with ALS in 2003 (Sapp et al 2003), mutations in FUS/TLS were first identified as causative of ALS in 2009 by Kwiatkowski et al. Mutations appear to be spread throughout the entire protein, with a few areas of clustering. As can be seen in Figure 8, the majority of the mutations occur in the C-terminal domain of the protein, largely affecting the nuclear localization sequence (NLS) and one of the arginine rich regions (RRG). However, similar to TDP-43, FUS contains a glycine rich region, which in tandem with the N-terminal domain forms a prion like domain, which also has a significant number of mutations clustered within it. The major effects of the mutations which cluster in the nuclear localization sequence would seem to impact the localization of FUS more than the function, FUS not being localized to the nucleus in ALS similarly to TDP-43. Mutations in the Prion-like domain affect the ability of FUS to form stress granules (Patel et al 2015).

Several mouse models of *FUS* mutations have been developed. In 2012, researchers overexpressed human FUS in mice, or expressed one of two human ALS causing *FUS* mutations (Verbeeck et al 2012). Mice expressing either of the two mutants displayed the clinical features of ALS including protein inclusions in cytoplasm of motor neurons and development of progressive motor neuropathy symptoms. In another group overexpressing human *FUS* in mice led to progressive neurodegeneration and symptoms similar to ALS including FUS proteinopathy in both motor neurons and associated glial cells (Mitchell et al 2013). In mice expressing human *FUS* mutations exclusively in neuronal tissue, aggregation of FUS protein in the spinal cord and motor neurons that was accompanied by neurodegeneration were seen (Deikin et al 2014). Further studies have expressed FUS lacking an RNA recognition motif, which led to the associated symptoms of FUS proteinopathy, leading to early death often with an accompanied muscle tremor in the days leading up to death. However, neuronal loss was not observed despite extensive FUS proteinopathy.

In general, all available data suggests that *FUS* mutations result in a toxic gain of function mechanism. Using mice, *FUS* was either knocked out, or a mutant lacking a nuclear localization sequence was knocked in. Both mice were embryonic lethal; however, analysis of neuronal density at death demonstrated that *FUS* mutant knockin mice displayed decreased motor neuron numbers (Scekic-Zahirovic et al 2016). Further studies have confirmed the likelihood of a toxic gain of function mechanism, via observations that fast axonal transport is impaired in mutant expressing cells and aberrant activation of the p38 MAPK pathway (Sama et al 2017).

An emerging area of research in the function of FUS has to do with its ability to induce processing of microRNAs via interactions with microRNA processing proteins (Dini Modigliani et al. 2014). Several of the mRNA that FUS targets have FUS as a target themselves, meaning that FUS has an auto-regulatory component that has not been studied very much, but could prove insight into the effect of levels of FUS in the cell. This autoregulatory component has led to many of the same issues in model development as with TDP-43, whereby the levels of protein and RNA are strictly controlled to prevent over-expression, limiting the development of disease in animal models. .

In addition, an association between FUS and mitochondrial dysfunction has been established, as FUS containing disease causing mutations has been seen to display increased localization in the mitochondria, which is in part regulated by the chaperone protein Hsp60 (Deng et al 2016). In cases of two different mutation, *in vitro* in cultured neurons and *in vivo* in transgenic Drosophila, fragmentation of the mitochondria was noted.

Rare Variants

***ALS2* (Alsin):**

Mutations in the gene *ALS2* demonstrate an autosomal recessive inheritance pattern, and are causative of several early onset forms of the disease, with phenotypes ranging from infantile-onset ascending spastic paralysis to severe juvenile ALS, with the possibility of juvenile primary lateral sclerosis. The *ALS2* gene encodes a guanine nucleotide exchange factor for the Rab 5 protein family, and is involved in endosomal trafficking, as it was seen to localize to early endosomes. It is thought that these mutations in the *ASL2* gene result in a loss of function phenotype, as no evidence of any Alsin or truncated forms could be detected in immortalized lymphoblast lines from patients with confirmed *ALS2* mutations (Yamanaka et al 2003). Mutations in this protein may lead to disease via changes in endosomal dynamics leading to motor neuron degeneration (Otomo et al 2003). In addition, loss of Alsin function has been associated with impaired mitochondrial dynamics, leading to fragmentation of mitochondrial membranes and clumping of mitochondria containing vesicles (Gautam et al 2015).

Several groups have attempted to generate an *ALS2* mouse model. Three groups in 2006 generated lines of mice with an *ALS2* knockout. All three groups reported that mice has subtle but noticeable defects in neuronal development, and differences in observed phenotype are likely related to the substrains of embryonic stem cells used in gene targeting. In Devon et al 2006, a decrease in the size of cortical neurons was noticed, along with changes in the balance of endosomal transport in neurons, particularly for BDNF and IGF1R proteins, which are involved in the growth and development of neuronal tissues. This group also demonstrated that there was a significant decrease in locomotor activities in *ALS2-/-* mice in a 3-minute open field test. Cai et al 2005 demonstrated that this decreased motor activity was not significant if the timeframe of the test was expanded to 30 minutes, indicative of an increased stress or anxiety response. In addition to this, multiple groups used the rotarod test for motor coordination, with mixed results; Cai et al and Deng et al observed that *ALS2* null mice demonstrated decreased motor coordination compared to their wild type littermates (Cai et al 2005; D eng et al 2007), while Hadano et al (2005) and Devon et al (2006) observed no change in motor coordination, possibly due to differences in conditions under which the tests were performed. The limited motor phenotype observed in mice is likely due to the presence of a gene with a redundant function, which limits the severity of the disease phenotype, despite the aggressive nature of ALS caused by *ALS2* mutations in humans.

***ALS3*:**

A rare form of ALS identified in one family with linkage mapping to chromosome 18q21. This autosomal dominant form of ALS displays a uniform clinical presentation within the affected population, with weakness of the legs and arms, bulbar symptoms, and an average age of onset of ≈45. The average life expectancy for an affected patient in this family is 5 years. This variant of ALS displays a classical clinical phenotype, with upper and lower motor neuron involvement, and no demonstrated dementia (Hand et al 2002). The gene that is causative of this variant of ALS has yet to be identified, and limited research has been performed on this variant.

ALS4 (*SETX*)

Analysis of DNA from members of a multi-generation family with a form of juvenile onset ALS characterized by both upper and lower motor neuron involvement and an average age of onset of 17 years led to the determination that the causative mutation for this form of ALS was present in a chromosomal region not previously associated with ALS: 9q34 (Chance et al 1998). Further studies led to the narrowing of the region (Blair et al 2000), before Chen et al (2004) were able to identify 3 mutations in the SETX gene encoding the protein senataxin. Despite the early development of symptoms, the progression of this variant of ALS is relatively mild, taking decades from onset to death; with difficulty walking, weakness, and atrophy of small muscles of the hand and face as primary symptoms, by 40 to 50 years of age, most affected persons will be wheelchair bound. Symmetrical weakening of muscles is commonly seen in this variant, while the involvement of bulbar symptoms is rarely seen.

Senataxin encodes a protein with homology to a yeast RNA and DNA processing protein Sen1p (Moreira et al 2004), and has been demonstrated to interact with RNA polymerase II, with knockdown of senataxin decreasing transcription of RNA Pol II gene targets (Suraweera et al 2009). In 2014, Fogel et al demonstrated that in fibroblasts taken from affected ataxia-oculomotor apraxia 2 patients, a disease also characterized by mutations in the *SETX* gene, that gene targets involved in neurogenesis, cell proliferation, and synaptic transmission had altered expression levels. Mutations in *SETX* may result in neuronal degeneration through dysfunction of helicase activity (Chen et al 2004), or by failing to resolve RNA/DNA hybrids that are formed during RNA Pol II transcription and are associated with transcriptional termination. This may also affect poly-adneylation of RNA Pol II transcripts, leading to a decreased stability of these transcripts and altering their expression levels, as senataxin has previously been demonstrated to interact with poly(A)-binding proteins 1 and 2.

ALS5 *(SPG11):*

Mutations in the *SPG11* gene, encoding the protein spatacsin, have been identified as causative of a rare form of autosomal recessive, familial juvenile ALS. This variant is a slow progressing phenotype, with an average age of onset of 16, and an average duration of 34 years (Orlacchio et al 2010). The presence of both upper and lower motor neuron symptoms are seen, with distal muscle weakness and atrophy as the primary symptoms, with hyperreflexia and spastic gait as associated symptoms. Significant variation is seen in the phenotypic expression of the disease in affected patients, with some expressing a rather mild phenotype with limited upper and lower motor neuron symptoms, while others were wheelchair bound with limited distal muscle function. In most cases, bulbar symptoms, including jaw spasticity, poor facial muscle reflexes, and a weak tongue muscle were observed. In all patients, the Hoffman sign is present; this involves tapping the nail or flicking the terminal phalange of the middle or ring finger to elicit a response of flexing the terminal phalanx of the thumb. In ALS patients, this reflex is commonly lost, or in some cases is exaggerated depending on the level of upper motor neuron versus lower motor neuron involvement. The involvement of bulbar symptoms led to the differentiation of this condition from spastic paraplegia, the primary condition associated with mutations in *SPG11*.

Spatacsin is ubiquitously expressed in neuronal tissue, with localizations to organelles such as protein trafficking vesicles, endoplasmic reticulum, microtubules (Murmu et al 2011), and lysosomes involved in autophagic lysosome formation via fusion with autophagosomes (Chang 2014). Recent evidence has suggested that a loss of spastacin function leads to alterations in lysosomal storage and clearance pathways, likely associated with autophagy defects, and that these alterations in lipid processing can lead to both upper and lower motor neuron degeneration in a mouse model with *SPG11* disrupted (Branchu et al 2017).

ALS7:

A rare variant of ALS with linkage to chromosome 20p3, demonstrated in 1 family from Boston with multigenerational autosomal dominant ALS. No other family with significant linkage to this region has been identified (Sapp et al 2003). The gene in which causative mutations lie in this family have yet to be identified.

ALS8 *(VAPB)*:

Mutations in the *VAPB* gene were identified as causative of ALS in a Brazilian Caucasian family in 2004 by Nishimura et al (Nishimura et al 2004). 26 affected patients across 3 generations were examined, with linkage mapping to chromosome 20q13.33. The average age of onset of the disease was approximately 39 years of age, with an average age of death of 49 years, indicating a 10 year progression for this autosomal dominant variant. In most patients, both upper and lower motor neuron symptoms are seen, though the lower motor symptoms appear more severe. Many patients presented with postural tremor, and in multiple cases the painful muscle cramps were observed, with approximately one fifth of the patients demonstrating bulbar involvement.

VAPB encodes the vesicle-associated membrane-protein associated protein B. Previous studies have demonstrated localization to mitochondria associated membrane regions, specialized regions of endoplasmic reticulum (De vos et al 2012). In mice, VAPB protein is expressed primarily in cell bodies and dendrites of neurons, with localization to regions of the endoplasmic reticulum (Larroquette et al 2015). Over-expression results in activation of the cellular unfolded protein response designed to clear excess proteins from the ER. Mutations in VAPB result in an insoluble protein that is localized to non-endoplasmic reticulum cellular locations, as can be seen in Figure 9. Groups have demonstrated that mutations in *VAPB* likely result in a dominant negative effect, as mutant VAPB expression limits the ability of wild type VAPB to trigger the unfolded protein response (Kanekura et al 2006). Members of this protein family have been demonstrated to be involved with membrane transport. Mutant VAPB proteins have been demonstrated to form cytoplasmic protein inclusions and ubiquitinated inclusions which ultimately lead to cell death (Chen et al 2010), and have been demonstrated to include ER tubule proteins (Teuling et al 2007). VAPB loss of function does not appear to be involved, as mice with loss of *VAPB* mutations do not display significant motor neuron degeneration phenotypes (Kabashi et al 2013), despite the fact that loss of function of a lipid binding domain known as a FFAT domain has been implicated in the progression of the disease.

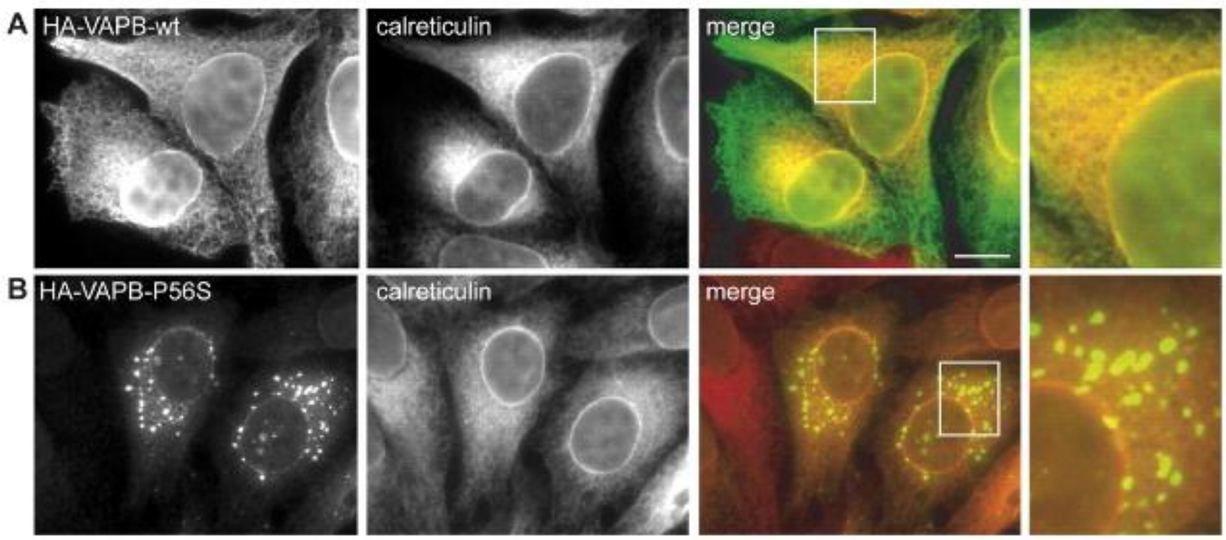


Figure 9. Altered expression patterns of VAPB seen in mutant mice (Teuling et al 2007). In wild type, VAPB is seen to be diffuse throughout the ER. When mutant VAPB is expressed, these proteins become localized to small insoluble protein inclusions within the ER. This may alter ER dynamics and the ER unfolded protein response triggering cellular stress.

Several groups have attempted to generate *VAPB* mutant ALS mice. In 2010, researchers created transgenic mice expressing human VAPB with the P56S mutation, and demonstrated at 18 months of age that mice expressing the human mutant developed TDP43 containing protein inclusions, demonstrating a possible link between these two proteins (Tudor et al 2010). Studies have also demonstrated that, in mice with ALS caused by expression of human *SOD1* mutants, levels of VAPB protein are decreased in end stage mice (Teuling et al 2007). Transgenic expression of mutant P56S *VAPB* led to multiple motor abnormalities, induced ER-stress, likely via affecting the unfolded protein response, and led to expression of pro-apoptotic proteins (Aliaga et al 2013). These mice demonstrated corticospinal motor neuron degeneration, but limited spinal motor neuron degeneration. A third group generated transgenic mice expressing the human mutant P56S *VAPB* gene with a *VAPB*-null genetic background via knockin, and observed multiple important characteristics of the resulting phenotype (Larroquette et al 2015). Cytoplasmic mislocalization of VAPB mutants was demonstrated, along with VAPB being predominantly expressed in lower motor neurons, transport into autophagosomes, and ER stress. Expression in predominantly lower motor neurons may explain the observation that affected patients present with primarily lower motor symptoms and limited bulbar involvement.

The involvement of VAP proteins in lipid synthesis and transport has also been observed, with groups identifying that VAPB is involved in sphingomyelin synthesis and transport (Kawano et al 2006, Perry and Ridgway 2006). This has been implicated in the progression of disease, as decreased transport of functionally important lipids to or from the ER may lead to decreased stability of these motor neurons and ultimately lead to cell death over a long disease course.

ALS9 *(ANG)*:

With a plethora of research demonstrating that angiogenic factors have importance in control and regulation of motor neuron degeneration (Lin et al 2000; Oosthuyse 2001; Azzouzz et al 2004), Greenway et al began to examine the VEGF-related protein angiogenin, encoded by the *ANG* gene (Greenway et al 2004). In follow up studies by the same group, mutations in angiogenin were confirmed in ALS patients of Irish and Scottish heritage displaying no other mutations identified as causative of ALS (Greenway et al 2006).

Patients with mutations in *ANG* present with classical signs of ALS, with distal motor weakness as the primary symptom. Associated symptoms include stiffness with cramping, upper and lower motor neuron signs, progressive muscle atrophy, brisk reflexes with normal sensory responses. There is large phenotypic variability within patients affected with this variant of ALS; some patients present with bulbar involvement, some with rapid degeneration progressing to a state of being wheelchair bound, difficulty breathing, presence of fasciculations, and in some cases painful muscle degeneration (Wu et al 2007). This variant of the disease was noted to be incompletely penetrant, as one obligate carrier of the mutation did not display any symptoms of ALS (Van es et al 2009), though the inheritance pattern appears to be predominantly autosomal dominant.

ALS11 *(FIG4):*

ALS10 is an autosomal dominant variant of ALS characterized by mutations in FIG4, a phosphatidylinositol phosphatase that is involved in the regulation of levels of $PI(3,5)P_2$. Alterations in signaling though $PI(3,5)P_2$ have been implicated in pathways associated with neurodegeneration (Chow et al 2009, Volpicelli-Daley et al 2007). FIG4 has also been demonstrated to be involved with retrograde endosome to trans-golgi network transport, which provides a link to the idea that an underlying issue in the pathogenesis of ALS is a disruption of intracellular transport.

FIG4 has been associated with multiple neurodegenerative disorders including ALS; in many of these diseases, FIG4 is found in intracellular inclusions, though in ALS associated with TDP-43 proteinopathy and formation of TDP-43 protein inclusions, FIG4 was not immunohistochemically detected in plaques (Chow et al 2009).

However, a line of mice known as the pale tremor (plt) mouse exists which is null for *FIG4* (*FIG4-/-*). Chow et al analyzed these mice in 2007 and found multiple issues. Fibroblasts, both isolated and cultured, had vacuole-filled cytoplasm with staining for LAMP-2, a lysosomal membrane associate protein (Chow et al 2007). This is indicative that these mice likely have an issue in the conversion of endosomes to lysosomes, which could be a possible explanation for the accumulation. These mice were also found to have profound motor neuron degeneration, displaying severe tremors and abnormal gait in early development, with abnormal limb postures, impaired motor coordination, muscle weakness, and continued abnormal gait. This ultimately culminates in juvenile death. The phenotype of these mice can be seen in Figure 10.

The pattern of neurodegeneration seen in these mice begins with sensory and autonomic neurons in neonatal stages. There are intermediate stages seen in this disease, with neuronal loss preceded by the development of vacuole filled neurons. Spinal neurons are normal at 3 weeks, but by 6 weeks display the presence of vacuolar inclusions. Peripheral neurons display decreased diameter, myelination, and conduction velocity (Chow et al 2007). When a human mutant *FIG4* is significantly overexpressed on a *FIG4 -/-* background, the mice experience dose dependent recovery of neuronal function, demonstrating decreased neurodegeneration, rescue of autophagy function, and significantly decreased accumulation of protein plaques containing p62 in astrocytes, which is one of the predominant features of *FIG4* mutations (Lenk et al 2011). This is indicative that *FIG4* mutations may result in a loss of function, or partial loss of function for the protein; the overexpression of the protein would rescue this if partial function is maintained by the mutants.

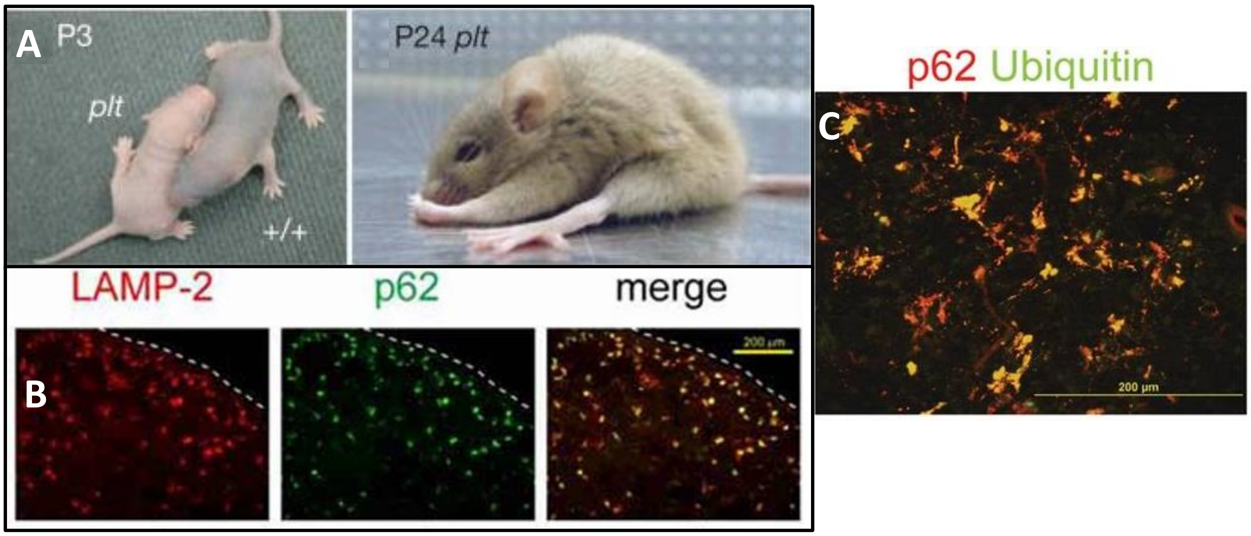


Figure 10: A) Chow et al (2007) Pale tremor mice displaying a “limp” phenotype; B) Lenk et al (2011) Cytoplasmic inclusions containing p62 and LAMP2, demonstrating localization to lysosomal associated membranes C) Ferguson et al (2009) p62/ubiquitin containing inclusions commonly seen in *FIG4* mutant associated ALS.

Chow et al 2009 first identified mutations in *FIG4* as causative of ALS. The features of this variant include a disease onset of ~55 years of age though there is significant variability in this. Approximately one quarter of patients present with bulbar onset, another two thirds with upper and lower extremity weakness, and approximately 5% with multiple sites of onset. In multiple examined patients, paralysis was asymmetric. Presence of fibrillations in muscle was detected via electrophysiology. Proximal muscles were primarily affected, asymmetrically, and no axonal length dependent affects were observed.

ALS12 *(OPTN)*:

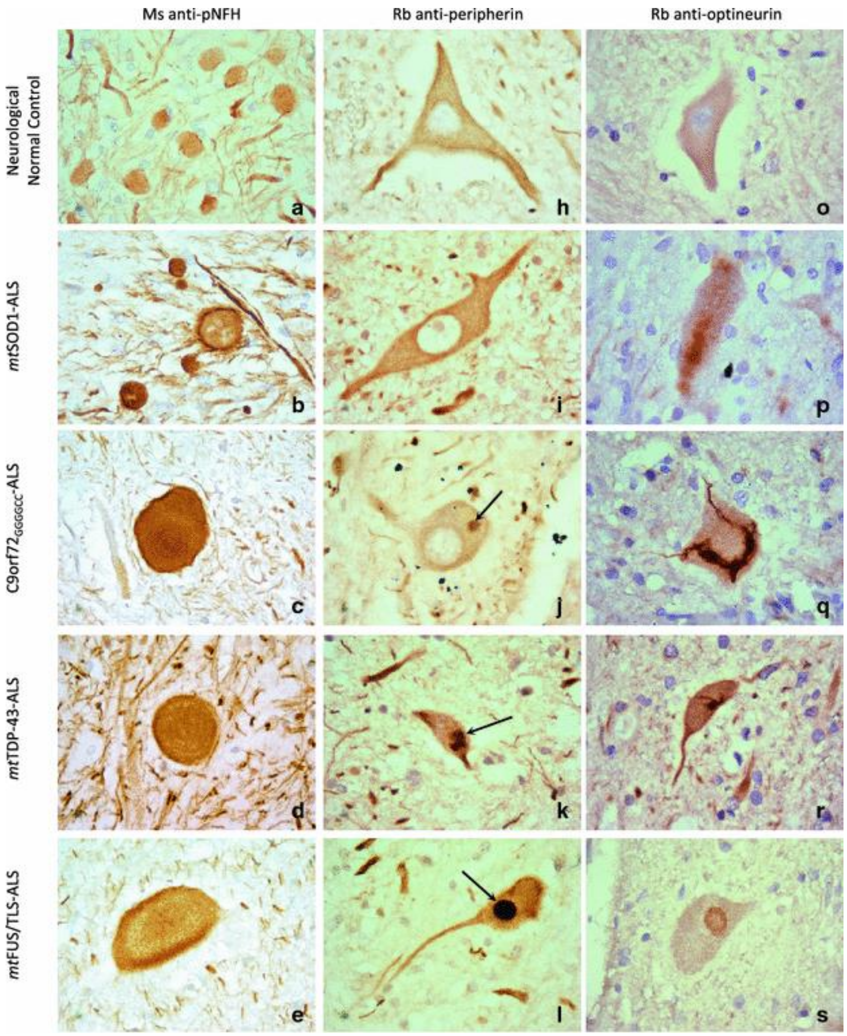


Figure 11. Protein inclusions staining for neurofilament subunit H (Left). Protein inclusions staining for peripherin, an intermediate filament (central). Protein inclusions staining for optineurin. In ALS associated with an array of different mutations (SOD1, C9orf72, TDP-43 and FUS), inclusions stain positive for these proteins (Keller et al 2012). This indicates that the functional role of optineurin may be interfered with in this disease, and establishes linkages to the structural components of the neuron, indicating that some intracellular transport pathways may be interfered with by this disease.

Mutations in optineurin were first identified as causative of ALS in 2010 (Maruyama et al 2010). In analyzing tissue samples from ALS patients, immunohistochemical labelling of SOD1 containing and TDP-43 containing inclusions respectively, demonstrated reactivity for optineurin when labelled with anti-optineurin antibodies. Genetic analysis of 6 individuals in a consanguineous Japanese family affected by ALS led to the identification of 3 different mutations in the *OPTN* gene. In multiple ALS variants, some of known origin and some of unknown origin, the presence of optineurin in protein inclusions has been observed (Keller et al 2012).

The significant expression of optineurin in protein plaques in ALS patients with other causative mutations implicates an important role of this protein in the progression of the disease. This can be seen in Figure 11.

The function of optineurin has been described as an adapter protein, as it has a myriad of cellular protein targets it can interact with. Optineurin inhibits NF-kB signaling, is involved in vesicle trafficking to the Golgi body via interactions with Rab8 or microtubule associated cytoskeletal motor proteins, may play roles in mitosis via interactions with CDK1, and has been seen to interact with Rab11 and the metabotropic glutamine receptor 1a, which indicate a functional involvement in vesicle trafficking (Paulus and Link 2014). Loss of optinerin *in vitro* leads to many functional changes in cells including increased apoptosis (Akizuki et al 2013), disruption and defects in Golgi structure and vesicle transport (Sahlender et al 2005) and disruption of autophagy (Wild et al 2011). It is important to note that the disruptions caused by optineurin changes all confer pathological changes to cells that have been implicated in ALS, suggesting that the role of optineurin in the development of ALS pathology is highly important (Keller et al 2012). This has also provided evidence that a loss of function of optineurin may be involved in the disease pathogenesis. In addition, loss of optineurin has been shown to lead to defective mitophagy of damaged mitochondria, potentially providing a mechanism for apoptotic neuronal death via improper mitochondrial disposal and cytochrome c release (Wong and Holzbaur 2014).

Alternatively, one group examining the role of ubiquitin and the ubiquitin binding domains of optineurin in the formation of inclusion bodies in neurodegenerative diseases demonstrated that mutations in optineurin decrease cellular autophagy responses against misfolded proteins (Shen et al 2015), which can induce cellular stress and increase apoptosis of these cells via the ER unfolded protein response. This provides the possibility that mutations in *OPTN* confer a dual loss of function and toxic gain of function (dominant-negative) advantage to the mutated protein. A second group analyzing the connection between *OPTN* mutations and autophagy identified that the ubiquitin binding domain of optineurin allows it to act as a selective autophagy receptor, and confirmed that mutations in the ubiquitin binding domain alter autophagy responses in the cell to poly-ubiqutinated proteins. In addition, optineurin can act as a regulator of the ubiquitin-proteasome system (UPS); when mutated, there is a decrease in the presence of regulatory subunits for the UPS (Ying and Yue 2016).

Links between multiple ALS-associated proteins and optineurin have been established. As shown in Figure 11, in ALS variants characterized by mutations in SOD1, TDP-43, FUS and C9orf72, cells contain protein inclusions that are positive for optineurin. In addition, a link between ubiquilin-2 and optineurin has been established, with observations *in vitro* that mutants of ubiquillin-2 localize to Rab 11 positive vesicles which are in part created by interactions with optineurin (Osaka et al 2015).

Recent studies have provided insight into the potential pathogenic mechanism of ALS associated with optineurin mutations, as Nakazawa et al (2016) identified that optineurin can normally bind caspase-8, and that mutations lead to increased TNF-a directed apoptosis (Nakazawa et al 2016). In a second study, Ito et al (2016) demonstrated interaction of optineurin with RIPK1, a protein that has been implicated in the induction of apoptosis and necroptosis (Ofengeim and Yuan 2013). In this study, Ito et al generated transgenic *Optn-/-* mice, and observed an age-dependent decrease in the number of motor neurons in the spinal cord (Ito et al 2016).

Clinical features of ALS caused by mutations in *OPTN* include a relatively short progression, similar to classical ALS, with variation in age and location of symptom onset. In most cases, hyperreflexia is present, with some bulbar involvement. Progressive weakening of proximal muscles has also been observed. In patient neuronal tissue, the presence of TDP-43 positive skein-like inclusions was commonly seen (Kamada et al 2013).

ALS13 *(ATXN-2):*

ATXN-2 contains a poly-glutamine repeat region that when expanded more than 33 times causes clinical symptoms of spinocerebellar ataxia-2 (SCA-2); expansions causative of SCA-2 were first identified by Trottier et al in 1995. Elden et al 2010 examined genetic changes that may enhance TDP-43 proteinopathy in yeast and *Drosophila*, and identified repeat expansions in *ATXN-2* as a risk modifier for the development of ALS. These studies demonstrated interactions between TDP-43 and ataxin-2 in an RNA dependent manner, meaning that when TDP-43 is bound to mRNA, ataxin-2 is able to interact with the same RNA and TDP-43. In addition observations that up-regulation of ataxin-2 led to an enhancement of neuronal degeneration were made. Mutations that ablate the nuclear localization signal of TDP-43 led to interaction with ataxin-2 and formation of intense staining protein plaques, which can be seen in Figure 12 (Elden et al 2010).

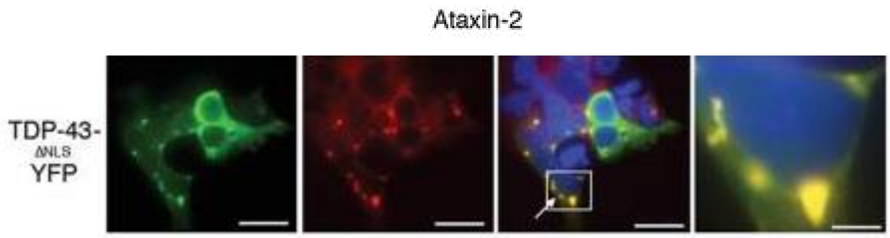


Figure 12: Elden et al (2010) Co-localization of TDP-43 with NLS mutations (yellow) and ataxin 2 (red), demonstrating that functional changes in TDP-43 are associated with mis-localization and can lead to interactions with other implicated ALS proteins.

It is important to note that intermediate expansion of the poly-glutamine repeat region is necessary to confer disease risk without development of symptoms of SCA-2. Expansions of greater than 35 repeats confer the clinical symptoms of SCA-2; expansions between 27-28 are normal; expansions in the range of 29-33 are considered intermediate, and modify the risk of ALS development, as well as enhancing the clinical features.

In patients with ALS, an analysis of repeat expansion in *ATXN-2* demonstrated that there is a significant increase in poly-glutamine repeat expansion between patients with ALS and control patients, implicating the role of ataxin-2 polyQ repeat expansions as a mechanism risk modification for the development of ALS phenotypes (Ross et al 2011).

In European populations with ALS and TDP-43 proteinopathy, a significant association was established between *ATXN-2* with 30-35 polyglutamine residue expansions and the development of ALS (Gispert et al 2012). A second group demonstrated similar results in an Italian population (Conforti et al 2012). Further studies have demonstrated that repeat expansions in ataxin-2 can enhance activation of caspase-3, indicative of stress-induced apoptosis, while also leading to an increase in the levels of hyperphosphorylated and cleaved TDP-43 fragments commonly found in protein inclusions seen in ALS. In the same study, it was also demonstrated that activation of caspases is involved in the development of these hyper-phosphorylated and cleaved fragments, as induction of caspase inhibitors in sample cells led to a decrease in disease phenotype (Hart and Gitler 2012). The cellular inclusions formed in ALS associated with ataxin-2 polyQ expansion differ morphologically from ALS without ataxin-2 polyQ expansion; when ataxin-2 intermediate polyQ expansions are present, skein-like inclusions resembling a roll of yarn are seen as opposed to the round Lewy-like bodies seen in ALS without ataxin-2 polyQ expansion.

In addition to enhancing TDP-43 proteinopathy in ALS patients, repeat expansions of *ATXN-2* have been demonstrated to enhance FUS proteinopathy in ALS with FUS causative mutations. Both mis-localization of FUS to the cytoplasm and enhanced ER stress responses were observed in the presence of intermediate polyQ expansions in ataxin-2 (Farg et al 2013). Multiple ubiquitin ligases have been seen to be interfered with by expansions in *ATXN-2* as well, indicating that ER stress and unfolded protein responses may be involved in the mechanism leading to cell death seen in these patients (Halbach et al 2015). Associations with C9orf72 repeat expansion and *ATXN2* repeat expansion have been reported as well, demonstrating an interaction between these two proteins, leading to increased TDP-43 proteinopathy and enhanced neuronal death (Ciura et al 2016).

Zhang et al 2017 identified the concomitant expression of C9orf72 repeat expansions and ATXN-2 repeat expansions in a family with combined phenotypes of ataxia, Parkinsonism, and dementia; this presents the possibility that, when coupled with previously identified interactions between C9orf72 and *ATXN2*, that combination of these mutations could pre-dispose patients to the development of ALS-FTD (Zhang et al 2017).

ALS14 *(VCP):*

Mutations in the *VCP* gene encoding valosin-containing protein were first identified as causative of ALS in 2010 (Johnson et al 2010). VCP protein appears to be an ATPase with multiple significant functions in cells, including the ability to associate with clathrin in coated pits, which are involved in endocytosis. Many of these functions have roles that have been implicated in the progression of disease, including ALS. One function of VCP includes protein quality control, in which it functions to help target ubiquitinated proteins to the proteasome and may be involved with autophagy in response to inappropriate processing of proteins (Meyer et al 2012). In addition, VCP is essential for maintenance of the outer mitochondrial membrane, and likely plays a crucial role in maintaining mitochondrial activity in cells (Xu et al 2011), and recruitment to the mitochondria leads to impairment of mitophagy in models of other neurodegenerative diseases (Guo et al 2016). Groups have also noted roles in the biogenesis of the ER and Golgi membrane networks (Uchiyama and Kondo 2005), as VCP can act to mediate membrane fusion, indicating a potential role in targeting as well via interactions with SNARE proteins. Further groups have established definitive link between VCP and autophagy roles (Dai and Li 2001), and in the ubiquitin-proteasome system in which it is involved in the degradation of proteins (Ju et al 2009). Based on all of these functions, it would appear that functionally VCP has roles in maintaining the stability of membranes and membranous organelles, likely through an endocytic regulatory process, or via interaction with targeting proteins that allow for correct localization of membrane components. Thus, it would be likely that mutations in VCP alter the ability of the cell to functionally maintain the ER, which would create cellular stress and potentially lead to apoptosis via similar pathways as many of the other involved proteins. Alternatively, the functional role in maintaining the mitochondrial associated membrane could alter the stability of both the ER and mitochondria leading to ER stress responses and upregulation of apoptotic signaling via mitochondrial membrane dysfunction and the related release of cytochrome C from the inner mitochondrial membrane.

The Kimonis research group has generated VCP mutant transgenic knockin mice to attempt to provide a disease model, and study the pathogenesis of the disease. In 2012, Yin et al created a transgenic VCP knockin mouse, and noted that it displayed a slow progressing neurodegenerative phenotype, underlined by slow and progressive weakening of the muscles, and the animals generally maintain normal behaviors throughout their life, making them different from many ALS mouse models which develop symptoms rapidly and do not model the course of the disease well. Motor neuron degeneration in the ventral horn neurons of the spinal cord was observed in an age dependent manner, with older age correlating to further neurodegeneration. In addition, the presence of TDP-43 positive protein inclusions were found in the neurons of the spinal cord. Uniquely, mitochondrial aggregates were identified in these tissues as well. Astrogliosis was commonly seen to affect these mice (Yin et al 2012). These mice were further studied in the following year, and similar results were demonstrated, along with the presence of ubiquitin containing inclusion bodies, though the inclusions did not contain VCP (Nalbandian et al 2012). These mice were observed to have an accelerated disease progression in specific homozygous knockin mutants (Nalbandian et al 2012), and the identification that autophagy and apoptosis were both increased (Nalbandian et al 2013).

Patients with VCP mutations present typically with lower limb weakness beginning in early middle age, which ultimately progresses to include all four limbs (Johnson et al 2010). Examination of several patients revealed upper and lower motor neuron signs, with abnormal electromyography readings demonstrating abnormal innervation and regions displaying typical signs of concurrent innervation and denervation. In one patient analyzed, the presence of bulbar symptoms as noted. In addition, the presence of frontotemporal dementia or frontotemporal dementia like symptoms has been observed in patients with ALS caused by mutations in VCP (Bersano et al 2009).

ALS15 *(UBQLN2)*

Ubiquilin proteins are involved at the interface between ubiquitinated proteins and the proteasome degradation system, physically linking ubiquitinated proteins to their target of the proteasome. Ubiquilin-2 has also been implicated as having roles in the cell cycle, based on re-distribution of the protein and increased expression in mitotic cells (Kleijnen et al 2000, Mah et al 2000). Ubiquilin-2 has also been suggested to have roles in autophagy, as it can interact with microtubule associate protein 1 light chain 3, which is a structurally and functionally important component of autophagosomes (N'diaye et al 2009). In other organisms, ubiquilin-2 has been demonstrated to play a role in assembly of the cytoskeleton by mediating interactions between cytoskeletal proteins (Wu et al 1999). In addition, it was recognized that Ubiquilin-2 has homology to yeast proteins involved in mitotic spindle development and maintenance, and elimination of this protein leads to stalling of yeast at the G2/M checkpoint, which was rescued by expression of human ubiquilin-2 in the system (Kaye et al 2000a, Kaye et al 2000b). In addition, ubiquilin-2 is involved in the proper disposal of mitochondrial proteins, and alterations in this pathway may be involved in disease pathogenesis (Itakura et al 2016).

Mutations in *UBQLN2* were first identified in 2011 by Deng et al. In analysis of one multi-generation pedigree, an unusual inheritance pattern displaying no transmission from affected father to son and incomplete penetrance in female carriers, implicating an X-linked mechanism for the manifestation of this variant. This led Deng et al to identify a region on the X chromosome containing approximately 200 genes, which via structural, expression, and functional analysis was found to contain mutations causative of this disease in the *UBQLN2* gene.

Clinical features of ALS caused by this variant result in sex-linked differences in development. In an analysis of 40 patients (5 obligate carriers not expressing full disease penetrance), on average, affected males develop symptoms almost 15 years earlier than their female counterparts. However, despite this age discrepancy, the time-course of the disease does not display sex-dependent effects. Approximately one fifth of the patients analyzed displayed symptoms of dementia; in some cases, symptoms of dementia proceeded symptoms of motor dysfunction; in all cases, motor dysfunction was ultimately evident. Protein aggregates in neuronal tissue samples taken from affected patient's post-mortem were analyzed for the presence of ubiquilin-2, and demonstrated strong immunohistochemical staining of ubiquilin in skein-like inclusions. In addition, in patients without mutations in ubiquilin-2, such as those with ALS caused by mutations in FUS, TDP-43 and optineurin, ubiquilin-2 IHC staining demonstrated the presence of ubiquilin-2 in protein inclusions.

Indeed, further work as demonstrated a potential link between ubiquilin-2 and optineurin. In wild type cells, ubiqulin-2 localizes to autophagosomes formed by optineurin, and that disease causing mutations in optineurin prevented this localization (Osaka et al 2015). Along with this, demonstration of a link between ubiquilin-2 and TDP-43 has been established with the observation that ubiquilin-2 aggregates that form in ALS are involved in the recruitment of TDP-43 to the plaques, possibly via ubiquitin binding domains (Picher-Martel et al 2015). Recent research has demonstrated a potential role in transcriptional control, as interactions between ubiquilin-2 and hnRNPA1 have been demonstrated in yeast, with the finding that mutations in the gene causative of ALS decrease interactions between ubiquilin-2 and hnRNPA1 (Gilpin et al 2015). hnRNAp1 is involved in binding to transcribed mRNA in the nucleus of human cells, and plays a role in alternative splicing of mRNA (Pollard et al 2002, David et al 2009)

Transgenic animal models of ALS containing mutations in *UBQLN-2* have been generated to study the *in vivo* effects of the mutations. In one study, expression of *UBQLN-2* mutants led to the development of neurological symptoms in mice, with limited motor involvement, more closely modeling the features of dementia or frontotemporal dementia seen in cases of ALS with *UBQLN-2* mutations (Gorrie et al 2014). The presence of ubiquilin-2 protein containing inclusions was commonly seen in the neuronal tissue of the mice, as well as displaying decreased numbers of dendritic spines. In electrophysiological studies, mice displayed diminished long term potentiation of neurons and enhance long-term depression of neurons, indicating a change in synaptic turnover and likely diminished signaling. Expected impairment of proteasome function was also observed. In a second group expressing *UBQLN2* mutants in mice, similar results were seen, including protein inclusions and dementia like features associated with neurodegeneration, but limited motor involvement (Ceballos-Diaz et al 2015). One final group performed electrophysiological analysis of neuronal cells taken from transgenic mice expressing mutant ubiquilin-2 protein and demonstrated that cells were hypo-excitable, had decrease glutamatergic current, and both increased time and reduced amplitude response in times immediately after hyperpolarization (Radzicki et al 2016).

ALS16 *(SIGMAR1):*

The *SIGMAR1* gene encodes the sigma-nonopioid receptor 1, which functions as a molecular chaperone in the ER, binding a range of ligands including neurosteroids, and as such is expressed at highest levels in motor neurons (Mavlyutov et al 2015). In cells, Sig-1R is involved in interactions with ion channels, predominantly K+ channels, can interact with inositol triphosphate receptors, and has been implicated in lipid transport and differentiation of stem cells into neuronal cells (Aydar et al 2002, Hayashi et al 2003a, Hayashi et al 2003b). Of functional importance is the ability of Sig-1R to form lipid-raft like microdomains in the cell membrane, as this plays a role in oligodendrocyte differentiation (Hayashi et al 2004). With identification of mutations in *SIGMAR1* as causative of frontotemporal lobar degeneration by Luty et al in 2010, researchers turned their attention towards other neurodegenerative diseases with similar phenotypes that may also have mutations in *SIGMAR1* and identified mutations as causative of one variant of juvenile ALS.

In 2011, analysis of a Saudi Arabian family with an autosomal recessive variant of juvenile onset ALS revealed mutations in the *SIGMAR1* gene via homozygosity mapping of affected individuals (Al-Saif et al 2011). Affected patients in the family present with noticeable weakness in the lower limbs with associated spasticity by the age of 1-2 years, with exaggerated tendon reflexes (hyperreflexia). By the age of 9-10, weakness of the hands and forearms was noticeable. Progression affected the proximal muscles of the upper arms, leading to weakness and denervation in the triceps and extensors of the forearm. No respiratory or bulbar weakness was noted. Two of the four patients analyzed were wheelchair bound by the age of 20. No dementia, frontotemporal dements, or frontotemporal lobar degeneration were observed in these patients.

Interestingly, analysis of protein distribution in neuronal cells transfected with human mutant Sig-1R protein on a knockdown background of wild type Sig-1R demonstrated that mutant Sig-1R is found in low-density membrane fractions, indicating that mutations may disrupt the propensity of this protein to form micro-domains with lipid enrichment. Due to the functional ability of Sig-1R to regulate lipid transport and interact with ion channels, it may be suspected that Sig-1R has a role in the generation of high density lipid microdomains in membranes, which could explain the altered localization of the mutant protein. Detergent resistant clusters of protein were also formed by the mutant Sig-1R, possibly due to dimerization of proteins, which would provide evidence for a mechanism of protein aggregation in patients expressing *SIGMAR1* mutations, as well as providing a mechanism for loss of function of Sig-1R in these patients, and demonstrating a toxic gain of function.

An unexpected function of Sig-1R is the ability to regulate apoptosis by modulating Ca^{2+} levels in the endoplasmic reticulum, which can have profound effects on cell survival and signaling. When mutant Sig-1R is expressed in cells, this increases the likelihood of apoptosis by approximately 1.7 fold, indicating that mutations in this protein alter the ability of the cells to deal with ER stress (Hayashi and Su 2007). Conversely, overexpression of Sig-1R leads to increased calcium uptake into mitochondria and increased synthesis of ATP (Shioda et al 2012). Prause et al (2013) noted that the sub-cellular localization of Sig-1R was altered in ALS.

Mitochondrial dysfunction has long been a proposed mechanism of cellular damage in ALS, with direct evidence of interactions between mutant SOD1 proteins and BCL-2, an anti-apoptotic protein associated with the mitochondrial membrane (Pasinelli et al, 2004). This likely prevents BCl-2 from acting in an anti-apoptotic manner, and may shift the balance of apoptotic signaling to favor apoptosis in neuronal cells that experience ER stress, altered proteasomal dynamics, or altered autophagy dynamics. This has led researchers to consider the role of mitochondrial dysfunction in many causative mutations associated with ALS, including Sig-1R mutations. In recent studies, expression of mutant Sig-1R in neuro2A cells causes dissociation of Sig-1R from IP3 receptors in the mitochondria, leading to decreased ER-calcium uptake and associated mitochondrial dysfunction, proteasomal dysfunction, and directed cytoplasmic localization while enhancing ubiquitination of TDP-43 (Tagashira et al 2014). Further studies have demonstrated similar results, with mutations in Sig-1R leading to dissociation from the mitochondrial membrane, altered calcium homeostasis, increased ER-stress induced apoptosis, and evidence of mitochondria-associated membrane collapse in *SIGMAR1* mutant ALS (Watanabe et al 2016). Treatment with methyl pyruvate, which acts to increase ATP production rescued proteasome activity even in the presence of ER stress (Tagashira et al 2014).

At least one group has generated and analyzed the effects of a Sig-1R knockout on mouse development, but noted no changes in mouse phenotype with the exception of the mouse hyper-excitability response (Langa et al 2003). However, knockout of Sig-1R in SOD1 G93A ALS mice leads to a more rapid and severe progression of the disease (Mavlyutov et al 2013). In SOD1 G93A ALS mice, administration of Sig-1R agonists improved locomotor function and decreased motor neuron degeneration (Peviani et al 2014).

ALS17 *(CHMP2B)*

CHMP2B encodes a member of the chromatin modeling protein/charged multivesicular body family of proteins, and appears to function as part of an endosomal sorting complex which aids in the internalization and degradation of surface receptors (Tsang et al 2006). Chmp2b appears to be expressed in all neuronal tissues, with mutations altering the expression pattern (Skibinski et al 2005).

Mutations in *CHMP2B* have previously been identified as causative of frontotemporal lobar degeneration (Skibinski et al 2005), and with the emerging idea of the existence of neurodegenerative disorders ALS and FTD and FTLD existing on a spectrum with overlap between diagnoses and involved genes, this led groups to determine if patients with ALS expressed mutations in *CHMP2B*. In 2006, Parkinson et al's group was able to demonstrate cases of mutations in an adult, rapid onset and progression variant of ALS.

Affected individuals demonstrated varied symptoms (Parkinson et al 2006). One affected male demonstrated bulbar symptoms with progression to dysarthria and dysphagia. In the same time frame, wasting and weakness of the hands was noticed. Widespread neurogenic changes were evident upon electrophysiological analysis. Rapid progression of the disease was observed. No evidence of dementia symptoms were noted. The second patient presented with inappropriate behavior at age 65 that was ultimately diagnosed as frontotemporal dementia. After 5 years, motor neuron involvement was observed with dysphagia as the presenting symptom. This developed into dysarthria with right arm and hand atrophy. In other patients, progression was similar, though age of onset can vary significantly. In all cases observed, the presence of bulbar symptoms was noted.

An interesting note about this variant is the rare upper motor neuron involvement; few to no neurons from the cerebral motor cortex demonstrate pathological lesions (Parkinson et al 2006).

Further studies have gone into elucidating the pathogenic mechanism of *CHMP2B* mutations, and have demonstrated widespread results. An analysis of genetic material from over 400 ALS patients demonstrated 4 with mutations in *CHMP2B*, as opposed to 0 in controls (Cox et al 2010). In addition, functional changes within cells provided detrimental effects. In addition to previously noted defects in autophagosome clearance and clearance of protein aggregates from autophagosomes leading to accumulation of autophagosomes (Lee et al 2007, Filimonenko et al 2007), multiple cellular processes are disrupted by mutations in *CHMP2B*. Expression of human mutant *CHMP2B* in HEK293 cells demonstrated significant alteration of RNA processing and transcriptional activity (almost 900 mRNA transcripts downregulated, almost 600 upregulated with respect to wild type). This is likely via altered downstream effects as this protein is involved in various signaling transcripts that regulate mRNA production. Of note, multiple mRNA transcripts pertaining to endosomal targeting, ER targeting, and Golgi targeting are altered, disrupting these processes. In addition, downregulation in microtubule stabilizing protein transcripts were noted, which combined with stathmin upregulation (a protein that functionally de-stabilizes microtubules) likely challenges cytoskeletal integrity in these cells. Proteins involved in autophagy are also downregulated (Cox et al 2010).

Importantly, modification of the MAPK/p38 system may provide a link between this variant and other rare variants, such as ALS16 caused by mutations in *SIGMAR1*, as Sig-1R has been seen to modulate p38 signaling in astrocytosis/gliosis associated with ALS (Moon et al 2014). Cox et al (2010) demonstrated a downregulation of Ras, Raf, and ERK2, with upregulation of inhibitors of these proteins such as NF-1, indicating that growth may be limited in these cells. In addition to decreased MAPK signaling, decreased p38 signaling was noticed, leading to downregulation of proteins involved in many processes, including one that has notable functions in RNA stability and actin cytoskeletal changes, MK2. Downstream targets of MK2 including Hsp27, a protein involved in prevention of apoptotic activation by cytochrome c, caspase-3 and caspase-9, indicating a balance shift in apoptotic signaling in these cells, and likely pre-disposing these cells to apoptosis under conditions of ER stress, which is likely to be observed based on defects in ER and Golgi related transport pathways. Lastly, changes in mitochondrial calcium uptake were noted, indicating that changes in cellular energetic and mitochondrial dysfunction may be contributing, if not underlying factors in this disease. Based on all of this, it would seem that alterations in *CHMP2B* result in downstream signaling pathway changes pre-disposing the cell to ER stress and apoptosis due to decreased activity of Hsp27, which likely triggers to ERAD unfolded protein response.

At the cellular level, cells with *CHMP2B* mutations develop multiple large vesicles. It appears that interactions with Rab8, a protein associated with defective proteins in ALS, may be involved in these aberrant signaling and endosomal storage pathways. Changes in lysosomal storage pathways have been observed, and may help explain the formation of vacuoles as well (Clayton et al 2015).

In 2016, Vernay et al developed a transgenic mouse model expressing *CHMP2B* human mutants under the control of a mouse, neuronal-specific promoter. Importantly, integration of this gene into the mouse genome did not alter production of mRNA transcripts from the gene it inserted into. Previously developed transgenic *CHMP2B* mouse lines displayed neuronal pathology with inclusion bodies when expressed under control of the hamster prion promoter but no neuronal dysfunction (Ghazi-Noori et al 2012), or displayed symptoms of frontotemporal lobar degeneration and FTD without motor dysfunction (Gascon et al 2014). The mice developed by Vernay et al display clinical signs of both ALS and FTD, including abnormal gait, neuromuscular junction abnormalities, and presence of neuronal inclusions in an age dependent manner, indicating a reasonable model of the disease progression. A second group in 2017 created a transgenic mouse model expressing mutant *CHMP2B* with a C-terminal truncating mutation, essentially the same as those previously used (Clayton et al 2017). Many of the same features were identified as in the previous study, with new findings that reactive gliosis in neuronal tissue likely leads to the development of neurodegeneration, and the observation of neurodegeneration as measured by brain mass showed signs of neurodegeneration in transgenic mice. These mice will likely allow researchers to further understand the mechanisms associated with neurodegeneration, and possibly provide insight into mechanisms by which RNAi knockdown of mutant *CHMP2B* lead to restoration of wild type phenotype in fibroblasts isolated from patient fibroblasts (Nielsen et al 2012).

ALS18 *(PFN1)*:

Mutations in the gene for profilin-1 are some of the rarest variants associated with ALS (Lattante et al 2013, Zou et al 2013, Chen et al 2013, van Blitterswijk et al 2013, Syriani et al 2014), with affected populations primarily being of Nordic and German ancestry. Mutations in *PFN1* were first identified by via analysis of genetic data from two large ALS families (Wu et al 2012).

Profilin-1 is ubiquitously expressed in the cytosol, and was first identified for its ability to bind and interact with actin, possibly as a sequestration mechanism for free actin monomers (Carlsson et al 1977). Later studies demonstrated that profiling-1 functions in seeding of actin monomers into filamentous actin (Yarmola et al 2009), and have also demonstrated that aside from actin dynamics, profilin-1 may have functional implications in signaling pathways, such as VEGF signaling, as profilin-1 has been shown to interact with VEGFR (Pronto-Laborinho et al 2014), and VEGF signaling has previously been shown to be involved in the development of neurons as well as demonstrating neuroprotective effects (Jin et al 2000).

Indeed, in mice with conditional knockout of profilin-1 in selected neuronal tissues to prevent embryonic lethality seen in full knockout mice, development of the brain was affected by knockout of profilin-1, leading to a decreased brain volume, decreased radial migration of neurons in the CNS, and lack of association with glial cells by neurons (Kullman et al 2011). In a second mouse model expressing *PFN1* mutants on a null background, mice experienced progressive and unrelenting loss of motor neurons and associated symptoms of muscle weakness, ultimately leading to paralysis and death of the mice, likely due to respiratory failure of inability to gather resources (Yang et al 2016). In addition, examination of neuronal tissue displayed inclusions containing profilin-1 mutants and a disruption of neuronal cytoskeletal architecture. It is important to note that when observing these mice, development of protein inclusions was proceeded by neurodegeneration, indicating that formation of protein inclusions in this variant is not the causative mechanism, but an underlying result, and directing the idea that other variations caused by profilin-1 mutants, such as altered cytoskeletal dynamics may be mechanistic in the pathogenesis.

Links between profilin-1 and other proposed ALS pathogenic mechanisms have been proposed as well. It has been observed that profilin-1 associates with stress granules, membrane bound inclusions containing translational machinery and transcriptional altering machinery, and that mutations in *PFN1* can alter the dynamics of stress granule formation and resolution (Figley et al 2014). In addition, interactions between profilin-1 and ataxin-2, another protein in which mutations cause rare variants of ALS discussed above, were seen with co-localization to stress granules; this may simply be correlative, as ataxin-2 is localized to stress granules normally. Interactions between mutant profilin-1 and TDP-43 have also been established, as mutant profilin-1 protein appears to provide a seed for TDP-43 containing inclusions, likely due to a gain of function mechanism (Tanaka et al 2016, Tanaka and Hasegawa 2016). TDP-43 proteinopathy is commonly observed in multiple variants of ALS, with incorporation into plaques and a mis-localization which likely allows profilin-1 to bind and seed the abnormal cytoplasmic TDP-43 into insoluble plaques.

ALS19 *(ERBB4)*:

Mutations in ERBB4 were identified as causative of an adult onset ALS with relatively slow progression in a Japanese family diagnosed with ALS that was cleared of having mutations in more common disease causing genes such as *SOD1, TARDBP, FUS,* and *ALS2* (Takahashi et al 2013). Affected individuals displayed an autosomal dominant inheritance pattern, with an age of onset usually in the 6^{th} or 7^{th} decade of life. Weakness in the upper limbs was the most commonly identified phenotype at onset of the disease. Interestingly, the disease appears to have a plateau effect, whereby a large change in symptoms is seen initially, yet later in the progression little to no continued development of symptoms is seen. In most cases, patients experienced respiratory failure and became dependent on ventilators. Average time-course of the disease appears to be about 6-7 years before death.

ERBB4 is a member epidermal growth factor receptor subfamily of receptor tyrosine kinases. Binding of neuregulins to the extracellular binding domain leads to autophosphorylation of tyrosine residues in an ERBB dimer pair, triggering downstream signaling consequences (Huang et al 2000). Mutations in ERBB4 causative of ALS decrease autophosphorylation activity (Fleisig et al 2004). This likely plays a role in the progression of the disease, potentially limiting out-growth of neurons or dendrites.

Generation of ERBB4 knockout mice resulted in embryonic lethality associated with altered motor-neuron development (Gassmann et al 1995). This led researchers to examine the effects of neuregulin-1 knockouts in mice, as neuregulin-1 is the primary ligand for ERBB4. Mice with mutant neuregulin-1 developed abnormally, with 60% spinal motor neuron reduction (Wolpowitz et al 2000). Conditional neuronal knockout mice for neuregulin-1 experience embryonic lethality and demonstrate retraction of spinal motor neurons, indicating a role for ERBB4 and neuregulin-1 in the outgrowth of spinal motor neuron axons (Yang et al 2001).

Functionally, ERBB4 and neuregulin-1 appear to important for the maintenance and development of C boutons, specialized neuronal structures containing a modified endoplasmic reticulum that exist at synaptic terminals and heavily express cholinergic activity. This modified endoplasmic reticulum structure has been referred to as a subsurface cistern, and appears to be a site of neuregulin-1 and ERBB4 accumulation in disease (Gallart-Palau et al 2014). This is functionally different than in normal mouse spinal motor neurons, which display neuregulin-1 as punctuate throughout the soma and dendrites, whereas ERBB4 is usually found in presynaptic nerve terminals. In SOD1 G93A mouse models of ALS, at disease onset, transient localization of NRG-1 to C boutons was observed with increase expression; this was not observed in mice at end stage of the disease. Further studies have identified similar results, while also noting a decrease in ERBB4 concentrations in motor neurons of ALS mice (Lasiene et al 2016). Treatment of SOD1 G93A ALS mice with neuregulin-1, the ligand for ERBB4, leads to a partial cessation of symptoms characterized by an increase in collateral sprouting and re-innervation (Mancuso et al 2016).

ALS20 *(hnRNPA1)*:

In 2013, Kim et al identified mutations in *hnRNPA1* as being causative of ALS. Via screening of 212 familial ALS patient exomes, identification of one autosomal dominant form of the disease with mutations in known ALS proteins excluded resulted in the identification of a mutation in *hnRNPA1* that was also associated with the development of Paget bone disease in patients without *VCP* mutations (VCP discussed above; see ALS14). The mutation replaces an aspartate that is highly conserved across species boundaries, indicating functional importance of the residue (Kim et al 2013).

hnRNPA1 encodes the heterogeneous nuclear ribonuclear protein A1, which is involved in maintaining stability of mRNA and likely in transport of synthesized mRNA to the cytoplasm for transport (Michael et al 1995). Further work has shown that hnRNPA1 has functional activity as a splice site repressor, and can likely cause disease via exon skipping in *SMN2, BRCA1,* and *FBN1* related disorders, as depletion of hnRNPA1 leads to restoration of transcription for *SMN2,* partial recovery for *FBN1*, and no recovery in *BRCA1*, demonstrating the specificity of hnRNPA1 in repressing cryptic exons (Kashim et al 2007). Proper splicing of SMN-2 is restored by depletion of hnRNPA1, leading to a functionally active and correctly processed mRNA transcript (Kashima et al 2007). hnRNPA1 may also be involved in a complex to dissociate proteins involved in telomere enhancement from single stranded telomeric DNA, allowing for repair (Flynn et al 2011).

Mutations in *hnRNPA1* have interesting, and in some cases unexpected effects for cells. Due to the fact that hnRNPA1 contains a prion-like domain, formation of fibrils and polymerization of hnRNAP1 into insoluble plaques has been readily observed in disease causing mutants (Kim et al 2013, Shorter and Taylor 2013). In addition, these mutations that lead to formation of fibrils and inclusions also lead to increaseed localization of hnRNPA1 to stress granules, indicating an alteration in the balance of mRNA transcripts being processed and transported from the nucleus (Shorter and Taylor 2013), as well as providing a possible link between pathologies observed in ALS with mutations in other disease causing proteins. One group attempted to model changes in hnRNPA1 using computer simulations to predict the effects of various mutations along the length of the protein; predicted results of common mutations matched visible pathologies, such as propensity to aggregate, increased stability, and changes in C-terminal interactions, important for the RNA processing functions of hnRNPA1 (Krebs and De Mesquita 2016)

Interactions with other disease causing proteins have been demonstrated in hnRNPA1. For example, mutations in *hnRNPA1* or *UBQLN2* decrease the ability of these two proteins to interact, which led to increased turnover rates of hnRNPA1, indicating a role in stabilization for ubiquilin-2. In addition, hnRNPA1 has been shown to interact with TDP-43 (Burratti et al 2005), and in patients with TDP-43 proteinopathy with skein-like inclusions, a reduction in the total amount of hnRNPA1 was seen in both the nucleus and cytoplasm (Honda et al 2014).

Mouse models of homozygous and heterozygous deletions of hnRNPA1, as well as knockdown models of hnRNPA1 were first generated in 2012 by Chang et al (2012; not published except as patent; US Patent 20130067605 A1). These mice display embryonic lethality and decreased embryonic development, as well as growth delays; heterozygotes for hnRNPA1 knockout display some expression of hnRNPA1, and were not characterized further by the authors. Recently, another group has generated both homozygous knockout and heterozygous knockout hnRNPA1 mice; homozygotes are embryonic lethal, and do not display the presence of alternative splicing transcripts of proteins involved in muscle development. Heterozygote hnRNPA1 +/- mice survive to birth, and display relatively normal phenotypes. Muscle development and neuronal control defects are observed, as abnormal electrical activity in the heart is a common phenotype. In addition, presence of alternative splicing variants of muscle structural proteins were identified in this study, indicating the importance of hnRNPA1 in the development of muscle fibers, and possibly in the correct innervation of these fibers (Liu et al 2017).

ALS21 *(MATR3)*:

Matrin proteins, such as Matrin 3, were first identified in 1990 by the work of Stuurman et al as proteins present in the nuclear matrix of multiple mammalian cells. These were differentiated into inner and outer matrix proteins depending on solubility after reduction (Stuurman et al 1990). In addition, matrin 3 has DNA and RNA binding properties, appears in a granular pattern throughout nuclei of spinal motor neurons, as well as in surrounding glial cells, and can interact with TARDBP (Johnson et al 2014).

Analysis of a large Caucasian family with familial ALS and no previously identified causative mutations identified mutations in matrin 3 as causative of ALS. This variant appears to express autosomal dominant inheritance pattern, and tends to display classical symptoms of ALS with a slow progression, with an onset in the 6th decade of life, and a progression to death within the following two decades (Johnson et al 2014). Severe respiratory failure was noted as the cause of death in most cases. Upper motor neuron lesions were established by the presence of brisk knee-jerk reflexes in some, and in others weakness in the upper limbs was established. All affected patients displayed a “split hand” pattern of muscle wasting in the hand, whereby the muscles of the thenar (lateral, associated with ulna and metacarpal/phalange 5 primarily) eminence are selectively wasted in comparison to those of the phenar (medial, associated with the radius and phalange 1 primarily) eminence (Johnson et al 2014).

Matrin 3 is known to interact with TDP-43 (Ling et al 2010, Salton et al 2011), and has been demonstrated to be capable of pulling down TDP-43 in co-IP experiments (Johnson et al 2014), providing a link for possible pathologies associated with matrin 3 mutations, which include TDP-43 positive protein inclusions and matrin 3 positive inclusions (Wang 2015; thesis from Boston University).

Transgenic mice have been developed in an attempt to model several disease associated with changes in *MATR3*. Homozygous knockout of *MATR3* results in embryonic lethality, while heterozygous deletion results in the development of cardiac abnormalities (Quintero-Rivera et al 2015). Recently, groups have expressed human matrin 3 protein on a background of mouse matrin 3 protein, and demonstrated incomplete ALS phenotypes with associated hindlimb paresis and paralysis and muscle atrophy (Moloney et al 2016). This may implicate a toxic gain of function mutation in pathogenesis of the disease, and definitively rules out loss of function as a cause of pathology. In addition, it was noted that there is significant differences in matrin-3 expression levels within tissues and within the nervous system, with lowest expression levels in the spinal cord; observation of expression levels demonstrated a decline during neuronal development before stabilization of levels (Rayaprolu et al 2016).

ALS22 *(TUBA4A)*:

Full exome sequencing was performed on over 350 patients from various locations with confirmed ALS but no genetic variants in known disease causing genes, leading to the identification of *TUBA4A* mutations as causative of ALS in these patients (Smith et al 2014).

Patients with *TUBA4A* mutations display classical spinal onset ALS with upper and lower motor neuron signs; in at least one case, the presence of frontotemporal like dementia or frontotemporal lobar degeneration were diagnosed.

TUBA4A encodes a tubulin subunit expected to be incorporated into microtubules. Based on the pathology of other ALS variants, Smith et al 2014 examined the localization of TUBA4A, and found that mutations identified as causative of ALS prevented incorporation of alpha-tubulin into microtubules, instead finding them labelled in ubiquitinated cytoplasmic inclusions. Analysis was performed to determine if dimerization was possible (indicative of the potential to polymerize in microtubules), and it was determined that one mutation, $TUBA4A^{W407X}$ was unable to form dimers, while other mutations led to dimerization at significantly lower levels than wild type alpha tubulin 4A.

It has been proposed that mutations in *TUBA4A* confer a dominant-negative (toxic gain of function) action to these proteins, allowing them to interfere with generation of normal microtubule formation.

This is one of the first clear cases of ALS being caused by de-stabilization of microtubule dynamics, opening up the possibility that treatments leading to microtubule stabilization may provide relief against symptoms of this ALS variant, and the few others that experience microtubule dysfunction (Clark et al 2016).

***TBK1*:**

In 2012, researchers studying *OPTN* mutations leading to the development of ALS discovered that TBK1 (TANK1 Binding Kinase 1) co-localized with optineurin in protein inclusions seen in a zebrafish model of

ALS (Korac et al 2012). TBK1 is involved in regulation of optineurin and plays a role in the interaction between optineurin and autophagy associated machinery.

Loss of function mutations in TBK1 were identified as causative of ALS in 2015 by two independent groups (Chase 2015, Freischmidt et al 2015). TBK1 was first identified in mice as a kinase with the ability to control NF-kB signaling (Tojima et al 2000), but more recently it was determined that this protein primarily plays a role in activation of interferon regulatory factor (IRF) (Chau et al 2008). TBK1 also has important roles in the process of autophagy via interactions with optineurin, and is necessary for maturation of autophagosomes into autophagolysosomes for degradation of protein components such as ubiquitinated proteins and p62, a common component of protein inclusions in ALS (Ahmad et al 2016). When mitochondria in cells become damaged, the process of mitophagy to remove and destroy damaged mitochondria necessitates the interaction of TBK1 and optineurin; mutations in either of these proteins alter binding to the other (Freishmidt et al 2015). Taken together, these findings implicate a likely role for defective autophagy and mitochondrial dysfunction as mechanisms underlying the pathogenesis of ALS in these cases.

***P62/SQSTM1*:**

Mutations in *p62/SQSTM1* have been implicated in the pathogenesis of ALS, and are potentially causative. Two patients were identified with sporadic ALS and mutations in *p62* (Teyssou et al 2013). Patients had increased presence of p62 containing and TDP-43 containing protein inclusions in motor neurons, and increased levels of TDP-43 in the spinal cord. In all identified patients, ALS is concomitant with Paget disease of bones (Tessyou et al 2013, Kwok et al 2013).

Researchers have been unable to determine if mutations in *p62* are causative of ALS or rather act to increase the likelihood of development of the disease. P62 has important roles in autophagy, and appears to act at the center of the cells protein degradation and autophagy pathway, possibly helping shuttle proteins to their correct locations for degradation via the proteasome or autophagy (Bjorkoy et al 2006, Pankiv et al 2007). In addition, p62 has been shown to interact with both TDP-43 and FUS, and is localized to cytoplasmic inclusions containing these proteins in disease samples and models (Mizuno et al 2006, Hiji et al 2008, Deng et al 2010).

Important associated protein- *RBM-45*:

RNA Binding Motif 45kD is a protein expressed in the nervous system implicated in neuronal development (Tamada et al 2002) that has been discovered to be incorporated into insoluble protein plaques visible in ALS, and is elevated in CSF of sporadic ALS patients (Collins et al 2012). It has also been demonstrated that RBM-45 is capable of interacting with TDP-43 C-terminal fragments in yeast, indicating linkage to another ALS-causative protein (Hans et al 2014). Importantly, interactions with TDP-43 require oligomerization of RBM-45 via a conserved linker domain between two DNA/RNA binding domains.

RBM-45 is predominantly a nuclear protein, and can be seen to localize to the nucleus via fluorescent tagging and immunohistochemical analysis (Li et al 2015). Co-IP of RBM-45 pulled down TDP-43 and FUS; at endogenous levels, only TDP-43 was pulled down, indicating an interaction between these two proteins. In the presence of oxidative stress, similarly to TDP-43 and FUS, nuclear localization of RBM-45 decreases with increased appearance in stress granules; RBM-45 was commonly seen in stress granules with TDP-43 but not FUS.

No causative mutations of ALS have been discovered in RBM-45, but its role as an interacting protein with TDP-43, ability to be incorporated into stress granules, and presence in TDP-43 positive inclusions may indicate an important role for this protein in the pathogenesis of the disease. Conversely, since mutations in TDP-43 tend to confer toxic gain of function/dominant negative properties to the protein, this could be a by-product of the mislocalization of TDP-43 into cytoplasmic inclusions, or RBM-45 may be physically dragged into cytoplasmic inclusions by TDP-43 plaque formation since they interact and are capable of pulling each other down at endogenous levels.

ALS-FTD associated with *C9orf72* hexanucleotide-repeat expansions:

In 2011, two groups independently identified expanded repeats of a GGGGCC sequence in chromosomal region 9p21 in a non-coding region of the gene *C9orf72* (DeJesus-Hernandez et al 2011, Renton et al 2011), based on previously reported data demonstrating linkage between an affected ALS family and alterations in chromosome 9p21 (Boxer et al 2011). Boxer et al was not able to demonstrate the presence of any causative mutations in the region demonstrating linkage to the disease phenotype, leading to attempts by DeJesus-Hernandez et al and Renton et al to sequence this chromosomal region and look for non-coding variants that may be involved in the pathogenesis and progression of this ALS variant. The families studied had confirmed frontotemporal lobar degeneration (FTLD) with TDP positive inclusions (FTLD-TDP), and had a family history of FTD, ALS, or the mixed phenotype disease affecting motor and non-motor neurons amyotrophic lateral sclerosis with frontotemporal dementia (ALS-FTD); the genomic sequencing demonstrated that almost two thirds of families in this proband demonstrating FTLD-TDP exhibit expanded repeat numbers within the non-coding region of *C9orf72* between exons 1a and 1b. To demonstrate the frequency of *C9orf72* repeat expansion associated pathologies in FTLD, ALS, and FTD, a group of 696 patients had genetic material analyzed for repeat expansions. 59 patients were identified in this screen as containing repeat expansions, including 22 with suspected sporadic FTLD. The authors admit that ALS was not the central focus of this study, leading to a slightly reduced cohort of ALS patients compared to FTLD. That being said, repeat expansions were noted as having significant association to ALS; 4.1% of sporadic ALS cases and 23.5% of familial ALS cases analyzed in this study demonstrated repeat expansion variants in *C9orf72*, clinically demonstrating *C9orf72* as one of the most common causative genes associated with ALS, ALS-FTD, and FTLD-TDP. It was proposed that repeat expansions interfered with production of *C9orf72* variants via defective alternative splicing, which would match the clinical data indicating a reduction in the levels of one full-length variant. The structure of the *C9orf72* gene can be seen in Figure 13. RNA foci formed from aggregation of repeat expanded mRNA were detected via FISH, and demonstrated the presence of RNA foci in approximately 25% of neuronal cells from affected patients, indicating a possible role in disease pathogenesis.

Analysis of 402 ALS cases compared to 478 controls from a Finnish population demonstrated that in 28.1% (113) of affected patients exhibited significant repeat expansion as compared to 0.4% (2) of the control cases (Renton et al 2011). Using FISH to determine repeat expansions length, it was concluded that the maximum demonstrated number of repeats seen in patient tissues was approximately 250. *C9orf72* transcripts were detected in multiple CNS tissues with highest expression in the cerebellum. No changes in mRNA levels for *C9orf72* were detected in patients compared to non-patients. To confirm results from the first population, a mixed German, Italian, and North American cohort was used which demonstrated repeat expansion in 38.1% of affected cases as compared to 0 in control tissue. In addition, comorbidity of ALS and FTD was observed in multiple patients with repeat expansion in C9orf72.

Neuronal protein inclusions containing TDP-43 are considered one of the hallmarks of most ALS, FTD/MND (ALS-FTD) and FTLD, and have been demonstrated consistently for many years (Leigh et al 1988, Lowe et al 1988). In addition, protein inclusions have been commonly demonstrated to contain p62, an important regulatory molecule in many pathways including autophagy, and ubiquitin. To determine whether patients harboring *C9orf72* repeat expansions had neuronal tissue with TDP-43 positive inclusions, 14 FTLD or FTD/MND patients with confirmed *C9orf72* hexanucleotide-repeat expansions were compared to 22 patients with confirmed lack of expansions, and 4 controls with no expansions (Al-Sarraj and King et al 2011). Analysis of neuronal tissues taken from the cerebellum and hippocampus revealed the presence of p62 positive protein inclusions that were predominantly TDP-43 negative in the neuronal cytoplasm and within the nuclei of neuronal tissue. In the spinal cord, p62 and TDP-43 positive inclusions were demonstrated. Ubiquitin was not stained for in this study, but a previous study performed before the identification of repeat expansions in *C9orf72* were observed used FTLD patients and demonstrated the presence of ubiquitin containing inclusions (Pikkarainen et al 2008). Notably, location dependent differences in the pattern of staining and inclusion were seen in ALS patient tissue used in the Al-Sarraj and King et al study. In the hippocampal layer, p62 positive inclusions were common, forming large star-shaped bodies, with rare instances of TDP-43 containing inclusions. In upper layers of the frontal and temporal lobes, semi-lunar and dot-like inclusions positive for p62 and TDP-43 were seen in addition to presence of large, p62 positive star-shaped inclusions, which were more common in lower layers of these regions. Within the spinal cord, p62 positive and TDP-43 positive skein-like inclusions were commonly seen.

Demonstrating the presence of TDP-43 positive, p62 positive, and ubiquitin positive inclusions in cases harboring *C9orf72* repeat expansions was one of the first steps in identifying a common pathologic mechanism, and essentially confirmed the concept that ALS exists as a part of a spectrum of disease including FTD and FTLD. This also led researchers to consider whether *C9orf72* interacted with other implicated proteins in ALS. Analysis of neuronal tissue samples from another group of 75 patients with clinically confirmed ALS and identified 23 patients with either ALS (14) or FTD/FTLD-TDP (9) with repeat expansion in *C9orf72* (Brettschneider et al 2012). IHC labelling for C9orf72 demonstrated similar patterns to those seen by DeJesus-Hernandez et al (2011), identifying coarse, but point-like staining of C9orf72 primarily in hippocampal synaptic terminals. IHC staining for ubiquilin-2 demonstrated pathological lesions in the hippocampal molecular layer. Dystrophic neurons with focal swelling and dot-like inclusions were seen. In non-*C9orf72* expansions patients, limited involvement of the hippocampal molecular layer was observed. In the cerebellum, round, Lewy-body like inclusions were stained with antibodies for ubiquilin-2 and seen commonly in the granular layer, and in neocortical regions showed dystrophic neurites and presence of inclusions throughout as compared to non-expansion cases limited to superficial neocortical layers. Based on the unique pattern of protein inclusions seen, researchers were able to predict whether samples contained a *C9orf72* repeat expansion or not. This interesting study provides insight into the central pathway that may be dysregulated in ALS, as it would be unexpected to find ubiquilin-containing inclusions without mutations in ubiquilin-2. Their findings suggest *C9orf72* likely encodes a protein involved in pathways associated with both ubiquilin-2 and p62, and that these interactions contribute to disease pathology.

Building upon this work, further groups analyzed the presence of other proteins whose genes harbor mutations causative of ALS. Keller et al (2012) demonstrated the presence of multiple ALS-associated proteins in neuronal inclusions from patients with a mutation in only one ALS-associated genes (mutations can be homozygous or heterozygous depending on the variant and inheritance pattern). In motor neurons from patients with *C9orf72* repeat expansion, the presence of TDP-43 in cytoplasmic inclusions was demonstrated, and the presence of FUS in both cytoplasmic inclusions and nuclear cytoplasmic inclusions was observed (Keller et al 2012). In patients with *C9orf72* repeat expansions, C9orf72 cellular distribution was normal; however in patients with SOD1 mutations, C9orf72 positive inclusions were commonly seen in motor neurons. Ubiquilin and p62 containing inclusions were demonstrated to be a clinical feature of *C9orf72* related pathologies. Large diameter round inclusions and skein-like inclusions positive for the neurofilament subunit H were seen in *C9orf72* related pathologies, implicating a possible destabilization of neuronal structure in the pathogenesis of the disease, and providing for overlap between other disease where neurofibrillary tangles and inclusions are commonly seen. Optineurin containing inclusions were also observed for patients with *C9orf72* repeat expansions. This work provides more insight into the clinical overlap between genes associated with ALS, and demonstrates that formation of protein inclusions is a relatively common feature of ALS.

Location in Brain	TDP-43 Positive Inclusions	p62 Positive Inclusions	Size and Shape
Cerebellum	No	Yes	Large, round, or irregular (star-shaped)
Hippocampus	No	Yes	
Fronto-temporal lobes	Yes	Yes	Semi-lunar or dot-like; Large round, or irregular as well
Spinal Cord	Yes	Yes	Skein-like

Table 2: A generalization of the locations and types of protein inclusions seen in *C9orf72* hexanucleotide repeat expansion associated ALS-FTD. While the picture is not this clear clinically, and many other proteins have been observed in inclusions, these are the most commonly observed results from the studies presented above.

Clinical Features:

Patients presenting with *C9orf72* repeat expansions demonstrate a wide variety of neuronal and glial involvement, age of onset, progression rate, and involvement of frontotemporal dementia symptoms. Commonly, initial symptoms of C9orf72 associated ALS is the presence of bulbar symptoms, such as dysarthria or dysphagia, trouble speaking and trouble eating respectively (Stewart et al 2012). Patients presenting with *C9orf72* repeat expansions and the clinical symptoms of ALS displayed expected TDP-43 pathologies with limited extrapyramidal involvement (Murray et al 2011). Atrophy of the frontal and temporal cortexes in the brains of ALS patients was not noted, but was highly prominent in patients displaying with frontotemporal dementia and frontotemporal dementia with associated motor neuron degeneration. All patients displayed nucleocytoplasmic inclusions containing ubiquitin in the cerebellar internal granular cell layer, which were less numerous in ALS cases than other associated pathologies. Gliosis was a prominent feature detected in ALS cases as compared to FTD cases, and sever hypoglossal neuronal loss was observed in all cases, indicating bulbar pathologies. No significant cortical neuron loss was observed in ALS patients as compared to FTD/FTLD cases. Glial cell inclusions are more commonly seen in ALS than FTD/FTLD, and were concentrated in hypoglossal regions and the medullary pyramid. In a study by Byrne et al (2012), it was noted that cognitive decline was significantly greater in patients with *C9orf72* repeat expansions than patients lacking this, and that over time gain of dementia like symptoms was commonly seen. In addition, shorter survival times for patients with repeat expansions and comorbid ALS-FTD were observed (Byrne et al 2012). Along with this, it was noted degeneration of the corticospinal tract and a loss of lower motor neurons from both the cortex and spinal cord (Stewart et al 2012). Small, round, ubiquitin and p62 containing inclusions were another common feature of *C9orf72* associated ALS. In FTD patients, the presence of ubiquitin and p62 positive cytoplasmic inclusions that do not stain for TDP-43 are commonly seen in the cerebellar cortex (Hsiung et al 2012), and these appear to be specific to *C9orf72* repeat expansion associated pathologies (Bigio et al 2013). In addition, this study noted the wide range in age of onsets (in 30 patients, age of onset ranges from 34-74). In further studies, it has been noted that the association of ALS and FTD is commonly seen in patients with *C9orf72* repeat expansions, and an older average age of onset has been demonstrated (Millecamps et al 2012), though in other studies, a younger average age of onset has been seen (van Blitterswijk et al 2012, Irwin et al 2013). In addition to a younger age of onset, a shorter disease time-course has been established (Irwin et al 2012), with the time-course before progression to death being approximately 1 year shorter in *C9orf72* repeat expansion cases.

Dipeptide Repeat Proteins

In large, repeated regions of DNA, translation of the repeated region will lead to a repeat protein, so long as a multiple of 3 nucleotides is repeated. In *C9orf72* hexanucleotide repeat expansions, improper processing of the RNA and translation of the intronic repeated sequence can lead to the production of dipeptide repeat proteins, which are proteins consisting of two amino acids repeated. Because of the sequence that is repeated GGGGCC, and depending on the reading frame used, at least 6 possible repeat proteins can be generated, each containing a repeat of two amino acids. The various dipeptide repeat proteins generated will be described below, as well as how they are generated from intronic sequences in this disease.

Initiation of translation is a highly regulated process, and the presence of an RNA transcript containing an AUG codon has largely been considered crucial to the process. However, alternative forms of translation exist, including one that has the potential to impact *C9orf72* hexanucleotide-repeat expansion patients. Repeat associated non-ATG translation (RAN translation) was first identified in 2011, when it was identified as a possible pathogenic mechanism in the disease progression of spinocerebellar ataxia type 8 (Zu et al 2011; Pearson 2011). In *C9orf72* hexanucleotide-repeat expansion, both the sense and anti-sense strands undergo transcription, resulting in GGGGCC and GGCCCC repeat mRNAs which are both translated to form dipeptide repeat proteins, with the exact composition of the repeats varying based on the reading frame (Gendron et al 2013a, Gendron et al 2013b, Mori et al 2013). These two RNA strands form unique secondary structures, with the GGGGCC repeat mRNA forming G-quadruplexes with hairpin turns, and the GGCCCC repeat mRNA likely forming a double helix (Fratta et al 2012, Reddy et al 2013). These unique secondary structures may be involved in the initiation of RAN translation, though the mechanism remains unclear. Ash et al 2013 was able to demonstrate the presence of dipeptide repeat proteins, most notably a poly (glycine-proline) dipeptide repeat protein based on immunohistochemical staining for 3 different possible dipeptide repeat proteins that could be formed from GGGGCC hexanucleotide repeat expansions (Ash et al 2013). These products were specifically detected in gray matter and within neurons, but not within white matter or support cells, indicating inclusion bodies in the soma and dendrites of neuronal tissue. Neuronal cytoplasmic inclusions possessed the characteristic star shaped appearance. Indeed, translation of the antisense GGGGCC transcripts also resulted in dipeptide repeat proteins in all three reading frames (Mori et al 2013). Further work has demonstrated translation of both positive and negative sense mRNA transcripts, as the presence of both poly (alanine-proline) and poly (proline-arginine) dipeptide repeat proteins was detected in p62 positive, TDP-43 negative cytoplasmic inclusions in neuronal tissue taken from the cerebellum and hippocampus of affected patients. In addition, cells transfected with a vector leading to the expression of dipeptide repeat proteins associated with RAN translation of *C9orf72* repeats, aggregates of poly (glycine-alanine) dipeptide proteins were significantly more prone to aggregation, forming large aggregates as compared to the other dipeptide repeat proteins, which remained diffuse or formed small aggregates (Zhang et al 2014). These inclusions differ from those commonly seen in TDP-43 pathology, which form Lewy like bodies or skein-like inclusions, and also different from previous results of dipeptide repeat protein aggregation into star-shaped inclusions. Through the use of a transfected cell line, researchers were able to avoid any RNA foci mediated toxicity, while examining the effects of dipeptide repeat protein accumulation on cellular dynamics. Using caspase-3 activity as a measurement of cellular stress, researchers identified that expression of poly-GA dipeptide repeat proteins increased apoptotic activity by a factor of approximately 8, indicating conditions of stress in these cells. In primary mouse neurons transfected to express poly-GA proteins, proteasome degradation of proteins was significantly reduced, indicating a likely role for ER-stress in the apoptotic response leading to cell death. Further research has demonstrated that in cultured cells transfected to express polyGA dipeptide repeat proteins, the formation of poly-GA containing, p62 containing, ubiquitinated inclusions were routinely seen in neuronal bodies (Yamakawa et al 2015). Expression of these proteins has been shown to alter the ubiquitin-proteasome system's ability to function (possibly by increasing the degradation burden of the system), including the ability to degrade TDP-43, and sensitivity to proteasomal inhibitors, both of which were increased in the presence of dipeptide repeat proteins formed from *C9orf72* hexanucleotide repeat expansion. Dipeptide repeat protein inclusions have since been demonstrated to include other nuclear proteins and DNA/RNA binding proteins, such as the presence of Drosha in dipeptide repeat aggregates containing p62 and ubiquilin (Porta et al 2015).

In one study looking at the effect of dipeptide repeat proteins on cell survival dynamics, it was noted that none of the dipeptide repeat proteins were aggregation prone, did not form insoluble protein plaques, and did not induce proteasomal stress or ER-stress (Tao et al 2015). Formation of aggregate like inclusions in the nucleus was seen for the arginine containing repeat proteins, which induced nucleolar stress and impaired formation of stress granules. These results are significantly different from previously observed results, and appear to be contradictory in nature to what has been previously observed. The propensity of these dipeptide repeat proteins to aggregate has been repeatedly established, and the development of ER–stress has been observed to be an implicated pathogenic mechanism in many different variants of ALS including *C9orf72*-associated ALS. Therefore, these results are very surprising, and in many ways detract from what was previously observed. This discrepancy from previously reported results may be due to differences in cell lines used, though it is not clear that this result is representative of the body of research as a whole, and has not been confirmed by the work of any other research groups.

Recently, it was observed in spinal motor neurons of ALS patients with confirmed *C9orf72* repeat expansion that dipeptide repeat proteins are almost completely absent (Gomez-Deza et al 2015). Due to the fact that these inclusions have been seen in the cerebellum and hippocampus, with expression in essentially all cortical regions, it is unexpected that these inclusions were not also found in spinal motor neurons. However, this might explain how *C9orf72* repeat expansion can lead to the symptoms of both FTD and ALS. Gain of function in the form of dipeptide repeat formation may be involved in neurotoxicity associated with FTD/FTLD, while loss of function in protein activity or gain of function in the formation of RNA foci may be implicated in neurotoxicity associated with ALS.

In a yeast screen, dipeptide repeat proteins were shown to affect RAN-mediated nucleocytoplasmic transport, indicating a potential role for aggregation of dipeptide repeat proteins in affecting cellular transport dynamics which are commonly altered in other ALS variants (Jovicic et al 2015). This result was confirmed in mice expressing transgenic poly-GA proteinopathy, whereby expression of poly-GA proteins led to the sequestration of proteins involved in nucleocytoplasmic transport, such as nuclear pore proteins (Zhang et al 2016). In rats, expression of dipeptide repeat proteins in neurons demonstrated that poly-GA proteins lead to inclusions that resemble the poly-GA/p62/ubiquitin positive aggregates seen in *C9orf72* ALS patients. As previously reported, arginine containing dipeptide repeat proteins largely localized to the nucleolus; these proteins co-localized with a transcriptional repressor, indicating a potential role for gene transcription defects in the pathogenesis of the disease. As previously reported, these inclusions were predominantly in the cortical neurons of the cerebellum, hippocampus, thalamus, and neocortex, with limited appearance in the brain stem or spinal neurons. Poly (proline-arginine) repeat protein inclusions were more commonly seen in FTLD patients, despite being rare in all cases, indicating a potential role for transcriptional control in neurodegeneration causative of FTLD symptoms. Levels of poly (glycine-proline) dipeptide repeat proteins have also been shown to be lower in patients with predominantly ALS phenotypes as compared to predominantly FTD/FTLD phenotypes, particularly in the cerebellar regions. In fact researchers were able to demonstrate a quantitative relationship between poly (GP) protein levels and cognitive score in FTLD/FTD patients (Gendron et al 2015). In addition, levels of poly (glycine-alanine) dipeptide repeat proteins were decreased in ALS patients as compared to FTLD/FTD patients when comparing levels in the cerebellum. These phenotypic differences between the levels of dipeptide repeat proteins, presence or absence of inclusions, and expression of distinct dipeptide repeat proteins in various regions of the brain may provide some insight into the pathogenesis of the disease, but currently, these mechanism remain to be elucidated.

Previous associations had demonstrated arginine dipeptide repeats proteins localized to the nucleolus. Further research has demonstrated that these dipeptide repeat proteins bind and sequester mRNA transcripts, bind and sequester translation initiation proteins, and block access of translation initiation proteins via the formation of insoluble protein plaques co-localized with mRNA and translation initiation factors (Kanekura et al 2016). This may explain the large scale dysfunction seen in many cells with ALS mutations, where alterations in one protein lead to widespread dysfunction in terms of cellular autophagy, mitochondrial dysfunction, and intracellular transport dynamics. In addition, this may explain why in some variants of ALS, mutations in proteins that have no effect on RNA processing, transcription, or translation result in down-regulation of large numbers of RNA targets.

Very recently, it has been demonstrated that the poly-GA dipeptide repeat protein forms protein plaques that have cell to cell transmissible properties, while confirming that poly-GA dipeptide repeat proteins form amyloid like fibrils. By adding poly-GA peptides, containing 15 GA repeats, to cell culture media, researchers were able to demonstrate the presence of poly-GA containing plaques in N2a cells that did not express the transgene for poly-GA proteins (Chang et al 2016). Further research performed in neurons created with directed differentiation from *C9orf72* ALS patient stem cell lines demonstrated evidence that dipeptide repeat proteins are able to be propagated between cells in a prion-like manner (Westergard et al 2016). In addition, poly (Glycine-Arginine) dipeptide repeat proteins can result in increased DNA damage as evidenced by activation of p53 in neuronal stem cells derived from *C9orf72* patients (Lopez-Gonzalez et al 2016). Expression of dipeptide repeat proteins in normal neuronal derived stem cells also led to increased DNA damage with age dependency as measurable by increased activation of p53. When measuring the level of reactive oxygen species in stem cells with *C9orf72* hexanucleotide repeat expansions, ROS levels were not increased at 4 weeks, but at 8 weeks ROS levels began to increase and continued to increase at 12 weeks. This observation adds credence to the observed age dependent DNA damage in these cells. When expressing poly-GR dipeptide repeat proteins in wild type neuronal tissues, mitochondrial dysfunction was also observed, as poly-GR proteins bound and sequestered mitochondrial proteins. Alterations in structures formed by dipeptide repeat proteins of various lengths may influence the pathogenesis of the disease by increasing the sequestration of involved proteins, as it has been demonstrated that different repeat length dipeptide proteins result in the formation of distinct intracellular inclusions with distinct protein binding and sequestration properties (Flores et al 2016). Of important note, in cells expressing poly-GA proteins at high levels, nuclear import of TDP-43 is impaired, possibly linking the observed pathology of TDP-43 positive inclusions to *C9orf72* hexanucleotide repeat expansion (Khosravi et al 2016). In addition, presence of poly (Proline-Arginine) dipeptide repeat proteins were demonstrated to bind to domains in nuclear pore forming proteins leading to defects in nuclear import and export (Shi et al 2017), which could possibly explain the presence of DNA/RNA foci commonly seen in this disorder (Freibaum and Taylor 2017).

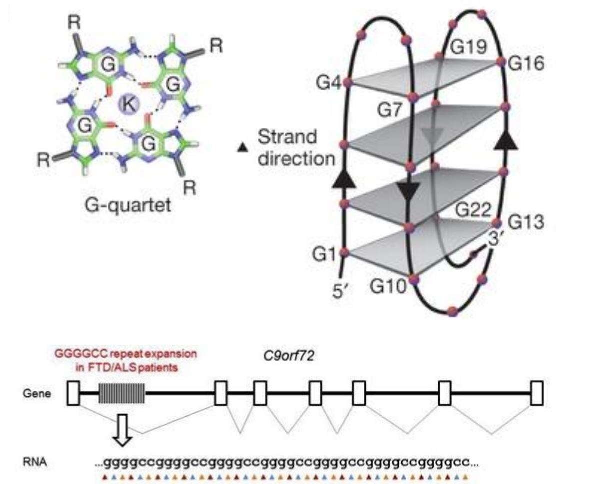


Figure 13: Top) Structure of G-quadruplex mRNA and formation of G-quadruplexes from repeat expanded mRNA (Haeusler et al 2014). Bottom) Gene structure of *C9orf72* showing repeat expansion region, which is in an intronic region of the gene. (Mori et al 2013).

Based on these studies, we can conclude that there is a significant role for dipeptide repeat proteins in the development of the disease. While protein inclusions are directly toxic to cells, these may or may not be causative of the associated neuronal cell death, and indeed are likely antecedent to neuronal death. The presence of dipeptide repeat proteins leads to major alterations in cellular phenotypes, including RNA processing and translational initiation, which may explain the multiple associated changes to neuronal cell physiology associated with the disease. The previously elucidated roles of dipeptide repeat proteins in clogging proteasomal degradation pathways, clogging and interfering with autophagy degradation, and sequestration of RNA may explain why large scale disruption of cellular processes leading to cellular death is observed.

G-Quadraplexes

RNA is a molecule capable of forming multiple, three dimensional structures and assuming a multitude of different conformations. One such structure is a G-quadruplex, which is a highly stable structure formed in guanine rich regions of DNA or RNA. The structure of these can be seen in figure 13. Using NMR, Fratta et al 2012 demonstrated the formation of G-quadruplex structures in DNA from patients with GGGGCC hexanucleotide expansions in *C9orf72*. In melting point tests, G-quadruplex DNA of *C9orf72* was found to be highly stable, retaining the same structure at 95° C, and when testing the effect of cation levels on stability, the role of potassium in maintaining G-quadruplex stability was noted (Fratta et al 2012). Formation of G-quadruplexes results in changes in the transcription of *C9orf72*, with increased repeat number leading to shorter transcripts, possibly due to stability of the G-quadruplex structure blocking RNA polymerase progress (Haeusler et al 2014). In addition, the formation of DNA/RNA hybrid structures was noticed, as treatment of cells with RNaseH led to decreases in the number of abortive transcripts, and an increase in the number of full length transcripts, indicating that the formation of DNA/RNA hybrids occurs and is one of the causes of abortive, short length *C9orf72* transcript formation. In this study, it was also noted that RNA foci forming in nucleoli of cells contain GGGGCC repeat mRNA, and that these foci induce stress in the nucleolus. One final important observation from this study is that the number of P bodies (stress granules) observed in cells containing *C9orf72* hexanucleotide-repeat expansions is significantly increased compared to controls, indicating that alterations in both RNA processing and translation result from *C9orf72* hexanucleotide repeat expansion. Via NMR techniques and the use of 8-deoxybromoguanosine substitution, a modified guanine derivative for detection in DNA, led to the resolution of the G-quadruplex structure for *C9orf72* mRNA specfically (Brcic and Plavec 2015), and was also resolved via NMR by a second group (Zhou et al 2015). The structures have all four strands in an antiparallel conformation, with linkages of cysteines, which allow for the formation of a very condensed structure. G quadruplexes have been demonstrated to sequester ribonuclear proteins leading to alterations in RNA processing, including splicing variants (Conlon et al 2016). Recently, it has been confirmed that C9orf72 mutations impair the formation and maintenance of membrane-less organelles, such as the nucleolus, via interactions with RNA binding proteins and proteins containing low complexity sequence domains which are involved in formation of these structures (Lee et al 2016).

In addition, G-quadruplex structures formed from hexanucleotide repeat expansion in *C9orf72* has been demonstrated to confer a toxic property of increasing the intrinsic oxidation and peroxidation properties of heme, which could lead to alteration of DNA sequences or alter transcription, as increased levels of reactive oxygen species in cells predispose DNA to damage via binding of ROS species to nucleotide bases or the phosphate backbone of DNA (Grigg et al 2014).

The formation of G-quadruplexes by *C9orf72* mRNA may be important in the pathogenesis of ALS, as these formations have been shown to sequester other mRNA, which could lead to reduced transcriptional levels of various proteins important to neuronal survival. The property of activating heme and increasing oxidative properties of the cell could lead to increased levels of reactive oxygen species, predisposing the cell to apoptosis. Lastly, due to the structure of these G-quadruplexes, which are capable of forming highly condensed structures and RNA foci, making the RNA foci formed in these cells much harder to clear.

RNA Foci

RNA foci forming inclusions are often observed in cells from ALS patients, with both nuclear and cytoplasmic RNA foci observed (DeJesus-Hernandez et al 2011; Donnelly et al 2013). It has been demonstrated that the use of anti-sense oligonucleotide therapies can reduce toxic phenotypes in neurons with *C9orf72* hexanucleotide repeat expansions. In a study looking at the effect of G-quadruplex RNA foci, it was determined that larger repeat expansions increase the formation of RNA foci, while reducing transport of large repeat RNA, indicating the possibility that RNA foci form soon after transcription and preclude processing or export (Lee et al 2013). In addition, in cells expressing longer repeat numbers, activation of apoptotic factors was increased as compared to wild-type, indicating that RNA foci may be causing toxicity in neurons. Long repeats are suspected to lead to nuclear retention of transcripts that form RNA foci that are RNase resistant and toxic to neurons. Due to the formation of RNA foci, sequestration of RNA binding and processing proteins could be expected. Indeed, RNA processing proteins such as hnRNP H are sequestered by *C9orf72* RNA foci and likely decrease the ability of RNA processing proteins to perform necessary functions such as the ability to create splicing variants. Additionally, interaction and possible sequestration with nucleolin, an essential nucleolar protein have been demonstrated, possibly providing a link for nucleolar stress and dysfunction observed in *C9orf72* associated ALS (Haeusler et al 2014). Accumulation of G-quadruplex containing RNA foci has been shown to decrease global levels of mRNA transport out of the nucleus, leading to increased nuclear mRNA concentration and a decrease in levels of translation (Rossi et al 2015). In patients with *C9orf72* repeat expansion, alterations in levels of splicing variants for other proteins have been observed (Cooper-Knock et al 2015). This may be explained by the sequestration of RNA processing enzymes into RNA foci by G-quadruplex containing mRNA. Recent evidence has suggested that *C9orf72* RNA may be localized to neuritic transport granules that are delivered along the length of the axon. (Burguete et al 2015). In addition, it was observed that expansion of *C9orf72* repeats led to branching defects in neuronal development, though this is unlikely to be the cause of degeneration associated with ALS, as this change confers neurodevelopmental problems, and we do not see significant neurodevelopmental defects in patients.

The presence of RNA foci is likely an important pathological feature, as this leads to multiple issues, including sequestration of various other mRNA, sequestration of mRNA processing proteins, and likely to stress responses in the cell that are involved in clearance of excess RNA. In addition, this sequestration of mRNA into foci limits the supply of rNTP molecules, and could induce a stress response, possibly increasing the propensity of these cells to undergo apoptosis due to changes in cellular energetics and a lack of mRNA degradation.

C9orf72: Loss of Function or (Toxic) Gain of Function

There is much controversy in the field of ALS as to how repeat expansion of *C9orf72* confers toxic properties to cells. Multiple groups have attempted to answer this question definitively, but conclusive answers have remained elusive.

Evidence for Loss of Function

Near the promoter region for *C9orf72* lies a CpG island, which can be hypermethylated (Xi et al 2014). Hypermethylation of DNA generally leads decreased gene expression. Hypermethylation of the CpG island has been associated with the presence of repeat expansions, is linked to a faster progression, and has been demonstrated to significantly associated with familial ALS caused by *C9orf72* repeat expansion. Further studies have demonstrated that hypermethylation of the CpG island leads to decreased levels of *C9orf72* transcripts and reduced formation of RNA foci (Liu et al 2014). In addition, removal of methylation pre-disposed cells containing repeat expanded C9orf72 in culture to oxidative and autophagic stress, implicating a role for C9orf72 in the regulation of these pathways. In Russ et al (2015), similar results were confirmed, but it was noted that increased hypermethylation was associated with a smaller repeat expansions (Russ et al 2015) This is contradictory to previously reported results that demonstrated that increased repeat number was associated with increased hypermethylation, and of the two is the less likely. It would make sense that in repeat expanded *C9orf72* the promoter is hypermethylated more frequently to provide protective effects to the cell against the formation of dipeptide repeat proteins, and hopefully simultaneously reduce the presence of RNA foci, given the general role of methylation in DNA sequestration. In addition, since hypermethylated genes are transcribed and translated less frequently, they are more prone to experience mutations, and this could possibly lead to continued repeat expansion during subsequent transcriptions, as genes that are not transcribed commonly are also not repaired commonly. Given the role of hypermethylation in gene suppression, and decreased expression of *C9orf72* noted in patient tissue samples of ALS and FTD/FTLD patients, this implicates haploinsufficiency/loss of function in the development of the disease. With the use of imaging and neuropathologic analysis from patients, further confirmation of the neuroprotective effects of promoter hypermethylation was demonstrated (McMillan et al 2015). In addition, it has been observed that methylation of the CpG island in the *C9orf72* promoter leads to a reduction in the number of RNA foci formed while also decreasing the levels of toxic dipeptide repeat proteins associated with RAN translation (Bauer 2016).

In a zebrafish model, knockdown of *C9orf72* led to motor deficits (Ciura et al 2013). However, as zebrafish C9orf72 is only expressed in development of the nervous system, these defects could be related to neurodevelopmental rather than neurodegenerative changes. In *C. elegans*, deletion of *C9orf72* was associated with an ALS like phenotype including progressive, age related motor dysfunction leading to paralysis, and an increased stress response to osmotic changes, indicative of an altered stress response in the neuronal tissue of these organisms (Therrien et al 2013).

It has also been noted that depletion of C9orf72 in neuronal tissues leads to impaired autophagy and increased sensitivity to ER-stress (Sellier et al 2016), indicative of a possible loss of function mechanism.

Lastly, the almost complete lack of dipeptide repeat protein containing inclusions seen in ALS patient neuronal tissue samples makes loss of function more likely, as the toxic properties conveyed by these dipeptide repeat proteins may not be involved in the pathogenesis of spinal motor neurodegeneration (Gomez-Deza et al 2015).

Taken together, all of these studies suggest that a loss of function mechanism may be implicated in *C9orf72* ALS. However, the results of these studies are less than convincing in many cases, and contradictory results have been observed in some cases. Due to the contradictory nature of many results, but taking into account the wealth of data from animal models generated, this in my opinion, is the less likely of the two possible options for what type of change is conferred to cells by the repeat expansion.

Evidence for Gain of Toxic Function:

The most prominent evidence for a gain of function mutation in *C9orf72* comes from observations on clinical phenotype of patients and observations from animal studies.

The formation of dipeptide repeat proteins has been discussed in great detail above. RAN translation of hexanucleotide repeat sequences leads to the production of dipeptide repeat proteins which accumulate into insoluble plaques, cause nucleolar and autophagy/ER stress and dysfunction, and have been demonstrated to have prion like propagation patterns. These properties are definitively a toxic gain of function type mutation.

Hypermethylation of the *C9orf72* promoter has been associated with protection from the associated symptoms of repeat expansion, indicating that loss of function is likely not the mechanism by which *C9orf72* repeat expansions lead to the development of pathology (Liu et al 2014). However, the results of hypermethylation studies can argue for either gain or loss of function depending on interpretation. Thus, these have little predictive value in determining the nature of the disease, but do need to be discussed. Because of the contradictory nature of results, we cannot say for sure how hypermethylation is affecting the gene or gene structure. It would make sense that, since hypermethylation leads to sequestration of DNA, that hypermethylation of the *C9orf72* promoter would protect the patient from the effects of the repeat expanded gene, while simultaneously allowing the repeat expansions to continue or worsen, and thus implicating the gain of function due to dipeptide repeat proteins or RNA sequestration as a more likely mechanism.

Most notably, in animal models, abalation of *C9orf72* does not lead to a phenotype similar to clinical ALS (Koppers et al 2015). In a recent study, mice were transgenically engineered to express *LacZ* in the coding region of *C9orf72*, resulting in a complete loss of C9orf72 expression and function (Atanasio et al 2016). These animals displayed slight differences in motor function compared to wild type, most notably progressive hind limb weakness and associated changes in gait. Mice were also less able to sit upright, behavior commonly seen in mice when eating, drinking, or grooming. Despite subtle motor defects, in rotarod tests, mice did not display time differences. Major associated symptoms of deletion of *C9orf72* in these mice included lymphadenopathy (formation of masses in lymph nodes) and splenomegaly. In addition, these mice display expansion of myeloid cells in lymphoid organs, mixed inflammatory infiltrate in various tissues, and increased activation of T cells and plasma cell generation, all indicative of immune hyperactivity. Resultantly, mice with *C9orf72* ablation develop increased levels of autoantibodies, and display glomerulonephritis, likely from autoantibody accumulation in glomeruli. These mice better model the clinical features of Systemic Lupus Erythematosus than ALS.

It is possible that loss of C9orf72 may increase the severity of other ALS mutation, but this does not provide more evidence for loss of function (Sellier et al 2016, Ciura et al 2016).

In reality, ALS and FTD/FTLD caused by repeat expansion of *C9orf72* may be the result of a “mixed bag” of gain and loss of function. Defects in autophagy pathways have been implicated in the disease progression, and likely stem from a loss of function. Alternatively, formation of RNA foci by *C9orf72* RNA lead to decreased levels of C9orf72 protein and could disrupt autophagy by sequestering proteins necessary for this process or by overloading the cells autophagy systems, which would be an example of a toxic gain of function. In addition, dipeptide repeats containing inclusions are commonly seen in the cerebral regions of patients with FTD/FTLD, but not in the neuronal tissue of ALS patients, indicating a possible dual gain of function and loss of function mechanism in different tissues and mediating different disease states. This could possibly be controlled by differential expression of this protein in tissues, or by variations in splicing profile that are cell lineage specific.

C9orf72 Normal Function

When first identified as causative of ALS, groups researching *C9orf72* mutations identified that the gene and protein have sequence homology to a class of proteins known as Differentially Expressed in Normal and Neoplasia (DENN) which is a protein encoding a GDP/GTP exchange factor (GEF) (Levine et al 2013). A role for C9orf72 as a GEF was confirmed by demonstrating significant interactions with proteins involved in endocytic transport (Farg et al 2014). Interactions with Rab1, Rab5, Rab7, and Rab11 were established via fluorescent microscopy. A reduction in transport from the membrane to the Golgi apparatus was observed upon knockdown of C9orf72 mRNA. In addition, C9orf72 co-localizes with another ALS implicated protein ubiquilin-2 which is also involved with autophagy, co-localized with the autophagosome marker LC3, and migrated with vessels labelled with lysosomal markers. In this study, it was also demonstrated that C9orf72 interacts with hnRNPA1, a splicing regulatory protein which has also been implicated in ALS. Based on these results, and the availability of sequence alignment data, these results strongly suggest that C9orf72 is a GEF for multiple Rabs.

More evidence of the role of C9orf72 as a GEF has been established with the observation that in *C9orf72* knockdown via CRISPR/Cas9 system resulted in a complete ablation of stress granule formation, indicating an effect on the ability of cells to regulate transcription and maintain protein dynamics. This result implicate the autophagy pathway in this process, as formation of stress granules involves formation of membrane bound vesicle like bodies containing transcribed mRNA and translational machinery (Maharjan et al 2016). This is in itself a form of autophagy, as the cell needs to “eat” these mRNA and translational proteins to sequester them. In addition, expressing hexanucelotide repeat expanded C9orf72 resulted in increased RNA foci formation and spontaneous formation of stress granules. These results have been confirmed in patient derived induced pluripotent stem cells from ALS patients with *C9orf72* hexanucleotide-repeat expansions which presented with common stress granule formation, increased endoplasmic reticulum calcium levels, which have been highly implicated in mitochondrial dysfunction associated with ALS. All of these factors implicate C9orf72 as having a central role in the process of intracellular transport, adding plausibility to the identification of C9orf72 as a GEF for Rabs.

Further research has demonstrated interactions between C9orf72 and GEFs for Rab8a and Rab39b, both of which have documented roles in the process of autophagy, specifically macroautophagy, the disposal of large cellular structures such as membrane bound organelles (i.e. mitochondria, ER/Golgi domains that become damaged) (Corbier and Sellier 2016). Knockdown of C9orf72 in primary mouse neuronal cultures and neoplastic neuronal tissue demonstrated a reduction in autophagy characterized by accumulations of p62.

This result was confirmed in a study using induced pleuripotent stem cells from C9orf72 hexanucleotide repeat expansion ALS patients with directed differentiation, which demonstrated that induction of autophagy was regulated by C9orf72. Interactions between C9orf72 and Rab1a, with a preference for GTP-bound Rab1a were also observed, as well as interactions with C9orf72 and ULK1, which is involved in initiation of autophagy (Webster et al 2016). Indeed, these results may implicate a loss of function of C9orf72 as causative of these cellular changes, as we do see a reduction in autophagy in neurons from affected patients. This fits with the concept that C9orf72 repeat expansions may confer multiple mechanisms of toxicity to the cell, with both loss of normal function interfering with the autophagy and cellular protein/organelle disposal pathway, and gain of function inducing dipeptide repeat protein and RNA foci mediated toxicity. In patients with ALS alone, if we were to see significant defects in autophagy as compared to FTD patients who express high levels of dipeptide repeat proteins, then this would add support for this hypothetical mechanism.

Altered mitochondrial morphology has been noted in cases of *C9orf72* repeat expansion. Morphologically, mitochondria in patients with *C9orf72* repeat expansions display an increased propensity for mitochondrial fragmentation. This is accompanied by downregulation of BCL-2, shifting the cellular balance of pro-apoptotic versus anti-apoptotic proteins in favor of apoptosis, and predisposing these neurons to undergo apoptosis under conditions of stress (Dafinca et al 2016).

In an analysis of C9orf72 and TDP-43 mutant containing patient derived fibroblasts demonstrated altered mitochondrial morphology with fragmenting of the mitochondrial network (Onesto et al 2016). In addition, increased oxygen consumption and mitochondrial hyperpolarization were observed in a background of elevated ROS and ATP content. Thus, the mitochondria are more prone to breakdown improperly, without having undergone mitophagy. In addition, these results suggest that in cells with C9orf72 repeat expansions, there is either damage to the mitochondrial membrane or lack of a protein involved in mitochondrial stability, such as BCL-2, which was previously demonstrated to be downregulated in this disease. All of these factors provide evidence that mitochondrial dysfunction of some sort is involved in the progression of the disease and damage to neuronal cells.

Interestingly, the involvement of *C9orf72* on the development of the immune system has been established in mice. In mice lacking the mouse analog of *C9orf72*, development of macrophages and other myeloid lineage cells was significantly impaired, leading to accumulation of large macrophage-precursor-like cells in the spleen and lymph nodes (O'Rourke et al 2015). This was further supported by the work of Atanasio et al (2016) who generated a knockout mouse for *C9orf72* and observed similar results. Human ALS patients have not been reported to have immune system deficits.

It appears that C9orf72 may have some function involved in neuronal growth, as knockdown of C9orf72 in primary culture of mouse neuronal cells led to a decrease in axonal length, whereas over-expression of C9orf72 led to an increase in the length of axons (Sivadasan et al 2016). A potentially novel interaction between C9orf72 and actin binding proteins such as cofilin was established (Sivadasan et al 2016). With mutations in *PFN1* as a known cause of ALS, the discovery of C9orf72 interactions with cofilin may add a new layer to the understanding of ALS. It was observed that actin dynamics were altered in motor neurons knocked down for C9orf72 expression or expressing hexanucleotide repeat expanded *C9orf72*. This could be due to observed changes in phosphorylation state of cofilin. In addition, it was demonstrated that actin filament assembly was significantly slower in cells with C9orf72 knockdown. This provides linkage to a previously poorly understood causative mutation in profilin-1, which may implicate a role actin cytoskeletal structure in disease progression. The most likely explanation for this would be that actin is necessary to maintain proper neuronal body structure, and changes in cell shape can result in alterations in cell signaling processes, though this is purely conjecture.

As an interesting note, it has been demonstrated that TDP-43 is capable of transporting G-quadruplex forming mRNA, thus providing a link between two of the most commonly seen causative changes associated with ALS (Ishiguro et al 2016). This could provide explanation for the observed TDP-43 proteinopathy characteristics observed in ALS patients that do not contain a TDP-43 mutation.

Animal Models:

As previously discussed, a knockout model of *C9orf72* was generated by Atanasio et al (2016). Knockout of *C9orf72* led to severe phenotypes in mice, but not of motor neuron degeneration or a progressive ALS phenotype. The mice generated displayed altered immunodynamics including increased activation of T and plasma cells, generation of autoantibodies, and lymphadenopathy/splenomegaly, on a background of slight motor dysfunction and muscle weakness.

In a zebrafish model, the importance of the poly (Glycine-Alanine) dipeptide repeat protein was analyzed, and was a key contributor to neurotoxicity as compared to normal. This phenotype was able to be rescued via morpholino mediated decay of *C9orf72* polyGA mRNA transcripts (Ohki et al 2017). This experiment was not neuronal specific, as morpholino injection occurred in the embryonic stage, and knocked down global expression of C9orf72. Knockdown of the polyGA transcript leads to knockdown of C9orf72 as a whole, as this intron is part of the original transcript. This result again provides evidence that gain of function in the form of toxic dipeptide repeat proteins is implicated in the progression of the disease. It is generally accepted that the overexpression of dipeptide repeat proteins is toxic to the cell; overexpression of dipeptide repeat proteins in this disease overloads the proteasomal degradation system, leading to increased cellular stress and a predisposition to apoptosis if the proteins cannot be cleared.

Chew et al (2015) expressed a 66 repeat containing version of *C9orf72* in the neural system of mice via adeno-virus targeting, and demonstrated the presence of dipeptide repeat protein containing inclusions, as well as TDP-43 positive inclusions. Cortical neuronal loss was observed to significantly alter the mass of the brain. Astrogliosis was noted as well, which has been described as an associated feature of ALS with more research determining if this is reactive to or causative of the disease. In addition, behavior phenotypes of hyperactivity, anxiety, and anti-social behavior were noted via various tests such as open field tests. While not necessarily relevant to ALS phenotypes, these are indicative of FTD/FTLD phenotypes in the mice, and indicate that these animals could be useful for the modelling of ALS-FTD.

A second group has generated a bacterial artificial chromosome (BAC) mouse model of ALS (Peters et al 2015). By expressing human *C9orf72* containing 500 GGGGCC repeat motifs, researchers were successfully able to demonstrate several of the characteristics of ALS. Notably, the mice did not display any phenotypic defects associated with ALS (no muscle weakness, wasting, or dementia-like symptoms). However, histopathological analysis of these mice demonstrated the presence of protein containing inclusions positive for dipeptide repeat proteins. At the same time as the Peters group was creating their model, another group used similar techniques to develop multiple different lines of mice expressing either normal human *C9orf72*, or *C9orf72* with repeat expansions ranging between 100-1000 GGGGCC repeats (O'Rourke et al 2015). These mice displayed typical RNA foci associated with expansions, and also displayed RAN translation dipeptide repeat protein containing cytoplasmic inclusions, which were able to be treated with antisense oligonucleotide administration. In addition, these mice displayed defective localization patterns of the nuclear protein nucleolin, suggesting that mitochondrial dysfunction seen in this disease is recapitulated in these mice. Shang et al (2017) also noticed altered distribution of nuclear pore proteins in both *C9orf72* repeat expansion transgenic mice, and in patient tissue, further implicating a role for nuclear dysfunction in the disease progression. These results provide some further evidence for the toxicity of RNA foci in cells as well as partially implicating nucleocytoplasmic transport in the disease process of ALS. A disturbance of nuclear pore dynamics could lead to decreased RNA export from the cell and increase the propensity to form RNA foci.

Recently, a transgenic mouse model expressing *C9orf72* hexanucleotide repeat expansions was generated; however, due to an error in experimental design, an incorrect gene construct containing 90CGG repeats associated with Fragile-X system was introduced to the oocytes by mistake, and led to the development of transgenic mice for fragile-X syndrome being mis-labelled as transgenic *C9orf72* repeat expansion mice. The article has since been retracted; however, if performed correctly, this experiment would provide significant insight into the development of ALS with *C9orf72* pathologies, as the inducible nature of the mouse allows for age dependent defects to be studies in a much simpler manner (Hukema et al 2016; retracted). One of the major issues in the design of ALS animal models is the concept of face validity. While many of these animals display the phenotypic features of ALS, they often develop the disease in a condensed time-course. With an inducible model, researchers could “turn on” the disease causing mutation after some period of time; since most of the models display an accelerated disease progression, if researchers were to allow mice to develop normally for a while before exerting the disease causing effects, we could gain much needed insight into the pathogenesis of the disease.

Several other groups have simultaneously worked to create transgenic mouse lines expressing *C9orf72* repeat expansions. Liu et al (2016) transfected mice with a BAC construct containing a *C9orf72* repeat expansion isolated from a patient containing approximately 1200 hexanucleotide repeats. As in humans, both the sense and anti-sense strands of the gene are transcribed. Gait abnormalities are observed in mice beginning at approximately 16 weeks. Some of the transfected female mice display an extremely condensed disease progression, characterized by rapid progression of muscle weakness, difficulty breathing, hindlimb paralysis, and decrease survival. In addition to the acute phenotype, other mice show a progressive phenotype with reduced activity and intermittent seizures. In animals with acute disease at end-stage, marked denervation of neuromuscular junctions was observed. These mice display dipeptide repeat protein aggregates in neuronal cytoplasm and neuronal nucleolar cytoplasm, as well as characteristic TDP-43 positive inclusions. C9orf72 expression levels appear to be normal, and many of these observations would be expected to be attributable to pathologies caused by *C9orf72* repeat expansion, such as protein aggregates and RNA foci formation. Overall, this is likely the best currently available model of neurodegeneration with regards to repeat expansion in C9orf72 (Liu et al 2016). This model provides more support for gain of function mutations in *C9orf72* hexanucleotide repeat expansion ALS, as transfection with repeat expanded *C9of72* led to the development of ALS-like symptoms that are not seen in *C9orf72* knockout mice.

Jiang et al (2016) in addition to creating a transgenic knockout mouse for comparison, used BAC to transfect mice with *C9orf72* repeat expansions. Length of the repeat led to differential incorporation into RNA foci. In addition, they observed age dependent effects of dipeptide repeat protein accumulation, which were also altered by repeat length. In mice expressing a 450 repeat variant of *C9orf72*, age dependent cognitive impairment developed in the relative absence of motor symptoms, with working memory deficits measured by maze trials, and was associated with neuronal loss in the hippocampus. Treatment with antisense oligonucleotides was able to reduce dipeptide repeat inclusion formation, while also reducing age dependent motor defects (Jiang et al 2016). This indicates that a toxic gain of function mechanism is likely the underlying result of neuronal damage, as ablation of dipeptide repeat proteins in these animals was able to partially rescue the phenotype. However, without full rescue of the phenotype, we cannot determine if other mechanisms are involved. Inability to fully rescue the phenotype may be due to advanced neurodegeneration alone, or it may be indicative that multiple functions are gained or lost by changes to *C9orf72*.

A likely reason for the difference in phenotype induced by expression of human *C9orf72* containing repeat expansions could be the presence of redundant gene functions, though this has not been confirmed. Another possibility that could be speculated is the presence of multiple autophagy pathways in mice, though this has not been confirmed by this author, and is purely conjecture.

Integration and Overview:

Within the diverse groups of proteins in which mutations causative of ALS lie, it is clear that there are several common pathways within cells that are disrupted.

Mitochondrial Dysfunction

Multiple of the genes containing mutations implicated as causative of ALS appear to be involved in maintaining the stability of mitochondria. The first protein within which mutations were identified as causative of ALS, SOD1, is heavily associated with the mitochondria and with scavenging of free radical species generated by the mitochondria. While in most cases, mutations do not seem to ablate the free radical scavenging abilities of SOD1, if the mutations lead to sequestration of SOD1 into plaques, this could alter the stability of the mitochondrial membrane, leading to increased oxidation of lipids and damage to the mitochondrial membrane, predisposing these cells to apoptosis. TDP-43 with disease causing mutations has been noted to localize to the mitochondria and increase mitochondrial dysfunction. Mitochondrial localization of FUS has been observed in disease causing mutants, with associated results of mitochondrial fragmentation. Mitochondrial fragmentation and clustering of mitochondria containing vesicles has been noted in Alsin mutations. VAPB is involved in maintenance of the mitochondrial associated membrane and may alter calcium dynamics in the ER and mitochondria, leading to mitochondrial dysfunction. Angiogenin has been previously shown to be involved with the cleavage of tRNA, and it has been demonstrated that these cleaved tRNA may bind and sequester cytochrome c (Saikia et al 2014). Thus, if mutations in angiogenin ablate this ability, this could result in mitochondrial dysfunction and pre-disposing the cell to apoptosis. The neuronal specific nature of this apoptosis may be due to how neurons respond to stress, may be related to the lack of mitotic abilities of neuronal versus many other tissues, or may be due to expression levels or expression variants seen exclusively in neurons, as many proteins have tissue specific variants based on alternative splicing profiles. Phosphoinositol signaling may be involved in mitochondrial function, and mutations in *FIG4* that alter PI signaling may lead to mitochondrial dysfunction. Optineurin has been demonstrated to be involved in the cellular process of mitophagy of damaged mitochondria. VCP has many functions in maintaining the stability of the mitochondrial associated membrane, and localization to the mitochondria has been shown to impair mitophagy. Ubiquilin has previously been shown to be involved in proper marking and trafficking of mitochondrial associated proteins for degradation. Sig-1R can interact with proteins associated with the mitochondrial membrane, such as SOD1 and BCL-2, and also has roles in ER calcium dynamics; therefore mutations could result in mitochondrial dysfunction by altering the influence of the anti-apoptotic protein BCL-2. CHMP2B is involved in signaling processes that control mitochondrial dynamics and initiation of the intrinsic apoptotic pathway. TBK1 interacts with optineurin to initiate mitophagy, meaning mutations that interfere with this interaction could result in decreased autophagy and release of mitochondrial proteins and components such as cytochrome c. Patient derived fibroblasts with *C9orf72* mutations have mitochondria that display altered morphology, increased ROS production, increased oxygen consumption, membrane hyperpolarization, and mitochondrial fragmentation.

Many of the proteins involved in ALS have been directly linked to the mitochondria, or to a pathway involved in degradation of the mitochondria. In addition, several of the proteins are transported to the mitochondria when mutated. All of this is indicative of mitochondrial dysfunction playing a central role in ALS. Because of these clear interactions and linkages to the function and dysfunction of the mitochondria in many of these proteins, it seems that mitochondrial dysfunction is both a likely and plausible etiology for the abnormal death of motor neurons.

Autophagy and Cellular Stress Responses

One of the most prominent areas of research into the pathogenesis of ALS currently is looking at the role of altered cellular autophagy and ER stress response dynamics. Many of the proteins implicated in ALS have well defined roles in the process of autophagy or in one of several cellular stress responses. Both TDP-43 and FUS have been implicated in stress granule dynamics, and abnormal sequestration of various mRNA may be involved in the pathogenesis of the disease. *FIG4* has been implicated in the process of autophagy, as expression of wild-type FIG4 on a knockout background, autophagy is rescued, whereas in the knockout, autophagy is severely limited. Optineurin is absolutely necessary for autophagy, and appears to be one of the central proteins involved in the process. Ataxin mutations have been demonstrated to alter ubiquitin ligase activity, leading to increased ER stress. VCP has a purported role in autophagy due to its role in targeting of proteins to the phagosome for degradation. Ubiquilin-2 has been seen to associate with proteins involved in the formation of autophagosomes. Sig-1R may play a role upstream of autophagy via its interaction with SOD1 and BCL-2. TBK-1 and the interaction between TBK-1 and optineurin are central to beginning the process of autophagy. P62 has a central role in the process of autophagy, and appears to help shuttle proteins to the correct disposal pathway. Depletion of C9orf72 has been shown to lead to altered autophagy responses and can induce ER stress; mutations in C9orf72 appear to disrupt the ability to help initiate autophagy. Alterations in essentially any of the proteins involved in autophagy leads to conditions of ER stress which can induce apoptosis.

Thus, an alteration of the autophagy pathway will lead to the development of ER stress. With the autophagy pathway not working correctly, the proteasome degrading as many proteins as possible, and the development of ER stress, the cell will be pre-disposed to undergo apoptosis, which could explain a possible mechanism for the cell death seen in ALS.

This is one of the currently most likely options; and indeed, alterations in autophagy will also lead to mitochondrial dysfunction, as an inability to initiate autophagy is likely accompanied by an inability to initiate mitophagy, which would allow for the release of mitochondrial proteins and compounds such as cytochrome c which would initiate the intrinsic apoptotic pathway and explain cell death. It is very likely that if either of these mechanisms is found to be pathogenic to ALS, both will be implicated in the progressions and development of the disease, as in the case of this disease, the dysfunction of the mitochondria is likely related to improper disposal oy mitochondrial products.

Intracellular, Axonal, or Nucleocytoplasmic Transport

In ALS, essentially every protein implicated as causative of the disease has been shown to be involved in some form of intracellular transport, with the major exception being SOD1, though looking large scale, alterations of ROS production could alter microtubule and cytoskeletal dynamics. TDP-43 and FUS are both RNA binding proteins; each has been shown to bind RNA molecules and be transported either within the neuronal cell body, from the nucleus to the cytoplasm, or via the length of the axon. Alsin encodes a GEF for Rab5 which is involved in recruitment of vesicles to endosome. SPG11 is commonly found localized to vesicles, indicating a significant role in protein transport, and likely in organelle transport. VAPB has been seen to associate with vesicles and with the ER, indicating a likely role in vesicular transport. FIG4 has been demonstrated to be involved in retrograde transport from the Golgi body to the ER. Optineurin is involved in vesicle trafficking to the Golgi body, is commonly seen to associate with Rab8, which is involved with transport from the trans-Golgi network to the basolateral surface of the membrane (Huber et al 1993), and has been shown to interact with microtubule associated proteins, indicating a very likely role in intracellular transport. Ataxin-2 binds to several mRNA that are also bound by TDP-43 in an RNA dependent manner (no interaction without RNA present), indicating that these two proteins may be simultaneously involved in maintaining mRNA stability during transport. VCP is seen to aid in the formation of lipid microdomains which are commonly involved in endocytic pathways, and is also involved in the biosynthesis of the ER and Golgi network, which involves membrane trafficking to establish. Ubiquilin-2 aids in cytoskeletal assembly, which is necessary for axonal and multiple types of intracellular transport to occur. Sig-1R has been shown to be capable of transporting lipids within the cell, and also demonstrates the ability to assemble lipid microdomains, indicating a potential role in endocytic pathways. CHMP2B interacts with Rab8, involved in transport from the Golgi network to the cell membrane; patients with CHMP2B mutations display large intracellular vesicles, likely indicative of an error in intracellular transport. Profilin is involved in maintenance of the actin cytoskeleton, which maintain the structure of the neuronal cell body, and thus without a direct role in intracellular transport is still associated with maintaining proper transport dynamics, as a failure of the actin cytoskeleton will result in deformation of the neuronal cell body and lead to transport defects. ERBB4 and its ligand neuregulin-1 transiently appear in the boutons of synapses, indicating that these proteins are either normally transported down the axon or are necessary for the development and transport of materials to the bouton. hnRNPA1 is involved in maintaining the stability of newly synthesized mRNA and is likely involved in transport from the nucleus, as it contains both a nuclear export and import signal, and transiently appears on both sides of the membrane. Matrin is involved in maintaining the structure of the nucleus and in maintaining proper nuclear pore complex structures, which indicates a role in nucleocytoplasmic transport. TUBA4A is a tubulin subunit, necessary for the formation of microtubules, which stabilize the axon and allow for much of the transport within the cell to occur. P62 has a well-established role as a protein shuttle between the proteasome and regions of the cell where autophagy is commonly taking place. RBM-45 can alter localization from the nucleus to the cytoplasm in response to stress, though this may occur in a complex with TDP-43, which is normally transported to the synapses of neurons. Lastly, C9orf72 has been demonstrated to be a GEF for Rab1, Rab5, and Rab7, all of which are involved in intracellular transport. In addition, roles have been established for transport of proteins to the Golgi, autophagosomes, and lysosomes.

Functionally, we can find the most proteins implicated in the disease that fall into the category of proteins involved in intracellular transport. In reality, these three mechanisms may not be distinct or disparate, and could very well be linked. If intracellular transport is altered, and proteins are not being shuffled to where they need to be, the cell will sense an excess of proteins in any of the ER, cytoplasm, or nucleus, or an excess of RNA within the nucleus or cytoplasm. This should lead to an activation of cellular stress responses, such as the misfolded protein response, ERAD protein clearance response, formation of stress granules, and an increase in autophagy. All of these will alter the dynamics of the ER, which could lead to changes in calcium signaling or associated changes in the mitochondrial associated membrane of the ER, which would in turn lead to mitochondrial dysfunction and the release of cytochrome c leading to apoptosis. In my opinion, this is the most likely pathogenesis of the disease; the mechanisms are not distinct, but are explained by a series of mishaps in the cell that all start with impairment of proper protein or RNA transport, lead to induction of cellular stress responses, and the failure of these stress responses to be cleared, or continuation of conditions suitable for the stress response ultimately lead to mitochondrial dysfunction via altered ER dynamics or lead to direct damage to the mitochondria and triggering of the intrinsic apoptotic pathway.

In addition, this author's proposed domino hypothesis centering on a breakdown of intracellular transport leading to alterations in mitochondrial function and ultimately an inability to clear misfolded proteins and damaged organelles from the cell, likely explains the observed ability of mutated proteins to be transferred to cells surrounding the damaged and dying cell. Since these neurons are dying via apoptosis and not necrosis, the membrane bound fragments of the cell are taken up by the surrounding cells, which will likely suffer from the same inability to clear the misfolded proteins, and lead to a protracted progression in following neurons. This could also explain the long "healthy period" in affected patients followed by a rapid disease course, as the disease does not typically onset until the 4th or 5th decade of life, in what I would refer to as a "domino hypothesis"- once one cell falls, the rest take up what was released from the dead neuron, and ultimately trigger the same issues as were seen in the cell that underwent apoptosis. While this has not been directly observed, based on everything that is known about ALS and its progression, this hypothesis would seem to encompass many of the issues commonly seen in ALS.

A table summarizing involved genes and implicated roles that map to the commonly seen pathological defects in cells displaying an ALS phenotype is presented below.

ALS Subtype	Gene	Mitochondrial Dysfunction	Cellular Stress Response	Intracellular, Axonal, or Nucleocytoplasmic Transport
ALS1	SOD1	Superoxide dismutase properties	Associated with BCL-2	Altered cytoskeletal dynamics due to ROS
ALS2	Alsin	Mitochondrial fragmentation		GEF for Rab5- recruitment of vesicles to endosome
ALS3	Unknown			
ALS4	SETX			
ALS5	SPG11			Localizes to vesicles and membranous organelles
ALS6	FUS	Abnormal mitochondrial localization	Stress granule formation	RNA binding protein that maintains RNA stability during axonal transport
ALS7	Unknown			

ALS8	VAPB	Mitochondrial associated membrane maintenance	Protein inclusions contain ER tubule proteins, indicating ER dysfunction	Associated with ER and vesicles
ALS9	ANG	Cytochrome c sequestration		
ALS10	TARDBP	Abnormal mitochondrial localization	Stress granule formation	RNA binding protein that maintains RNA stability during axonal tr
ALS11	FIG4	PI signaling	Decreased occurrence of autophagy	Retrograde Golgi to ER transport
ALS12	OPTN	Central protein in mitophagy	Central protein in autophagy, largescale dysfunction	Vesicle trafficking to Golgi, associates with Rab8 (transport from trans-Golgi network to basolateral cell membrane
ALS13	ATXN-2		Altered ER stress response	Associates with TDP-43 bound to mRNA
ALS14	VCP	Mitochondrial associated membrane maintenance	Protein targeting to phagsome	Formation of lipid microdomains which are common in endocytosis
ALS15	UBQLN2	Trafficking of mitochondrial proteins for degradation	Formation of autophagosome	Trafficking of proteins for degradation; cytoskeletal assembly
ALS16	SIGMAR1	Interactions with anti-apoptotic proteins	Interaction with upstream components in apoptotic pathway	Lipid transport and formation of lipid microdomains commonly seen in endocytosis
ALS17	CHMP2B	Mitochondrial signaling, mitophagy	Altered ER stress response	Interactions with Rab8, transport from Golgi network to cell membrane
ALS18	PFN1			Actin cytoskeleton dynamics; maintain neuronal cell body structure for proper transport
ALS19	ERBB4			Transiently transported to synapses during disease progression
ALS20	hnRNPA1		Altered mRNA dynamics of ER proteins	Maintaining stability of newly synthesized mRNA, transport from nucleus to cytoplasm
ALS21	MATR3		Altered nuclear stability and mRNA accumulation	Maintain nuclear structure and pore structure for proper nucleocytoplasmic transport
ALS22	TUBA4A			Tubulin subunit necessary for microtubule dynamics
	TBK1	Mitophagy is important role	Central protein in autophagy, largescale dysfunction	
ALS-FTD1	C9orf72	Altered morphology and oxygen consumption dynamics; mitochondrial fragmentation	Initiator of autophagy, involved in transport of proteins to autophagosome	Functions as a GEF for multiple Rab proteins associated with intracellular transport

Table 3: The genetic and pathogenic heterogeneity of ALS. All functions listed are proposed normal functions of these proteins based on the demonstrated interactions, phenotypes of knockdowns, and rescue of knockdowns via wild type gene transfection. The majority of the proteins demonstrated to be causative of ALS have functions in intracellular transport; many have functions in mitochondrial regulation and control or autophagy and cellular stress responses.

Discussion and Future Directions:

Significant recent research has been focused on defining a link between the cellular autophagy pathway and development of ALS. This has been established repeatedly with many of the implicated proteins, sometimes via downstream consequences, such as altering proteasome degradation of proteins, by binding of RNA into foci, via interference with alternative splicing, protein aggregation, and direct disruption of autophagy machinery. In addition much work has gone into understanding altered cellular energetics displayed in induced stem cell lines of ALS and in some animal models of ALS, both of which display altered mitochondrial morphology or function. Autophagy is an important cellular function that allows cells to dispose of damaged or dying organelles, and can also deal with misfolded or improperly disposed of proteins to prevent induction of cellular stress. Taken together, the process of autophagy appears to be central to proper maintenance of homeostasis for neuronal tissue. This is especially important due to the non-mitotic nature of neuronal tissue; in many other tissues, if stress becomes too great, cells can die and be replaced, but not neurons. Many tissues have a pool of stem cells which can replace damaged tissue, which would likely be less affected by damage caused by this disease due to their nature as stem cells and their capacity for repair and replacement. In addition, neurons necessarily have a tightly controlled environment both intracellularly and extracellularly, and changes in this environment can pre-dispose neuronal cells to death both *in vitro* and *in vivo*. Lastly, neurons perform autophagic and pahgocytic functions commonly due to their need to scavenge neurotransmitters which are released. Taken all together, the neuronal specific nature of the disease can likely be explained by several of the factors which appear to be driving the disease. Because many of the observed pathological issues in ALS can be explained by disruption of autophagy as well, this is a likely avenue to continue researching.

Future directions should be focused on elucidating how a disruption of autophagy leads to neuronal cell death, or demonstrating proposed hypotheses. It would make sense if disruption of autophagy increased ER stress in the cell, altered ER dynamics, including calcium uptake, leading to mitochondrial dysfunction, and eventually cellular apoptosis. However, little research has gone into observing if there is a link between altered autophagy and altered mitochondrial function, and this should be an area of upcoming research. In addition, further research needs to be performed to determine if protein inclusions in neuronal cells are causative of ALS, or whether they are simply a result of the pathogenic mechanism underlying the development of the disease.

In addition, the continued development of animal models of ALS is necessary. We now have several models that recapitulate the disease in a very similar manner to humans, and these will absolutely be necessary for demonstrating the underlying pathogenesis of the disease. If there is one common pathogenesis for all involved causative mutations or different pathogenic mechanisms involved in each of the subtypes, identifying functional or structural changes in involved proteins that allow for grouping based on interactions and functions should allow researchers to continue to elucidate information about the disease. As it currently stands, profilin-1 mutations are essentially the “odd man out” though groups have established interaction between other ALS associated proteins and other actin binding proteins opening up the potential that even ALS caused by mutations in *PFN1* may fit into the overall disease picture. In addition, when developing animal models, I believe that we need to focus more on fully recapitulating the disease state as seen in humans. Many of the currently available animal models display an accelerated or condensed disease time course to help improve the timeframe for generation of results. While the accelerated time course may be useful for research purposes, if all three of the commonly proposed mechanisms are occurring either in series or simultaneously, in an accelerated model we may only see one of the three mechanisms at play, which does not give researchers a true model of the disease state.

If I could perform one experiment to determine the pathogenesis of ALS, I would first develop a transgenic animal model that fully models the disease processes in human ALS. By this, I suggest that the mouse should display symptoms beginning around two-thirds of the way throughout life, with relatively normal life before, and a disease time-course equivalent to between 5 and 10 weeks This might be difficult, as many of the currently generated animal models display an accelerated progression of the disease, with death around 20 weeks at most. However, with the normal lifespan of a mouse being approximately 2-4 years, a time course of half a year reflects at best one quarter of the mouse's life which is significantly less than the between half and two-thirds life-expectancy onset of most ALS variants. If this could be achieved, and a mouse who developed the clinical features ALS beginning around 80 weeks of life was successfully bred, then we could use these mice to examine the changes in neurons leading up to onset of the disease. This would likely need to be performed using a BAC construct that allowed an inducible nature of the disease such that researchers can induce the disease shortly after birth and monitor the progression. In addition, if possible, we could design mice where the disease state can be induced after normal development, as ALS does not appear to be a neurodevelopmental disorder. This would currently provide some of the best insight into the disease mechanism. We need to see what is going wrong with cells before outright symptoms of the disease are observed, such that we can see what processes are fundamentally altered to allow for the death of motor neurons. I would examine mitochondrial structure, ER dynamics, proteasomal degradation, axonal, intracellular, and nucleocytoplasmic transport, and the ability of the cell to clear proteins and organelles via autophagy. In addition, I would monitor these animals to determine if any clinical or molecular biomarkers of the disease development can be seen in the symptom free stage, which would further allow researchers to increase our understanding of the disease. If we could identify an early marker for the development of ALS that does not require genetic testing, this would greatly improve our diagnostic and research capabilities, as the ability to monitor suspected ALS patients before the onset of the disease would allow us to further improve our understanding of how and why this condition develops, how and why the specific subsets of neurons are affected, and how and why we don't see widespread cellular deficits if a central process in cell homeostasis is altered.

This author would like to see research move away from the prion-like hypothesis; while necessary characteristics for this have been demonstrated and may be involved in the reactive astrocytosis and gliosis seen in ALS patients, currently, the prion hypothesis does little to add to the knowledge of the disease. With the growing amount of information available on the types of mutations undergone by these proteins, and the growing realization that many of the autosomal dominantly inherited phenotypes likely have mutations in causative proteins conferring a toxic gain of function mechanism, altered binding to associated proteins and dominant negative mechanisms may explain most of the incorporations into protein inclusions that are seen. In addition, neurons in ALS die via apoptosis, meaning that the products of the dead cell are absorbed by those around it, which could explain the appearance of insoluble plaques in cells surrounding the dying neuron. As there are other simpler, and in this author's opinion, more likely hypotheses for the spread of proteins from cell to cell, now might be as good a time as any to lay this hypothesis to rest. In addition, only 3 of the 25 proteins where mutations have been associated as causative of ALS contain a prion-like domain despite all being able to be transferred from dying cells to healthy surrounding cells.

This author would also like to see research focused on understanding the role of intracellular, nucleocytoplasmic, and axonal transport on the pathogenesis of ALS, as many of the proteins involved display common patterns of mis-localization, which would be explained by the presence of altered intracellular transport mechanisms, a function which many of the implicated proteins have been associated with. With the developments provided recently linking the functional role of TDP-43 in C9orf72 localization in cells (by binding *C9orf72* mRNA for axonal transport), this continues to provide evidence for the involvement of the intracellular transport system in the pathogenesis of ALS (Ishiguro et al 2016). At this current stage in research, we have not elucidated whether alteration in intracellular transport are causative or a result of the pathology, and arguments for both sides can be made. In my opinion, intracellular transport dynamics are likely altered first, leading to alterations in autophagy or proteasomal dynamics because proteins are not being sent where they need to be, allowing them to accumulate, and necessitating clearance by the cell. The stress on the ER and protein clearance mechanisms likely also leads to changes in the mitochondria, as the mitochondria are associated with the ER, and the dynamic exchange of ions and proteins between the two keeps the mitochondria functioning correctly. This alteration of protein clearance dynamics and mitochondrial function could ultimately lead to activation of the intrinsic apoptotic pathway as the mitochondria begins to break down. With impaired autophagy, the damaged mitochondria cannot undergo mitophagy and be safely cleared from the cell. All in all, it is likely that a combination of all three commonly researched mechanisms contribute to the pathogenesis of ALS.

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