A Fruitful Endeavor: Modeling ALS in the Fruit Fly

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Abstract

For over a century *Drosophila melanogaster*, commonly known as the fruit fly, has been instrumental in genetics research and disease modeling. In more recent years, it has been a powerful tool for modeling and studying neurodegenerative diseases, including the devastating and fatal amyotrophic lateral sclerosis (ALS). The success of this model organism in ALS research comes from the availability of tools to manipulate gene/protein expression in a number of desired cell-types, and the subsequent recapitulation of cellular and molecular phenotypic features of the disease. Several *Drosophila* models have now been developed for studying the roles of ALS-associated genes in disease pathogenesis that allowed us to understand the molecular pathways that lead to motor neuron degeneration in ALS patients. Our primary goal in this review is to highlight the lessons we have learned using *Drosophila* models pertaining to ALS research.

Keywords

Amyotrophic lateral sclerosis; ALS; *Drosophila*; Fly models; Animal models; Neurodegeneration; Motor neuron diseases; FTLD; SOD1; TDP-43; FUS; C9ORF72; VCP

1.0 Introduction

Amyotrophic lateral sclerosis (ALS), also called Lou Gehrig's disease, is an adult onset, fatal, neurodegenerative disorder, for which there is currently no available cure. ALS was first described in the late 1860's and early 1870's by Jean-Martin Charcot (Charcot, 1874;Charcot and Joffroy, 1869), and a clear understanding of its pathogenesis at cellular and molecular levels is still eluding researchers today, nearly 150 years later. ALS is primarily characterized by the loss of upper and lower motor neurons, and as a result, death is usually due to respiratory failure (Gordon, 2013;Hardiman et al., 2011;Rowland, 1998). Clinically, ALS can be categorized into three primary types depending on the origin of onset

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(Hardiman et al., 2011). Approximately 65% of ALS patients are diagnosed with limb onset, 30% with bulbar and the remaining 5% with respiratory onset (Hardiman et al., 2011). In general, bulbar and respiratory have a worse prognosis than limb onset (Chio et al., 2009). Occurrence of the disease is highest between approximately 60 and 85 years of age (Alonso et al., 2009;Fang et al., 2009;Logroscino et al., 2010;O'Toole et al., 2008), with males typically having an overall higher rate of incidence than females (Alonso et al., 2009;Johnston et al., 2006). Disease progression is very rapid, and the average life expectancy post-diagnosis is 2-5 years (Byrne et al., 2012;Huisman et al., 2011;Lee et al., 2013;Logroscino et al., 2008;Mortara et al., 1984;Pugliatti et al., 2013).

Approximately 5% of all ALS cases show signs of inheritance and are referred to as familial ALS (FALS) (Byrne et al., 2011). The remaining 95% are not inherited, and are referred to as sporadic ALS (SALS). To date, several genes have been linked to both FALS and SALS, including *SOD1* (Jones et al., 1994a;Jones et al., 1994b;Rosen et al., 1993), *ALS2* (Hadano et al., 2001;Yang et al., 2001), *VAPB* (Nishimura et al., 2004a;Nishimura et al., 2004b), *TARDBP* (Arai et al., 2006;Mackenzie et al., 2007;Neumann et al., 2006), *FUS* (Belzil et al., 2009;Corrado et al., 2010;DeJesus-Hernandez et al., 2010;Kwiatkowski, Jr. et al., 2009;Vance et al., 2009), *VCP* (Johnson et al., 2010), *OPTN* (Del et al., 2011;Iida et al., 2012;Maruyama et al., 2010), *SQSTM1* (Fecto et al., 2011), *UBQLN2* (Deng et al., 2011), *C9ORF72* (DeJesus-Hernandez et al., 2011;Gijselinck et al., 2012;Majounie et al., 2012;Renton et al., 2011), *PFN1* (Wu et al., 2012a), *hnRNPA1* and *hnRNPA2B1* (Kim et al., 2013), and most recently *MATR3* (Johnson et al., 2014), reviewed in (Leblond et al., 2014;Renton et al., 2014). In order to study these genes, researchers have developed animal models that recapitulate several of the cellular and molecular phenotypic features of ALS pathogenesis and neuronal cell death that are observed in human patients. Some of these features include the formation of cellular aggregates (Chou et al., 1996a;Chou et al., 1996b;Kato et al., 1997;Niwa et al., 2002;Shibata et al., 1996a;Shibata et al., 1994;Shibata et al., 1996b), mitochondrial abnormalities (Hirano et al., 1984a;Nakano et al., 1987;Swerdlow et al., 1998;Wiedemann et al., 2002), axonal abnormalities (Hirano et al., 1984a;Hirano et al., 1984b), dysfunctions in glutamate signaling and subsequent excitotoxicity (Kawahara et al., 2004;Rothstein et al., 1990;Takuma et al., 1999), and oxidative damage (Bowling et al., 1993).

2.0 History

It was more than 100 years ago that researchers began to realize the usefulness of *Drosophila melanogaster* in genetics research. Shortly after the turn of the 20th century, many key discoveries were made in *Drosophila* that helped shape our understanding of chromosome structure and heredity, reviewed in (Arias, 2008), (Bellen et al., 2010) and (Rubin and Lewis, 2000). From there it became instrumental for studying genetic mutations, and today researchers around the world use *Drosophila* as a model system for studying human diseases, including neurodegeneration. Many key discoveries that have helped in advancing the neurodegenerative disease field have been made using flies as a model system including, but not limited to, the identification of candidate genes as causes and therapies of disease (Fernandez-Funez et al., 2000;Steffan et al., 2001;Warrick et al., 1999), *in vivo* evidence for RNA-mediated neurodegeneration (Jin et al., 2003;Li et al., 2008), the

identification of ER-golgi trafficking inhibition as a contributor to alpha-synuclein-associated toxicity in Parkinson's disease (Cooper et al., 2006) and the identification of a molecular link between the ubiquitin-proteasome system and autophagy (Pandey et al., 2007).

Thomas Morgan, Alfred Sturtevant, Calvin Bridges and Hermann Muller were among the first pioneers of genetic research in *Drosophila*. They worked together in Morgan's "Fly Room" at Columbia University (Sturtevant, 1965) where they studied heredity and clarified the relationships between genes, chromosomes and phenotypes, and ultimately won Morgan the 1933 Nobel Prize in Physiology or Medicine. In 1913, just a few short years after beginning his work with *Drosophila*, Sturtevant published the first ever genetic map (Sturtevant, 1913). A year later Bridges showed that genes and chromosomes were not separate entities, but that genes existed within chromosomes (a concept that was unclear at the time) (Bridges, 1914). Muller was instrumental in the development of balancer chromosomes that allowed researchers to stably and indefinitely maintain stocks of flies as heterozygotes for a desired mutation that is otherwise lethal to homozygotes (Muller, 1918). Several years later, Muller also discovered that gene mutations could be caused, as well as intentionally induced, by x-radiation (Muller, 1927). This finding won him the 1946 Nobel Prize in physiology or medicine.

With the work performed by Morgan and his colleagues, *Drosophila* was thrust to the forefront of genetics research. Researchers moved beyond using fruit flies to understand basic genetic concepts, and began identifying genes and understanding molecular pathways that are relevant to human development and functional processes. Though the importance of these discoveries cannot be overstated, some of the most important milestones in *Drosophila* genetics were those that led to its establishment as a model for studying human diseases. In 1982 Gerald M. Rubin and Allan C. Spradling were the first to use the P transposable element to insert exogenous DNA into the *Drosophila* genome (Spradling and Rubin, 1982). They used this method of creating transgenic flies to successfully integrate an exogenous gene and rescue a mutant phenotype (Rubin and Spradling, 1982). In 2000, the fully sequenced *Drosophila* genome was published (Adams et al., 2000;Myers et al., 2000). Since then, it has been estimated that as many as 77% of the human disease-associated genes reported in the Online Mendelian Inheritance in Man (OMIM) database have similar, likely orthologous, genes in *Drosophila* (Pandey and Nichols, 2011;Reiter et al., 2001). These discoveries laid the foundation upon which modern *Drosophila* models were created. Diseases are now being studied in an animal that is cheaper, faster, and easier to maintain than other models such as mice, rats and primates. The earliest *Drosophila* models of neurodegenerative diseases were developed in the 1990's, and more are created every year (Bonini, 1999;Jackson et al., 1998;Lloyd and Taylor, 2010;Min and Benzer, 1997;Rincon-Limas et al., 2012;Warrick et al., 1998). In this review, we will highlight the *Drosophila* models that have been developed over the past five years for studying ALS.

3.0 *Drosophila* models of ALS

3.1 Superoxide dismutase (SOD1)

SOD1 is a protein that, under normal conditions, protects cells against oxidative stress by catalyzing the conversion of superoxide anions into oxygen and hydrogen peroxide (Fridovich, 1986). As it has been hypothesized that oxidative damage is linked to the aging process (HARMAN, 1956), *SOD1 Drosophila* models have been used to determine whether altering SOD1 expression levels can extend or shorten lifespans (Klichko et al., 1999;Parkes et al., 1998;Sun and Tower, 1999). Indeed, overexpression of SOD1 does appear to improve lifespans of *Drosophila*, although this effect appears to be tissue-specific, and does not occur in cells of the nervous system or in muscle cells (Martin et al., 2009). SOD1 may also play less of a role in the aging of organisms with naturally longer lifespans, as experiments in ants have shown lower SOD1 expression in queens than in male and worker ants, even though queens live much longer (Parker et al., 2004). In 1993, *SOD1* became the first gene responsible for causing ALS (Rosen et al., 1993). We now know that *SOD1* mutations account for approximately 1% of SALS cases and 12% of FALS cases (Renton et al., 2014). After the initial report in 1993, researchers were quick to utilize *Drosophila* to understand how *SOD1* mutations lead to ALS (Table 1). By 1995, a fly model was developed to study the effects of mutant SOD1 protein subunits on wild type (WT) and mutant protein dimerization (Phillips et al., 1995). In more recent years a model was made by comparing transgenic flies overexpressing either *Drosophila SOD1* (*dSOD1*), human *SOD1* (*hSOD1*), or mutant *hSOD1* (A4V or G85R) (Watson et al., 2008). Flies expressing WT *hSOD1* or either mutant had a reduced climbing ability compared to *dSOD1* flies, suggesting a motor neuron dysfunction due to expression of either WT or mutant versions of *hSOD1*. The differences in climbing did not become apparent until day 14, an observation that suggests a progressive loss of motor function in the WT and mutant *hSOD1*-expressing flies. In addition, these flies exhibited reduced synaptic transmission in dorsal longitudinal muscles of the giant fiber motor pathway (Watson et al., 2008). Consistent with *SOD1*-associated ALS pathogenesis in mouse models (Bruijn et al., 1997;Bruijn et al., 1998;Watanabe et al., 2001), the motor neurons developed aggregates of hSOD1 protein. Altogether, this model provided evidence that WT and ALS-causing mutant *hSOD1* are toxic to motor neurons.

ALS is thought to be a non-cell autonomous neurodegenerative disease (Boillee et al., 2006). Therefore, researchers have also studied non-neuronal cells in animal models of ALS. The *Drosophila* model developed in 2008 was utilized a few years later by another group to examine how different cell-types expressing mutant and WT *hSOD1* are affected by ALS-linked environmental insults (Islam et al., 2012). One advantage to using *Drosophila* as a model organism is the ability to direct expression of transgenes to a wide range of specific tissue types using the UAS-gal4 system (Brand and Perrimon, 1993;McGuire et al., 2004). Flies expressing *hSOD1* specifically in glial cells showed a decrease in lifespan when exposed to the neurotoxin β-N-methylamino-L-alanine (BMAA) compared to *dSOD1*-expressing controls. Interestingly, however, flies expressing either mutant or WT *hSOD1* specifically in motor neurons survived longer than controls when exposed to BMAA. This is an unexpected and intriguing result, considering the role of motor neurons in ALS pathogenesis. Conversely, expression of the *hSOD1-G85R* mutant in either motor neurons or

glia reduced *Drosophila* lifespan compared to controls when exposed to a different environmental insult, hydrogen peroxide (Kumimoto et al., 2013). In the absence of induced environmental factors, expression of *hSOD1-G85R* in either motor neuron or glial cells led to a decrease in lifespan, but expression in both cell types simultaneously increased lifespan compared to controls. However, another study showed that expression of the FALS SOD1 mutant, *hSOD1-G41S*, in *Drosophila* motor neurons increases lifespan when compared to controls (Elia et al., 1999). Although the differences caused by the G85R and G41S mutants may be due to their locations in different domains of the protein (Turner and Talbot, 2008), these observations highlight the complexities of studying ALS in animal models, as well as the variability that is observed among different fly models.

Mitochondrial dysfunction is a pathological hallmark of ALS in humans (Sasaki and Iwata, 1999;Wiedemann et al., 2002;Wiedemann et al., 1998), and may be caused by altered protein activity of *SOD1* mutants (Ferri et al., 2006;Liu et al., 2004;Pasinelli et al., 2004). ALS-associated mutations can hinder binding of SOD1protein to zinc ions, resulting in cellular toxicity (Crow et al., 1997;Lyons et al., 1996). Recently, researchers using transgenic *Drosophila* expressing zinc-deficient hSOD1 obtained evidence supporting a link between mutant *SOD1* and mitochondrial dysfunction (Bahadorani et al., 2013). These flies harbored a D83S mutation in a zinc-binding domain of exon 4 that inhibits binding of SOD1 protein to zinc, without affecting binding to its other cofactor, copper. They found that ubiquitous expression of zinc-deficient hSOD1 led to age-dependent locomotor dysfunction that was not present in control flies. However, these flies exhibited normal lifespans and showed no brain or eye degeneration. When they examined the effects of zinc deficiency on mitochondria, they found that mitochondrial structure was altered in flight muscles of mutant flies. ATP production was also reduced in the fly heads compared to controls, but not in fly bodies, possibly suggesting a reduction of ATP production specifically in neurons. In addition, mutant hSOD1 D83S expression resulted in an increased sensitivity to paraquat and zinc exposure based on declines in lifespan of these flies (Bahadorani et al., 2013). Taken together, this model provides evidence that zinc deficiency due to hSOD1 mutations may result in ALS pathogenesis through reduced mitochondrial function.

3.2 Alsin

In 2001, mutations of the gene, *ALS2*, were identified in individuals with ALS, specifically ALS2 (Hadano et al., 2001;Yang et al., 2001). Alsin (also called ALS2), the protein encoded by *ALS2*, is a guanine nucleotide exchange factor for Ras-related in brain 5 (Rab5) (Otomo et al., 2003). Since Rab5 is a GTPase involved in the endocytic pathway (Bucci et al., 1992), it has been proposed that loss of Alsin function due to ALS-causing mutations perturb endosomal dynamics, thereby implicating these processes in the pathogenesis of the disease (Otomo et al., 2003).

A *Drosophila* model for Alsin-associated ALS was recently developed using the *Drosophila* homolog of Alsin, dALS2 (Table 1) (Takayama et al., 2014). Consistent with the disease state in ALS2 patients, where mutations of Alsin result in premature cessation of translation and subsequent production of truncated protein products (Yamanaka et al., 2003), the mutations used in the *Drosophila* model were *dALS2* constructs lacking approximately 30%

of their coding sequence. Mutant flies did not show significant phenotypic changes at the NMJ compared to WT controls, apart from a slight increase in the bouton number of abdominal motor axons of adult mutant flies. They did, however, show a significant age-dependent reduction in locomotion when compared to WT flies. This reduction was rescued by ubiquitous WT dALS2 overexpression, suggesting dALS2 loss-of-function as the cause of the phenotype. Interestingly, however, this rescue was not as significantly recapitulated by overexpressing WT dALS2 in motor neurons (Takayama et al., 2014). It is possible that differences in transgene expression levels between larvae and adult flies, depending on whether expression is ubiquitous or directed to motor neurons, might explain these results. The locomotor reduction observed with ubiquitous expression, however, may prove to be a useful output in future experiments aimed at identifying modifiers for the dALS2-related phenotype with this model (Takayama et al., 2014).

3.3 VAMP-associated protein B (VAPB)

VAMP (vesicle associated membrane protein)-associated protein B (VAPB), encoded by the gene, *VAPB*, localizes to both the endoplasmic reticulum (ER) and mitochondrial-associated membranes, where it is involved in the ER-associated unfolded protein response (Kanekura et al., 2006) and ER-mitochondria calcium exchange (De Vos et al., 2012) processes, respectively. A missense mutation in the coding region for the major sperm protein (MSP) domain of VAPB was first identified in human ALS patients in 2004 (Nishimura et al., 2004a;Nishimura et al., 2004b). Since then, *Drosophila* models have been used by researchers to better understand WT VAPB function in various tissue-types, as well as how different ALS-linked mutations in VAPB lead to toxicity and subsequent pathogenesis of the disease.

3.3.1 Overexpression and Knockdown—Several *Drosophila* models have been developed to elucidate the function of WT human VAPB (hVAPB) and its *Drosophila* homolog DVAP33-A (referred to as dVAP in this review), encoded by the gene, *DVAP33-A* (Chai et al., 2008;Forrest et al., 2013;Han et al., 2012;Moustaqim-Barrette et al., 2014;Ratnaparkhi et al., 2008;Tsuda et al., 2008;Yang et al., 2012). hVAPB and dVAP proteins are structurally and functionally similar, sharing approximately 62% homology, and one protein can compensate for the loss of the other in maintaining normal cellular processes (Chai et al., 2008). In addition, creating equivalent human disease-causing mutations in *dVAP* leads to neurodegenerative phenotypes that mimic those seen when hVAP with ALS-causing mutations are expressed in *Drosophila* (Chai et al., 2008;Chen et al., 2010;Forrest et al., 2013;Oyanagi et al., 2008;Ratnaparkhi et al., 2008;Sanhueza et al., 2014;Tsuda et al., 2008).

Models utilizing either homolog have shown that altering their expression levels in neurons results in functional and morphological changes of the neuromuscular junction (NMJ). Loss of dVAP function in dVAP-null mutant flies exhibit an increase in bouton size, but a decrease of overall bouton number at the NMJ, compared to normal flies. Exogenous expression of hVAPB in dVAP-null flies rescues this loss of boutons, providing evidence to support functional overlap between the two proteins (Chai et al., 2008). When dVAP is overexpressed in neurons, bouton size decreases, with a concurrent decrease in the number

of vesicles, but the overall bouton number increases (Chai et al., 2008;Pennetta et al., 2002;Ratnaparkhi et al., 2008). These changes are also observed in neurons overexpressing hVAPB (Chai et al., 2008). Synaptic transmission is also affected by hVAPB/dVAP expression, suggesting a possible role of these proteins in maintaining synaptic functions. Loss of dVAP function in dVAP-null mutants reduces the quantal content at the NMJ by approximately 40%, compared to normal control flies. Conversely, overexpressing dVAP increases the quantal content, consistent with an increase in the bouton number, but the overall quantal size is reduced (Chai et al., 2008). It was concluded that quantal changes are a response to the morphological changes occurring at the NMJ in these fly lines, in an effort to keep the synaptic transmission at normal levels (Chai et al., 2008). Loss of dVAP function increases the number of presynaptic active zones at the NMJ (Chai et al., 2008). Different groups looking at the effects of dVAP overexpression have reported differences in the number of active zones, although these differences may be due to the different techniques used to visualize the NMJ's. One group, utilizing electron microscopy, has reported no change in the number of active zones upon overexpression of dVAP (Chai et al., 2008), whereas a second group, utilizing confocal microscopy with antibodies specific to the active zone protein, Bruchpilot, reported a decrease in active zones (Ratnaparkhi et al., 2008).

Mitochondrial dysfunction has become a hallmark of ALS pathogenesis in humans (Sasaki and Iwata, 1999;Wiedemann et al., 2002;Wiedemann et al., 1998). Similarly to a recently developed *SOD1* model (Bahadorani et al., 2013), dVAP-null mutant flies have morphological defects of their muscle mitochondria (Han et al., 2012), suggesting that mitochondrial dysfunction may be a cause of toxicity in VAPB-associated ALS.

Pan-neuronal overexpression of dVAP P58S in *Drosophila* (equivalent to the ALS-causing mutant, P56S, in humans) appears to be toxic, causing a decrease in larval mobility and an increase in larval central neuron cell death (Chai et al., 2008;Nishimura et al., 2004a;Nishimura et al., 2004b) . One possible mechanism for dVAP P58S toxicity is through its ability to induce morphological changes at the NMJ. Similarly to dVAP-null flies, P58S mutant expression in neurons increases bouton size at the NMJ. Not surprisingly, therefore, although WT dVAP is capable of rescuing the bouton phenotype in the dVAP-null background, dVAP P58S is not (Ratnaparkhi et al., 2008). dVAP P58S also hinders microtubule organization at the NMJ, although the mutant protein does not appear to have an effect on presynaptic active zones or the number of vesicles (Ratnaparkhi et al., 2008).

It has been proposed that dVAP P58S might exert toxicity through a dominant negative effect of sequestering normal proteins into cytoplasmic granules (Ratnaparkhi et al., 2008). dVAP P58S mutant protein forms ubiquitin-positive, cytoplasmic aggregates in larval neurons and muscle cells (Ratnaparkhi et al., 2008;Tsuda et al., 2008). Interestingly, WT dVAP is mislocalized from the cell membrane into these aggregates, suggesting that mutant dVAP is capable of recruiting WT protein, thereby hindering its normal activity in the cell (Chai et al., 2008;Ratnaparkhi et al., 2008;Tsuda et al., 2008). Under normal conditions, WT dVAP localizes to the cell membrane, where the N-terminal portion that includes the major sperm protein (MSP) domain is secreted as a ligand for Eph receptors (Tsuda et al., 2008). Secretion of the MSP domain is decreased in cells that are expressing dVAP P58S and are

positive for cytoplasmic aggregates (Tsuda et al., 2008). In addition, evidence for a dominant negative effect can be seen on an organismal level. dVAP-null mutant pupae have a reduced eclosion rate, compared to normal controls. Expression of WT dVAP in these pupae rescues the eclosion rate. However, co-expression of WT dVAP with P58S mutant protein in the dVAP-null background does not rescue the phenotype, suggesting that mutant dVAP can suppress WT function, possibly through dimerization of WT and mutant proteins (Ratnaparkhi et al., 2008).

Another VAPB mutation was identified in FALS patients in 2010, VAPB T46I (Chen et al., 2010). A model was subsequently developed to characterize the *Drosophila* homolog of the VAPB mutant, dVAP T48I (Chen et al., 2010). Expressing dVAP T48I in adult fly eyes causes structural degeneration not present in control flies, confirming the deleterious nature of the mutation. When WT dVAP is expressed pan-neuronally, neurons of larval brain and nerve fibers show WT protein localized to cell and ER membranes. In contrast, dVAP T48I mutant protein localizes to bright aggregates. Interestingly, mutant dVAP expression in the brain and in muscle cells causes structural changes of the ER, including fragmentation, similarly to what has been observed in ALS patients (Oyanagi et al., 2008). The Hsp70 chaperone protein is also found to be elevated in these cells. Taken together, this model has provided evidence that expression of the dVAP T48I mutant leads to toxicity, and has thus provided insight into the nature of the VAPB T46I mutant in humans. Specifically, these results have identified stress response and morphological changes of the ER as possible modes of mutant protein toxicity in VAPB-related ALS (Chen et al., 2010).

A *Drosophila* model was recently developed to characterize a third VAPB mutation identified in 2012 in a single FALS patient with an additional *C9ORF72* repeat expansion (Sanhueza et al., 2014;van et al., 2012). The human VAPB mutant protein, VAPB V234I, has an equivalent mutation in *Drosophila*, dVAP V260I. The experiments performed in this model focus on comparing overexpression of mutant dVAP with WT dVAP. dVAP V260I and WT dVAP overexpression exhibited similar phenotypic results, with the mutant dVAP producing more severe phenotypes (Sanhueza et al., 2014). Expression of both WT and dVAP V260I cause morphological changes at the NMJ including decreased bouton size with increased bouton number, as well as the formation of satellite boutons. The formation of these satellite boutons appears to be the result of alterations in microtubule architecture (Sanhueza et al., 2014). On a cellular level, both WT and mutant protein expression form aggregates in muscle cells. These cells also exhibit altered nuclear morphology including elongation, an increase in overall volume and clustering of nuclei with neighboring cells. Interestingly, the dVAP V260I mutant protein localizes to the muscle nuclei (Sanhueza et al., 2014). Altered nuclear morphology is also observed in neurons of larval brains, and dVAP V260I protein forms large cytoplasmic, not nuclear, inclusions in these cells. Similarly to dVAP T48I overexpression (Chen et al., 2010), WT and dVAP V260I expression in muscle cells induces a heat shock stress response, identified by an increase in Hsp70 expression and localization to nuclei (Sanhueza et al., 2014). Experiments focused on changes on the organismal level revealed reduced larval locomotion, pupal eclosion rates and altered adult wing posture when WT and dVAP V260I were neuronally expressed. Altered wing posture was also observed upon expression in muscle cells. When targeted to

adult eyes, both WT and mutant protein caused structural degeneration (Sanhueza et al., 2014). It has been observed that both WT and mutant protein expression induced the altered phenotypes, with the dVAP V260I mutant producing more severe phenotypes, suggesting a possible gain of mutant protein function. The formation of aggregates in muscle cells and possible protein sequestration, however, may suggest a loss-of-function, so it was suggested that dVAP V260I toxicity may be due to both a loss and a gain-of-function (Sanhueza et al., 2014).

3.3.2 Modifiers of VAPB-Associated Phenotypes—Recently, proteins that interact with endogenous dVAP have been identified, including Down syndrome cell adhesion molecule (Dscam) (Yang et al., 2012), Suppressor of Actin 1 (Sac1) (Forrest et al., 2013) and Oxysterol binding protein (Osbp). Pan-neuronal, RNAi-mediated knockdown of the endogenous phosphoinositide phosphatase, Sac1, in *Drosophila* causes morphological changes at the NMJ, as well as structural degeneration of the adult eyes (Forrest et al., 2013). Interestingly, Sac1 and WT dVAP colocalize in larval brains, eye imaginal disks and salivary glands. When the ALS-associated dVAP P85S mutant is overexpressed in neuronal cells of these tissue-types, Sac1 is sequestered into mutant dVAP-induced cytoplasmic aggregates. Furthermore, RNAi-mediated knockdown of dVAP and Sac1in these tissues, as well as overexpression of dVAP P85S mutant protein, elevated expression of phosphatidylinositol 4-phosphate (PtdIns4P). Increased PtdIns4P expression results in degenerative phenotypes at the NMJ (Forrest et al., 2013). It has therefore been proposed that dVAP P85S mutant toxicity occurs through Sac1 interaction and sequestration into cellular aggregates, resulting in increased expression of PtdIns4P and subsequent morphological changes at the NMJ (Forrest et al., 2013). Taken together, these results identify Sac1 and PtdIns4P proteins as candidates for involvement in ALS pathogenesis, as well as possible therapeutic targets for developing effective therapies for ALS in the future.

The lipid binding protein, Osbp, interacts with WT dVAP, but not the dVAP P85S mutant (Moustaqim-Barrette et al., 2014). In normal cells, Osbp colocalizes with dVAP at the cell and ER membranes. In dVAP P85S mutant-expressing cells, however, Osbp mislocalizes to cytoplasmic aggregates, similarly to dVAP (Moustaqim-Barrette et al., 2014). Interestingly, in dVAP-null mutants, Osbp mislocalizes to the golgi. Since overexpression of human Osbp (OsbpL8) in ER is sufficient to rescue loss-of-function-associated phenotypes of dVAP-null flies, it has been proposed that the P85S mutation of dVAP incurs a loss-of-function to the protein in disease pathogenesis (Moustaqim-Barrette et al., 2014).

3.4 TAR DNA-binding protein 43 (TDP-43)

TDP-43 is a 43 kDa protein encoded by the gene *TARDBP*. It was first identified in 1995 as a transcriptional regulator that bound to pyrimidine-rich motifs of the human immunodeficiency virus 1 (HIV-1) regulatory element, TAR (Ou et al., 1995). TDP-43 is a primarily nuclear protein, and was first linked to ALS in 2006 when it was identified as a component of ubiquitinated, cytoplasmic granules in the neurons of patients with ALS and FTLD (frontotemporal lobar degeneration) (Neumann et al., 2006). Shortly thereafter, researchers began identifying several *TARDBP* mutations in FALS and SALS patients (Kabashi et al., 2008;Kuhnlein et al., 2008;Rutherford et al., 2008;Sreedharan et al.,

2008;Van Deerlin et al., 2008). To date, a total of 47 missense mutations and 1 nonsense mutation in *TARDBP* have been identified (Lattante et al., 2013), some of which have been directly linked to cytoplasmic mislocalization of the TDP-43 protein (Barmada et al., 2010;Winton et al., 2008). We now know that TDP-43 is involved in regulating multiple aspects of RNA metabolism, including alternative splicing (Buratti et al., 2001), mRNA stability (Volkening et al., 2009) and likely mRNA transport (Alami et al., 2014;Wang et al., 2008a), thus implicating aberrations of these processes in disease pathogenesis.

Several *Drosophila* models for TDP-43-associated ALS have been generated and extensively characterized (Table 1). One of the major goals of researchers has been to determine if TDP-43-associated ALS pathogenesis is due to a gain or a loss of protein function. *Drosophila* models have provided evidence to support both hypotheses, and the truth may actually be a combination of the two.

3.4.1 Overexpression and Knockdown—TDP-43 is an RNA-binding protein that is highly conserved across organisms, including *Drosophila*. Researchers, therefore, commonly use *Drosophila* models to observe the effects of overexpression or knockdown of either human TDP-43 (hTDP-43) or the drosophila ortholog, dTDP-43, which is encoded by the gene *TBPH* (TAR DNA-binding protein-43 homolog). These gain-of function/loss-of-function experiments have been helpful for elucidating TDP-43 function as well as validating new models through recapitulation of key phenotypic features of ALS.

It has been demonstrated, with the use of both exogenous and endogenous WT TDP-43 in *Drosophila*, that maintaining normal TDP-43 protein levels in various tissue-types is critical for proper physiological functions of the organism. Pan-neuronal reduction of endogenous *Drosophila* TBPH levels, either through deletion mutants or through dTDP-43 RNAi, reduces adult fly lifespan and locomotion (typically walking and climbing) (Diaper et al., 2013b;Feiguin et al., 2009). Interestingly, co-expressing human TDP-43 rescues these phenotypes, suggesting a possible natural feedback mechanism for maintaining expression levels, that is conserved in these orthologs (Feiguin et al., 2009). Pan-neuronal overexpression of dTDP-43 has also shown a reduction in adult lifespan, as well as a reduction in both adult fly and larval locomotion (Diaper et al., 2013b). In sensory neurons of *Drosophila* larvae, lowered expression of dTDP-43 reduces dendritic branching, and overexpression increases branching (Lu et al., 2009). In mushroom bodies of the *Drosophila* brain, both overexpression of human TDP-43, as well as reduced *Drosophila* TDP-43 expression, causes loss of axons and neuronal death (Li et al., 2010). When dTDP-43 is overexpressed in this tissue-type, there is a reduction of lobe size, as well as the formation of TDP-43-positive cytoplasmic aggregates (Lin et al., 2011).

Since ALS affects motor neurons in humans, several experiments in *Drosophila* models are carried out on larval and adult fly motor neurons. When dTDP-43 levels are decreased specifically in larval motor neurons, dysmorphologies of the NMJ are observed, including reductions in axonal branching (Feiguin et al., 2009). There are, however, differences in reported bouton numbers at the NMJ between different groups. Some have reported a decrease in bouton number when endogenous dTDP-43 levels are reduced via deletion mutants in a *W1118* fly strain (Feiguin et al., 2009). Others have reported an increase in

bouton number when using a $y^1 w^{67c23}$ fly strain carrying a different deletion of dTDP-43 (Lin et al., 2011). It has been suggested that the conflicting observations may be due to the differences in genetic backgrounds of the flies used in the experiments, as well as the differences in the dTDP-43 deletion constructs (Lin et al., 2011). Expression of human TDP-43 in larval motor neurons resulted in reduced axonal branching and fewer synaptic boutons, in addition to axon swelling, motor neuron cell death and the formation of hTDP-43-positive cytoplasmic aggregates (Estes et al., 2011;Li et al., 2010). Some groups, however, have reported only small amounts of cytoplasmic hTDP-43, as well as little to no aggregation (Hanson et al., 2010;Voigt et al., 2010). Therefore, it is possible that a small amount of cytoplasmic TDP-43 is enough to cause toxicity in motor neurons, even without the presence of large aggregates. TDP-43-positive cytoplasmic aggregates, a hallmark of TDP-43-associated ALS in humans (Arai et al., 2006;Mackenzie et al., 2007;Neumann et al., 2006;Tan et al., 2007), have also been reported in adult *Drosophila* motor neurons overexpressing endogenous TDP-43 (Lin et al., 2011).

It has previously been shown that overexpressing human TDP-43 in adult fly motor neurons causes a significant reduction in lifespan (Hanson et al., 2010) compared to controls. Interestingly, overexpression of hTDP-43 with either the human caspase inhibitor, P35, or the *Drosophila* homolog, dIAP (*Drosophila* inhibitor of apoptosis), in adult motor neurons does not improve lifespan compared to flies expressing hTDP-43 alone (Zhan et al., 2013). This result suggests that human WT TDP-43 toxicity may not be due to neuronal apoptosis through programmed cell death mechanisms. However, when overexpressing endogenous *Drosophila* TDP-43 in pupae, caspase inhibition rescues CCAP/bursicon neuron apoptosis, suggesting that in these cells, at this stage of development and using endogenous protein, loss of neurons does occur through programmed cell death (Vanden Broeck et al., 2013).

Adult eyes are often used for transgene expression in *Drosophila* models of neurodegenerative diseases to produce easily observable eye phenotypes (Cushman-Nick et al., 2013;Hindle et al., 2013;Mohan et al., 2014;Yadav and Tapadia, 2013). Death of neurons in the eyes produces degenerative phenotypes that can be seen both inside and on the surface of the eye. These phenotypes can range from structural changes to loss of pigmentation. Expressing human TDP-43 in adult fly eyes causes age and dose-dependent structural degeneration (Elden et al., 2010;Estes et al., 2011;Hanson et al., 2010;Li et al., 2010;Ritson et al., 2010). This degeneration has also been directly correlated with mislocalization of hTDP-43 to the cytoplasm, and the mislocalization alone is sufficient to cause the degeneration (Ritson et al., 2010). In contrast, however, overexpression of endogenous *Drosophila* TDP-43 in eye imaginal disks of third instar larvae does not appear to cause cytoplasmic mislocalization, despite the presence of observed neurodegenerative phenotypes in adult flies (Diaper et al., 2013b).

Muscle atrophy and excitotoxicity from aberrant glutamate signaling are hallmarks of ALS pathogenesis in humans (Kawahara et al., 2004;Rothstein et al., 1990;Takuma et al., 1999). Recently, endogenous *Drosophila* TDP-43 expression has been identified in muscle and glial cells (Diaper et al., 2013a). Specifically knocking down dTDP-43 in adult muscle cells causes an overall reduction in motor function, based on an observed reduction in physical activity, compared to controls expressing normal protein levels. Overexpressing dTDP-43 in

larval muscle cells results in protein mislocalization from nuclei into sarcoplasmic aggregates. These larvae also exhibit reduced locomotion and early lethality compared to controls. Targeted overexpression in glial cells is lethal to larvae. However, unlike in muscle cells, knockdown of protein levels in adult glial cells causes motor dysfunction that is only apparent in aged flies (Diaper et al., 2013a).

It has also been shown that overexpression and loss of function of dTDP-43 in glial cells and neurons alters the mRNA levels of dEAAT1 and dEAAT2, the *Drosophila* homologs of the glutamate transporters EAAT1 and EAAT2(Diaper et al., 2013a). EAAT2 mRNA has previously been identified as a possible target of TDP-43 (Sephton et al., 2011;Tollervey et al., 2011). This transporter is important for the clearance of glutamate released at the NMJ, and its dysfunction or loss has been linked to ALS through the identification of increased glutamate levels in the plasma and cerebral spinal fluid of ALS patients, potentially resulting in neuron excitotoxicity (Couratier et al., 1993;Plaitakis and Caroscio, 1987;Rothstein et al., 1992;Rothstein et al., 1995;Shaw et al., 1995;Spreux-Varoquaux et al., 2002;Trotti et al., 2001;Trotti et al., 1999). Consistent with the disease state in humans, dTDP-43 null mutant flies express lower overall levels of both dEAAT1 and dEAAT2 mRNA compared to controls. WT dTDP-43 decreases mRNA levels when overexpressed in glial cells, but increases mRNA levels when overexpressed in neurons (Diaper et al., 2013a).

Recent experiments using *Drosophila* models to study the effects of expressing human TDP-43 in glial cells have provided evidence to support its toxicity in this cell-type. The results have shown that WT hTDP-43, as well as A315T, D169G, G298S and N345K mutants, mislocalize to the cytoplasm when overexpressed (Estes et al., 2013). WT and mutant hTDP-43 overexpression in both glial and motor neuron cells also reduces bouton number at larval NMJ's and hinders larval motor function, specifically their ability to turn over after being flipped upside down. When expressed in glial cells, WT and hTDP-43 mutants cause an increase in the number of postsynaptic glutamate receptors, but have no effect on the number of presynaptic active zones. In contrast, when expressed in motor neurons, WT and mutant proteins increase the number of active zones, but only WT hTDP-43 concurrently increases the number of glutamate receptors. The effect of these changes in both cell types is an imbalance in the ratio of active zones to glutamate receptors at the NMJ. This imbalance coincides with the motor dysfunction observed in the larvae, suggesting that this may be the mechanism that leads to toxicity of the overexpressed proteins. It was hypothesized that the increase in both active zone and glutamate receptor number, caused by overexpression of WT hTDP-43 in motor neurons, suggests a different mechanism of toxicity from the mutants in this cell type (Estes et al., 2013). However, the fact that WT and mutant TDP-43 exert similar effects in glial cells makes it difficult to identify the consequences of the ALS-linked mutations. These results suggest that the mutations themselves are not necessary for toxicity in glial cells. Taken together, however, these experiments provide further evidence linking glial and muscle cells to ALS, and support a role of glutamate transport dysfunction in disease pathogenesis.

In addition to studying overexpression and knockdown of WT TDP-43, *Drosophila* models have been used to study ALS-associated TDP-43 mutants. Interestingly, in many of these experiments, mutant and WT toxicity is either not compared, or cannot be distinguished

from each other due to having comparable effects. As a result, it is difficult to determine what ALS-associated mutations actually do to normal protein function, and how large of a role they play, if any, in the actual pathogenesis of the disease. Overexpression of human TDP-43 M337V in *Drosophila* eyes causes mild degeneration and shows both full-length TDP-43, as well as a 25kD fragment, similar to what has been identified in FTLD patient samples (Neumann et al., 2006;Ritson et al., 2010). Dose and age-dependent adult eye degeneration is also seen in flies expressing D169G, G298S and N345K mutants, when compared to non-hTDP-43 expressing control flies (Estes et al., 2013). This phenotype is accompanied by axonal aggregation of these mutant proteins in eye neuroepithelium, providing further evidence for a link between the aberrant mislocalization and aggregation of hTDP-43, and neurodegeneration (Estes et al., 2013). The *Drosophila* TDP-43 Q367X mutant reduces miR-9a expression in larvae compared to WT TDP-43-expressing flies, and subsequently results in improper specification of sensory organ precursor cells(Li et al., 2013). This suggests that dTDP-43 controls specification through miR-9a, and that a loss of function through an ALS-associated mutation causes dysregulation of the process (Li et al., 2013).

Pan-neuronal expression of A315T, G348C, A382T, G287S, or N390D reduces adult lifespan compared to control flies expressing driver alone, the extent of which varies between different mutants (Voigt et al., 2010). However, these results do not directly implicate the mutations themselves in toxicity, as WT hTDP-43 overexpression caused the largest reduction in lifespan compared to the mutant and control flies. Interestingly, RNA-binding-deficient hTDP-43 only mildly reduced adult lifespan, essentially rescuing the WT hTDP-43 phenotype (Voigt et al., 2010). These results were supported by later experiments performed in adult eyes (Ihara et al., 2013). Overexpression of WT hTDP-43 caused retinal degeneration that was rescued by inhibiting RNA-binding function through both removal and mutation of the RNA recognition motif 1 (RRM1). Degeneration caused by G298S and M337V mutants was also rescued by inhibiting the RNA-binding ability of these proteins. Removal of the nuclear localization signal (NLS) in WT hTDP-43 and G298S and M337V mutants resulted in "forced" cytoplasmic mislocalization when expressed in adult eyes that corresponded to increased degeneration compared to WT hTDP-43 controls with intact NLS's. When the RNA-binding ability of these proteins was removed, cytoplasmic mislocalization remained, but the degeneration was rescued (Ihara et al., 2013). Taken together, the experiments performed by these groups provide evidence that the RNA-binding function of TDP-43 may be necessary for toxicity, as mislocalization alone does not appear to be sufficient for retinal degeneration.

Another group has also used human TDP-43 lacking a functional NLS to further study the effects of cytoplasmic mislocalization in disease pathogenesis (Miguel et al., 2011). The NLS-mutant hTDP-43 in these experiments causes severe degeneration in adult eyes, as well as reduced adult lifespan when expressed in neurons, and lethality when expressed in larval neurons (Miguel et al., 2011). Interestingly, increased death of adult flies begins prior to the formation of cytoplasmic aggregates, lending support to the conclusions of others that aggregation of TDP-43 is not necessary for toxicity (Hanson et al., 2010;Miguel et al., 2011). In contrast, however, studies of FTLD show a link between cellular toxicity and

aggregates that are positive for either full-length TDP-43 or the disease-linked, 25kD, C-terminal fragment, TDP-25 (Igaz et al., 2009;Igaz et al., 2008;Neumann et al., 2006;Zhang et al., 2009). In fact, it was recently shown in a *Drosophila* model that upregulating endogenous chaperone proteins in various tissue types can reduce TDP-43 and TDP-25 aggregation, a result that coincides with rescue of their ALS-associated phenotypes in the flies (Gregory et al., 2012). Furthermore, altering expression levels of the stress granule-associated PEK, ROX8 and GADD34 proteins in adult neurons affects TDP-43-associated toxicity (Kim et al., 2014). Specifically, knocking down PEK and ROX8, two proteins involved in stress granule formation and the *Drosophila* homologs of mammalian PERK and TIA1, respectively, (Brand and Bourbon, 1993;Farny et al., 2009) inhibits stress granule formation and subsequently suppresses TDP-43 toxicity. Knocking down GADD34, a phosphatase that prevents the formation of stress granules by dephosphorylating the critical stress granule component, eIF2α, (Novoa et al., 2001) effectively promotes stress granule formation and subsequently enhances TDP-43 toxicity (Kim et al., 2014). Therefore, due to the discrepancies in the data obtained by these different groups, it remains unclear as to whether or not cytoplasmic mislocalization and aggregation of TDP-43, a phenotype linked to ALS in humans, is a primary or secondary cause of toxicity.

Drosophila and human TDP-43 overexpression and knockdown flies were utilized to analyze global gene expression changes in various tissue types and stages of *Drosophila* development (Hazelett et al., 2012;Vanden Broeck et al., 2013;Zhan et al., 2013). These studies have specifically led to the identification of genes, including *Hey, Nup50* (Zhan et al., 2013) and *Map205* (Vanden Broeck et al., 2013), as well as pathways such as Notch (Zhan et al., 2013), Wnt and BMP (Hazelett et al., 2012), that may be linked to TDP-43-associated ALS pathogenesis. In a more general sense, they have shed light on the role and importance of TDP-43 in development, and the need for an organism to maintain normal TDP-43 protein levels for physiological functions. Changes in *Drosophila* TDP-43 expression levels in the larval central nervous system (CNS) result in largely non-overlapping gene expression patterns. Specifically, dTDP-43 overexpression leads to downregulation of one set of genes, whereas larvae carrying loss-of-function mutations in dTDP-43 exhibit an upregulation of a different set of genes (Hazelett et al., 2012). However, when dTDP-43 levels are altered ubiquitously at the late pupal stage, there is a significant overlap between upregulated and downregulated genes (Vanden Broeck et al., 2013). Taken together, the results of these studies provide evidence that TDP-43 plays a large role in gene expression that may change throughout *Drosophila* development. Interestingly, a ubiquitous reduction of dTDP-43 in the late pupal stage causes increased expression of the endogenous microtubule associated protein, Map205. Aberrant expression of this protein, in turn, appears to induce cytoplasmic mislocalization of the ecdysteroid receptor (EcR), which may result in a failure to transition from pupal to adult gene expression patterns and subsequent neuronal death (Vanden Broeck et al., 2013). Overall, these *Drosophila* models have identified candidate genes for future study, and suggest that the pathogenesis of TDP-43-associated ALS is a very complex process, involving many genes and biological processes.

It is still an enigma if WT or mutant TDP-43 toxicity is due to a gain or a loss of function. Observing WT toxicity can be helpful to identify how perturbations of normal TDP-43

levels affect disease pathogenesis. Unfortunately, it is challenging to assess the effects of ALS-associated TDP-43 mutants when WT protein is itself toxic. WT toxicity in and of itself suggests a gain of function. However, loss of function models have also been developed (Hazelett et al., 2012). As mentioned previously, flies overexpressing WT *Drosophila* TDP-43 produce different expression profiles in the CNS during larval development than flies carrying *dTDP-43*-null mutations (Hazelett et al., 2012). Therefore, it is possible that both a gain and a loss of TDP-43 function are toxic, but for different reasons, and through different mechanisms. Taken together, however, *Drosophila* models have shown that WT and ALS-associated mutant TDP-43 expression level changes, cellular mislocalization, and protein functions such as RNA-binding are all factors that may play a role in ALS pathogenesis.

3.4.2 Modifiers of TDP-43-Associated Phenotypes—*Drosophila* has been a useful model organism for performing genetic screens to identify genes and pathways that modify phenotypes associated with neurodegenerative diseases, including spinocerebellar ataxia (Fernandez-Funez et al., 2000;Ren et al., 2011;Shieh and Bonini, 2011), Wolfram syndrome (Jones et al., 2014), Alzheimer's disease (Moran et al., 2013;Shulman and Feany, 2003) and Huntington's disease (Kazemi-Esfarjani and Benzer, 2000). Screens can be targeted, testing the effects of an array of known genes on a particular phenotype (Jones et al., 2014;Ren et al., 2011), or they can be unbiased, looking for any genes that modify a phenotype. Unbiased screens are typically performed using either deficiency lines that carry heterozygous deletions of mapped regions of the chromosome (Li et al., 2014;Simonsen et al., 2007), or lines with random enhancer-promoter (EP)-transposable element insertions (Fernandez-Funez et al., 2000;Shulman and Feany, 2003). *Drosophila* at any stage of development can be used, depending on the phenotype of interest (Batlevi et al., 2010;Rorth et al., 1998). Eye degeneration is a commonly used phenotypic output (Kazemi-Esfarjani and Benzer, 2000;Moran et al., 2013), although genetic screens have also been performed using survival (Li et al., 2014), wing posture (Shieh and Bonini, 2011), climbing (Shieh and Bonini, 2011) defects and axonal and synaptic degeneration (Wishart et al., 2012). Here we highlight the genes that have been identified as modifiers of TDP-43-associated neurodegeneration in *Drosophila* models of ALS.

Histone deacetylase 6 (HDAC6) is a human class II histone deacetylase that has previously been linked to neurodegenerative diseases (Grozinger et al., 1999;Pandey et al., 2007). Unlike other histone deacetylases, HDAC6 functions primarily in the cytoplasm where it plays a critical role in the clearance of cytoplasmic, ubiquitinated, misfolded proteins (Hubbert et al., 2002;Kawaguchi et al., 2003;Verdel et al., 2000). Homozygous knockout of *TBPH* in 1st-instar larval brains, as well as heterozygous knockout in adults, shows a reduction in HDAC6 mRNA levels (Fiesel et al., 2010). These results suggest that altering TDP-43 levels in neurons also affects HDAC6 levels, and may therefore result in toxicity by inhibiting the removal of accumulated cytoplasmic proteins in patients with ALS (Fiesel et al., 2010). Another possible mechanism of toxicity is through aberrant synaptic transmission. Bruchpilot (BRP), a protein that is part of the presynaptic dense body and regulates synaptic transmission by gathering synaptic vesicles together for release, was recently identified as a target of HDAC6 deacetylation in the neurons of adult *Drosophila*

(Miskiewicz et al., 2014). Increasing HDAC6 expression reduces BRP acetylation, thereby increasing its binding to vesicles and creating a larger vesicle pool for release. Consistent with the identification of HDAC6 as a target of TDP-43 (Fiesel et al., 2010), this phenotype is recapitulated when human TDP-43 is overexpressed, with a larger effect observed in flies harboring ALS-associated TDP-43-mutations (A315T and A382T) (Miskiewicz et al., 2014). Conversely, *TBPH*-null mutant flies show an increase in BRP acetylation and subsequently smaller vesicle pools. Following these observations, it was found that reducing HDAC6 levels in the A315T mutant flies rescues the acetylation levels. In addition, reducing HDAC6 expression also rescues motor dysfunction observed in A315T mutant adult flies (Miskiewicz et al., 2014). Taken together, these results suggest that TDP-43-associated ALS pathogenesis may be due to aberrant synaptic transmission, resulting from increased HDAC6 expression and subsequently reduced BRP acetylation.

Ubiquilin is a cytosolic protein capable of binding both poly-ubiquitinated chains and 19s proteosome subunits. It is therefore believed to play a role in the degradation of misfolded proteins (Ko et al., 2004). A *Drosophila* model was developed in 2010 to show the effect of ubiquilin overexpression on TDP-43 associated neurodegeneration. Consistent with other models, overexpression of hTDP-43 in motor neurons reduces the lifespans of adult flies, reduces pupal eclosion rates and causes wing malformations. Co-expression with ubiquilin enhances these phenotypes. Interestingly, however, human TDP-43 in cells co-expressing ubiquilin is almost exclusively nuclear, and is not found in cytoplasmic aggregates, despite the overall enhanced degeneration in these flies. In addition to linking ubiquilin to TDP-associated pathology in a model of ALS, these results provide more evidence that aggregation of mislocalized hTDP-43 is not necessary for progression of the disease (Hanson et al., 2010).

Valosin-containing protein (VCP) is an ATPase that has been linked to the pathogenesis of ALS (Johnson et al., 2010), as well as other neurodegenerative diseases including inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia (IBMPFD) (Higashiyama et al., 2002;Watts et al., 2004). The *Drosophila* homolog of VCP is encoded by the gene, *ter94*. One mutation of VCP that causes IBMPFD, R155H, has an equivalent mutation in *Drosophila*, dVCP R152H (Ritson et al., 2010). A drosophila model for TDP-43-associated ALS has revealed that coexpression of dVCP R152H and WT human TDP-43 in fly eyes enhances the structural degeneration and cytoplasmic mislocalization that is observed when WT hTDP-43 is overexpressed alone. This enhancement is also produced when WT *Drosophila* VCP is expressed with the ALS-associated TDP-43 mutant, hTDP-43 M337V. Expression of dVCP alone causes mild degeneration that is suppressed when *Drosophila* TDP-43 is knocked down, and enhanced when dTDP-43 is overexpressed (Ritson et al., 2010). Taken together, these results identify VCP is a modifier of TDP-43 pathogenesis, and provide *in vivo* evidence linking a single mutation to the pathogenesis of both ALS and IBMPFD.

CAG repeat expansion within the coding region of ataxin-2, *ATXN2*, causes spinocerebellar ataxia type 2 (SCA2) (Imbert et al., 1996;Lorenzetti et al., 1997;Pulst et al., 1996;Sanpei et al., 1996). The *Drosophila* homolog of ataxin-2, dAtx2, enhances eye degeneration and decreases adult lifespan when coexpressed with WT human TDP-43 in eyes and motor

neurons, respectively (Elden et al., 2010). When endogenous dAtx2 levels are reduced, eye degeneration and lifespan improve in flies expressing hTDP-43. It is important to note that altering dAtx2 in these experiments does not alter hTDP-43 levels. Parallel work in cell culture has shown that ataxin-2 and TDP-43 physically interact in an RNA-dependent manner (Elden et al., 2010). Thus, ataxin-2 appears to be a modifier of ALS-related TDP-43 toxicity. As with IBMPFD, these experiments have identified a possible link between ALS and SCA2.

Inositol-1,4,5-trisphosphate receptor type 1 (ITPR1), encoded by the gene *ITPR1*, is an IP3 receptor bound to the endoplasmic reticulum, and is involved in controlling the release of intracellular calcium ions (Nucifora, Jr. et al., 1995). A recent high content RNAi screen in HeLa cells identified ITPR1 as a modifier of TDP-43 cytoplasmic mislocalization (Kim et al., 2012). Specifically, knockdown of ITPR1 increased TDP-43 cytoplasmic mislocalization. These results were confirmed in *Drosophila* using the ortholog of *ITPR1*, *Itp-r83A*. Expression of *Itp-r83A* mutants in motor neurons resulted in a very mild increase in cytoplasmic mislocalization of human TDP-43. These mutants also partially rescued adult climbing and lifespan phenotypes associated with hTDP-43 overexpression toxicity. It was thus proposed that TDP-43 overexpression toxicity is due to high nuclear concentrations. The toxic phenotypes are rescued when nuclear concentrations are reduced through the reduction of calcium signaling and subsequent cytoplasmic localization and degradation of TDP-43 (Kim et al., 2012).

Cacophony, a voltage-gated calcium channel encoded by the gene, *cac*, was recently identified as a potential modifier of *Drosophila* TDP-43 toxicity (Hazelett et al., 2012). Cacophony calcium channels have been identified at presynaptic active zones of the NMJ (Kawasaki et al., 2004) of *Drosophila*, and play an important role in regulating function, development and plasticity of neurons through calcium signaling (Peng and Wu, 2007). More recent analyses in a *Drosophila* loss of function model of TDP-43-associated ALS have shown that flies homozygous for *Drosophila TDP-43*-null mutations exhibit lower CAC expression levels than control flies (Chang et al., 2013). Both pan-neuronal and motor-neuron-specific overexpression of CAC in these mutant flies rescues the crawling defects that have been previously reported (Chang et al., 2013;Hazelett et al., 2012). In addition, loss of dTDP-43 reduces CAC levels at the NMJ. Furthermore, in normal flies, WT dTDP-43 associates with CAC mRNA transcripts, and loss of dTDP-43 function in dTDP-43-null flies results in mild alternative splicing of CAC mRNA (Chang et al., 2013). Considering the role of CAC at the NMJ, and previous reports linking abnormalities of the NMJ to ALS (Estes et al., 2013;Feiguin et al., 2009), these results provide evidence that CAC dysfunction may be involved in ALS pathogenesis and is a potential therapeutic target.

Recently it has been shown that expression of hTDP-43 can suppress the neurodegenerative phenotypes of a *Drosophila* model of Fragile X-associated tremor/ataxia syndrome (FXTAS) in an RNA-dependent manner. Interestingly, it was found that ALS-causing mutations of TDP-43 enhanced the phenotypes associated with FXTAS. In addition, phenotypic suppression by TDP-43 appears to be through an interaction with Heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1), suggesting a link between these two RNA-binding proteins and FXTAS (He et al., 2014).

3.4.3 Posttranslational Modifications—Hyperphosphorylated TDP-43 in cytoplasmic aggregates was reported in neurons of patients with TDP-43-associated ALS (Neumann et al., 2006). It has been proposed that aberrant phosphorylation of TDP-43 is associated with progression of the disease (Arai et al., 2006). Phosphomimetic TDP-43 shows a diffuse pattern in neurons of *Drosophila*. In contrast, phospho-deficient mutants form aggregates in perikarya and dendrites (Li et al., 2011). These results suggest that hyperphosphorylation prevents aggregation of TDP-43, which does not appear to coincide with what is observed in ALS patients. Recently Doubletime (DBT), the *Drosophila* homolog of casein kinase I⟨, was identified as a specific TDP-43 kinase that phosphorylates Ser409/410 of WT human TDP-43 as well as Q331K and M337V mutants (Choksi et al., 2014). Coexpression of DBT with TDP-43 Q331K in adult fly eyes produces severe retinal degeneration that does not exist when DBT is coexpressed with either WT TDP-43 or the M337V mutant. In addition, only the Q331K mutants form aggregates in neurons when phosphorylated by DBT (Choksi et al., 2014). It is unclear as to why toxicity and aggregate formation, following phosphorylation by DBT, is specific to TDP-43 Q331K mutant proteins in these experiments. However, in contrast to the earlier phosphorylation study (Li et al., 2011), the effects that DBT phosphorylation has on the Q331K mutant more accurately reflect the aggregation and toxicity observed in ALS patients (Hasegawa et al., 2008;Inukai et al., 2008).

3.5 Fused in sarcoma/translocated in liposarcoma (FUS/TLS)

FUS/TLS, often referred to as FUS, is a DNA/RNA binding protein (Baechtold et al., 1999;Zinszner et al., 1997). FUS regulates gene transcription (Tan and Manley, 2010;Uranishi et al., 2001;Wang et al., 2008b) and, similarly to TDP-43, plays a role in regulating different aspects of RNA metabolism, including nucleocytoplasmic RNA transport (Zinszner et al., 1997) and alternative splicing (Lagier-Tourenne et al., 2012;Rogelj et al., 2012;Yang et al., 1998). As its name suggests, FUS was originally linked to cancer (Crozat et al., 1993;Rabbitts et al., 1993). In more recent years, however, mutations of FUS have been identified in patients with neurodegenerative diseases including FALS (Kwiatkowski, Jr. et al., 2009;Vance et al., 2009), SALS (Belzil et al., 2009;Corrado et al., 2010;DeJesus-Hernandez et al., 2010) and FTLD (Neumann et al., 2009). The first *Drosophila* models for FUS-associated ALS were developed in 2011 (Chen et al., 2011;Lanson, Jr. et al., 2011;Wang et al., 2011) (Table 1). Similarly to TDP-43 and SOD1, these models, and those that followed (Table 1), have shown that expression of WT or mutant human FUS (hFUS) in various *Drosophila* tissue-types and stages of development recapitulates key pathological features of ALS.

3.5.1 Overexpression and Knockdown—Given their functional similarities and mutual link to ALS, it is logical to suspect that FUS and TDP-43 have overlapping mechanisms underlying their toxicity in neurons. FUS overexpression and knockdown in various tissue types has shown similar effects to TDP-43 experiments, and has provided some interesting new insights that may be applicable to TDP-43 pathogenesis as well. Similarly to TDP-43, expression of WT human FUS and ALS-associated mutants (R518K, R521C, R521H, R524S and P525L) in *Drosophila* eyes leads to degeneration that increases with age and expression level (Chen et al., 2011;Lanson, Jr. et al., 2011;Xia et al., 2012).

We and others have also shown evidence that the degeneration is greater in hFUS mutant-expressing flies than in WT-expressing flies (Chen et al., 2011;Lanson, Jr. et al., 2011). Removal of the nuclear export signal (NES) in mutant constructs, thereby containing their localization to nuclei, rescues eye degeneration phenotypes (Lanson, Jr. et al., 2011). These results suggest that FUS mutations may confer either a toxic gain of cytoplasmic function, or a loss of nuclear function to the protein. In contrast, when targeted to mushroom bodies, both WT and mutant hFUS (R524S and P525L) cause decreased lobe sizes (Chen et al., 2011). Pan-neuronal expression of WT hFUS and R518K, R521C and R521H mutants in adult flies leads to reduced locomotion compared to controls, with mutant locomotion lower than WT flies. In addition, WT and R521H-expressing flies have a shorter lifespan than controls, with mutant flies having shorter lifespans than WT flies (Lanson, Jr. et al., 2011).

As ALS is primarily a motor neuron disease, researchers in the last few years have focused heavily on studying this cell type in *Drosophila*. It was recently shown that overexpression of hFUS WT and mutant protein causes presynaptic structural changes at the NMJ of *Drosophila* larvae (Shahidullah et al., 2013). Consistent with previous reports (Lanson, Jr. et al., 2011), bouton number is unaffected. However, contrary to mutant hTDP-43 expression in motor neurons (Estes et al., 2013), hFUS R521C-expressing larvae do have a reduced number of presynaptic active zones, as well as dysmorphology of the structures that form the active zones (Shahidullah et al., 2013). These structural abnormalities are accompanied by reduced synaptic transmission in the NMJ's, a phenotype that is also observed in *Drosophila* FUS-null mutants. Despite these abnormalities, excitability of the neurons and propagation of the action potentials are unaffected in these mutant flies. Therefore, presynaptic abnormalities at the NMJ of motor neurons may in fact be involved in the initial stages of ALS pathogenesis (Shahidullah et al., 2013).

When expressed in larval motor neurons, WT hFUS cellular distribution is primarily nuclear, whereas ALS-associated mutant hFUS mislocalizes to the cytoplasm (Chen et al., 2011;Lanson, Jr. et al., 2011). These results are consistent with the disease state in ALS-patients (Kwiatkowski, Jr. et al., 2009;Vance et al., 2009). There is also a reduction of larval mobility, and an enlargement of the neurons themselves in both WT and mutant hFUS-expressing larvae (Chen et al., 2011;Lanson, Jr. et al., 2011;Xia et al., 2012). Interestingly, however, it appears that not all ALS-associated hFUS mutants produce the same morphological changes at the NMJ. R521G, R524S and P525L mutants cause a reduction in bouton number, but R518K, R521C and R521H mutants do not (Chen et al., 2011;Lanson, Jr. et al., 2011;Xia et al., 2012).

Over time, there is an accumulation of insoluble, non-aggregated WT hFUS when expressed in adult neurons that coincides with neurodegenerative phenotypes (Miguel et al., 2012). Reducing insoluble hFUS levels with coexpression of the chaperone protein, HSPA1L, reduces eye degeneration and improves lifespan. WT hFUS is primarily localized in the nucleus of these cells. These results suggest that the toxicity observed from WT hFUS overexpression is the result of nuclear, not cytoplasmic function (Miguel et al., 2012), consistent with the recently proposed cause of TDP-43-associated toxicity (Kim et al., 2012).

The *Drosophila* homolog of FUS is cabeza, encoded by the gene *caz*. The two proteins share approximately 53% identity (Stolow and Haynes, 1995). dFUS-null mutant flies show reduced pupae eclosion, adult lifespan, and adult locomotion compared to controls (Wang et al., 2011). Interestingly, expressing WT dFUS, WT human FUS, or ALS-associated human FUS mutants (R522G or P525L) rescues the eclosion phenotype. Adult lifespan and locomotion is rescued by WT dFUS and hFUS, but not by the human FUS mutants (Wang et al., 2011). Pan-neuronal expression of dFUS RNAi does not affect adult lifespan, but does inhibit climbing. RNAi knockdown in motor neurons also reduces bouton number and length of synaptic branches at the NMJ (Sasayama et al., 2012). Additionally, knockdown in fly eyes causes external degeneration (Azuma et al., 2014;Shimamura et al., 2014). As with TDP-43, it is still unknown whether FUS toxicity is due to a gain of function or a loss of function. As mentioned previously, some early experiments with human FUS mutants suggest a possible toxic gain of function (Chen et al., 2011;Lanson, Jr. et al., 2011). However, these studies using dFUS show that a loss of function recapitulates ALS-associated phenotypes similarly to overexpression models. Interestingly, both WT dFUS overexpression and null-mutant loss of function are capable of causing similar phenotypes in *Drosophila*, with the exception of motor neuron apoptosis (Xia et al., 2012). Overexpression of WT dFUS causes apoptosis of motor neurons, but dFUS-null mutants do not. The conclusion of these results is that although perturbations of dFUS homeostasis are toxic to flies, the mechanisms of toxicity may not always be the same (Xia et al., 2012). Taken together, it is possible that, similarly to TDP-43, FUS toxicity is due to a combination of both a loss of nuclear function and gain of cytoplasmic function.

We have recently shown evidence that the RNA-binding ability of FUS is necessary for ALS-associated toxicity in both *Drosophila* and yeast model systems (Daigle et al., 2013). We expressed WT hFUS and hFUS mutant proteins lacking RNA-binding capabilities in *Drosophila* and found that ALS-associated phenotypes were reduced compared to normal WT and mutant hFUS-expressing flies. Removing RNA-binding function resulted in reduced eye degeneration in adults, improved larval eclosion and mobility, reduced brain atrophy in larvae, reduced cytoplasmic mislocalization, and reduced incorporation into cytoplasmic stress granules following treatment with sodium arsenite (Daigle et al., 2013). These results are consistent with the previously discussed results showing that RNA-binding may also be necessary for TDP-43-associated toxicity in ALS pathogenesis (Ihara et al., 2013;Voigt et al., 2010).

3.5.2 Modifiers of FUS-Associated Phenotypes—In addition to similar pathological phenotypes in ALS, there is evidence that FUS and TDP-43 have similar and potentially overlapping mechanisms of toxicity (Kim et al., 2010;Ling et al., 2010). Our laboratory has previously shown that WT and mutant hTDP-43 modifies the hFUS-associated ALS phenotypes observed in *Drosophila* models. Both WT and ALS-associated mutant hTDP-43 (M337V) enhance WT and hFUS R521H mutant degeneration in fly eyes (Lanson, Jr. et al., 2011). Others have shown that *Drosophila* TDP-43 modifies the dFUS phenotype, and the results suggest that *TBPH* and *caz* function in a similar pathway (Wang et al., 2011).

In addition to modifying TDP-43-associated ALS phenotypes in *Drosophila*, VCP has recently been shown to modify FUS-associated ALS phenotypes as well (Azuma et al.,

2014). RNAi mediated knockdown of dFUS in adult fly eyes causes external degeneration that can be partially rescued by coexpression with WT dVCP. Conversely, overexpressing dFUS in *dVCP*-null mutant flies enhances the degeneration. dVCP also affects dFUS levels in neurons. Reduction of nuclear levels of dFUS via pan-neuronal, RNAi-mediated knockdown in larvae is enhanced in *dVCP*-null mutant flies compared to knockdown in normal flies. Coexpression with WT dVCP, however, increases nuclear dFUS levels compared to dFUS knockdown alone. Adult flies expressing dFUS RNAi in neurons exhibit reduced climbing capabilities that gets worse with age. This climbing dysfunction is enhanced in *dVCP*-null mutants, but is mildly rescued by overexpressing WT dVCP. Consistent with these results, dVCP also modifies dFUS toxicity at NMJ's (Azuma et al., 2014). Larvae expressing dFUS RNAi in neurons show a reduction in presynaptic terminal branching and number of boutons. Expressing dFUS RNAi in a *dVCP*-null mutant fly enhances these reductions. Both phenotypes are rescued by coexpression with WT dVCP, with bouton numbers even surpassing control larvae (Azuma et al., 2014). Not only do these results identify dVCP as a modifier of ALS-associated dFUS phenotypes, they provide further evidence, in addition to TDP-43 studies (Ritson et al., 2010), that VCP may be involved in ALS pathogenesis and may be a viable target for therapy.

Recent evidence suggests that dFUS is involved in the epidermal growth factor receptor (EGFR) signaling pathway through the EGFR-associated genes *rhomboid-1*, *rhomboid-3* and *mirror* (Shimamura et al., 2014). Reduced expression of any one of these genes in adult eyes is sufficient to suppress the eye degeneration observed in dFUS knockdown flies. These results are consistent with previous reports linking perturbations of the mitogen-activated protein kinase (MAPK) signaling pathway to ALS (Ackerley et al., 2004;Bendotti et al., 2004;Bendotti et al., 2005;Dewil et al., 2007;Holasek et al., 2005;Kim and Choi, 2010;Shimamura et al., 2014;Tortarolo et al., 2003).

3.5.3 Posttranslational Modifications—Protein arginine methyltransferases (PRMT's) catalyze the methylation of arginine residues of proteins. This is important for regulation and maintenance of protein function, reviewed in (Bedford and Richard, 2005;Fackelmayer, 2005). PRMT1 methylates FUS, regulating its function and cellular distribution (Dormann et al., 2012;Du et al., 2011;Tradewell et al., 2012;Yamaguchi and Kitajo, 2012). Using a *Drosophila* model previously developed in our laboratory (Lanson, Jr. et al., 2011) in combination with mammalian cell cultures, it has been recently shown that PRMT1 interacts with WT hFUS, as well as the ALS-associated hFUS mutant, R521H (Scaramuzzino et al., 2013). Targeted coexpression of PRMT1 RNAi with either WT hFUS or hFUS R521H in *Drosophila* eyes enhances the degeneration that occurs with WT or mutant hFUS alone. The results suggest that reduced arginine methylation of FUS by PRMT1 may be associated with ALS pathogenesis (Scaramuzzino et al., 2013).

3.6 TAF15 and EWSR1

The structural and functional similarities between TDP-43 and FUS, including their shared RNA-binding abilities, have led to the hypothesis that RNA metabolism plays a critical role in the pathogenesis of ALS (Lagier-Tourenne and Cleveland, 2009). For this reason, a yeast functional screen was recently performed on 133 human RNA-binding proteins, in an effort

to identify ALS-causing candidates that exhibit cytoplasmic aggregation and toxicity similarly to TDP-43 and FUS (Couthouis et al., 2011). Of the proteins examined, TAF15 (RNA polymerase II, TATA box binding protein (TBP)-associated factor, 68kDa) and EWSR1 (EWS RNA-binding protein 1) not only exhibited aggregation and toxicity, but are also in the same protein family as FUS (Bertolotti et al., 1996;Couthouis et al., 2012;Couthouis et al., 2011). Therefore, TAF15 and EWSR1 were selected as candidates for further study.

TAF15 and EWSR1 are RNA-binding proteins that function in transcriptional regulation (Bertolotti et al., 1996;Lee et al., 2004;May et al., 1993) and alternative splicing (Ibrahim et al., 2013;Paronetto et al., 2011). Traditionally, *TAF15* and *EWSR1* have been associated with cancer through the formation of fusion genes that result in aberrant transcription (Law et al., 2006). Both genes are linked to extraskeletal mixoid chondrosarcoma (Attwooll et al., 1999;Labelle et al., 1995) and acute leukemia (Martini et al., 2002). Following their identification in the recent yeast functional screen, *TAF15* and *EWSR1* were found to harbor mutations in patients with SALS (Couthouis et al., 2012;Couthouis et al., 2011). *Drosophila* models using human WT and mutant forms of each protein were subsequently developed (Table 1) (Couthouis et al., 2012;Couthouis et al., 2011). In the TAF15 model, overexpression of WT protein caused eye degeneration and an age-dependent decrease in climbing ability, when expression was targeted to the eyes and motor neurons, respectively, compared to normal control flies. Overexpressing WT TAF15, as well as two different ALS-linked mutants, R408C and G391E, in motor neurons reduces lifespan compared to normal controls, with the mutants exhibiting shorter lifespans than the WT-expressing flies (Couthouis et al., 2011).

In the EWSR1 model pan-neuronal expression of WT protein reduces lifespan of the flies, and causes an age-dependent reduction of climbing ability compared to normal controls (Couthouis et al., 2012). Directed expression of human WT EWSR1 to the adult eyes caused dose-dependent structural degeneration. Interestingly, two different ALS-linked mutants, G511A and P552L, produced relatively equivalent levels of degeneration. This group suggests that observing similar effects of both mutant and WT protein expression in the *Drosophila* eyes may represent a limitation of the model, or may be evidence that mutant EWSR1 is not the cause of pathogenicity in ALS patients (Couthouis et al., 2012). Further experimentation with this model will be necessary to determine the reason for this observation. Taken together, these models have provided *in vivo* data confirming the function of both TAF15 and EWSR1, and further implicate RNA metabolism in disease pathogenesis.

3.7 C9ORF72

One of the more recently discovered genes to be linked to ALS is *C9orf72*. It was first identified in ALS patients in 2011, and its toxicity is due to a GGGGCC hexanucleotide repeat expansion within the first intron of the gene (DeJesus-Hernandez et al., 2011;Gijselinck et al., 2012;Renton et al., 2011). A recent study has shown this hexanucleotide repeat expansion of *C9orf72* present in ALS patients in populations all over the world. The combined data showed an overall frequency of approximately 6.3% of SALS

cases and 37.6% of FALS cases, making it the most common known genetic cause of SALS worldwide (Majounie et al., 2012). The first *Drosophila* model for *C9orf72*-associated ALS was developed in 2013 (Table 1) (Xu et al., 2013). This model shows that expression of 30 riboGGGGCC-repeat constructs recapitulates ALS-associated phenotypes when compared to control flies expressing 3 riboGGGGCC repeats. Expression of 30-repeat constructs in *Drosophila* eyes causes severe degeneration that increases with age and is not seen in control flies. When targeted to motor neurons, the GGGGCC expansion also reduces adult locomotion over time compared to controls that show no effect at all. The RNA-binding protein, Pur α, was concurrently found to bind to the expanded riboGGGGCC repeats in mammalian cell culture experiments performed by this group (Xu et al., 2013). Co-expression of Pur α with expanded riboGGGGCC in *Drosophila* eyes suppressed the degeneration phenotype. In addition, Pur α colocalizes with ubiquitin in cellular inclusions in the flies expressing the expanded riboGGGGCC repeats, but not in controls. Taken together, these results not only add further *in vivo* evidence to support the role of GGGGCC repeats in the pathogenesis of *C9orf72*-associated ALS, but they also provide evidence to support the hypothesis that sequestration of RNA-binding proteins is a mechanism for disease development and progression (Xu et al., 2013).

There is a growing body of evidence that GGGGCC repeat toxicity may also be due to the production of dipeptide-repeat (DPR) proteins through repeat-associated non-ATG (RAN) translation (Ash et al., 2013;Baborie et al., 2014;Kwon et al., 2014;Mizielinska et al., 2014;Mori et al., 2013;Zhang et al., 2014;Zu et al., 2011;Zu et al., 2013). Through this process, both the sense and antisense GGGGCC repeat sequences can be translated into five different DPR proteins (proline:arginine, glycine:arginine, proline:alanine, glycine:alanine and glycine:proline/proline:glycine) that vary based on the reading frame of the transcripts (Kwon et al., 2014). A *Drosophila* model was recently developed in which adult flies expressing proline:arginine and glycine:arginine DPR proteins in the eyes exhibited eye degeneration that was not present in proline:alanine or glycine:alanine-expressing flies (Table 1) (Mizielinska et al., 2014). Survival from egg to adult stages was also decreased in the flies expressing the arginine-rich DPR proteins. Adult lifespans are reduced when the arginine-rich DPR proteins are expressed pan-neuronally, compared to the proline:alanine and glycine:alanine-expressing flies (Mizielinska et al., 2014). Consistent with the previous *Drosophila* model (Xu et al., 2013), toxicity is also observed in flies expressing expanded GGGGCC-repeat RNA, compared to controls (Mizielinska et al., 2014). These results suggest that both DPR proteins and expanded GGGGCC-repeat RNA are sufficient for cellular toxicity.

3.8 Heterogeneous nuclear ribonucleoprotein A1 and A2B1 (hnRNPA1 and hnRNPA2B1)

hnRNPA1 and *hnRNPA2B1* were first linked to ALS in 2013 (Kim et al., 2013). hnRNP's are nuclear, RNA-binding proteins involved in mRNA processing (Hutchison et al., 2002). hnRNPA1 and hnRNPA2B1 have been shown previously to bind directly to TDP-43 for mRNA splicing (Buratti et al., 2005). Mutations within the prion-like domains of *hnRNPA1* and *hnRNPA2B1* were found in familial cases of multisystem proteinopathy and ALS (Kim et al., 2013). A newly developed model (Table 1) has shown that one of the identified hnRNPA2 mutants, D290V, causes severe degeneration when expressed in indirect flight

muscle cells of *Drosophila* (Kim et al., 2013). This degeneration is rescued by removing a portion of the prion-like domain. In addition, D290V mutants form cytoplasmic aggregates, whereas WT hnRNPA2, as well as mutant proteins lacking prion-like domain function, are localized to nuclei. These results, in combination with yeast and cell culture data, add *hnRNPA1* and *hnRNPA2B1* to the list of ALS-causing genes, and suggest that toxicity is specifically due to the activity of the prion-like domains of the proteins (Kim et al., 2013). These results also imply that other proteins with prion-like domains may be involved in ALS pathogenesis.

4.0 Translation from Flies to Mammalian Models

Drosophila melanogaster is a very useful organism for modeling human disease. As an animal model, *Drosophila* has an advantage over cell culture models of disease, in that the data obtained is more relevant to a living organism. *Drosophila* are less expensive to maintain than other animal models such as mice, rats and non-human primates. They also have shorter lifespans than these larger organisms, allowing for faster matings and production of experimental progeny, as well as shorter run-times on lifespan experiments. For these reasons, fruit flies are a fast, inexpensive tool for the initial studies of a disease. However, on a physiological level, *Drosophila* models may not be the best model of choice per se, as the mammalian nervous system is much more complicated than the fly nervous system. Therefore, it is critical that the studies performed in flies are translated and verified in mammals.

Most of the mammalian models of *SOD1*-associated ALS are mice and rats, although dog models have also been developed (Joyce et al., 2011). ALS-linked *SOD1* mutations have been more thoroughly modeled in rodents than in *Drosophila*. More than 10 of these mutant genes are expressed in mouse models compared to two in *Drosophila*, reviewed in (Joyce et al., 2011). In both flies and mice, expressing human SOD1 A4V and G85R mutant proteins results in the development of neurodegenerative phenotypes that are reminiscent of those seen in ALS patients, including the formation of SOD1-positive inclusions (Bruijn et al., 1997;Deng et al., 2006;Watson et al., 2008). Interestingly, however, SOD1 A4V protein-positive inclusions are only formed in mice when WT human SOD1 is also expressed, an observation not seen in flies (Deng et al., 2006;Watson et al., 2008). SOD1 G85R-expressing mice also show an acceleration of SOD1-positive inclusion formation when WT human SOD1 is concurrently expressed (Wang et al., 2009). Although both *Drosophila* and mammalian models will continue to be useful in studying SOD1-associated ALS, the more extensive set of mouse models available will likely make them the most useful to researchers in the near future.

Several mammalian model systems have been generated for TDP-43-associated ALS since TDP-43 has been linked to neurodegenerative diseases. Interestingly, these systems reflect the same gain of function/loss of function dilemma that exists in *Drosophila* models (Ihara et al., 2013). Overexpression of WT and ALS-associated mutant human TDP-43 causes loss of neurons in mice (Igaz et al., 2011;Wegorzewska et al., 2009;Wils et al., 2010), suggesting a gain of protein function as the cause of the disease. However, loss of endogenous TDP-43 expression in mouse spinal motor neurons causes accumulation of ubiquitinated,

cytoplasmic proteins in these cells, as well as cell death, suggesting a loss of protein function as the cause of disease pathogenesis (Wu et al., 2012b). Furthermore, ALS-like neurodegenerative phenotypes are observed in rats overexpressing ALS-linked mutant human TDP-43, but not WT protein (Zhou et al., 2010). Although seemingly contradictory, these mammalian models, as with the *Drosophila* models of TDP-43 associated ALS, provide evidence that both a loss and gain of protein function are toxic, although possibly through different mechanisms.

The observation that WT and ALS-linked mutant human FUS are toxic in *Drosophila* (Chen et al., 2011;Daigle et al., 2013;Lanson, Jr. et al., 2011) has been confirmed in rats (Huang et al., 2012;Huang et al., 2011) and in mice (Qiu et al., 2014). The toxicity also appears to be greater in the mutant FUS-expressing rats than in the WT-expressing animals (Huang et al., 2011). These results are also consistent with work done in ALS-patient skin fibroblasts harboring mutations in the 3' untranslated region of *FUS*, in which these mutations caused overexpression of WT hFUS and are likely the cause of ALS in these patients (Sabatelli et al., 2013).

GGGGCC hexanucleotide repeat expansion of *C9ORF72* was found to be toxic in *Drosophila* and mammalian cell models, simultaneously (Xu et al., 2013). Expression of C9ORF72 with as few as 30 repeats was sufficient to cause ALS-associated phenotypes in the flies, and cell death of neuro-2a cells, a mouse-derived neuronal cell line (Xu et al., 2013), and suggested that whatever we learned from a fly model of C9ORF72 could be translated to a mammalian system.

5.0 Summary and Future Directions

Drosophila melanogaster has been established as a useful model organism for studying ALS through its ability to recapitulate phenotypic features of the disease. This, in addition to a fully sequenced genome, has made *Drosophila* models well-suited for unbiased genetic screens that identify modifiers of ALS-associated phenotypes. These screens helped in discovering candidate genes that may be involved in mediating the disease process as well as in modifying neurodegenerative phenotypes. The ability to manipulate the expression of genes with and without ALS-causing mutations in specific tissue types, and at different stages of development, has also been useful for understanding the roles of those genes and tissues in disease progression. In spite of its usefulness in ALS research, however, *Drosophila melanogaster* is a non-mammalian organism and therefore has its limitations as a physiological model system of human disease. It is critical that the knowledge gained from *Drosophila* continues to be translated into mammalian models, followed by validation in human patient samples. The overexpression/knockdown paradigm is overused in *Drosophila*, and it is possible that expressing high levels of human protein in flies may not be completely relevant to the disease process in humans. Translating the knowledge gained from flies into mammalian and human model systems will turn these insights into a usable understanding of ALS that will lead to effective treatments, and eventually cures.

Acknowledgments

Abbreviations

ALS	Amyotrophic Lateral Sclerosis
OMIM	Online Mendelian Inheritance in Man
SOD1	Superoxide dismutase
FALS	Familial ALS
SALS	Sporadic ALS
WT	Wild type
dSOD1	*Drosophila* SOD1
hSOD1	Human SOD1
BMAA	β-N-methylamino-L-alanine
Rab5	Ras-related in brain 5
VAPB	VAMP (vesicle-associated membrane protein)-associated protein B
ER	Endoplasmic reticulum
MSP	Major sperm protein
hVAPB	Human VAPB
dVAP	*Drosophila* VAPB (DVAP33-A)
NMJ	Neuromuscular junction
Dscam	Down syndrome cell adhesion molecule
PtdIns4P	Phosphatidylinositol 4-phosphate
Osbp	Oxysterol binding protein
TDP-43	TAR DNA-binding protein 43
dTDP-43	*Drosophila* TDP-43
hTDP-43	Human TDP-43
HIV-1	Human immunodeficiency virus 1
FTLD	Frontotemporal lobar degeneration
TBPH	TAR DNA-binding protein-43 homolog
dIAP	*Drosophila* inhibitor of apoptosis
EP	Enhancer promoter
HDAC6	Histone deacetylase 6

BRP	Bruchpilot
VCP	Valosin-containing protein
IBMPFD	Inclusion body myopathy associated with Paget disese of bone and frontotemporal dementia
SCA2	Spinocerebellar ataxia type 2
RRM	RNA recognition motif
NLS	Nuclear localization signal
FUS/TLS	Fused in sarcoma/translocated in liposarcoma
NES	Nuclear export signal
EGFR	Epidermal growth factor receptor
MAPK	Mitogen-activated protein kinase
PRMT	Protein arginine methyltransferase
CNS	Central nervous system
ITPR1	inositol-1,4,5-trisphosphate receptor type 1
FXTAS	Fragile X-associated tremor/ataxia syndrome
TAF15	RNA polymerase II, TATA box binding protein (TBP)-associated factor, 68kDa
EWSR1	EWS RNA-binding protein 1
RAN	Repeat-associated non-ATG
DPR	Dipeptide-repeat protein
hnRNP	Heterogeneous nuclear ribonucleoproteins
EcR	Ecdysteroid receptor
RRM1	RNA recognition motif 1
CAC	Cacophony

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Highlights

- Over a century *Drosophila melanogaster* has been a powerful model organism for genetics research

- *Drosophila* models have been used in ALS research for understanding the molecular basis of disease

- Identifying modifiers of ALS by genetic and drug screening would help in understanding the disease

Table 1

ALS Drosophila Models Developed Since 2008

Disease	Phenotype	Reference
SOD1	Expression of WT or mutant hSOD1 caused climbing defect, reduced synaptic transmission and hSOD1 aggregation in motor neurons.	Watson *et al.* 2008
	Ubiquitous expression of zinc-deficient SOD1 results in locomotor dysfunction, altered mitochondrial structure & reduced ATP levels.	Bahadorani *et al.* 2013
Alsin	Age dependent reduction in adult locomotion in flies homozygous for mutant dALS2 compared to WT controls.	Takayama *et al.* 2014
VAPB	dVAP-null mutant flies exhibit morphological defects at the NMJ, quantal content and size and the number of presynaptic active zones.	Chai *et al.* 2008
	WT dVAP overexpression exhibit morphological changes at the NMJ. dVAP P58S mutant protein expression causes morphological changes at the NMJ and induced the formation of cytoplasmic aggregates.	Ratnaparkhi *et al.* 2008
	Only N-terminal-WT dVAP that includes an MSP domain is cleaved at the cell membrane and secreted as a ligand for Eph receptors.	Tsuda *et al.* 2008
	dVAP T48I mutant protein expression causes external eye degeneration and elevated Hsp70 expression in brain.	Chen *et al.* 2010
	dVAP-null mutant flies exhibit morphological defects of muscle mitochondria.	Han *et al.* 2012
	Endogenous dVAP protein interacts with Down syndrome cell adhesion molecule (Dscam).	Yang *et al.* 2012
	dVAP P85S mutant protein sequesters Sac1 in cytoplasmic granules, increasing PtdIns4P levels, causing degenerative phenotypes.	Forrest *et al.* 2013
	Oxysterol binding protein (Osbp) interacts with WT dVAP. Osbp localizes to cytoplasmic aggregates in dVAP P85S mutant-expressing cells, and localizes to golgi in WT dVAP-expressing cells.	Moustaqim-Barrette *et al.* 2014
	dVAP overexpression caused eye degeneration, wing posture, pupal eclosion rates, protein aggregation and defects at the NMJ.	Sanhueza *et al.* 2014
TDP-43	Reduced lifespan, adult locomotion, axonal branching at neuromuscular synapses and reduction in the number of synaptic boutons upon reduced expression of endogenous dTDP-43.	Feiguin *et al.* 2009
	Reduction of dendritic branching of larval sensory neurons upon reduced expression of WT hTDP-43 or WT dTDP-43. Increase of dendritic branching upon overexpression of dTDP-43.	Lu *et al.* 2009
	Reduced HDAC6 mRNA levels in *TBPH* knockout flies.	Fiesel *et al.* 2010
	Axon loss and neuronal death with hTDP-43 overexpression and dTDP-43 knockdown in mushroom bodies. Overexpressing hTDP-43 in motor neurons causes cytoplasmic accumulation of TDP-43 aggregates, cell death, neuron swelling and NMJ morphological defects.	Li *et al.* 2010
	hTDP-43 overexpression in flies caused age-dependent eye degeneration, reduced lifespan and adult wing malformations.	Hanson *et al.* 2010
	Mild degeneration caused by dVCP is enhanced by coexpression with dTDP-43, and suppressed with knockdown of dTDP-43. Coexpression of either dVCP R152H with WT hTDP-43 or WT dVCP with hTDP-43 M337V causes enhanced eye degeneration.	Ritson *et al.* 2010
	Flies exhibit suppressed ALS-associated phenotypes when expressed TDP-43 mutants lack RNA-binding ability.	Voigt *et al.* 2010
	dAtx2 overexpression enhances eye degeneration and decreases adult lifespan when coexpressed with WT hTDP-43.	Elden *et al.* 2010
	Larvae expressing dTDP-43 in neurons as well as larvae carrying dTDP-43 null mutants exhibit changes at the NMJ. Overexpression in mushroom bodies leads to a decrease in lobe size, as well as the formation of TDP-43-positive cytoplasmic aggregates.	Lin *et al.* 2011
	Hyperphosphorylation of hTDP-43 reduces TDP-43 cytoplasmic aggregation in neurons. Hypophosphorylation increases aggregation.	Li *et al.* 2010

Disease	Phenotype	Reference
	hTDP-43 NLS-deficient mutants cause severe degeneration in adult eyes, lethality when expressed in larval neurons, and reduced adult lifespan when expressed in adult neurons.	Miguel *et al*. 2011
	Expressing hTDP-43 A315T mutant causes eye degeneration. Expressing either WT or A315T mutant TDP-43 causes mislocalization and aggregate formation in axons of eye imaginal disks, with WT TDP-43-expressing flies exhibiting more severe aggregation.	Estes *et al*. 2011
	Upregulation of endogenous chaperones reduces TDP-43 and TDP-25-associatied phenotypes, including cellular aggregation.	Gregory *et al*. 2012
	Larvae overexpressing dTDP-43 produce a different expression profile in *Drosophila* CNS than larvae carrying dTDP-43-null mutants.	Hazelett *et al*. 2012
	Expression of *Drosophila Itp-r83A* mutants in motor neurons causes a mild increase of hTDP-43 cytoplasmic mislocalization, as well as a partial rescue hTDP-43-associated lifespan and climbing defects in adult flies.	Kim *et al*. 2012
	Overexpressing human TDP-43 in adult motor neurons causes a reduction in lifespan that is not rescued by expression of the human caspase inhibitor, P35, or the *Drosophila* homolog, dIAP.	Zhan *et al*. 2013
	Pan neuronal overexpression and reduced expression of dTDP-43 both result in a reduction of adult lifespan, as well as reduced adult and larval locomotion. Overexpression of dTDP-43 in third instar larvae eye imaginal disks does not result in cytoplasmic mislocalization of the protein.	Diaper *et al*. 2013b
	Reducing dTDP-43 protein levels in glial and muscle cells causes movement disorders in adult flies. Overexpressing dTDP-43 resulted in early lethality and formation of sarcoplasmic aggregates. Altering dTDP-43 protein levels in glia and neurons also alters dEAAT1 and dEAAT2 mRNA levels.	Diaper *et al*. 2013a
	In glial cells, WT hTDP-43 as well as A315T, D169G, G298S and N345K mutants mislocalize to the cytoplasm, reduce larval motor function, reduce bouton number at the NMJ, and increase the number of postsynaptic glutamate receptors.	Estes *et al*. 2013
	WT hTDP-43 and G298S and M337V mutants cause retinal degeneration in adult eyes. WT and mutant proteins lacking a NLS undergo cytoplasmic mislocalization that corresponds to severe retinal degeneration.	Ihara *et al*. 2013
	Altering levels of stress-granule associated proteins, ROX8, PEK and GADD34, causes changes in TDP-43 toxicity in neurons of adult flies. Inhibiting stress granule formation reduces toxicity.	Kim *et al*. 2014
FUS	Expression of WT hFUS and mutant hFUS (R524S and P525L) causes eye degeneration in adult flies. Neurons for both WT and mutant are enlarged, bouton number is reduced, and larval locomotion is decreased.	Chen *et al*. 2011
	Conditional expression of WT hFUS and mutant hFUS (R518K, R521C and R521H) in adult neurons reduces lifespan and locomotion. hFUS R521C mutants have a reduced number of presynaptic active zones at the NMJ.	Lanson, Jr. *et al*. 2011 Shahidullah *et al*. 2013
	dFUS-null mutant causes reduced adult lifespan and locomotion, and reduced pupae eclosion when expressed pan-neuronally. Coexpression with dFUS or hFUS rescues all phenotypes.	Wang *et al*. 2011
	Cytoplasmic accumulation of insoluble, non-aggregated hFUS occurs in neurons over time, and corresponds to neurodegenerative phenotypes. Reduction of insoluble FUS with the chaperone protein reduces phenotypes.	Miguel *et al*. 2012
	RNAi knockdown of dFUS in neurons causes reduced adult climbing, bouton number and synaptic branches.	Sasayama *et al*. 2012
	Expression of WT dFUS causes motor neuron (MN) apoptosis. dFUS-null mutants do not cause MN apoptosis.	Xia *et al*. 2012
	Perturbing the RNA-binding ability of WT and mutant FUS strongly suppresses toxicity, including eye degeneration, larval eclosion and mobility and cytoplasmic mislocalization into stress granules.	Daigle *et al*. 2013
TAF15	Expression of WT human TAF15 in *Drosophila* eyes and neurons causes eye degeneration and locomotor dysfunctions respectively.	Couthouis *et al*. 2011
EWSR1	WT and mutant (G511A and P552L) EWSR1 expression induce degeneration in adult eyes, reduced	Couthouis *et al*. 2012

Disease	Phenotype	Reference
	life span and climbing ability.	
C9orf72	30-repeat expansion of riboGGGGCC causes eye degeneration, reduced adult locomotion, and colocalization of the RNA-binding protein, Pur α, with ubiquitin in cellular inclusions.	Xu *et al.* 2013
	Flies expressing arginine-rich dipeptide-repeats exhibit eye degeneration, reduced eclosion rate, and reduced adult lifespan.	Mizielinska *et al.* 2014
hnRNPA2	hnRNPA2 D290V mutant expression causes degeneration of indirect flight muscles and mislocalizes to cytoplasmic aggregates. Removal of prion-like domain function rescues these phenotypes.	Kim *et al.* 2013