Understanding Motor Disorders Using Flies

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Abstract

The fruit fly, *Drosophila melanogaster*, is an attractive model for studying human disease. The popularity of the model is a consequence of its well-developed toolbox for genetic engineering and the finding that 75% of genes that cause human disease have orthologs in the fly. Diseases of the human nervous system have been modeled extensively in the fly, taking advantage of a complex, well mapped out nervous system. A popular strategy to model a disease is to identify the fly ortholog of a disease gene and develop an experimental model, based on the ortholog, to gain insight into the mechanisms of gene function and malfunction. The lessons learned from the fly can then be used to dissect out the cellular and molecular basis of the disease in humans.

In this chapter, we highlight research using *Drosophila* to gain insight into mechanisms that underlie neurodegenerative diseases, with a focus on amyotrophic lateral sclerosis (ALS). Till date, 31 familial genetic loci have been identified in ALS, with each gene involved in cellular processes that are widely divergent from each other. This divergence of function has hampered efforts to elucidate a common model for the initiation and progression of ALS. Here we describe well-established fly models for *C9ORF72, SOD1, TDP-43, FUS, VAP,* and *VCP*. We explore the alterations in protein and RNA homeostasis, metabolic changes, intracellular and intercellular signaling, and transport, stress, and immune response concerning each of these genetic loci as well as architectural changes that occur during development and aging of the fly. Studies that provide

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evidence for common themes between these loci through genetic, epistatic, or physical interaction have been highlighted.

Many cellular hallmarks of these diseases can be recapitulated in *Drosophila,* providing a platform to conduct further sophisticated genetic and chemical perturbations to gain a better understanding of the human disease. In this chapter, we speculate on the possibility of a gene regulatory network that underlies the breakdown in motor function in ALS, composed of ALS causative genes, which reveal critical mechanistic features that can be targeted for therapy.

Keywords

Amyotrophic lateral sclerosis · Familial · *Drosophila* · Gene regulatory network · Neurodegeneration

Introduction

In humans, a subset of neurodegenerative diseases that affect motor functions is collectively termed as motor diseases. Amyotrophic lateral sclerosis (ALS), hereditary spastic paraplegia (HSP), Charcot-Marie-Tooth (CMT) disease, and spinal muscular atrophy (SMA) are a few examples of clinically described motor diseases. Amyotrophic lateral sclerosis, also known as Lou Gehrig's disease, is a late-onset, slowly progressive disorder that culminates in the death of motor neurons of the brain cortex, brain stem, and the spinal cord. This causes loss of signaling between motor neurons and voluntary muscles, causing paralysis and subsequent death of the patient. Although the debilitating clinical features of SMA are similar to ALS, SMA is found to manifest as early as during infancy, while ALS sets in above the average age of 55 years. SMA can be classified as Type 1–4, depending on the age of onset. Similar to SMA, HSP and CMT are found in juveniles as well as in adolescents. HSP is more pleiotropic in origin characterized by corticospinal dysfunction, muscular weakness, and spasticity, which may involve cerebral atrophy, speech, and cognitive defects and even optic defects. CMT affects the peripheral nervous system, both motor and sensory, causing atrophy of long axonal projections and nerve endings, as also the protective myelin sheath, without being a fatal disorder.

Amyotrophic lateral sclerosis is a motor neuron disease first identified as a neurological condition in 1874 by Jean-Martin Charcot. The term "A-myo-trophic" (In Greek, A: not, myo: muscle, trophic: nourishment) refers to lack of nourishment to the muscle. Death of motor neurons leads to disruption of signaling to the voluntary muscles, which leads to atrophy of the muscles. The term "lateral" refers to the lateral region of the spinal cord whose motor neurons are affected. "Sclerosis" refers to the scarring caused due to the degeneration of the motor neuron. A case of

typical ALS disorder shows clinical symptoms by an average age of 55 years. The prognosis of the disease thereafter is rapid and results in death within 3–5 years of onset. The disease could commence either as "bulbar" or as "spinal," affecting upper motor neurons from the cortex or lower motor neurons from the brain stem and spinal cord, respectively. The symptoms that follow include muscular weakness, fasciculation, spasticity, speech defects, and, finally, paralysis. A common reason for death is respiratory failure owing to the loss of control over the thoracic and diaphragm muscles. In most cases, the sensory functions remain unaffected. ALS may also manifest atypically, such as in the case of juvenile ALS, which is early onset (25 years or younger), ALS with fronto-temporal dementia (FTD), and ALS with spinal muscular atrophy (Andersen and Al-Chalabi 2011).

The origin of the manifestation of ALS in the cell is hard to pinpoint. The disease works by destabilizing the general homeostasis in the motor neuron, as well as its communication with the neighboring glial cells and muscle cells that form the tripartite junction. At the cellular level, ALS is marked by a number of stereotypic hallmarks of neurodegeneration. A prime feature shown in ALS patient tissue samples is the presence of proteinaceous, ubiquitinated cellular inclusions, identified clinically as "skein-like" or "Lewy body-like," "Bunina bodies," hyaline inclusions, as well as TDP-43-positive RNA foci (Blokhuis et al. 2013). Several other homeostasis mechanisms in the cell get affected in the course of the disease, such as ER stress, unfolded protein response, mitochondrial dysfunction, oxidative stress, glutamate excitotoxicity, ubiquitin proteasomal machinery, and autophagy, to name a few. Cellular structures such as neurofilaments, microtubules, and neuromuscular junctions also become disrupted. Axonal transport, ER-to-Golgi trafficking, and vesicular trafficking are other processes that are impaired in motor neurons (Ferraiuolo et al. 2011). The disease pathogenesis has also been shown to involve cell non-autonomous factors such as crosstalk of motor neurons with voluntary muscle cells at the synapse and neuronal neighbors such as astrocytes and microglia that can signal and evoke an immune response (Boillée et al. 2006). The kind of response particularly generated at the cell autonomous and cell non-autonomous level might concur with selective susceptibility of motor neurons in this disease.

Around 90% of the cases known are sporadic, whereas around 10% of the cases are found to be familial. Thirty-one genetic loci have been linked to familial ALS with or without other associated conditions such as FTD in various cohorts of families throughout the world. Superoxide dismutase1 (SOD1) is the first known genetic loci in ALS (Rosen 1993). Since the advent of genome-wide association studies (GWAS) and linkage and sequencing studies, a variety of genetic loci with specific mutations have been identified. These mutations that are known for each of these genetic loci show different levels of prevalence and penetrance. Certain loci are also associated with other neurodegenerative diseases such as C9ORF72 with fronto-temporal dementia, VAPB with spinal muscular atrophy, and ataxin-2 with ataxia. Thus, there exists a pleiotropy in the manifestation of the disease pertaining to different loci (Andersen and Al-Chalabi 2011). The progressions of sporadic or familial cases have not been shown to be clinically different. Indeed, recent reports show that relatives of patients with sporadic ALS are susceptible to the disease and that SALS may have a

genetic basis for the pathogenesis of the disease (Andersen and Al-Chalabi 2011). Several genome-wide association studies and linkage and sequencing studies have analyzed the genetic makeup of SALS (Sporadic ALS) patients and demonstrated that around 26 susceptibility loci might be involved. Among these, several loci have been shown to be common between FALS (Familial ALS) and SALS, among which the most abundantly found in population studies in ALS are the hexanucleotide repeats at C9ORF72, superoxide dismutase1 (SOD1), TAR DNA-binding protein-43 (TDP-43), and fused in sarcoma/translocated in liposarcoma (FUS/TLS) (Renton et al. 2014).

Modeling Motor Disorders in Flies

Drosophila serves as a simple, yet elegant model to study varied aspects of human diseases ranging from genetic to cellular to phenotypic characteristics. For example, counterparts of about 75% of human disease-causing genes are found in *Drosophila*, whose functional relevance can be studied using a plethora of genetic tools developed in the fly. According to FlyBase, of the 31 loci involved in typical ALS, 15 orthologs have been identified and modeled in flies (Table 1). Additionally, transgenic flies expressing human orthologs for these genetic loci have been developed to model ALS (Table 1). This is particularly a useful strategy to study loci that are not conserved in *Drosophila*, the best example being that of the hexanucleotide expansion of C9ORF72. The UAS-GAL4 system has been extensively used for expression or knockdown of ALS loci as well as expression of its associated mutations in specific tissues. This approach allows for understanding the role of these genes and subsequent manifestation of a disease condition in a cell-specific manner. Reverse genetics screens have been designed using this strategy to study the interactors of these loci to identify genetic interactomes and gene regulatory networks (GRNs) that govern the disease. Various pathways affecting disease progression have been identified through these studies such as MAP kinases, BMP, Notch, and TOR signaling. Owing to ease of maintenance of large populations, along with genetic manipulations, *Drosophila* also serves as a platform for large-scale drug testing. A variety of phenotypic readouts such as NMJ defects, aggregation, ubiquitination, retinal degeneration, motor defects, and lifespan defects mimic classical ALS phenotypes mapping different stages of disease progression. With the advent of CRISPR-Cas9 technology, genome edited models are now being developed to study disease-causing genes that are physiologically more significant, bearing a closer resemblance to human disease initiation and progression.

SOD1

Superoxide dismutase 1 is the first known ALS locus, identified in 1993 (Rosen et al. 1993), and till date, more than 150 different mutations have been reported in both familial and sporadic cases of the disease. SOD1 is an antioxidant enzyme that is responsible for containing the ROS levels in the cell by converting superoxide species

Table 1 *Drosophila* models for human ALS. Tabulated below are lists of genes for which fly models have been generated

Label	Symbol	FlyBase ID	Name	Function	References
ALS1	*Sod1*	FBhh0000019	Superoxide dismutase 1	Cellular redox regulation	Elia (1999), Mockett et al. (2003), Watson et al. (2008), Bahadorani et al. (2013) and Şahin et al. (2017)
ALS2	*Als2*	FBhh0000033	Amyotrophic lateral sclerosis 2	Rho guanine nucleotide exchange factor for RABs	Takayama et al. (2014)
ALS4	*Hsap\SETX, CG7504*	FBhh0000611	Senataxin	DNA/RNA helicase	Mushtaq et al. (2016)
ALS6	*Hsap\FUS, caz*	FBhh0000018	Fused in sarcoma, cabeza	RNA binding and regulation	Lanson et al. (2011), Sasayama et al. (2012), Machamer et al. (2014) and Matsumoto et al. (2018)
ALS8	*Vap33*	FBhh0000020	VAMP-associated protein 33 kDa	Lipid homeostasis, vesicular trafficking	Chai et al. (2008), Ratnaparkhi et al. (2008), Chen et al. (2010), Moustaqim-barrette et al. (2013) and Sanhueza et al. (2014, 2015)
ALS10	*Hsap\ TDP-43, TBPH*	FBhh0000017	TAR DNA-binding protein-43 homolog	RNA binding and regulation	Li et al. (2010), Voigt et al. (2010), Miguel et al. (2011), Diaper et al. (2013a), Romano et al. (2015) and Chang and Morton (2017)
ALS11	*FIG4*	FBhh0000335	FIG4 phosphoinositide 5-phosphatase	Phosphoric ester hydrolase activity	Bharadwaj et al. (2016)
ALS14	*Hsap\VCP, TER94*	FBhh0000021	Valosin-containing protein	ER-associated degradation	Chang et al. (2011), Wang et al. (2016) and Kushimura et al. (2017)
ALS15	*Ubqln*	FBhh0000823	Ubiquilin	Ubiquitin chaperone	Kim et al. (2018a)
ALS17	*Hsap\ CHMP2B*	FBhh0000022	Charged multivesicular body protein 2B	Component of the heteromeric ESCRT-III complex	West et al. (2015)
ALS18	*Hsap\ Profilin, chic*	FBhh0000023	Chickadee/ Profilin	Binds to actin polymers	Matsukawa et al. (2016) and Wu et al. (2017)

(continued)

Table 1 (continued)

Label	Symbol	FlyBase ID	Name	Function	References
ALS20	*Hrb98DE*	FBhh0000034	Heterogeneous nuclear ribonucleoprotein at 98DE	RNA binding and regulation	Romano et al. (2014)
FTDALS4	*Hsap\TBK1*	FBhh0000148	TANK-binding kinase 1	Inhibition of I-kappa B	Kuranaga et al. (2006)
FTDALS1	*Zzzz\ GGGGCC*	FBhh0000024	GGGGCC hexanucleotide	–	Xu et al. (2013), Freibaum et al. (2015), Tran et al. (2015), Zhang et al. (2015), Lee et al. (2016) and Moens et al. (2018)
Postulated	*CG14718*	FBhh0000408	EWSR1/TAF15	RNA binding and regulation	Couthouis et al. (2011, 2012)
SCA/ALS13	*Hsap\ ATXN2, Atx2*	FBhh0000062	Ataxin-2	RNA binding, translational regulation	Elden et al. (2010)

to free oxygen and peroxide. SOD1-immunoreactive puncta are observed in SOD1-ALS patients. Most of the SOD1 mutations tested in model systems render the protein to form cellular oligomeric inclusions. The nature of these aggregates is shown to be variable; some mutations have been shown to form thioflavin-reactive insoluble amyloids, while others have been shown to form soluble inclusions (Sheng et al. 2012). Different mutations have been shown to render the protein to form aggregates with different propensities (Prudencio et al. 2009). The study shows that mutations that lower the net charge on SOD1 protein or increase the hydrophobicity of the molecule have an increased propensity for aggregation in comparison with wildtype (Sheng et al. 2012). The study has also correlated increased aggregation propensity to faster progression of disease and death post-diagnosis (Prudencio et al. 2009). Most of the mutations appear to functionally impair the protein. Most, but a few, SOD1 mutants lose their ability to bind to Cu and/Zn ions responsible for its catalytic activity and stability. This could be a possible reason for increased ROS levels in SOD1 patients. However, SOD1 knockdown mice have been shown to not develop ALS, and disease mutants such as SOD1-G93A and SOD1-A4V that do not lose their catalytic activity have also been identified, indicating that oxidative stress may be triggered through other homeostatic defects in the disease. Instead, this observation shows that these might be gain-of-function mutations (Prudencio et al. 2009).

Being the oldest known locus in ALS, over the last 25 years, various fly lines to model ALS1 have been generated using older techniques to generate null mutations, P-element insertions, tissue-specific inducible overexpression and knockdown, and, more recently, CRISPR-Cas9 genome editing tools. Studies performed during the 1990s have favored oxidative stress generated due to the loss of function of SOD1 in ALS1 as a disease mechanism. Indeed, SOD1 null mutation or feeding hydrogen peroxide or paraquat, a ROS-generating drug, caused a decrease in the lifespan of flies. This decrease could be rescued by motor neuron-specific overexpression of SOD1 using the UAS-GAL4 system as well as heat shock-induced expression of SOD1 using the FLIP-FRT system (Parkes et al. 1998; Elia 1999; Sun and Tower 1999; Kumimoto et al. 2013). However, while both *Drosophila* (dSOD1) and human SOD1 (hSOD1) could rescue the lifespan of null mutants, activity levels of human SOD1 were significantly lower than *Drosophila*. A number of ALS mutants of hSOD1, such as G93C and G37R, showed partial rescue, while A4V and G41D showed marginal rescue, and I113T showed no rescue of lifespan of SOD1 null mutants (Mockett et al. 2003). Glial-specific dSOD1 expression, but not hSOD1 G84R, could also rescue peroxide toxicity more prominently in older flies (Kumimoto et al. 2013). Although a sudden decline of activity levels and motor defects of these older mutant rescue lines correlated with lifespan, the activity levels and motor functions of young flies were found to be comparable with wildtype rescue line. Taken together, these results indicated that lowered function of SOD1 below a certain threshold could induce oxidative stress and subsequent death in *Drosophila* (Mockett et al. 2003). While oxidative stress is central to neurodegenerative diseases, loss of SOD1 activity itself may not be directly responsible for ROS toxicity given that other antioxidants such as SOD2 or catalase could compensate for its activity. Reciprocally, it was tested whether SOD1 and other antioxidants

could enhance the lifespan of the fly. Different reports based on the use of different transgenic flies have yielded contradictory results. Ectopic overexpression of SOD1 in motor neurons alone showed increased enzymatic activity and lifespan (Parkes et al. 1998; Elia 1999; Sun and Tower 1999). This claim was refuted in a report that compared the effect of several antioxidants in combinations from across various studies along with their experiments. Their findings indicated that overexpression of antioxidants in long-lived strains did not drastically change lifespan as compared to short-lived strains (Orr et al. 2003). More recent studies in the last ten years have detailed the motor function and lifespan changes with an expression of dSOD1 or hSOD1 or mutants of hSOD1 in different cell types. Ubiquitous expression or knockdown of SOD1 could, respectively, increase or decrease the lifespan of the flies. While overexpression did not produce a change, knockdown could drastically reduce motor function (Martin et al. 2009). Pan-neuronal, motor neuronal, and muscle-specific hSOD1 expression or knockdown only appeared to produce a minor or no improvement in lifespan with limited reduction in motor function (Watson et al. 2008; Martin et al. 2009; Bahadorani et al. 2013). Although dSOD1 and hSOD1 are evolutionarily conserved, given these motor defects, hSOD1 does not appear to be a functional equivalent when expressed in *Drosophila.* However, glial, but not motor neuron-specific, expression of dSOD1 reduced lifespan and motor function (Kumimoto et al. 2013). Zinc-deficient loss-of-function mutant of hSOD1 D83S does lead to motor defects associated with mitochondrial dysfunction with only a marginal effect on lifespan when expressed under motor neuronal, pan-neuronal, or glial promoters, but not in muscles (Bahadorani et al. 2013). On the other hand, motor neuronal expression of toxic gain-of-function mutant, hSOD1 G85R, showed reduced lifespan and motor function in an age-dependent manner (Watson et al. 2008). This was accompanied by a reduction in motor neuron number, increase in electrophysiological defects as well as accumulation and aggregation of SOD1 with an increase in age. Not only mutant but wildtype hSOD1 also showed similar defects in motor neurons. Curiously, a simultaneous increase in chaperone, HSP70 staining was observed in the surrounding glial cells, indicating a non-cell-autonomous response (Watson et al. 2008). In another study, this G85R mutant, when expressed simultaneously in glia and motor neurons, could increase the lifespan and climbing activity of the fly (Kumimoto et al. 2013). Genes involved in metabolisms such as pentose-phosphate pathway, NADP, and glutathione metabolism seem to be downregulated in G85R flies, indicating a direct effect on oxidative stress (Kumimoto et al. 2013). Given these conflicting results, the study of the importance of glia in the development of these phenotypes is crucial in the disease. Recently, a knock-in line using CRISPR cas9 strategy was created that harbored mutations such as H48R, H71Y, G85R, G51S, and G37R, and characterized (Şahin et al. 2017). These mutants showed reduced eclosion rates and lifespan, increased motor and muscle defects accompanied by reduced motor neuron number. These mutants, while forming dimers and higher molecular weight complexes, showed reduced expression in an increase in age. These knock-in lines have validated the toxic gain-of-function effects associated with these mutants in flies serving as a *Drosophila* model that most closely mimics the development of the disease (Şahin et al. 2017).

C9ORF72

In 2011, C9ORF72 was discovered as the most commonly found locus in the ALS-FTD spectrum, accompanied with increased glutamate excitotoxicity thus underlining a strong link between these diseases. The locus essentially represents the expansion of the non-coding hexanucleotide, GGGGCC, to several hundred repeats in the disease, in contrast to the 2–25 repeats found in normal conditions in the C9ORF72 gene. The pathological conditions associated with this locus are multifaceted. Reduced expression of the C9ORF72 gene owing to the presence of repeat expansions was hypothesized to lead to neurodegeneration due to haploinsufficiency. However, knockout mice models failed to develop any neurodegeneration proving haploinsufficiency to be an unlikely course of action. The hexanucleotide repeats at the molecular level acquire very stable DNA/DNA or DNA/RNA G-quadruplex conformations along with DNA/RNA hybrid R-loops. Such secondary structures have been shown to stably bind nucleolar proteins such as nucleolin (NCL) and hnRNPs in a conformation-dependent manner forming nuclear inclusions that can cause protein mislocalization and nuclear stress. It appears that disease mechanisms are centered more toward the gain-of-function phenotypes arising with the sense and anti-sense RNA quadruplexes of G4C2 repeats that lead to the formation of nuclear RNA foci that could potentially sequester RNA-binding proteins and cause nuclear toxicity. Abortive transcripts of variable lengths that get generated from this locus are translated through a non-AUG translation mechanism (Zhang et al. 2014). RNA products undergo non-AUG translation to form five different dipeptide repeats (DPRs) of polyGR and polyGA from sense RNA, polyPR and polyPA from antisense RNA, and polyGP from both, culminating in protein aggregation. A key question of what drives disease progression, RNA toxicity or DPR aggregates or both, has been addressed using flies as a model.

Since 2013, there have been several reports focused on the use of different construct designs to overexpress variable lengths of G4C2 repeats in the 5'UTR or in the intron under an upstream activating sequence (UAS) followed by a downstream SV40 3′UTR containing a polyA tail, enabling the selective expression of RNA and/or DPRs to delineate the pathological cause for the disease. Few studies have favored the RNA toxicity hypothesis leading to retinal degeneration with eye-specific expression or a reduction in the number of active zones in larval neuromuscular junctions with motor neuron-specific expression, suggesting impairment in RNA metabolism and nucleocytoplasmic transport as major causes of cellular defect in ALS (Xu et al. 2013; Zhang et al. 2015; Celona et al. 2017). In these studies, toxicity associated with RNA complexes that sequester RNA binding proteins, such as Pur alpha or RANGAP or Zfp106, could be rescued by the overexpression of these proteins. However, these studies have not accounted for the presence of DPR aggregation as a possible disease mechanism. Several studies have shown that RAN expression of DPRs, in addition to RNA repeats, but not intronically expressed RNA repeats alone, leads to retinal defects, reduced lifespan, reduced bouton number at the NMJ, and reduced muscle size along with increased nucleolar volume (Mizielinska et al. 2014, 2017; Freibaum et al. 2015; Tran et al. 2015) In fact, a recent study demonstrated that presence of

interspersed stop codons prevent the non-ATG translation of the G4C2 repeats but retain formation of cytoplasmic as well as nuclear RNA foci of around 1000 repeats, but do not show drastic lifespan defects or eye defects, proving that the effects arise from DPR pathology (Moens et al. 2018). When DPRs of 50 copies of polyGR, polyGA, polyPR, or polyPA were conventionally expressed in the eye, using a codon-optimized sequence to prevent the formation of any stable secondary structures of RNA repeats, dramatic eye degeneration was observed (Boeynaems et al. 2016). Two genetic screens have identified a number of modulators of eye degeneration phenotype involved in nucleocytoplasmic transport placing it as a core mechanism in C9ORF72-mediated pathology (Freibaum et al. 2015; Boeynaems et al. 2016). Impairment of nuclear transport allows for leakage of RNA repeats into the cytoplasm promoting the expression of toxic DPRs. Inhibition of nuclear export via SRSF1 could rescue the eye phenotype as well as motor functions in flies (Hautbergue et al. 2017). Consistently, it has been shown that arginine containing DPRs, polyGR, and polyPR appears to cause more aggressive phenotypes as compared to polyGA, polyPA, and polyGP (Mizielinska et al. 2014; Wen et al. 2014; Freibaum et al. 2015; Tran et al. 2015; Yang et al. 2015; Boeynaems et al. 2016). When 36 repeats of toxic GR/PR species were expressed in a narrow subset of neurons that are glutaminergic, NMJ phenotypes of increased synaptic vesicles and active zones, accompanied by increased glutamate excitotoxicity and intracellular calcium, were observed (Xu and Xu 2018). Inhibition of vGLUT, a glutamate transporter, in this background could rescue the associated motor defects and shortened lifespan (Xu and Xu 2018). The arginine containing DPRs has been shown to disturb the phase transition of low complexity domain (LCD) proteins into ribonucleoprotein (RNP) complexes such as nucleolus, stress granules, and Cajal bodies (Lee et al. 2016). The field currently favors DPR pathology to be the driving force in ALS/FTD via disruption of RNA bodies, RNA processing, and nucleocytoplasmic transport. Elucidating the differential outcome of C9ORF72 pathology between these two diseases remains a challenge.

TDP-43

Drosophila has been extensively used to model and study TDP-43 pathology in ALS. TAR DNA-binding protein 43 is shown to form ubiquitinated cytoplasmic inclusion in SALS and ALS linked with fronto-temporal dementia (ALS-FTD) cases (Neumann et al. 2006). It is a DNA/RNA binding protein that is usually found to be present in the nucleus. It binds to intronic and 3' UTR of RNA, thereby playing an essential role in RNA metabolisms such as processes like RNA splicing, transcriptional control, and RNA trafficking. Due to mutations in TDP-43 in a diseased condition, proteinopathy is observed in the cytoplasm of the spinal cord and brain tissue, bringing a possible loss-of-function phenotype (Blokhuis et al. 2013). This associated aggregation is conferred by the C-terminal region of TDP-43, which is a low-complexity domain that harbors most of the mutations associated with the disease. Till date, 47 missense mutations and one nonsense mutation have been

found in the TDP-43 locus. TDP-43 immunoreactivity is now being used as a clinical marker to detect ALS/FTD conditions.

The initial hypotheses to address how TDP-43 causes the disease revolved around loss-of-function versus toxic gain-of-function mechanisms. TDP-43 (TBPH in *Drosophila*) null flies generated through classical genetic methods yielded phenotypes such as lowered lifespan, motor defects, disrupted NMJ, and lowered dendrite branching (Feiguin et al. 2009; Lu et al. 2009). Flies lacking TDP-43 showed impaired mTOR signaling through its regulation of the levels of the raptor, a member of the TORC1 complex, with a direct effect on genes involved in autophagy (Xia et al. 2016). Null mutants of TDP-43 led to the increased post-synaptic accumulation of glutamate. This excitotoxicity appeared to be a result of the loss of function of glutamate acid decarboxylase (GAD1) (Romano et al. 2018). Glia-specific knockdown of TDP-43 could increase glutamate excitotoxicity by affecting axon wrapping and glutamate receptor clustering via glutamate transporter, EAAT1 (Romano et al. 2015). TDP-43 null flies also showed lowered levels of *cacophony*, a voltage-gated calcium channel, leading to loss of motor function, which could be rescued by the overexpression of *cacophony* even in a subset of motor neurons alone (Lembke et al. 2017).

Overexpression of wildtype fly or human TDP-43 gene leads to defects in NMJ, eye, locomotion, and lifespan, suggesting gain-of-function roles (Li et al. 2010; Voigt et al. 2010; Estes et al. 2011; Miguel et al. 2011). Intriguingly, despite similarities between phenotypes of null and overexpression, a high-throughput RNA sequencing has shown that there is little overlap in the gene expression patterns between these genotypes (Hazelett et al. 2012). The effect of wildtype overexpression appears to be more exacerbated than overexpression of point mutants for C-terminal, RRM, NLS, or nuclear export signal (NES) (Li et al. 2010; Voigt et al. 2010; Estes et al. 2011; Miguel et al. 2011). Surprisingly, although point mutations in the RRM cause nuclear puncta, retinal, and lifespan defects, deletion of RRM domain does not cause any neurodegeneration, but abrogates the deleterious effects of wildtype and ALS-linked mutant TDP-43 overexpression (Li et al. 2010; Ihara et al. 2013). Along with its roles in the nucleus, TDP-43 also regulates RNA packaging, splicing, and transport in the cytoplasm. It is proposed that in the presence of RRM deletion mutant, TDP-43 mutants cannot sequester RNA targets, thus preventing ALS pathology (Ihara et al. 2013). An example supporting RNA binding as a mechanism involved in ALS is the regulation of translation and localization of *futsch* mRNA by TDP-43 via a stretch of UG-rich region in its 5' UTR (Coyne et al. 2014; Romano et al. 2016). In normal conditions, TDP-43 transports *futsch* mRNA for translation at the NMJ. However, overexpressed TDP-43 or its mutant in the CTD sequesters the *futsch* mRNA into RNP complexes altering its localization and expression (Coyne et al. 2014). Overexpression of *futsch* could reverse the effects of TDP-43 pathology, including RNA transport and aggregation (Coyne et al. 2014). Another modifier of TDP-43, identified in reverse genetics screen in a mammalian cell line, is inositol-1, 4, 5-triphosphate receptor, inhibition of which could increase nuclear export of TDP-43, thereby reducing its nuclear dosage and further rescuing climbing defects and lifespan in flies (Kim et al. 2012).

Furthermore, ALS-linked mutations in TDP-43 have also been shown to impair anterograde transport of TDP-43 RNA granules and, subsequently, its mRNA targets (Alami et al. 2014). While several studies (Li et al. 2010; Voigt et al. 2010; Miguel et al. 2011; Diaper et al. 2013a) have detected nuclear accumulation but not mislocalization upon overexpression of TDP-43 in neurons, others (Estes et al. 2011, 2013; Gregory et al. 2012) have reported the presence of cytoplasmic accumulation in the eye disc and glial cells. These TDP-43 defects and aggregation could be lowered by pharmacological upregulation of heat shock response and chaperone activity (Gregory et al. 2012). A chaperone, HSPB8, in particular, has been shown to rescue against toxic aggregation of various TDP-43 mutants and truncated forms, TDP-25 and TDP-35, through autophagic degradation (Gregory et al. 2012; Crippa et al. 2016). Clusterin, an extracellular chaperone, localizes to the cytoplasm in the presence of ER stress, countering motor, and lifespan defects by aiding the clearance of cytosolic TDP-43 aggregates (Gregory et al. 2017). Peptides flanking the mutation A315T in TDP-43 have been shown to form amyloid structures in vitro that were found to be infectious and neurotoxic in *Drosophila* neuronal cells in culture. This study has demonstrated the prion-like behavior of aggregate formation and propagation of the disease (Guo et al. 2011).

The phenotypes of TDP-43 overexpression are suggested to show dose-dependent increase, implying that accumulated TDP-43 renders the protein ineffective leading to loss of function or a dominant negative effect in case of mutants, which determines the extent of neurodegeneration. The consensus in the field favoring dosage-dependent neurodegeneration was thought to be the defining factor in TDP-43 pathology in *Drosophila,* initiating with synaptic defects followed by loss of neuronal connections and neuronal death (Diaper et al. 2013b). In normal conditions, the levels of TDP-43 are maintained by an alternative splicing mechanism through the action of three splicing factors, SF2, Rbp1, and Sf3b1. This effect was inferred by expressing a transgenic construct of TDP-43 with a region of the 3'UTR responsible for autoregulation, which could reduce the TDP-43 mRNA levels and subsequent protein levels in the cell (Pons et al. 2017). With the age of the fly, it appeared that TDP-43 regulation is affected, leading to a decrease in TDP-43 levels before the onset of motor defects (Cragnaz et al. 2015). A genome-edited version in *Drosophila*, replacing the fly homolog with a human TDP-43 gene or its mutants G294A or M337 V, serves as a potential model to study the outcome of ALS pathology in flies. TDP-43 expressed under the endogenous promoter appears to be autoregulated, phosphorylated, and ubiquitinated, without drastic defects in motor function or lifespan. Further analysis would shed light on the functional aspects of human TDP-43 in flies (Chang and Morton 2017).

FUS

Fused in sarcoma was first described as a proto-oncogene involved in liposarcoma. In 2009, it was found to be another RNA-binding protein involved in ALS and FTD (Vance et al. 2009; Neumann et al. 2010). FUS binds pre-mRNA at intronic regions,

non-coding RNA, exons, and 3'UTRs, and is involved in processes such as DNA repair, miRNA processing, transcription, splicing, and mRNA transport. Mutations in FUS, mainly in the nuclear localization sequence (NLS), have been shown to cause the formation of skein-like cytoplasmic aggregates in large cohorts of ALS cases, with diffused nuclear signal causing loss of function of the protein (Vance et al. 2009). FUS consists of a prion-like sequence in its N-terminal region that has been shown to promote aggregation even in the wildtype protein in yeast (Sun et al. 2011). It is intrinsically prone to aggregate in vitro (Blokhuis et al. 2013). In patients of both sporadic and familial cases, FUS is a part of cytoplasmic aggregation that may or may not be TDP-43 positive (Neumann et al. 2010). FUS pathology does not seem to be limited to ALS/FTD as FUS-positive cellular puncta have been observed in other neurodegenerative diseases as well as Huntington's disease and spinocerebellar ataxia. FUS pathology was found to be similar to that of TDP-43 in that it affected RNA processing and nucleocytoplasmic transport. As in TDP-43, FUS consists of an RRM domain, a low-complexity domain glycine-rich region, and a zinc finger domain. Cabeza (caz) is the *Drosophila* homolog of FUS. Expression of domain deletion mutants of caz showed changes in the levels of the endogenous caz protein. Indeed, overexpression of wildtype FUS could lower the expression of the endogenous caz, emphasizing the presence of an autoregulatory function (Machamer et al. 2014). In flies, this reduction appears to be attributed to the active degradation of *caz* via the ubiquitin-proteasomal machinery (Yamamoto et al. 2018). While the complete deletion mutant of *caz* showed motor defects and reduced lifespan, neuronal knockdown of *caz* also showed NMJ disturbances and motor defects but not reduced lifespan (Sasayama et al. 2012). This loss-of-function effect could be rescued by the cell-specific overexpression of human FUS or *Drosophila caz* but not mutant FUS-P525L (Sasayama et al. 2012; Machamer et al. 2014). Motor neuron-specific overexpression of caz or FUS wildtype or disease mutants also led to phenotypes similar to loss of function such as lowered bouton number, impaired synaptic function, and motor defects (Chen et al. 2011; Lanson et al. 2011; Xia et al. 2012; Shahidullah et al. 2013). However, unlike loss of function, overexpression of FUS wildtype or mutants shows retinal degeneration and mushroom body defects with axonal degeneration as well (Chen et al. 2011; Miguel et al. 2012). While overexpression of FUS alone only showed low cytoplasmic localization, expression of the mutants such as R524S and P525L showed cytoplasmic inclusions reminiscent of the disease (Chen et al. 2011). Nuclear accumulation of the insoluble form of FUS leading to the manifestation of neurodegenerative phenotypes suggests that ALS symptoms may be triggered before cytoplasmic proteinopathy (Miguel et al. 2012). Improving the solubility of the protein using molecular chaperone HSPA1L could reverse some of the retinal degenerative effects. Alternatively, overexpressed FUS may behave in an altered manner, such as a change in post-translational modifications like phosphorylation may cause a toxic gain of function (Miguel et al. 2012). A recent screen performed to identify modulators of FUS-R521G mutant in a class of motor neurons of abdominal ganglion revealed genes involved in nucleocytoplasmic transport such as exportin-1 and Nup154 as suppressors of phenotype. Cytoplasmic aggregation of FUS mutants could be solubilized in the absence of

exportin-1, preventing its sequestration into stress granules, thus providing a neuroprotective role (Steyaert et al. 2018). These studies suggest that while perturbations of wildtype FUS or caz affect neuronal well-being, it is the mutant protein that forms persistent cellular aggregation in flies. *Drosophila* primary neuronal cell co-culture studies could be used to demonstrate a prion-like cell-to-cell transfer of FUS P525L and FUS R524S aggregates, but not of wildtype FUS (Feuillette et al. 2017). However, wildtype FUS is an intrinsically disordered nuclear protein whose expression, localization, and solubility are affected by RNA binding. One such example is hsrωb belonging to a class of long non-coding RNA called architectural RNA that forms the nucleoplasmic ω-speckles compartment (Jolly and Lakhotia 2006). Knockdown of hsrω downregulates *caz* transcription as well as leads caz protein to be mislocalized into cytoplasmic inclusions (Lo Piccolo and Yamaguchi 2017; Lo Piccolo et al. 2017). The phase separation property of FUS is essential for the formation of RNA complexes. A recent study used domain deletion mutants of FUS in an attempt to understand the property of phase separation of this RNA binding protein into stress granules. Deletion of the QGSY motif in the N-terminal LCD and the RGG2 motif in the C-terminal LCD both reduce toxicity in *Drosophila* (Bogaert et al. 2018). Mutation of QGSY to GQ in the N-terminal LCD could act as dominant active by rescuing the eye degeneration phenotype of C-terminal NLS mutant FUS P525L, without being sequestered to the cytoplasmic aggregates, upon coexpression. This proved that the N-terminal LCD was important for self-assembly of FUS (Matsumoto et al. 2018). LCDs form strong synergistic interaction in the formation of liquid droplets as well as hydrogels in vitro, suggesting that point mutations in these domains might make the protein more susceptible to phase separation leading to aggregation-induced toxicity in the disease (Bogaert et al. 2018).

VAPB

In 2004, Mayan Zats group identified another ALS locus as a point mutation, P56S, in a gene coding for VAMP-associated protein B (VAPB) in eight Brazilian families, of Portugal origin. Several members of these families harboring this mutation developed motor diseases in the form of not just ALS, but also SMA (Nishimura et al. 2004). The reason for the differential manifestation of these diseases is unknown, bearing no correlation with age or gender. Other isolated cases featuring VAPB(P56S) were found in families in Japan, Germany, and the USA (Funke et al. 2010; Millecamps et al. 2010). Since then, four more mutations, T46I, S160Δ (Landers et al. 2008; Chen et al. 2010), V234I – associated with C9ORF72 (van Blitterswijk et al. 2012), and P56H (Sun et al. 2017), have been identified through sequencing studies. VAPB is an ER membrane protein that integrates into the membrane via its C-terminal domain. The protein works as a homodimer or a heterodimer with VAPA. Through its N-terminal MSP domain, VAPB interacts with several proteins that contain an FFAT motif, displaying roles in membrane tethering between organelles, vesicular transport, and lipid transport. VAP localizes in the ER membrane as well as membrane contact sites between organelles and intracellular vesicles. VAPB

plays an important role in cellular homeostasis by regulating calcium signaling and proteostasis. VAP mutant, owing to change in conformation, leads to misfolding and aggregation of the protein. Overexpression of VAP(P58S), VAP(T48I), and VAP(V260I) in the *Drosophila* homolog, VAP33a (after that mentioned as VAP), led to the formation of cellular puncta (Ratnaparkhi et al. 2008; Chen et al. 2010; Sanhueza et al. 2014). Coexpression of tagged VAP and VAP(P58S) protein showed colocalization, suggesting the dominant negative effect of the VAP(P58S) that interacts with and sequesters the wildtype VAP into its ubiquitinated aggregates. VAP null mutation and expression of other disease-related mutants, VAP(P58S), VAP(T48I), and VAP(V260I), are accompanied with ER stress in the adult brain of the fly as suggested by aggregation and mislocalization of ER luminal resident proteins, Boca, PDI, chaoptin, SERCA, and Hsp70, and increase in puncta of chaperone upregulated in UPR, Hsc3, and XBP1-GFP (Tsuda et al. 2008; Chen et al. 2010; Sanhueza et al. 2014; Yadav et al. 2018). Upon neuronal expression, the N-terminal MSP domain of VAP can be cleaved and secreted out of the neurons possibly, as a ligand for ephrin or Robo/Lar-like receptors on the muscle, thereby affecting cytoskeleton and mitochondrial morphology (Tsuda et al. 2008; Han et al. 2012). The secretion of MSP domain does not seem to occur in the presence of VAP(P58S) aggregation, such that neuronal overexpression of VAP causes myofibril disruption in the muscle while VAP(P58S) does not (Tsuda et al. 2008). Neuronal overexpression of VAP leads to dosage-dependent changes in the NMJ, including smaller bouton size and increase in bouton number (Pennetta et al. 2002; Chai et al. 2008; Ratnaparkhi et al. 2008). Human and *Drosophila* VAP appear to be phenotypically similar at the NMJ, suggesting evolutionarily conserved functionality (Chai et al. 2008). The mutant VAP(P58S) appears to have the opposite effect with a lesser number of larger boutons similar to VAP null phenotype, showing disruption of microtubule organization, lowered number of active zones, and reduced retrograde BMP signaling (Ratnaparkhi et al. 2008; Forrest et al. 2013).VAP(V260I), on the other hand, shows an increased number of smaller boutons with an affected microtubule architecture similar to increased VAP expression (Sanhueza et al. 2014). VAP null mutants in *Drosophila* show defects in dendritic localization and axonal transport of Down syndrome cell adhesion molecule (Dscam) protein involved in self-recognition and avoidance in DA neurons (Yang et al. 2012). Phosphoinositide levels appear to be increased in ALS8, leading to axonal and synaptic defects. Sac1, the phosphoinositide phosphatase, the enzyme required to regulate phosphoinositide metabolism, was found to interact with VAP physically. Downregulation of Sac1 or expression of VAP(P58S) affects synaptic microtubule organization that could be rescued by reducing the levels of phosphoinositide (PI) (Forrest et al. 2013). A phosphatidylinositol transfer protein (PIPT) domain-containing protein, RDGBα, responsible for PIP2 metabolism, is recruited to the ER:PM contact sites via its interaction with VAP in photoreceptor cells (Yadav et al. 2018). Despite lowered synaptic function, both neuronal overexpression of VAP and VAP(P58S) could rescue VAP-deficient flies. This suggested that while VAP(P58S) appears to be dominant negative and phenocopies VAP null at the NMJ upon overexpression, it does not seem to be non-functional. While ubiquitous and muscle-specific expression of

VAP and VAP(P58S) caused lethality at 29 °C, at 25 °C, VAP, but not VAP(P58S), showed lethality (Ratnaparkhi et al. 2008). This suggested that above a certain threshold VAP protein, but not VAP(P58S), could develop toxic functions in the cell. Muscle-specific expression of VAP(V260I) showed a change in the shape, size, and position of muscle nuclei, leading to a disruption of nuclear envelop architecture (Sanhueza et al. 2014). Pan-neuronal and glial cells appeared to be more tolerant of the overexpression of these proteins, as they did not lead to the lethality of the fly at either temperature (Ratnaparkhi et al. 2008). However, neuronal VAP(P58S) overexpression did seem to cause motor defects and neuronal death in the larval brains according to one transgenic model (Chai et al. 2008). Eye-specific expression of VAP(P58S) indeed showed retinal degeneration that could be rescued by the overexpression of inhibitor of apoptosis, DIAP2 (Forrest et al. 2013; Sanhueza et al. 2015). Expression, in sensory organ precursor cells, of wildtype VAP but not mutant VAP, reduced the number of thoracic macrochaetae. Coexpression of VAP and VAP (P58S) could recover the thoracic bristle number. A reverse genetic screen designed to identify interactors of VAP using macrochaetae as a read-out helped identify 103 genes that formed a part of gene regulatory network consisting of 406 genes including physical interactors (Deivasigamani et al. 2014). This screen identified the TOR pathway as a modulator of VAP as well as VAP(P58S). Downregulation of TOR appears to rescue morphological defects at the NMJ associated with VAP(P58S), while upregulation of TOR could rescue the effects associated with VAP (Deivasigamani et al. 2014). Members of the TOR pathway were also identified as modulators of VAP(P58S) aggregation through a S2R+ cell-based screen. This interaction could be based on ROS regulation coupled with proteasomal degradation of VAP(P58S) aggregates (Chaplot et al. 2019). Another reverse genetics screen around the same time identified a large network of genes modulating of retinal degeneration associated with eye-specific expression of VAP(P58S). Genes involved in vesicular and endocytic trafficking (Rab5, Rab7), proliferation (Ric) and apoptosis (Diap2), proteolysis and lipid biogenesis were identified as a part of the network. VAP(P58S) aggregates expressed in the fly brain clustered with Rab5, similar to that found in patient motor neuron samples (Sanhueza et al. 2015). In 2013, constructs of the genomic region of VAP as well as VAP containing the P58S mutation were generated and site-specifically inserted into the third chromosome to generate transgenic flies expressing VAP or its mutation under its promoter (Moustaqim-barrette et al. 2013). Both the wildtype and the mutant genomic construct could rescue the lethality associated with VAP null mutant. While wildtype VAP could rescue the entire length of the *Drosophila* lifespan, the VAP(P58S) genomic rescued flies survived only up to 25–30 days post-eclosion. Curiously, when expressed at endogenous levels, the heterozygous combination of one copy each of wildtype and mutant construct could survive for as long as wildtype flies. The expression of VAP(P58S) at endogenous level does not compromise the functional VAP protein unlike its overexpression using the UAS-GAL4 system. It appears that the threshold of VAP(P58S), as well as VAP protein level, determines the extent to degeneration in the fly. This suggests that the reduction in lifespan of VAP(P58S) genomic-rescued flies is a result of partial loss of function of VAP(P58S)

mutant protein. Oxysterol binding protein (OSBP), a physical interactor of VAP, normally present in the ER and responsible for cholesterol transport, is mislocalized to the Golgi in VAP null flies. The shortened lifespan of the VAP(P58S) genomic-rescued flies could be increased to wildtype levels by the overexpression of human OSBP, specifically in the motor neuron. Overexpression of hOSBP restored OSBP localization to the ER in VAP null flies, lowering accumulation of ER proteins and ER stress associated with VAP loss of function (Moustaqim-barrette et al. 2013). The genomic-rescued flies display ER stress and disruption of ER quality control compartment, demonstrating the partial loss of function of VAP(P58S), which is also observed with VAP(P58S) overexpression (Tsuda et al. 2008; Moustaqim-barrette et al. 2013).

Other Genetic Loci

A set of ALS loci involved in degradative mechanisms, such as valosin-containing protein (VCP), ubiquilin-1/ubiquilin-2 (UBQLN1/2), TANK-binding kinase (TBK1), and senataxin (SETX), have been modeled in *Drosophila*. VCP is a hexameric AAA ATPase that forms a part of the ER-associated degradation complex responsible for the translocation of ER-based proteins for proteasomal degradation. Pathogenic mutations in VCP have been identified in several neurodegenerative diseases such as ALS and inclusion body myopathy with Paget's disease of bone and fronto-temporal dementia (IBMPFD). VCP is conserved in *Drosophila* as TER94. Dominant active pathogenic mutations of VCP involved in IBMPFD cause midline crossing of β/γ lobes of the mushroom body in the brain, muscle disruption, and retinal degeneration, which is sensitive to cellular ATP levels (Chang et al. 2011). It plays a role in dendritic pruning promoted by ecdysone signaling via Mical, actin-severing enzyme, in class IV DA neurons. The regulation of Mical mRNA and subsequent dendritic pruning in pupal stages is controlled by RNA-binding proteins such as TDP-43, whose localization is dependent on VCP (Rumpf et al. 2014). Stress-induced sumoylation of VCP has suggested a mechanism for its nuclear transport, stress granule recruitment and promotion of ERAD pathway; reduced sumoylation in pathogenic mutants could result in altered co-factor binding and function (Wang et al. 2016). VCP mutations associated with ALS expressed in motor neurons lead to NMJ defects such as the appearance of ghost boutons and decrease in bouton number, coupled with crawling defects. In muscles, VCP mutant protein leads to sarcomere and mitochondrial defects similar to that in *PINK* and *parkin* mutant. VCP appears to be essential for mitochondrial quality control and is recruited to the mitochondria via parkin (Kim et al. 2013b; Kimura et al. 2013).

Mutations in the proline-rich region of an X-lined ALS locus, UBQLN2, cause juvenile as well as adult-onset ALS and FTD. UBQLN1/UBQLN2 are ubiquitin chaperones that participate in both proteasomal and autophagic degradation mechanisms. UBQLN interacts with both ubiquitin ligases and the proteasome via its ubiquitin-associated domain and ubiquitin-like domain. Mutations in its proline-rich region caused misfolding and cytosolic aggregation of UBQLN2 that appear to

be both ubiquitin and p62 positive. Mutant UBQLN2 proteins showed an age-dependent decrease in solubility and increase in sensitivity to chymotryptic cleavage. Eye-specific expression of mutant UBQLN2proteins caused hyperpigmentation, while neuronal expression leads to changes in NMJ morphology and climbing defects. Proline mutants possessing enhanced binding to ubiquitin and toxic gain of function, clubbed with changes in folding and subsequent aggregation, appear to be the cause for toxicity (Kim et al. 2018a).

The *Drosophila* homolog of the recently identified gene in ALS, TBK1, has been studied previously as a regulator of an inhibitor of apoptosis, DIAP2, levels via its phosphorylation and subsequent degradation, in developing sensory organ precursor cells, thereby controlling the non-apoptotic functions of caspases (Kuranaga et al. 2006). A DNA/RNA helicase, SETX, has been shown to modulate NMJ structural organization by affecting the number of futsch loops and actin puncta associated with the boutons. The effect of SETX and its mutants at the bouton showing a decrease in several active synaptic zones could be a result of increased BMP signaling and decreased highwire activity (Mushtaq et al. 2016). Highwire is an E3 ubiquitin ligase that negatively regulates BMP signaling (Mccabe et al. 2004).

A few ALS loci are involved in endosomal trafficking such as Alsin2, FIG4, CHMP2B, and actin polymerization regulator, profilin, with essential roles in membrane remodeling. Alsin2 is a GTP exchange factor (GEF) involved in the activation of the early endosomal protein, Rab5. ALS-linked mutations in Alsin2 led to a reduction in its GEF activity in *Drosophila* S2 cells. Knockout of Alsin2 caused defects in NMJ and dendritic morphologies similar to Rab5 knockout along with climbing defects, which could be rescued by expression of Alsin2 under ubiquitin-GAL4, but not with motor neuron-specific expression (Takayama et al. 2014). FIG4, a phosphoinositide phosphatase, was found as a locus in not only ALS but also CMT and Yunis–Varon syndrome. Mutation known in CMT has been studied using *Drosophila* FIG4 protein in larval muscles. FIG4 null mutation caused an accumulation of lysosomes, which could be partially or entirely rescued by mutant FIG4 and wildtype FIG4 overexpression, respectively. This phenotype could also be rescued by inhibiting the upstream Rab7 and HOPS complex function, preventing fusion of late endosome with lysosomes. FIG4, in complex with VAC14 and FAB1, showed a non-catalytic function, involved in the maintenance of lysosomal size (Bharadwaj et al. 2016). A member of the ESCRT-III complex, CHMP2B, was found be involved in ALS-FTD. An FTD-associated mutant of CHMP2B developed NMJ and eye defects that could be modulated by members of recycling endosome machinery, RAB8, which was in turn regulated by JNK and BMP pathway (West et al. 2015). Profilin regulates actin polymerization through its interaction with formin. Neurodegenerative effects of mutant forms of human profilin expression in *Drosophila* appeared to be a result of partial loss of function in nature. These mutants do not seem to aggregate, as seen in the case of the disease and mice models. Overexpression of both wildtype and mutant forms of human profilin lowered satellite boutons along with decreased synaptic vesicles. However, wildtype overexpression led to the formation of several ghost boutons and an increased number of active zones, as compared to mutants. Nevertheless, they were able to rescue pupal

lethality associated with the knockdown of the endogenous *Drosophila* profilin, *chickadee* (Wu et al. 2017).

Studies on RNA pathology have gained momentum in the field of ALS and FTD concerning other RNA binding proteins, as well. A functional screen in yeast revealed RNA binding proteins such as EWSR1, TAF15, HNRNPA0, and DAZ1 as potential ALS loci that had a propensity for cytoplasmic aggregation similar to that seen in TDP-43 and FUS. Expression of TDP-43, FUS, HNRNPA0, and DAZ1 proved to be highly toxic to yeast, whereas EWSR1 and TAF15 showed milder toxicity. These genes, when tested in the *Drosophila* eye, developed retinal degeneration in a dose-dependent manner. RGG mutants of EWSR1 and TAF15 also showed rough eye phenotypes. Pan-neuronal overexpression of these genes lowered the lifespan and climbing ability of flies. Finally, puncta of these proteins were found in sporadic cases of ALS, further emphasizing the impact of RNA pathology in ALS (Couthouis et al. 2011, 2012). In flies, hnRNPA2 mutants have been shown to cause mild myotubule organization defects as well as cytoplasmic inclusions (Kim et al. 2013a). Like TDP-43 and FUS, EWSR1, TAF15 hnRNPA1, and A2 are examples of proteins that contain low-complexity domains or prion-like domains enabling them to phase separate into functional membrane-less organelles of RNP complexes. In ALS, mutations identified in these loci make the protein more susceptible to aggregation, altering the dynamics and function of these RNP complexes.

A Unifying Genetic Network in ALS and Other Neurodegenerative Disease

Death of motor neurons is often viewed as the core feature responsible for ALS. However, a collection of cell autonomous and non-cell autonomous events lead to the onset, progression, and death of the patient in ALS. The motor neurons engage in cell-to-cell communication with different cell types such as glial cells, intermediate neurons, and muscles. Perturbations in external cues and downstream signaling lead motor neurons to develop a higher level of susceptibility that manifests in ALS. These perturbations can be genetic as well as environmental. Several essential genes identified as ALS loci perform important housekeeping functions such as RNA processing, protein quality control, axonal transport, vesicular and endosomal trafficking, ER, mitochondrial and oxidative stress regulators. A major class of loci is the RNA binding proteins containing a prion-like domain (QGSY-rich), RRM, glycine-rich domain, RGG domains, and NLS site (PY motif). Mutations identified in ALS have been mapped to all these regions of these genes making these proteins more prone to mislocalization and aggregation. RNA-binding proteins are generally responsible for RNA packaging and trafficking. This property becomes more crucial in stress where these proteins form reversible protective RNP complexes such as P-bodies and stress granules in order to process mRNA degradation. The formation of these RNP complexes is often regulated by post-translational modifications such as phosphorylation and ubiquitination (Li et al. 2013).

Around ~95% of ALS cases appear to display TDP-43 pathology in both familial and sporadic cases. TDP-43 pathology is observed in the background of other mutations such as FUS, C9ORF72, hnRNPA1/2, VCP, and UBQLN. *Drosophila* studies have explored the interaction between RNA binding proteins that cause similar disease manifestations. Caz null flies and TBPH null flies show a shortened lifespan and motor defects with increased bouton number in larval NMJ. While Caz overexpression in motor neurons can rescue these phenotypes of these null flies, TBPH overexpression was not sufficient to rescue caz null phenotypes, suggesting a robust epistatic relationship between these genes. Expression levels of neither protein were dependent on one another. Caz and TBPH appeared to interact physically but only in the presence of RNA. Caz mutant proteins were found to be physical interactors of TBPH, even though caz mutant proteins mislocalized to the cytoplasm (Wang et al. 2011). Mutants of FUS and TDP-43, when overexpressed in the eye, show rough eye phenotypes and retinal degeneration. This effect is exacerbated when mutants of both genes are coexpressed (Lanson et al. 2011).

Similarly, a null mutant of ataxin-2, a protein containing polyglutamine (polyQ) expansion involved in spinocerebellar ataxia type 2, worsens the phenotypes associated with TDP-43 overexpression in the eye, motor function, and lifespan (Elden et al. 2010). *Drosophila* homolog of hnRNPA1, Hrp38, is also shown to be a physical interactor of TBPH as well as TDP-43, involved in processing TBPH mRNA by inhibiting the splicing of exon 3. Knockdown of Hrp38, as well as *TBPH* mutant with deletion in exon 3, caused neuropil degeneration, motor defects, and reduced lifespan, effects that are enhanced in combination (Romano et al. 2014).

Aggregation of proteins in the disease scenario can have a severe effect on the regulation of protein turnover and corresponding gene expression. As mentioned previously, overexpression of TDP-43 or FUS could downregulate the endogenous counterparts of these proteins in a feedback loop, while the protein product itself was also tagged for degradation in response to various cellular cues. TDP-43 pathology, but not FUS pathology, was induced in *Drosophila* eyes upon expression of mutants of profilin, probably owing to a shift in TDP-43 localization (Matsukawa et al. 2016). This change in localization of TDP-43 from the nucleus to the cytoplasm, coupled with the rough eye phenotype, was observed in the presence of VCP(R152H) mutant expression as well, as opposed to wildtype VCP expression. This mislocalization and subsequent degeneration was also observed with the coexpression of TDP-43(M33V) with wildtype VCP and even more prominent with VCP(R152H). This led to the hypothesis that VCP could be responsible for TDP-43 nucleocytoplasmic shuttling as well as its degradation, processes that may be stalled when either one of the proteins is mutated. This would culminate in the accumulation of TDP-43 in the cytoplasm, raising toxic gain-of-function effects, and being depleted in the nucleus, causing loss of function.

Interestingly, knockdown of *Drosophila* homolog, ter94, could rescue the degenerative eye effects of polyglutamine-induced aggregates, while overexpression could enhance it, relaying a plausible role for VCP in cell death mechanisms (Higashiyama et al. 2002). However, another study showed that VCP could be sequestered into polyQ aggregates of huntingtin and ataxin-1, preventing its nuclear

role in DNA repair. In this case, overexpression of VCP could bypass any modulation of polyQ aggregates, reaffirming its role in double-stranded break repair (Fujita et al. 2013). VCP appears to inhibit the rhodopsin (Rh) pathology in retinitis pigmentosa in another mechanism. Rh mutant P37H expressed in the eye misfolds and forms non-toxic aggregates, which in the presence of wildtype Rh promote light-sensitive retinal degeneration. It is also rescued by the knockdown of VCP that triggers the unfolded protein response in the eye, as also by chemical inhibition of the ERAD and proteasomal pathway (Griciuc et al. 2010a, b).

Another well-studied locus involved in degradation is ubiquitin, which shows genetic interactions with proteins involved in Alzheimer's disease. For instance, overexpression of presenilin (Psn), a γ-secretase protein, led to peculiar defects in the eye about the interommatidial bristles that correlated with decreased Notch signaling (Li et al. 2007). These defects were exacerbated with knockdown of UBQLN and partially rescued by its overexpression along with notch signaling (Li et al. 2007). UBQLN was found to physically interact with Psn via the UBA domain (Ganguly et al. 2008). UBQLN overexpression could, however, lead to an age-dependent degeneration in the eye. This feature could be rescued by the overexpression of Psn (Ganguly et al. 2008). UBQLN knockdown showed similar wing defects as seen in notch pathway downregulation, further corroborating a link between UBQLN and Psn (Li et al. 2007; Ganguly et al. 2008). Similar to VCP and profilin, UBQLN overexpression could also reduce the expression levels of TDP-43 in the eye. However, change in localization of TDP-43 or colocalization with UBQLN was not observed, questioning the mechanism of TDP-43 degraded in the presence of UBQLN (Hanson et al. 2010). However, another study showed that UBQLN could physically interact, alter solubility and ubiquitination, and delegate TDP-43 from nucleus to cytoplasm (Jantrapirom et al. 2018). Decreased solubility of TDP-43 with UBQLN knockdown could be recovered with VCP overexpression (Jantrapirom et al. 2018). Increased soluble ubiquitinated TDP-43 appears to be the toxic force in ALS pathology in *Drosophila* determined by motor assay (Jantrapirom et al. 2018). A *Drosophila* chaperone CG5445 could increase solubility and enhance the proteasomal degradation of TDP-43 protein (Uechi et al. 2018). It could physically interact with TDP-43, probably via its ubiquitin-associated domain. This interaction and solubilization were retained even in TDP-43(M33V) mutant, but not in FUS(R521C) mutant. A possible ortholog of this gene in humans, C6ORF106, can act as a potential therapeutic option (Uechi et al. 2018).

Mitochondrial dysfunction and oxidative stress are determining factors in ALS pathology. Mitochondrial morphology in indirect flight muscles and axons of leg motor neurons appeared to be fragmented with TDP-43, FUS, and TAF15 overexpression, an effect that could be rescued by the knockdown of mitochondrial fission proteins, Drp1 and Marf, or overexpression of the fusion protein, Opa1. This fragmentation could be a result of lowered marf levels degraded via the activity of E3 ubiquitin ligase, parkin (Altanbyek et al. 2016). In another study, the parkin-initiated degradation of TAF15 could rescue its degenerative effect by decreasing aggregation, retinal degeneration, motor defects, and shortened lifespan (Kim et al. 2018b). Proteasomal degradation of overexpressed VAP(P58S) is driven by ROS activation

via SOD1 knockdown as well as TOR downregulation in third instar larval brain. These modulators of VAP(P58S) aggregates were identified in a cell-based RNAi screen. Surprisingly, ROS could also decrease expression levels of endogenous VAP (Chaplot et al. 2019). VAP overexpression, as in the case of *sod1* and *sod2* null mutant, leads to the increase in several boutons at the NMJ, a phenotype correlated with oxidative stress (Pennetta et al. 2002; Milton et al. 2011). Indeed, VAP overexpression in *Drosophila*, in a cell-type specific manner, appears to be more toxic than VAP(P58S) and is accompanied by increased ROS (Ratnaparkhi et al. 2008; Chaplot et al. 2019). Synapse development is regulated by oxidative stress via MAP kinase pathways such as JNK and p38, as are TOR (target of rapamycin) pathway and autophagy (Collins et al. 2006; Milton et al. 2011; Deivasigamani et al. 2014). TDP-43 overexpression also caused ROS toxicity that could be attenuated by JNK signaling and accentuated via p38b signaling downstream of MAP kinase, Wallenda (Zhan et al. 2014). The extent of oxidative stress developed in the fly correlated with shortening of lifespan. In a relationship similar to the change in ROS, these signaling pathways also regulated antimicrobial peptide (AMP) production in response to the innate immune pathways, Toll/Dif and Imd/Relish, invoked by TDP-43 toxicity (Zhan et al. 2014).

Summary

ALS is a debilitating disease that occurs in 1 among 50,000 people per year. The late onset of the disease is coupled with a rapid prognosis of 3–5 years before patients succumb to death due to respiratory failure. Treatment in ALS is limited to two FDA-approved drugs, riluzole, and edaravone. Riluzole, the only approved drug for ALS in the last 20 years, acts by decreasing glutamate excitotoxicity, improving the life of the patients by only a few months. Edaravone, on the other hand, is involved in the reduction of oxidative stress and was approved as a treatment option by FDA in 2017. A large number of processes involved in the disease provide a battery of potential drug targets. Rapamycin has been shown to have beneficial effects in fly models of TDP-43 and VAP, as well as in models of zebrafish and mice. Rapamycin is now in phase II drug trial for ALS (Mandrioli et al. 2018). RNA therapy is another example that has been shown to work in animal models successfully. Its use has already helped in splicing correction in SMN2 pre-mRNA in children suffering from SMA (Chiriboga et al. 2016). A *Drosophila* study has helped validate the use of small binding molecules targeting the G-quadruplex of G2C4 repeats in C9ORF72, thereby increasing its lifespan by inhibiting RNA toxicity and DPR production (Simone et al. 2017). Use of siRNA and genome editing techniques against targets, such as SOD1, TDP-43, Ataxin-2, are the new methods of treatment currently being explored in animal models (Mathis and Le Masson 2018).

Drosophila research has shed light on certain unifying factors among ALS loci that agree with other disease models and patient data as well (Fig. 1). In most cases, overexpression, as well as knockdown of these loci, proved to cause morphological changes, motor defects, and lethality in the fly. While the use of null mutants and

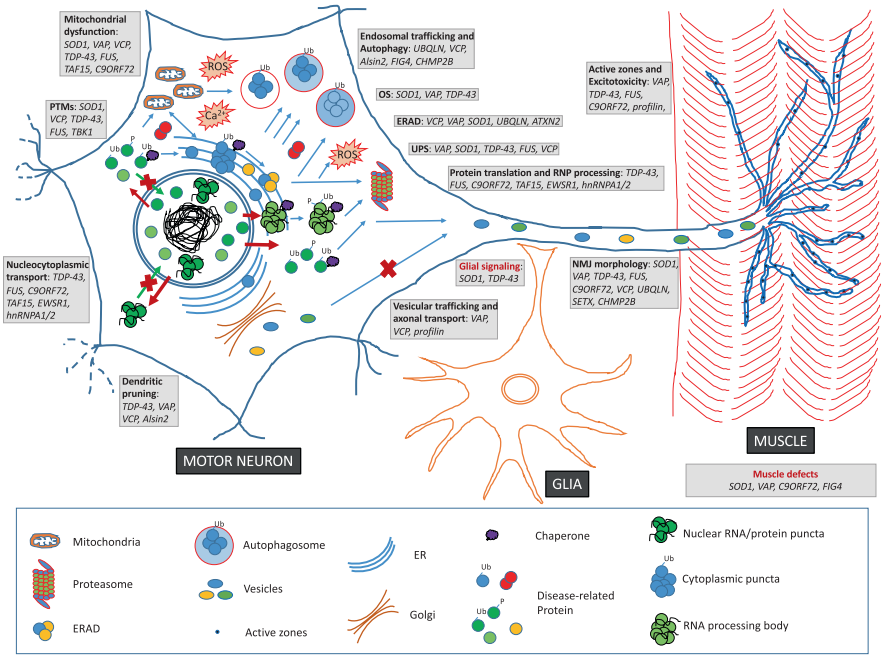

Fig. 1 Schematic representation of known functions of genes that have been identified as causative loci for motor neuron disease. Seventeen genes are listed, classified based on their function and site of action. Fifteen of these genes have *Drosophila* orthologs that have been modeled in flies (Table 1). FTDALS1 is studied using overexpression systems as G4C2 repeats and DPRs. CG14718 is identified as fly orthology of the postulated loci, EWSR1 and TAF15. Disease-causing mutations in these genes presumably cause a loss-of-function, or in some cases, a toxic gain-of-function. A class of these loci is RNA-binding proteins, DPRs, TDP-43, FUS, hnRNPA1/2, EWSR1, and TAF15. Defects associated with these loci include nuclear toxicity, impaired nucleocytoplasmic transport, altered RNA binding, and trafficking, disrupted protein translation, and toxic RNA-protein complex formation. Expression of some of these proteins such as TDP-43 and FUS also appears to be autoregulated. Another arm severely affected at the cellular level in ALS is proteostasis. This includes ER stress, unfolded protein response (URP), oxidative stress (OS), chaperone activity, ER-associated degradation (ERAD), proteasomal degradation and autophagy. Loci actively involved in these mechanisms are *SOD1, VAP, VCP,* and *UBQLN*. Mutant proteins in ALS can act as monomers with toxic gain-of-function and form toxic RNP complexes and protein aggregates. Monomeric, oligomeric, or aggregated forms of these proteins are subjected to post-translational modifications like phosphorylation and ubiquitination. Oligomers and aggregates can also be solubilized through chaperone activity. PTMs and solubilization can prime these proteins for degradation through proteasome or autophagy. Certain loci such as *SOD1, VAP, VCP, TDP-43, FUS, TAF15,* and *C9ORF72* can affect the mitochondria triggering mitochondrial fragmentation, energy imbalance, oxidative stress, autophagy, and calcium signaling defects. Transport machinery such as vesicular trafficking, endosomal recycling, and axonal trafficking can be disrupted due to microtubule disorganization along the axon and at the synapse in the case of VAP, UBQLN, VCP, Alsin2, FIG4, CHMP2B and profilin. This can lead to NMJ morphology and function defects in bouton shape and size, active zones, and glutamate release. This is directly related to perturbation of signaling across the NMJ, such as JNK, BMP, and mTOR among others. NMJ morphology is a feature most commonly affected in almost every model of ALS studied in *Drosophila*. In a few cases like TDP-43, VAP, VCP, and Alsin2, a similar disruption is also observed at dendritic nerve endings that synapse with interneurons. Although most of the functions are neuronal, a few genes contribute to the disease because of their function/malfunction in muscle and glia. Sarcomeric disorganization, myotubule disruption, and nuclear envelop defects are some of the effects accompanied by muscle expression of ALS loci such as SOD1, VAP, C9ORF72, and FIG4. Glial expression of loci such as SOD1 and TDP-43 directly affects oxidative stress, axonal wrapping, and glutamate excitotoxicity.

knockdown studies directly aid in identifying functional roles, overexpression studies demonstrate a deliberate effect of toxicity manifesting through the proteins themselves. Overexpression of these proteins could lead to degenerative phenotypes as a result of the gain-of-altered function as in the case of VAP, TDP-43, and FUS. This meant that stringent regulation of these proteins was required for their optimal function. Disease-causing mutations gave rise to a wide range of functional consequences such as dominant active, dominant negative, loss-of-function, and gain-of-function phenotypes. The mutant protein could lead to misfolding (e.g., VAP), mislocalization (TDP-43, FUS), or altered physical interaction (UBQLN), ultimately leading to aggregation. Oligomeric or aggregated forms of these proteins can tend to sequester binding partners such as proteins or RNA preventing their normal function, further adding to the toxic nature. Processes involving the movement of cellular components, such as nucleocytoplasmic transport (C9ORF72, TDP-43, FUS), vesicular trafficking (VAP, VCP, UBQLN), and axonal transport (VAP, profilin) are found to be prominently disrupted, promoting mislocalization and accumulation of mutant proteins. Thus, cytoplasmic accumulation of protein aggregates appears to act as a sink for functional protein and associated binding partners, abetting the breakdown of cellular processes. Mutant protein could also change post-translational modifications such as phosphorylation (TDP-43), ubiquitination (most loci) or sumoylation (VCP), or changes in binding proteins (VAP), proving to be more toxic as a monomer or oligomer than in an aggregated form. In response to various triggers ranging from RNA binding to ROS to chaperone activity (TDP-43, FUS, VAP), ubiquitinated mutant proteins are targeted for proteasomal degradation or autophagy. Degradation mechanisms are severely affected in cases of proteins directly involved in the process (VCP, UBQLN) as a result of ER stress and unfolded protein response.

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