Drosophila as an *In Vivo* Model for Human Neurodegenerative Disease

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ABSTRACT With the increase in the ageing population, neurodegenerative disease is devastating to families and poses a huge burden on society. The brain and spinal cord are extraordinarily complex: they consist of a highly organized network of neuronal and support cells that communicate in a highly specialized manner. One approach to tackling problems of such complexity is to address the scientific questions in simpler, yet analogous, systems. The fruit fly, *Drosophila melanogaster*, has been proven tremendously valuable as a model organism, enabling many major discoveries in neuroscientific disease research. The plethora of genetic tools available in *Drosophila* allows for exquisite targeted manipulation of the genome. Due to its relatively short lifespan, complex questions of brain function can be addressed more rapidly than in other model organisms, such as the mouse. Here we discuss features of the fly as a model for human neurodegenerative disease. There are many distinct fly models for a range of neurodegenerative diseases; we focus on select studies from models of polyglutamine disease and amyotrophic lateral sclerosis that illustrate the type and range of insights that can be gleaned. In discussion of these models, we underscore strengths of the fly in providing understanding into mechanisms and pathways, as a foundation for translational and therapeutic research.

KEYWORDS FlyBook; *Drosophila*; ALS; FTD; genetic pathways; polyglutamine disease; protein toxicity

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THE World Health Organization, the World Bank, and the Harvard School of Public Health describe neurological disease as one of the greatest burdens to public health, and they expect this burden to advance to a globally unmanageable problem (Aarli *et al.* 2006). Multiple scientific approaches are required to understand disease etiology, progression, and management, to aid understanding of onset and risk factors, as well as treatment and intervention design. With >600 neurological disorders listed by the National Institute of Neurological Disorders and Stroke—categories include neurodevelopmental, stroke, traumatic injury, cancer, and neurodegenerative—the disease range is complex. An approach toward disease insight is to model disease mechanisms and identify disease-modifying pathways in less complex, yet analogous, organisms. The fruit fly *Drosophila melanogaster* has yielded great advances into the underpinnings of many neurological and neurodegenerative diseases, not only providing understanding of biological pathways impaired in disease, but also the foundation for strategies for intervention approaches in mammalian systems.

The fly was introduced into biological research >100 years ago and quickly became a valuable tool that has been paramount to our understanding of genes, chromosomes, and the inheritance of genetic information (St Johnston 2002; Bellen *et al.* 2010). Ionizing and chemical mutational techniques were developed that allowed researchers to probe gene function by analyzing the biological impact of mutations. Given the relatively rapid lifespan of the fly, coupled with its propensity to produce large numbers of progeny, large-scale mutagenic screens identified many critical genes in a vast range of biological processes, including embryogenesis, neural development, and complex behaviors; many of these genes were later found to be conserved in sequence and function in mammals. One of the early genetic screens was designed to identify mutants with abnormal behavior, including the so-named *drop dead* (*drd*) mutant with a shortened lifespan and a degenerate brain (Benzer 1971). The discovery that the *drd* mutant underwent brain deterioration suggested that the fly could model not only puzzling behaviors like phototaxis and circadian cycles, but also human late-onset degenerative disorders. Later studies led to the isolation of additional fly brain mutants with features reminiscent of human brain disease pathology: *spongecake* and *eggroll* develop membrane vacuoles and multilamellar structures reminiscent of the pathological structures that form in Creutzfeldt-Jakob disease and Tay Sachs disease (Min and Benzer 1997). These genes have yet to be defined; however, the gene encoding *swiss cheese* (*sws*), a mutant with an age-dependent loss in motor activity and brain degeneration shows homology to

the human gene encoding neuropathy target esterase *PNPLA6* (Kretzschmar *et al.* 1997; Lush *et al.* 1998). Mutations in *PNPLA6* have since been identified as causal for motor neuron disease and the neurodegenerative disorders Boucher-Neuhauser and Gordon Holmes syndromes (Rainier *et al.* 2008; Deik *et al.* 2014; Synofzik *et al.* 2014). These and other studies (for reviews, see Lessing and Bonini 2009; Zu *et al.* 2011) underscore the extraordinary contributions that the fly can make toward a fundamental understanding of gene function in brain maintenance through forward genetic screens.

Although powerful, the use of classical genetic screens to provide insight into human neurodegenerative disease can be slow because of several limitations. For example, mutations may not be evident in the precise fly homolog of the human disease gene; rather, the fly highlights the pathway or process that is reflected by the human disease (Reiter and Bier 2002). Classical fly mutant screens also typically isolate alleles that are loss of function, whereas in human disease, mutations can have complex presentations, including loss of function, gain of function, new functions, or a combination of these.

The sequencing of the fly and human genomes revealed remarkable gene and pathway conservation between the fly and human (Rubin *et al.* 2000), with an estimated two-thirds of known human disease-causing genes being present in the fly (Rubin *et al.* 2000; Reiter *et al.* 2001). With the genome information, a different approach to disease could be introduced whereby the fly can be “given” the human disease: by expressing the disease form of the human gene in the animal, the animals can be analyzed in detail to assess the biological effects and pathways involved in the disease process (Figure 1). Among ways to do this is the popular approach of the *GAL4-UAS* binary system (Brand and Perrimon 1993). *GAL4* is a yeast transcription factor that can be used to control both the spatial and temporal expression of target genes, to direct gene activity at a specific time (for example, the adult), and a specific cell type (for example, neurons). Alternatively, the human disease gene can be directly expressed in a nonessential tissue such as the eye, given the frequently dominant nature of such human disease mutations and their lethality. Initial fly models of this type recreated human polyglutamine (polyQ) disease in the animal by expressing normal and mutant versions of the genes associated with spinocerebellar ataxia type 3 (SCA3) (Warrick *et al.* 1998) and Huntington’s disease (Jackson *et al.* 1998). The explosion in the application of the fly as a model for such human neurodegenerative diseases since that introduction is aided by the vast array of available genetic tools that allow for the up- and downregulation of gene expression and ability to perform genome-wide unbiased screens (Perrimon *et al.* 2010; Venken and Bellen 2014): modifiers can be rapidly identified

Figure 1 Steps for generating and characterizing a *Drosophila* model for a human neurodegenerative disease. Here, we outline what we consider to be basic steps in the generation and characterization of a *Drosophila* neurodegenerative disease model. These steps generally apply to any human disease. Genes that are known to be involved in disease, based on genetic studies or pathological examination of human neural tissue usually serve as a starting point. *Drosophila* orthologs are identified to enable loss-of-function and gain-of-function gene manipulation. Alternatively, or in addition, the human gene or the genetic lesion associated with the disease (e.g., hexanucleotide repeat expansion as found in ALS/FTD) can be expressed in the fly. Multifarious strategies for genetic manipulation exist in the fly, allowing tissue-specific and age-specific expression of transgenes for both up- and downregulation of gene expression. Examples for loss-of-function studies include hypomorphic or null alleles, deficiency lines in which a stretch of DNA that includes all or part of the gene of interest or a region of the genome covering many genes has been deleted, and shRNA and dsRNA transgenes to knockdown gene expression. For upregulation, a commonly used strategy is expression of a transgene under the control of the upstream activating sequence (UAS). In this bipartite system, the GAL4 transcriptional activator is placed under a cell-type-specific promoter, such as *gmr-GAL4* for expression in the fly eye, enabling spatial and/or temporal control of transgene expression. Genes can also be directly driven by a promoter in a tissue of interest, and techniques can be used to create the human mutation within the fly ortholog under endogenous regulatory control. A plethora of GAL4 lines driven by defined DNA fragments for distinct cellular patterns in the developing and adult brain are available for very select expression patterns (Jenett *et al.* 2012; Li *et al.* 2014). Upon establishment of a model, the effects of the disease gene are examined and compared to known manifestations and features of the human disease, and those observed in other models. Detailed functional studies of the fly model can reveal new insight into the disease process. Functional characterization and genome-wide screens in the fly can allow additional insight into disease pathways and mechanisms. Genes and processes identified through these studies can then be examined in human samples or mammalian models to aid discovery of novel disease-associated genes, risk factors, and pathological hallmarks. Fly models can also be used for compound screening.

to define genes and biological pathways that impact disease susceptibility, onset, and progression.

A combination of several key factors makes the fly a uniquely powerful animal model for neuroscience research.

First, *Drosophila* has a short life cycle of ~10 days to the adult and is easily and relatively inexpensively maintained in the laboratory in large numbers. Since the days of Thomas Hunt Morgan who used *Drosophila* to discover the role of chromosomes in hereditary, and of Hermann J. Muller who found that irradiation causes an increase in the mutation rate in *Drosophila* (Morgan *et al.* 1915), tools to manipulate and control *Drosophila* genetics have been developed, offering researchers a comprehensive toolbox. One example are so-called balancer chromosomes that allow homozygous lethal mutations to be maintained in heterozygous “balanced” fly stocks and are typically marked with visible dominant markers.

Second, gene manipulation in *Drosophila* can be fast and relatively simple. Over the years, the fly community has developed a staggering number of mutant and transgenic fly lines. *Drosophila* stocks cannot be frozen, and all fly lines are maintained alive and are commonly shared. The Bloomington *Drosophila* Stock Center at Indiana University (Cook *et al.* 2010) maintains a comprehensive collection of mutant, RNAi, misexpression, as well as other stocks. During 2014, this stock center maintained >53,000 *Drosophila* stocks and shipped >230,000 fly cultures. Additional stock centers, such as the Harvard Transgenic RNAi Project (TRiP) (Ni *et al.* 2011) and the Vienna *Drosophila* Research Center (VDRC) (Dietzl *et al.* 2007), have generated genome-wide RNAi collections. This availability of tools to exquisitely manipulate expression of nearly every gene dramatically facilitates research capabilities. Precise spatiotemporal control over gene expression is easily attained in *Drosophila*, using large collections of cell-type-specific drivers, as well as compound- or temperature-activated driver lines. The versatile genetic toolbox of *Drosophila* surpasses that of any other multicellular organism and is constantly being expanded, allowing more elaborate manipulation of the *Drosophila* genome. FlyBase (www.flybase.org)—the website of *Drosophila* genes and genomes—curates published phenotypes, gene expression, genetic and physical interactions, and many other datasets concerning *Drosophila* genetics (St Pierre *et al.* 2014), allowing researchers access to high-quality and comprehensive information.

Third, for the study of the nervous system, the fly also offers unique advantages. While less complicated than the mammalian brain, the fly has a complex central nervous system with neurons and glia, is protected by a blood–brain barrier, and shares striking organizational similarities with the vertebrate brain. An example of such similarities, which were even observed by the great neuroscientist Ramon Y. Cajal, are the neuronal circuits of vision: as noted by Sanes and Zipursky (2010), Cajal turned to the fly with hopes of finding a simple circuitry to allow easier tracing of neuronal connectivity. Instead, he found a complicated system rivaling that of vertebrates. Functional, developmental, and molecular similarities between these systems further testify that basic principles of neural circuitry are conserved from flies to humans.

Fourth, the smaller size of the *Drosophila* genome (1.2×10^8 base pairs) compared to the human genome (3.3×10^9

base pairs) and the smaller number of genes (~14,000 vs. ~20,000–25,000 protein-coding genes) have an important practical implication: many genes in humans are part of gene families composed of paralogues with redundant and/or overlapping functions. In *Drosophila*, the reduced genome complexity allows easier interpretation of loss-of-function studies. Taken together, these advantages exemplify the reasons for which *Drosophila* has made paramount contributions to biological research in general and to neuroscience research in particular.

The approach of recreating a simplified version of human neurological disease by expressing (or knocking down) the human mutant gene in the fly has now been used to model features of many neurological and neurodegenerative disorders beyond polyQ disease. These include, but are not limited to, Parkinson's disease (Online Mendelian Inheritance of Man, OMIM no. 168600), Alzheimer's disease (OMIM no. 104300), fragile X syndrome (OMIM no. 300624), fragile X tremor/ataxia syndrome (FXTAS; OMIM no. 300623), and additional spinocerebellar ataxias (Feany and Bender 2000; Fernandez-Funez *et al.* 2000; Wittmann *et al.* 2001; Dockendorff *et al.* 2002; Morales *et al.* 2002; Jin *et al.* 2003; Mutsuddi *et al.* 2004). When combined with findings from human patient pathology, human population genetics and other biological understanding, such fly models provide mechanistic insight into the molecular pathways of disease that in turn may be of importance for future translational applications.

Given the current vast breadth of the field of fly models of neurodegenerative disease, here we focus on two disease classes for illustrative purposes: polyQ diseases and amyotrophic lateral sclerosis (ALS; OMIM no. 105400)/frontotemporal dementia (FTD; OMIM no. 600274) spectrum. These studies were selected to highlight how a disease model can be established in the fly, and how studies in *Drosophila* can be combined with basic research in human genetics and patient pathological findings to provide novel understanding. These examples also include emphasis on both protein-based disease toxicity models and contrasting RNA-pathway based models. These highlight two of the biologically diverse pathways known to contribute to major human neurodegenerative diseases (although also reveal that pathways may be more similar than initially suspected).

Our goal in this review is to illustrate the template and work flow of such studies using this extraordinary model system, by highlighting the methodology, as well as approaches that lead to surprising discoveries. We have selected studies that illustrate key points, as the foundation for developing and using fly models of disease. Because the study of neurodegenerative disease in *Drosophila* is a vigorous and broad field, many outstanding reviews are available that highlight additional disorders and aspects of study (see Shulman *et al.* 2003; Bier 2005; Bilen and Bonini 2005; Marsh and Thompson 2006; Lessing and Bonini 2009; Lu and Vogel 2009; Bakhoun and Jackson 2011; Jaiswal *et al.* 2012).

We include a figure illustrating the strategy plan, with the various steps that can be involved in development and char-

acterization of a human disease model (Figure 1) and highlight biological assays that are frequently used (Figure 2). In addition, for readers' reference, we include a table of the range of neurodegenerative diseases modeled in the fly, beyond polyQ and ALS, with an initial citation that presents the model (Table 1). Models include those for Alzheimer's disease, Parkinson's disease, motor neuron disease, fragile X tremor/ataxia syndrome, myotonic dystrophy, as well as a range of polyQ diseases, ALS, and frontotemporal dementia. Thus, this area of research is broad and vigorous, with many diverse and important models. As noted, here we focus on select studies, often launching from work in our laboratory, to illustrate how the fly can be used and how studies in the fly can be instrumental hand in hand with other studies to provide key insight into mechanism.

Modeling Protein-Based Toxicity in *Drosophila* with PolyQ Disease

Establishing a model

The human polyQ diseases encompass a set of diseases whereby a CAG-trinucleotide repeat expansion occurs within the open reading frame of the respective gene. These diseases include Huntington's disease (HD; OMIM no. 143100), spinal and bulbar muscular atrophy (SBMA; OMIM no. 313200), dentatorubral-pallidoluysian atrophy (DRPLA; OMIM no. 125370), and spinocerebellar ataxias types 1 (OMIM no. 164400), 2 (OMIM no. 183090), 3 (OMIM no. 109150), 6 (OMIM no. 183086), 7 (OMIM no. 164500), and 17 (OMIM no. 607136) (Gusella and MacDonald 2000; Orr and Zoghbi 2007). In the ataxias, regions of the brain that control motor coordination and balance degenerate, and patients often become permanently disabled. Spinocerebellar ataxia type 3 (SCA3) is the most common dominantly inherited ataxia (Ruano *et al.* 2014). The mutation in SCA3 is an expansion of the CAG-trinucleotide repeat in the coding region of the *ATAXIN 3* (*ATXN3*) gene and results in an expanded polyQ tract within the protein (Kawaguchi *et al.* 1994). The pathological hallmark of polyQ disease is the presence of insoluble ubiquitinated protein inclusions in the brain that contain the expanded polyQ protein, as well as proteins such as molecular chaperones (which normally facilitate protein folding) and components of protein degradation pathways (Paulson *et al.* 1997; Cummings *et al.* 1998; Gusella and MacDonald 2000; Orr and Zoghbi 2007).

The generation of transgenic flies (flies that stably carry exogenous DNA elements) has been instrumental to the generation of the vast array of tools available to *Drosophila* researchers (Venken and Bellen 2012). To provide *in vivo* models for polyQ disease, transgenic flies were generated for SCA3 and HD, such that the proteins encoded by the *ATXN3* gene and HD gene *HUNTINGTIN* (*HTT*), respectively, with normal or expanded mutant polyQ repeat lengths, were stably inserted into the fly genome (Jackson *et al.* 1998; Warrick *et al.* 1998). The SCA3 and HD transgenes were

Figure 2 Examples of robust assays to assess neural degeneration and dysfunction in *Drosophila*. We illustrate assays that can be used in disease models for a neurodegenerative effect. We note that similar approaches, but geared toward the features of the disease of interest, can be used in the characterization of fly models for other classes of human disease. (A) Using an eye-specific GAL4 driver, both the disease gene and candidate modifiers can be expressed in the eye. As the eye is a nonvital tissue, the effects of highly toxic genes can be assessed in adult flies without concerns of lethality. For large-scale screens, the external eye (top panel) offers a rapid readout. Compared with the normal eye, the degenerative eye can show disruption of ommatidial structure, reduced size, and loss of pigmentation. Internal sections (bottom panel) allow examination of the retinal tissue. The degenerative eye shows collapse of the retina (arrows). (B) The pseudopupil assay allows a quantitative measure of degeneration in adult flies by assessing the structure of the photoreceptor cells by counting the number of intact rhabdomeres (the specialized organelles of the photoreceptor cells). Each ommatidium is composed of eight photoreceptor cells. Using back illumination of the head, seven rhabdomeres of each ommatidium can be visualized (left panel) and their structural deterioration over time due to degeneration can be monitored (right panel). (C) Labeling of specific neuronal cell types, either by using selective antibodies (such as antityrosine hydroxylase to label dopaminergic neurons) or by expression of markers using the GAL4-UAS system can allow one to assess the integrity of

neurons in the brain [reprinted from Auluck *et al.* (2002)]. (D) Expression of disease genes in motor neurons, followed by immunostaining for presynaptic (red, anti-HRP) and postsynaptic (green, anti-Dlg1) neuromuscular junction (NMJ) markers can reveal NMJ abnormalities such as reduced bouton numbers and increase in “ghost boutons” that lack opposing postsynaptic structure (Kim *et al.* 2013b). Images are courtesy of N. C. Kim and J. P. Taylor (St. Jude Medical Center). (E) Lifespan analysis of flies expressing disease-related genes. Transgene expression can be limited to the nervous system, to specific neuronal cell types, or to the adult stage to bypass developmental effects by using the range of driver lines available. (F) The negative geotaxis climbing reflex of *Drosophila* can be used to examine motor deficits with age. Flies are tapped to the bottom of an empty vial and the number of flies that can climb above a certain height is recorded. (G) Brain degeneration in *Drosophila* is often accompanied by formation of vacuoles as shown here upon expression of a CAG-repeat RNA [reprinted from Li *et al.* (2008)]. Image of *Drosophila* in center, is courtesy of wiki commons.

expressed in specific cell types of the fly to determine the cellular consequences of the pathogenic polyQ protein. The fly eye is composed of thousands of neuronal and nonneuronal cells, and the *glass multiple reporter* (*gmr*) promoter and *gmr-GAL4* driver are active in the photoreceptor neurons and other cell types of the eye throughout development (Ellis *et al.* 1993). To test whether the pathogenic SCA3 and HD transgenes resulted in degeneration, they were expressed in the eye and disease-associated toxicity was measured through analysis of the integrity of the adult retina.

Expression of an Ataxin-3 expanded polyQ protein (SCA3trQ78) in the eye by *gmr-GAL4* conferred toxicity that was remarkably reminiscent of human polyQ disease: whereas a polyQ repeat within the normal range (SCA3trQ27) had no effect, expression of the disease form with the long expansion conferred late-onset progressive degeneration (Warrick *et al.* 1998) (Figure 3). Similarly, expression of a fragment of the HD protein (called exon1) with a short polyQ repeat (Q2) had no effect, whereas expression of HD exon1 with disease-associated polyQ expansions (Q75 and Q120) induced late-onset, progressive retinal deterioration (Jackson *et al.* 1998). In addition to gross degeneration of the eye,

these studies showed that the fly could recapitulate detailed molecular and neural pathological aspects of human polyQ disease. For example, in polyQ disease, there is a general correlation of disease severity with the length of the CAG-repeat expansion; this could be seen with the fly HD model. In humans, a neuropathological hallmark of polyQ disease is the accumulation of proteinaceous neuronal inclusions that contain the pathological polyQ protein, a feature shared not only by mouse models, but also by the fly (Jackson *et al.* 1998; Warrick *et al.* 1998). These initial studies of HD and SCA3 revealed the striking similarities between the fly with mouse models and human disease (Davies *et al.* 1997; DiFiglia *et al.* 1997; Paulson *et al.* 1997; Sapp *et al.* 1997) and launched the fly as a robust and remarkable model for approaching human degenerative disease.

Modifier pathways: molecular chaperones in polyQ toxicity and beyond

These initial polyQ models illustrated the generation of fly models that recapitulate pathological features of human disease. Subsequent studies include the generation of a wide array of fly disease models (Table 1) and also genetic modifier

Table 1 A listing of various fly models for human neurodegenerative disease

Gene or protein	Some initial references
Alzheimer's disease models	
APP	Fossgreen <i>et al.</i> (1998); Greeve <i>et al.</i> (2004); Merdes <i>et al.</i> (2004); Muhammad <i>et al.</i> (2008); Stokin <i>et al.</i> (2008); Chakraborty <i>et al.</i> (2011); Mhatre <i>et al.</i> (2014)
A-beta peptide	Finelli <i>et al.</i> (2004); Iijima <i>et al.</i> (2004); Cao <i>et al.</i> (2008)
PSEN 1 and 2	Ye and Fortini (1999); Seidner <i>et al.</i> (2006)
MAPT (Tau)	Wittmann <i>et al.</i> (2001); Jackson <i>et al.</i> (2002)
ALS disease models	
Ataxin-2	Satterfield <i>et al.</i> (2002); Kim <i>et al.</i> (2014)
EWSR1	Couthouis <i>et al.</i> (2012)
FUS	Chen <i>et al.</i> (2011b); Lanson <i>et al.</i> (2011); Miguel <i>et al.</i> (2012); Xia <i>et al.</i> (2012)
GGGGCC repeat	Xu <i>et al.</i> (2013); Mizielinska <i>et al.</i> (2014)
HNRNPA2B1	Kim <i>et al.</i> (2013a)
SOD1	Elia <i>et al.</i> (1999); Watson <i>et al.</i> (2008)
TAF15	Couthouis <i>et al.</i> (2011)
TARDBP (TDP-43)	Chen <i>et al.</i> (2009); Ambegaokar and Jackson (2010); Elden <i>et al.</i> (2010); Hanson <i>et al.</i> (2010); Ritson <i>et al.</i> (2010); Voigt <i>et al.</i> (2010); Ambegaokar and Jackson (2011); Estes <i>et al.</i> (2011); Miguel <i>et al.</i> (2012)
VABP	Chai <i>et al.</i> (2008); Ratnaparkhi <i>et al.</i> (2008); Chen <i>et al.</i> (2010)
VCP	Ritson <i>et al.</i> (2010)
Parkinson's disease models	
LRRK2	Liu <i>et al.</i> (2008); Chen <i>et al.</i> (2009); Venderova <i>et al.</i> (2009); Stempfle <i>et al.</i> (2010)
Parkin (loss of function)	Greene <i>et al.</i> (2003)
Pink1 (loss of function)	Clark <i>et al.</i> (2006); Park <i>et al.</i> (2006); Yang <i>et al.</i> (2006)
SNCA (α -synuclein)	Feany and Bender (2000)
Polyglutamine disease models	
Androgen receptor/SBMA	Takeyama <i>et al.</i> (2002)
Atrophin/DRPLA	Nisoli <i>et al.</i> (2010)
Huntington's disease	Jackson <i>et al.</i> (1998); Romero <i>et al.</i> (2008); Nishimura <i>et al.</i> (2010)
PolyQ domains	Kazemi-Esfarjani and Benzer (2000); Marsh <i>et al.</i> (2000)
SCA1	Fernandez-Funez <i>et al.</i> (2000)
SCA2/(ALS)	Satterfield <i>et al.</i> (2002); Kim <i>et al.</i> (2014); also above in ALS/Ataxin-2
SCA3/MJD	Warrick <i>et al.</i> (1998, 2005)
SCA7	Jackson <i>et al.</i> (2005); Latouche <i>et al.</i> (2007)
SCA8	Mutsuddi <i>et al.</i> (2004)
SCA17	Hsu <i>et al.</i> (2014)
Some additional disease models	
CTG toxicity (myotonic dystrophy type 1)	Houseley <i>et al.</i> (2005); de Haro <i>et al.</i> (2006)
CCTG toxicity (myotonic dystrophy type 2)	Yu <i>et al.</i> (2015)
FXTAS/CGG disease	Jin <i>et al.</i> (2003)
SMN (loss of function)	Chan <i>et al.</i> (2003); Rajendra <i>et al.</i> (2007)
PrP prion proteins	Gavin <i>et al.</i> (2006); Thackray <i>et al.</i> (2012)
Yeast Sup35 prion protein	Li <i>et al.</i> (2007)

Listed are a range of fly models for various human neurodegenerative diseases. These models highlight the approaches and understanding achieved by the variety of disease models, and the range of diseases and disease genes that have been expressed in *Drosophila* to provide insight into pathways involved, hand-in-hand with analysis in mammalian or patient tissue systems. Although there are many human disease genes, and many different models (we apologize that not all could be referenced here), this table can serve as a launching point for examples of the generation and characterization of disease models in *Drosophila* for readers.

screens that paved the way for the discovery of novel pathways involved in the disease process. We delineate these approaches below for polyQ disease, highlighting the overall steps in Figure 1 and Figure 2. Such an approach can be applied not only to neurodegenerative and neurological disease, but to other disorders as well.

Studies in mammalian cell culture systems showed that polyQ inclusions could be cleared by the upregulation of molecular chaperones (Cummings *et al.* 1998; Chai *et al.* 1999; Stenoien *et al.* 1999)—but could these pathways have an effect *in vivo* in the organism, and could they modulate neurotoxicity? To address this, the molecular chaperone Hsp70 was analyzed in the SCA3 fly model (Warrick *et al.*

1999). The nuclear inclusions that form in the fly by the SCA3trQ78 protein were found to colocalize with Hsp70, signifying that the polyQ inclusions in the fly have similar pathology to polyQ inclusions that form in human brain tissue (Paulson *et al.* 1997; Cummings *et al.* 1998). Importantly, the upregulation of chaperones in the SCA3trQ78 fly model powerfully protected against the ability of the protein to induce toxicity: the retinal degeneration induced by the pathogenic polyQ protein was dramatically prevented, restoring an eye to nearly normal structure (Warrick *et al.* 1999) (Figure 3). Interestingly, these studies also revealed that a compromise of chaperone activity can accelerate and enhance toxicity of the polyQ protein. This finding

Figure 3 Expression of pathogenic human polyQ protein in *Drosophila* causes severe eye degeneration that is suppressed by Hsp70. Eyes (A–C) and retinal sections (D–F) of flies expressing expanded polyQ protein alone or with human Hsp70, using the *gmr-GAL4* driver to express *UAS-transgenes*. (A and D) Control eye structure. These are flies expressing human *hsp70* gene and the eye structure is nearly normal. (B and E) Flies expressing the expanded polyglutamine protein SCA3tr-Q78. These flies have severely degenerate eyes that lack pigment and show severe loss of structure. (C and F) Flies expressing both SCA3tr-Q78 and Hsp70. Hsp70 expression mitigates the degenerative effects of the pathogenic polyQ protein to restore the external eye completely and the internal eye back toward normal [reprinted from Warrick *et al.* (1999)].

of dosage sensitivity of pathogenic polyQ protein to chaperone activity indicates that molecular chaperone function is a critical player in the presentation of toxicity *in vivo*: not only does upregulation modify the toxicity of the protein, but chaperone function normally contributes to the disease process.

The above studies examined the role of a specific player in the polyQ disease process: molecular chaperones. They illustrate how molecular genetic approaches can assess whether up- and downregulation of target genes of special interest can modulate the toxicity of a disease protein and thereby contribute to disease progression. These and other studies have revealed a number of effects of various molecular chaperone proteins, chaperone helper proteins, and protein quality control pathways on disease pathogenesis in fly models (Warrick *et al.* 1999; Chan *et al.* 2000; Kazemi-Esfarjani and Benzer 2000; Vos *et al.* 2010; Kuo *et al.* 2013). These studies have also revealed links between the ubiquitin–proteasome pathway and autophagy pathways, as these processes dynamically interact *in vivo* to affect protein degradation, normally and in disease (Pandey *et al.* 2007). Studies on the role of molecular chaperones in neurodegenerative disease have revealed that genes of the heat shock response pathway, as well as protein degradation pathways, which help to handle misfolded proteins in times of stress, are a plausible approach for mitigating disease protein toxicity. Intriguingly, compromise of just chaperone activity

alone was also noted to cause effects reminiscent of degeneration (Elefant and Palter 1999; Bonini 2002), suggesting that too little chaperone activity—on its own in the absence of a specific disease protein—is deleterious.

The yeast heat shock protein 104 (Hsp104) is a highly potent disaggregase that resolubilizes protein aggregates (Shorter 2008). Despite being absent from the metazoan genome, the disaggregase activity of Hsp104 is effective *in vivo* at mitigating the toxicity of the HD protein in the mouse and the toxicity of proteins associated with Parkinson’s disease in the rat (Vacher *et al.* 2005; Lo Bianco *et al.* 2008). The ability of Hsp104 to mitigate toxicity of a variety of different disease proteins highlights the possibility of a common therapeutic strategy to human neurodegeneration. The studies of Hsp104 in the mouse and rat involved coexpression of the disease protein with the Hsp104 disaggregase. But the question arises as to how effective would such a modifier be at mitigating protein toxicity after disease onset? The fly is an amenable system to address such a question. A drug-inducible adaptation of the GAL4 binary system called GAL4 GeneSwitch (GS) activates the GAL4 transcription factor in the presence of the drug mifepristone (RU486) (Osterwalder *et al.* 2001; Roman and Davis 2002). Cushman-Nick *et al.* (2013), used a *gmrGS-GAL4* driver to show that Hsp104 was able to not only reduce Ataxin-3 aggregation when expressed prior to inclusion formation, but also to suppress

toxicity of the pathogenic polyQ protein when expressed after the onset of degeneration (Cushman-Nick *et al.* 2013). Unexpectedly, whereas Hsp104 mitigated toxicity of the truncated Ataxin-3 protein, Hsp104 enhanced the toxic effect of full-length pathogenic Ataxin-3 protein, highlighting the importance of protein context in which the polyQ repeat is embedded (discussed below), even for interactions with cellular pathways such as molecular chaperones.

The introduction of the fly as a model for neurodegenerative disease rapidly led to additional models; among these was a Parkinson's disease model mimicked by the expression of α -synuclein (Feany and Bender 2000), a protein associated pathologically and by mutation with Parkinson's disease (OMIM no. 168600) (Kruger *et al.* 1998; Spillantini *et al.* 1998; Singleton *et al.* 2003; Zarranz *et al.* 2004; Lesage *et al.* 2013; Proukakis *et al.* 2013). Here we illustrate how findings from study of polyQ disease in the fly led to insight into Parkinson's disease. Parkinson's disease is the most common movement disorder and is characterized by resting tremor, bradykinesia (slowed movement), impaired balance and muscular rigidity. Pathologically, the disease involves the degeneration of dopaminergic neurons in the substantia nigra and is accompanied by widespread intracellular protein accumulations of α -synuclein called Lewy bodies and Lewy neurites (Lees *et al.* 2009; Goedert *et al.* 2013; Irwin *et al.* 2013). Causal association of α -synuclein is underscored by mutations in, and increased copy number of, the *ALPHA-SYNUCLEIN* (*SNCA*) gene in patients with Parkinson's disease (Kruger *et al.* 1998; Singleton *et al.* 2003; Zarranz *et al.* 2004; Lesage *et al.* 2013; Proukakis *et al.* 2013). The neurodegenerative capacity of α -synuclein was assessed in the nervous system of the fly using both global neuronal expression and expression directed selectively to dopaminergic neurons (Feany and Bender 2000). Expression of α -synuclein in flies confers age-dependent motor difficulties and a compromise of dopaminergic neurons, observed by a loss of tyrosine hydroxylase staining (Feany and Bender 2000; Trinh *et al.* 2008). α -Synuclein accumulations, reminiscent of Lewy bodies, occur in a small percentage of fly neurons and, similar to human Parkinson's, these accumulations are not restricted to the dopaminergic neurons (Feany and Bender 2000; Goedert *et al.* 2013). Although it is not clear that the neurons are gone in all models of α -synuclein expression (Auluck *et al.* 2005; Trinh *et al.* 2008), the neurons appear compromised for tyrosine hydroxylase immunostaining, allowing study of the pathological processes and pathways involved.

A common feature of many neurodegenerative diseases, including Parkinson's disease (Irizarry *et al.* 1998; Spillantini *et al.* 1998), is the ubiquitination of the protein aggregates. With the finding of chaperone suppression of degeneration conferred by toxic polyQ proteins, one could ask whether similar pathways may protect against the toxicity of other human disease proteins. Auluck *et al.* (2002) hypothesized that because the Lewy body and Lewy neurites are ubiquitinated, molecular chaperone pathways may be involved. To test this, Hsp70 was coexpressed with α -synuclein in the fly;

indeed, this led to mitigation of the effects of α -synuclein on tyrosine hydroxylase activity of the dopaminergic neurons (Auluck *et al.* 2002). In addition, α -synuclein aggregates in the fly brain were found to be ubiquitinated and immunostained for Hsp70. This provided the impetus to determine whether immunostaining for molecular chaperones was also a feature of human disease associated with Lewy pathology. Pathological analysis of human postmortem Parkinson's disease brain confirmed that Lewy bodies and Lewy neurites are similarly associated with molecular chaperones (Auluck *et al.* 2002; Klucken *et al.* 2006; Uryu *et al.* 2006; Durrenberger *et al.* 2009). Taken together, these studies suggest that several pathways involved in the cellular response to protein misfolding and aggregation appear conserved in their role in degenerative disease between human and flies. Such studies raised the prospect that chemical compounds that boost the stress response may be of benefit. A naturally occurring antimicrobial ansamycin, geldanamycin, promotes the heat shock response pathway (Onuoha *et al.* 2007). Flies expressing α -synuclein were fed geldanamycin, which led to protection of the dopaminergic neurons compared to vehicle-fed animals (Auluck and Bonini 2002). Geldanamycin has since been shown to reduce toxicity of α -synuclein in several other model systems and in additional disease models (McLean *et al.* 2004; Shen *et al.* 2005; Waza *et al.* 2005, 2006; Batulan *et al.* 2006; Thomas *et al.* 2006; Liu *et al.* 2009). Derivatives of the ansamycin family are being generated to identify inhibitors with reduced inherent toxicity for future therapeutics (Kitson *et al.* 2013).

Taken together, these studies illustrate how a modulator pathway can be identified in the fly system, how its normal role in the disease process can be revealed, and then how the pathway can be targeted with compounds for the hope of therapeutic intervention. The specific approach of protein quality control pathways originally defined in these studies is the subject of much additional research, focusing on the involvement of heat shock response proteins in brain diseases, as well as other processes such as the biology of ageing (see Powers *et al.* 2009 for a review). In addition, many more exciting studies have been performed on the toxicity pathways of α -synuclein in flies for important new insight, including the role of protein modifications of α -synuclein (Takahashi *et al.* 2003; Chen *et al.* 2009) and interactions between α -synuclein with proteins of other diseases, such as Alzheimer's and HD (Roy and Jackson 2014; Pocas *et al.* 2015).

Modifier pathways: histone acetyltransferase activity in HD toxicity

Pathogenic polyQ inclusions can form in nuclei and disrupt nuclear protein–protein interactions, such as those involved in transcriptional regulation (Huang *et al.* 1998; Steffan *et al.* 2000; Nucifora *et al.* 2001). Remarkable insight was revealed when studies to assess mechanisms by which the HD protein causes degeneration were pursued. CREB-binding protein (CBP) and CBP-associated factor (p300) are two ubiquitously expressed histone acetyltransferases that acetylate lysine residues within histone tails, facilitating the

recruitment of transcriptional regulators (Valor *et al.* 2013). Intriguingly, a specific interaction between CBP and the HD protein was found, such that CBP becomes sequestered in polyQ accumulations in HD, leading to the loss of CBP activity and repression of CBP/p300-associated gene expression (Steffan *et al.* 2000; Nucifora *et al.* 2001). Added CBP suppresses polyQ-associated degeneration, indicating that CBP is functionally important for polyQ-associated toxicity (McCampbell *et al.* 2000). Additionally, in HD mammalian cell models, histone acetylation is decreased (Steffan *et al.* 2001).

These studies raised the possibility that restoring histone acetylation to normal levels could be a promising strategy. To pursue this approach, histone deacetylase (HDAC) inhibitors, sodium butyrate and SAHA, which increase the global levels of acetylated histones, were tested for ability to prevent polyQ-associated neurodegeneration by feeding flies these compounds and assessing the effect on neurodegeneration. This showed that HDAC inhibitors had the ability to mitigate the degenerative disease by protecting against retinal deterioration, providing the first evidence of HDAC inhibitors as a potential treatment option (Figure 4) (Steffan *et al.* 2001). This direction has been pursued with many additional studies to define the specific HDACs involved, as well as pioneering combinations of agents that may be appropriate for mitigation of disease pathogenesis (Agrawal *et al.* 2005; Kazantsev and Thompson 2008; Pallos *et al.* 2008). Misregulation of histone acetylation to impact gene expression has been confirmed in a number of additional polyQ models, as well as several other diseases of the central nervous system, including motor neuron disease (reviewed in Kazantsev and Thompson 2008; Valor *et al.* 2013; Valor and Guiretti 2014). The use of HDAC inhibitors has been well advanced in the field of cancer, with many tested in clinical trials, and three approved for cancer therapy (reviewed in Mottamal *et al.* 2015). Phase II clinical trials for the use of HDAC inhibitors for the treatment of the motor neuron disease, spinal muscular atrophy (SMA), yielded initial encouraging results, although these were not confirmed or extended by later studies (Mercuri *et al.* 2004, 2007; Swoboda *et al.* 2010; Kissel *et al.* 2011, 2014). More research on the specific targets of acetylation, the specific enzymes involved, and the development of more directed compounds are likely required. Nevertheless, these studies highlight how the fly can be employed to provide data to accelerate research on potential therapeutic pathways and approaches.

Genome-wide screens to define modifier players

The cellular and molecular pathways involved in polyQ-mediated neurodegeneration are diverse and include autophagy, cell death pathways, the ubiquitin proteasome pathway, as well as transcriptional regulation (Fernandez-Funez *et al.* 2000; McCampbell *et al.* 2000; Chan *et al.* 2002; Ravikumar *et al.* 2004; Sang *et al.* 2005). Large-scale and candidate-based genetic-modifier screens can be used to identify novel pathways involved in the disease process. The availability of the vast array of fly reagents for both upregulation and loss of

function of the genes (see Introduction) make the fly highly conducive for such screens. One type of upregulation screen involves crossing the disease-bearing animals to fly lines that contain a transposon insertion that can direct the expression of the downstream gene in the presence of the GAL4 transcription factor, a so-called *EP* screen (Rorth 1996). Downregulation screens can also be performed, one popular approach being to use the *GAL4-UAS* system to direct reagents to reduce gene expression, such as double-stranded RNA (dsRNA) or short hairpin RNA (shRNA) (Dietzl *et al.* 2007; Ni *et al.* 2011). The approaches of knocking down and upregulating genes yield different, but complementary, information. If the knockdown of a gene affects the degenerative phenotype, then that gene is expected to normally function in this process. Thus, this tells you about the normal biology involved in pathogenesis, as well as pathways to target for interference. A modifier found through upregulation, on the other hand, suggests that added activity can impact the process, although the modifier may or may not normally contribute to pathogenesis. Modifier screens are distinct from classical forward genetic screens: for example, it is often difficult to discern whether the modifier pathway is acting directly in the pathway of disease toxicity or if the modifier pathway functions in parallel. Additional cell biological studies (such as the observations noted that polyQ protein accumulations normally immunostain for molecular chaperone activity or that the HD protein interacts directly with CBP/p300) can help discriminate these possibilities. We note that such understanding can be helpful for basic biological insight, but regardless of direct or indirect involvement, the uncovered pathway could still be a potential therapeutic target.

Large-scale and candidate-based genetic-modifier screens have been extremely successful in revealing pathways novel to polyQ disease protein toxicity (Fernandez-Funez *et al.* 2000; Kazemi-Esfarjani and Benzer 2000; Bilen and Bonini 2007; Nedelsky *et al.* 2010). Examples include a large-scale *EP* screen for polyQ modifiers that revealed new roles for microRNAs (miRNAs) in maintaining the integrity of the nervous system (Bilen *et al.* 2006; Liu *et al.* 2012). Studies on the function of the Atrophin gene, and of other miRNAs, have led to intriguing roles for additional such pathways to modulate neural function and integrity that may extend to mammals (Karres *et al.* 2007; Verma *et al.* 2015). Other types of insight from large-scale modifier screens include new toxic components of the gene mutation. For example, the toxicity of the CAG-trinucleotide mutation was believed to be conferred solely by the polyQ protein domain within the disease gene. However, a modifier screen using the SCA3 fly revealed that the RNA binding protein Muscleblind could modulate polyQ toxicity (Li *et al.* 2008). Muscleblind is an important modifier of myotonic dystrophy, in which a noncoding CTG repeat expansion in the 3' UTR of the *DMPK* gene (myotonic dystrophy protein kinase) leads to disease through RNA toxicity mechanisms that include sequestration of key players such as Muscleblind (Miller *et al.* 2000; Mankodi

Figure 4 Neurodegeneration induced by the Htt exon1 protein, assayed using the pseudopupil in the fly eye, is suppressed by treating the flies with HDAC inhibitors. (A) The retinal structure of the eye rhabdomeres seen with the pseudopupil assay shows degeneration from the normal number of seven, with expression of a Q48 htt exon1 transgene. (B) Feeding the flies HDAC inhibitors SAHA (2 μ M) or butyrate (100 mM) mitigates the degeneration seen at 6 days. (C) Photographs of the pseudopupil from Q48-expressing flies with and without added HDAC inhibitors [reprinted by permission from Macmillan Publishers: Nature (Steffan *et al.* 2001), copyright 2001].

et al. 2001; Jiang *et al.* 2004). Similar to CTG repeat RNAs, CAG repeat RNA will form unusual hairpin structures (Sobczak *et al.* 2003) that can bind and sequester RNA binding proteins; notably, Muscleblind binds CAG repeat RNA as well as CUG repeat RNA (Ho *et al.* 2005). Toxicity of the CAG repeat RNA transcript was tested by disrupting the hairpin structure of the CAG repeat: CAA codon interruptions of the CAG-trinucleotide repeat prevent the RNA from forming a hairpin structure, while maintaining the protein coding sequence for polyQ. Expression of such a CAA/G repeat in the fly brain led to the same polyQ protein expressed at the same level, yet the biological outcome was a twofold delayed onset of degeneration. These data indicate that toxicity associated with a CAG-trinucleotide expansion arises from both the polyQ protein and aspects of the CAG-repeat RNA (Li *et al.* 2008). Additional intriguing mechanisms of RNA toxicity have more recently been revealed, including that such hairpin repeats can be translated into proteins despite lacking conventional protein coding indicators (Zu *et al.* 2011; discussed below).

Small-scale genetic modifier screens that target specific biological processes have also been fruitful in uncovering disease-associated pathways. In human brain tissue and mouse models of HD, SCA1, and SCA3, abnormalities in dendritic structure are a prominent pathology (Graveland *et al.* 1985; Clark *et al.* 1997; Guidetti *et al.* 2001; Cemal *et al.* 2002). The dendritic arborization neurons in the *Drosophila* nervous system are well suited for the examination of disease-associated neuritic abnormalities (Grueber *et al.* 2002). Expression of mutant Ataxin-3 and Ataxin-1 in the dendritic arborization neurons results in morphological defects that precede neuronal cell loss (Lee *et al.* 2011). Careful examination of the fly revealed that the abnormalities arose from the preferential disruption of F-actin cytoskeletal structures. The defects were partially suppressed by the upregulation of F-actin polymerization through the Rac-PAK signaling pathway (Lee *et al.* 2011). These find-

ings provide an excellent launching ground for future studies into the disease process and possible therapeutic strategies targeted to the cytoskeleton in more complex model systems.

Integrative approaches that combine the fly model system with other model systems are being used to rapidly ascertain disease-associated pathways that are likely to be affected in the human. In a mouse model for SCA1, the phosphorylation of Ataxin-1 at serine position 776 (S776) leads to enhanced toxicity (Emamian *et al.* 2003). An integrative approach to elucidate potential kinases involved in Ataxin-1 toxicity combined a screen for kinases that could decrease the protein levels of Ataxin-1 in human cells in culture and a fly modifier screen of *Drosophila* kinases that could modify Ataxin-1 toxicity (Park *et al.* 2013). The overlap between these two screens identified the RAS-MAPK-MSK1 signaling pathway as a regulator of Ataxin-1 levels and toxicity, and, stunningly, this was via the phosphorylation of Ataxin-1 at S776 by the MSK1 kinase (Park *et al.* 2013). Further work in a mouse model expressing Ataxin-1 with an expanded polyQ repeat confirmed that reduction of MSK1 and MSK2 kinases mitigates Purkinje cell loss in the cerebellum (Park *et al.* 2013). These studies highlight the power of the fly at recapitulating very precise disease features, such as specific protein phosphorylation sites, and how the fly can be applied in combination with other approaches and model systems to define key players in the disease process.

How normal protein function and protein context relates to disease phenotype

A polyQ expansion mutation is causative for distinct human neurodegenerative diseases (Gusella and MacDonald 2000; Orr and Zoghbi 2007); yet, despite the same molecular mutation and common neuropathological hallmarks of ubiquitinated polyQ inclusions, the polyQ disorders can be clinically distinct. The protein context of the polyQ

mutation likely defines the observed differences, with cell-specific factors that selectively interact with the specific disease protein, and the expanded polyQ version, influencing the disease state. The fly has been powerfully utilized to investigate the differences in toxicity produced by a “raw” polyQ tract and a polyQ tract within the context of a disease-associated protein, as well as the effect of the mutant polyQ tract in the distinct disease proteins.

Intriguing initial studies of a *Drosophila* polyQ-containing protein, Dishevelled, addressed whether the polyQ mutation is sufficient for toxicity or whether the toxicity of a polyQ mutation is the result of an altered function of the protein in which the polyQ resides (Marsh *et al.* 2000). These data showed that expansion of the polyQ repeat from Q27 to Q108 in Dishevelled compromised the normal function, although not to null activity. Removal of the polyQ repeat had very mild loss-of-function effects. Importantly, these studies also showed a striking mitigation of the toxicity of an exceedingly long, highly toxic, “raw” polyQ tract upon its insertion into the Dishevelled protein (Marsh *et al.* 2000). This highlights the critical importance and impact of protein context to the toxicity conferred by a polyQ expansion. This has since been pursued in a number of ways in other systems, including how modulation of flanking sequences by post-translational protein modification as noted in the section above with Ataxin-1, among other studies, profoundly impacts polyQ toxicity (Emamian *et al.* 2003; Aiken *et al.* 2009).

Mounting evidence suggests that in several of the polyQ syndromes, including SCA3, the disease protein undergoes cleavage to produce toxic polyQ fragments (Wellington *et al.* 1998; Berke *et al.* 2004; Haacke *et al.* 2006; Evers *et al.* 2014). To establish *in vivo* evidence for how polyQ protein cleavage modulates toxicity of the Ataxin-3 protein, a combination of insect cell culture analysis with *in vivo* fly models was used to reveal that Ataxin-3 with an expanded polyQ undergoes a caspase-dependent cleavage, which can be prevented by mutation of putative caspase sites (Berke *et al.* 2004; Jung *et al.* 2009; Liman *et al.* 2014). Interestingly polyQ-associated toxicity is alleviated in transgenic fly lines expressing uncleavable Ataxin-3 with a polyQ expansion (Jung *et al.* 2009), lending support to the notion that the generation of polyQ fragments promotes disease severity.

Studies on the Ataxin-3 protein also revealed that the normal protein has activity to mitigate the toxicity of the mutant protein, leading to reduced levels of the pathogenic form (Warrick *et al.* 2005). Detailed molecular domain studies identified the ubiquitin protease domain as critical: the Ataxin-3 protein with an expanded polyQ repeat retains this protective activity, suggesting that in disease, cleavage of Ataxin-3 to remove the protective ubiquitin protease domain would enhance pathogenesis (Warrick *et al.* 2005). Further molecular analysis in mammalian cells identified the Ubiquitin Binding Site 2 (Ubs2) of Ataxin-3 to negatively regulate the protein's turnover, via interaction with Rad23A and Rad23B (Blount *et al.* 2014). Interestingly, decreasing the

levels of Rad23 reduces SCA3 disease-associated toxicity. These studies hint at complex interactions and regulation of the proteasome with normal and mutant Ataxin-3, which may play a critical role in disease. These studies also highlight how detailed analysis in mammalian model systems can be rapidly translated to the fly to gain functional *in vivo* insight.

To date there is minimal evidence to support that protein cleavage occurs in SCA1 (Klement *et al.* 1998). This suggests that pathogenesis in the context of Ataxin-1 may occur by other mechanisms. Particularly intriguing results emerged with studies of the normal and disease Ataxin-1 protein using *Drosophila*. These studies showed that expression of the normal Ataxin-1 protein with a normal length polyQ repeat was capable of producing deleterious effects in the fly that appear similar to Ataxin-1 protein with an expanded polyQ repeat (Figure 5) (Fernandez-Funez *et al.* 2000). This finding was confirmed in the mouse, where upregulation of the normal protein led to features reminiscent of disease (Fernandez-Funez *et al.* 2000). These data suggest that the polyQ repeat, at least in this case, may lead to increased levels of the protein, contributing to or causing neurodegeneration. Such findings underscore the importance of regulation of the expression and accumulation level of these proteins. Many other important studies have been performed on the Ataxin-1 protein, integrating fly with mammalian studies to gain important functional insight (Tsuda *et al.* 2005; Lam *et al.* 2006; Tong *et al.* 2011).

Additional findings in *Drosophila* have been highly revealing for the normal function of these disease-associated proteins. For example, *Drosophila* that have the homolog of the Huntington's disease gene deleted develop normally, but show age-related neurodegenerative phenotypes (Zhang *et al.* 2009) and reveal a regulatory role for the HD protein in autophagy (Ochaba *et al.* 2014; Rui *et al.* 2015). Studies on the full-length rather than the truncated version of the HD protein bearing a pathogenic expansion in the fly have shown that there are early effects to enhance neurotransmission and that these impacts on neurotransmission may be central to subsequent degenerative effects (Romero *et al.* 2008).

Finally, a stunning example of the critical importance of protein context was illustrated with *Drosophila* models for the Androgen receptor, whose pathogenic repeat expansions are associated with SBMA. In this situation, expression of the androgen receptor protein containing a pathogenic expanded repeat completely failed to confer any deleterious effect on the animal—despite robust expression of a presumably highly toxic protein bearing an expanded polyQ domain—unless the animals were fed an androgen receptor ligand that translocated the protein into the nucleus (Takeyama *et al.* 2002). These studies highlight not only the fundamental—and astounding—impact that protein context can have, but also indicated a remarkable feature of localization of the protein within the cell for pathogenesis—in this case in the nucleus. In the fly, both agonists and antagonists translocated the protein and thus allowed the protein to convey toxicity. Subsequent studies revealed that nuclear

Figure 5 The effect of the Ataxin-1 protein with a normal and expanded polyQ domain in the fly eye. (A–C) Scanning electron microscopic images of the eyes of flies that are control (A), expressing a normal Ataxin-1 protein (B), or an Ataxin-1 protein with a pathogenic expanded polyQ repeat (C). Insets show higher magnification view of the ommatidia. (D–F) Sections through the retina, show that the interior of the eye is affected. Degeneration is very severe with Ataxin-1 bearing an expanded polyQ and mild with the normal protein. Detailed studies as highlighted in the text, have revealed much insight into the critical role of protein context in polyQ pathogenesis in studies of the Ataxin-1 protein [reprinted by permission from Macmillan Publishers: Nature, (Fernandez-Funez *et al.* 2000) copyright 2000].

translocation and native functions of the protein were both required to confer toxicity, indicating that not only nuclear translocation, but also DNA binding of the protein is required (Nedelsky *et al.* 2010). Ligand-dependent toxicity was supported by antiandrogen treatments in transgenic mouse models for the disease, which mitigated degeneration; there are now data to support clinical endeavors, with mixed outcomes, for the testing of antiandrogen agents as treatments for SBMA (Katsuno *et al.* 2002, 2003; Chevalier-Larsen *et al.* 2004; Fischbeck and Bryan 2009).

Taken together, these studies highlight some of the striking findings regarding how normal function of the disease proteins can be uncovered using the fly system. We have attempted to illustrate some of the dramatic ways in which the context/molecular domains of the disease proteins have been shown to influence toxicity. Whereas early findings suggested a pathogenic polyQ expansion was the signature of disease, as in many fields, detailed studies have revealed a more complex story with protein context and disease protein function playing critical roles, with fly studies contributing importantly toward this understanding.

Modeling RNA-Based Mechanisms of Toxicity of ALS/FTD in the Fly

Modeling amyotrophic lateral sclerosis in the fly

ALS (OMIM no. 105400) is the most prevalent motor neuron disease, with a prevalence of ~3.9 cases per 100,000 individuals (Mehta *et al.* 2014). A devastating adult-onset disease, it impacts motor neurons in the motor cortex and spinal cord,

leading to paralysis and death. The first gene mutations associated with ALS were identified in *SOD1*, a metabolic gene encoding for copper/zinc ion binding superoxide dismutase (Rosen *et al.* 1993). In 2006, insight into ALS changed dramatically with the discovery that TDP-43 (transactive response (TAR) DNA binding protein of 43 kDa) is mislocalized to the cytoplasm in disease, where it forms phosphorylated and ubiquitinated accumulations in nearly all cases of ALS, as well as about half of a related disorder, FTD (OMIM no. 600274) (Neumann *et al.* 2006). Rare mutations associated with disease have been identified in TDP-43, with a preponderance within the C-terminal low complexity prion-like domain (Lagier-Tourenne and Cleveland 2009; Mackenzie *et al.* 2010). Since this discovery, enhanced genomic technologies have led to an explosion in the identification of genes associated with ALS (Ling *et al.* 2013; Renton *et al.* 2014). Similar to TDP-43, many of these genes encode proteins involved in RNA processing (Table 1), including proteins with similar domains, such as FUS (Andersson *et al.* 2008; Kwiatkowski *et al.* 2009; Vance *et al.* 2009; Bentmann *et al.* 2012). The association of several genes involved in RNA processing with ALS suggests that RNA biogenesis and regulation is involved in the pathology of the disease.

Here we focus on TDP-43 and a recently identified mutation that is the most common mutation associated with ALS and FTD in European ethnic populations—a GGGGCC-hexanucleotide repeat expansion within the *C9orf72* gene (DeJesus-Hernandez *et al.* 2011; Renton *et al.* 2011; Majounie *et al.* 2012). Again, we focus on use of the fly to reveal new insight and highlight

Figure 6 TDP-43 toxicity in *Drosophila* leads to eye degeneration and loss of climbing ability. (A) Expression of human TDP-43 in *Drosophila* causes degeneration, seen on the external eye and internal structure. Expression is moderate (M) or strong (S). Notice that with moderate expression, despite a mild effect on the external eye, the internal retina is severely disrupted. (B) TDP-43 causes climbing defects when expressed in motor neurons with the *D42-GAL4* driver. A mutant form of TDP-43, TDP-43.Q331K, causes a more severe loss of motility than the normal protein at the same level of expression [reprinted from Elden *et al.* (2010)].

how these studies have led to a convergence of mechanism, and gene overlap, with polyQ protein-based diseases. Our goal is to illustrate with select examples the power of the application of *Drosophila* to the study of disease and its mechanisms. As highlighted previously, many models of many diseases, including other genetic contributors to ALS, have been generated that provide exceptional insight (Table 1).

Modeling TDP-43 toxicity in the fly and the discovery of novel ATXN2 mutations

Several TDP-43 fly models have been generated and characterized for TDP-43-associated pathways. In summary, expression of human TDP-43 in the fly causes gross pathologies similar to those observed in ALS patients: neuronal degeneration, motor neuron deficits, and shortened lifespan (Figure 6) (Elden *et al.* 2010; Hanson *et al.* 2010; Li *et al.* 2010; Ritson *et al.* 2010; Voigt *et al.* 2010; Estes *et al.* 2011; Kim *et al.* 2014). At a more detailed level, the TDP-43-expressing flies also mimic molecular pathologies such as mislocalization of the protein to the cytoplasm and disease-associated phosphorylation (Hanson *et al.* 2010; Li *et al.* 2010; Estes *et al.* 2011; Choksi *et al.* 2014). Mutant forms of TDP-43, that lack the ability to bind RNA confer little toxicity in the fly compared to the normal protein (Voigt *et al.* 2010), indicating that it is not aggregation, but the disruption to RNA pathways that is important to TDP-43 toxicity.

As highlighted with polyQ disease models, the fly has become a well-embraced model to address complex questions of disease-associated cytotoxicity. Coupling these screens with in-depth analysis of human genetics, as well as pathological examination of patient tissue, can reveal new pathways relevant to the human condition. We use the following examples to illustrate how studies in the fly, along with other model organisms, can lead to defining new associations of mutations with human disease.

An integration of genetic modifier screens in yeast and fly identified *ATXN2*, the spinocerebellar ataxia type 2 (SCA2) gene, as a modifier of TDP-43: downregulation of Ataxin-2 mitigated TDP-43 toxicity, and upregulation of Ataxin-2 en-

hanced TDP-43 toxicity (Figure 7) (Elden *et al.* 2010). Subsequent analyses in human ALS spinal cord revealed abnormal accumulation of Ataxin-2, suggesting that *ATXN2* could be central to TDP-43-associated disease (Elden *et al.* 2010). In SCA2, an expanded CAG-trinucleotide repeat leads to a polyQ expansion of Q34 or longer in the Ataxin-2 protein (Imbert *et al.* 1996; Pulst *et al.* 1996; Sanpei *et al.* 1996; Lastres-Becker *et al.* 2008). However, the interaction between TDP-43 and *ATXN2* raised the possibility of *ATXN2* repeat expansions in ALS: indeed, sequence analysis revealed a significant association of CAG-repeat expansions of intermediate length—higher than normal (~Q22–23) but below the threshold for SCA2 (~Q27–33) associated with patients with ALS (Figure 7) (Elden *et al.* 2010). This finding has been confirmed in many different patient populations (Chen *et al.* 2011a; Ross *et al.* 2011; Conforti *et al.* 2012; Gellera *et al.* 2012; Gijssels *et al.* 2012; Gispert *et al.* 2012; Liu *et al.* 2013; van den Heuvel *et al.* 2014). Importantly, SCA2 and ALS have been reported in the same family where both the SCA2 and ALS cases had *ATXN2* repeat expansions, implicating *ATXN2* as a *bona fide* ALS disease gene; the findings suggest screening for such *ATXN2* mutations in family pedigrees that present with both ALS and SCA2 (Tazen *et al.* 2013). The fly studies showed that repeat expansions of only 10 CAG repeats, into the ALS disease range, are sufficient to enhance TDP-43 interactions with *ATXN2* (Kim *et al.* 2014). More recent human patient studies suggest that a longer repeat in Ataxin-2 of >Q31 is associated with shorter survival in ALS (Chio *et al.* 2015). Moreover, the fly studies indicate that Ataxin-2 could be an important target in ALS, as normal Ataxin-2 function promotes TDP-43 neural toxicity. These studies highlight the tremendous impact that relatively rapid genetic analyses in simple model organisms can have on human genetic understanding.

The Ataxin-2 protein bears two evolutionarily conserved protein domains: an RNA binding motif (LSM domain), and a PolyA binding protein (PABP)-interacting motif 2 (PAM2 motif) (Satterfield and Pallanck 2006). A direct link of a physical association of Ataxin-2 with RNA came from

Figure 7 Studies in fly and yeast led to testing for Ataxin-2 polyQ expansions in ALS human disease. (A) The fly Ataxin-2 homolog modulates the toxicity of TDP-43. Flies expressing TDP-43 or *Drosophila* Ataxin-2 (dAtx2) alone (dAtx2^{EP}; latter not shown here) have a mild effect on retinal structure, but TDP-43 toxicity is markedly more severe upon added upregulation of dAtx2 (dAtx2^{EP314}) and markedly reduced by heterozygous reduction of dAtx2 (null allele dAtx2^{X1}). (B) The *ATXN2* gene is a polyQ disease gene, with a repeat region normally of 22–23Q units. Expansions of >Q34 are associated with SCA2. Fly and yeast data revealed a striking interaction between TDP-43 and Ataxin-2 suggesting that expansions longer than normal, but less than that associated with SCA2, may be found in ALS. (C) The distribution of Ataxin-2 polyQ repeat lengths in ALS and controls. In Elden *et al.* (2010), polyQ lengths ≥ 27 were found to be significantly associated with ALS. Figure panels are adapted from Elden *et al.* (2010).

studies in the fly that found that the fly *Ataxin-2* (dAtx2) protein physically interacts with *pABp* and the polyribosome via the PAM2 motif (Satterfield and Pallanck 2006). In the fly, deletion of the PAM2 motif in *ATXN2* eliminates the enhancement of TDP-43 toxicity, indicating that the interaction between Ataxin-2 and TDP-43 is via PABP; indeed downregulation of PABP in the fly mitigates TDP-43 toxicity (Kim *et al.* 2014).

Upon stress, TDP-43 has been reported to localize to cytoplasmic stress granules—cytoplasmic accumulations containing translationally repressed RNA, abortive initiation complexes, and messenger RNA (mRNA) binding proteins such as Ataxin-2 and PABP (Anderson and Kedersha 2006; Colombrina *et al.* 2009; Freibaum *et al.* 2010; Cohen *et al.* 2011; Bentmann *et al.* 2012). Given the association of these proteins, among others in ALS such as FUS, stress granule formation may play a role in disease. PABPC1 also pathologically

mislocalizes in ALS motoneurons (Figure 8) (Dormann *et al.* 2010; Bentmann *et al.* 2013; Kim *et al.* 2014; McGurk *et al.* 2014). Intriguingly, therapeutic modulation of the stress granule pathway is a possible avenue for therapeutic research. A nucleating factor for stress granule formation is the phosphorylation of elongation initiation factor 2 α (eIF2 α) by stress-activated kinases such as protein kinase R (PKR) and PKR like-ER-kinase (PERK) (Anderson and Kedersha 2006). A small molecule inhibitor PERKi (GSK2606414) was developed by GlaxoSmithKline that specifically inhibits PERK function (Axten *et al.* 2012). Strikingly, feeding flies this inhibitor mitigates TDP-43 toxicity, such that animals retain locomotor ability over time (Kim *et al.* 2014). Extending this to mammalian cortical neurons showed that treatment by the PERKi inhibitor mitigates risk of death of primary rat cortical neurons expressing TDP-43 (Kim *et al.* 2014). The approach of targeting stress granule formation is

Figure 8 Fly studies highlighting interactions with PABP led to testing for PABPC1 mislocalization in motor neurons of ALS patient tissue. Fly data suggested that PABP may be involved in ALS; here human patient tissue was examined to assess that possibility. (A–D) Immunostaining for PABPC1 (cytoplasmic PABP) of spinal cord motor neurons in control and ALS patients. PABPC1 is present and localized throughout the cytoplasm in normal motor neurons, but shows striking accumulation in motor neurons of patients with ALS. Bars, 30 μm. (E) Quantitation of the accumulations [reprinted from Kim *et al.* (2014)].

a potentially rich area for investigation as stress granule modulation pathways have been implicated in mouse models of Alzheimer's (Ma *et al.* 2013) and prion disease (Moreno *et al.* 2012, 2013); in the latter case, treatment by the PERK1 strikingly mitigates degeneration (Moreno *et al.* 2013). The association of these processes with several different disease models, and perhaps normal memory (Sidrauski *et al.* 2013, 2015; Sekine *et al.* 2015), suggests that inhibition of translation via phosphorylation of eIF2α coupled to stress granule formation, may be a unifying pathway common to a number of disease and other situations.

In ALS, the accumulation of TDP-43 in the cytoplasm coincides with a clearance of TDP-43 from the nucleus (Neumann *et al.* 2006), raising the possibility that neurodegeneration could result from either a loss-of-function or gain-of-function mechanism, or contributions of both mechanisms. Whereas the aforementioned studies highlighted toxic mechanisms upon expression of TDP-43, studies in the fly on the endogenous TDP-43 homolog, *TBPH*, indicate that normal *TBPH* function is critical for the development and maintenance of the nervous system. Flies lacking *TBPH* have motor defects and reduced lifespan—features markedly similar to humans with ALS (Feiguin *et al.* 2009; Lu *et al.* 2009; Lin *et al.* 2011; Diaper *et al.* 2013). These data from the fly support the idea that disease-associated toxicity of TDP-43 could involve both loss-of-function and gain-of-function mechanisms (reduced nuclear function and accumulation in the cytoplasm, respectively). Interestingly, gene expression analysis using the fly has uncovered similar transcriptional alterations when TDP-43 is upregulated or deleted (Vanden Broeck *et al.* 2013), suggesting that nuclear clearing and cytoplasmic accumulation could potentially reinforce similar patterns of pathological gene expression.

A GGGGCC repeat expansion associated with ALS/FTD

As noted, FTD, the second most common presenile dementia, shares clinical and pathological overlap with ALS (Gunnarsson *et al.* 1991; Lomen-Hoerth *et al.* 2002; Mackenzie and Feldman 2005; Neumann *et al.* 2006; Murphy *et al.* 2007; Geser *et al.* 2009). In 2011, the most common mutation in European ethnic-based populations associated with autosomal dominant FTD and ALS was defined as a hexanucleotide repeat expansion of GGGGCC in the first

intron of the *C9orf72* gene (DeJesus-Hernandez *et al.* 2011; Renton *et al.* 2011; Gijssels *et al.* 2012; Majounie *et al.* 2012). Although variable in length, the repeat is typically 3 or 12 units in controls, with expansions of ~30 to far greater (>1000 units) in disease. The GGGGCC repeat expansion leads to an accumulation of RNA containing the repeat in nuclear foci, translation of dipeptide repeat proteins through repeat associated non-ATG translation, and may also be associated with reduction of *C9orf72* gene expression. These data underscore that both loss-of-function of the gene, as well as gain-of-function mechanisms associated with the accumulation of the expanded repeat RNA may underlie toxicity associated with the GGGGCC expansion mutation (DeJesus-Hernandez *et al.* 2011; Renton *et al.* 2011). Studies of the role of orthologs of the *C9orf72* protein will be important (Levine *et al.* 2013); however, here we focus on the underlying biology of the RNA gain-of-function mechanisms, describing two examples by which the fly was used to tease out contributing processes.

TDP-43 pathological inclusions characterize cases harboring GGGGCC expansions in the *C9orf72* gene (reviewed in Mackenzie *et al.* 2014). However, pathology specific to *C9orf72* carriers include RNA foci hybridizing to both the sense GGGGCC repeat RNA, as well as the antisense GGCCCC repeat RNA, in spinal cord and multiple brain regions, implicating an RNA gain-of-function mechanism (DeJesus-Hernandez *et al.* 2011; Simon-Sanchez *et al.* 2012; Donnelly *et al.* 2013; Gendron *et al.* 2013; Lagier-Tourenne *et al.* 2013; Lee *et al.* 2013; Mizielinska *et al.* 2013). An additional pathology found in GGGGCC repeat carriers is the cerebellar, hippocampal, and cortical accumulation of TDP-43 negative inclusions that are positive for proteins in the ubiquitin–proteasome pathway (Mackenzie *et al.* 2014). The underlying mechanism is interesting and suggests protein translation from the accumulated repeat RNA in the absence of canonically considered protein translation sequences. This finding stems from studies of the disease-causing mutation of spinocerebellar ataxia type 8 (SCA8) (OMIM no. 608768), which revealed that this CAG-trinucleotide repeat RNA underwent repeat associated non-ATG (RAN) translation. That is, the accumulated expanded repeat RNA could undergo protein translation,

and in all three reading frames, to produce polyQ, polyserine (polyS), and polyalanine (polyA), with polyQ and polyA accumulations detectable in patient brain tissue (Zu *et al.* 2011). The RNA lacks an AUG start codon, but nevertheless, repeat-containing RNAs were shown able to produce protein when expressed in cultured cells (Zu *et al.* 2011). As polyQ protein can be toxic, evidence was provided to suggest that these various generated proteins, polyQ and the others, could be toxic to cells. The ability to generate proteins from such accumulated disease RNAs was shown for a number of different expanded repeat RNAs, including CAG repeat RNAs. This raised a question of what mechanisms of RNA toxicity are conferred by the GGGGCC repeat expansion in patients.

Translation of the open reading frames of the sense and the antisense expanded repeat RNA associated with the *C9orf72* mutation is predicted to give rise to five different polypeptides [referred to as dipeptide repeat proteins (DPRs)]: glycine-alanine (GA), glycine-arginine (GR), and glycine-proline (GP) from the sense strand and proline-alanine (PA) and proline-arginine (PR) from the antisense strand. Upon raising antibodies specific to these DPRs, various DPRs have been detected in the TDP-43-negative accumulations in the cerebellum, hippocampus, and neocortex of *C9orf72* patient tissue (Ash *et al.* 2013; Gendron *et al.* 2013; Mackenzie *et al.* 2013; Mann *et al.* 2013; Mori *et al.* 2013a,b; Zu *et al.* 2013). Cell culture studies indicate that GR and PR peptides can be toxic to cultured neurons (May *et al.* 2014; Wen *et al.* 2014; Zhang *et al.* 2014; Yamakawa *et al.* 2015). Although it is still unclear if DPRs are toxic in disease *in vivo*, these pathological and cell culture findings indicate that the expanded GGGGCC repeat RNA could confer toxicity either because the RNA on its own may be toxic and also because the RNA produces RAN-translated DPRs at least some of which may be toxic. Fly models for GGGGCC-associated toxicity have provided important mechanistic insight into this question.

Insight Into GGGGCC-associated toxicity from *Drosophila*

Expression in *Drosophila* of a repeat of 30 GGGGCC units confers a disrupted eye morphology, and, when expressed in motor neurons, an age-dependent decline in motor function (Xu *et al.* 2013). Studies *in vitro* on the repeat RNA identified Pur- α as an RNA binding protein that could bind the expanded GGGGCC repeat RNA. To investigate the significance of this interaction, directed genetic tests revealed that downregulation of Pur- α mitigated GGGGCC toxicity (Xu *et al.* 2013). Immunohistochemical analysis showed that Pur- α was sequestered into the GGGGCC RNA foci in the fly eye. The relevance of these findings to disease is underscored by studies of human postmortem brain tissue, where Pur- α colocalized with GGGGCC RNA foci. These studies support the idea that RNA toxicity of the accumulated GGGGCC RNA may play a role in disease by sequestering RNA binding proteins. In this manner, the RNA toxicity may be akin to that of Muscleblind and other RNA binding protein interactions with

Figure 9 *Drosophila* was used to parse possible mechanisms of toxicity by the hexanucleotide GGGGCC repeat expansion in the *C9orf72* gene. Expansion of a GGGGCC repeat in the first intron of the *C9orf72* gene is a major genetic cause of ALS and FTD. The underlying mechanisms may include RNA-mediated toxicity due to sequestration of RNA-binding proteins, and also dipeptide repeat (DPR) protein-mediated toxicity due to repeat associated non-ATG (RAN) translation. To assess potential contributions of these distinct mechanisms, Mizielska *et al.* (2014) generated transgenic flies expressing either “RNA-only” GGGGCC repeats, which contained interrupting stop codons to prevent protein translation, or “protein only” transgenes of the predicted translated DPRs. Expression of these transgenes in the fly eye indicates that while the RNA-only repeats and the glycine/alanine (GA) or proline/alanine (PA) fail to confer toxicity, glycine/arginine (GR) and proline/arginine (PR) show robust toxicity. Fly lines are from the Bloomington Stock Center. Images were generated and kindly shared by Lindsey Goodman (Bonini laboratory).

an expanded CTG repeat, where the RNA that accumulates in disease binds and sequesters key proteins, thereby disrupting their function (Ranum and Cooper 2006; Wheeler and Thornton 2007).

A second hypothesis is that the RNA is indeed driving the expression of polypeptides that are toxic. This was examined using *Drosophila* models for a range of features of the GGGGCC RNA. Fly models were generated that expressed a series of constructs that probed both the potential toxic role of a GGGGCC RNA that lacked the ability to be translated, as well as the toxicity of the predicted protein products produced by such an RNA. First, pathogenic length repeats of GGGGCC were created bearing stop codon interruptions within the sequence, rendering the RNA incapable of being translated into DPRs. The phenotypes of these animals were compared to flies expressing pure, uninterrupted repeats of GGGGCC RNA that could be translated into protein (Mizielska *et al.* 2014). Remarkably, while both pure and stop-codon interrupted repeats could form RNA foci reminiscent of human disease, the interrupted GGGGCC repeats of up to 288 unit repeats did not confer degenerative eye effects, whereas

pure repeats of 33 or 103 conferred toxicity (Mizielinska *et al.* 2014). These data indicate that a pure repeat capable of being translated into protein is toxic. Flies were then generated that could express DPR proteins, although not by a pure GGGGCC repeat RNA, but by an RNA of alternate codons such that the RNA sequence was not a GGGGCC repeat (along similar lines to earlier studies on the pure vs. CAA interrupted CAG repeat). Comparison of these flies expressing various DPRs predicted to be encoded by the GGGGCC repeat and its antisense showed that the GR and PR peptides have toxicity (lengths of 36 and 100), while PA and GA peptides in lengths up to 100 DPR units do not (Figure 9). Thus, peptides of GR and PR can confer marked toxicity *in vivo* in the nervous system.

The strategy of using *Drosophila* to study mechanisms of toxicity that could be conferred by the GGGGCC mutation has provided important insight into potential pathogenic pathways that may function *in vivo* in patients bearing the C9orf72 repeat expansion mutation. Additional studies of fly models for this mutation promise to be highly revealing for biological pathways involved, as well as to dissect the mechanisms of this presumed atypical protein translation. Taken together, these studies of ALS disease genes exemplify how the fly can be used to discover new associations of mutations in human disease, as well as mechanisms at play, utilizing an *in vivo* system that is highly amenable to genetic manipulations.

Concluding Remarks

The fly is an exceptional research tool for providing insight into many biological processes; here we have emphasized how it has been used for directed studies of neurodegenerative disease. As a well-established model, it mirrors—with remarkable similarities—mammalian neurodegenerative disease pathologies. We selected examples that emphasize the power of the fly in this endeavor, and have also highlighted examples of genetic modifier screens that uncover the pathways perturbed (see Figure 1 and Figure 2). We have also tried to give examples of how the fly system has been integrated with mammalian studies, human genetics, and patient samples, for a new breadth of understanding.

With improvements in human genomic sequencing technologies, *Drosophila* can provide functional support in additional ways for human molecular genetic findings. One recent example integrated disease-associated mutations discovered by exome sequencing and linkage analysis in heterogeneous nuclear ribonucleoproteins (hnRNPs) A2B1 and A1 in families with multisystem proteinopathy and ALS, with fly data for functional effects (Kim *et al.* 2013a). These proteins have a low complexity prion-like domain like TDP-43 and FUS, and the mutations identified accelerate aggregation of the protein. The fly was instrumental in showing the pathogenicity of the disease-associated mutations and the association of toxicity with the prion domain.

Genome-wide association studies (GWAS) are important to reveal modifiers that may affect disease risk in humans; however, a challenge with GWAS is that the association domains are broad and include many loci, making it difficult to identify the actual gene implicated in the disease process. Beyond that is how the gene associated with disease-risk is involved in disease outcome—Does it mitigate or enhance? One approach to defining the important factors is to test the impact of the genes within a GWAS region in a disease model: whereas the human data identifies genomic regions of interest, coupling that with fly genetics to examine functional interactions can narrow down gene targets to reveal those with the potential to have an impact on the disease process (Shulman *et al.* 2014). Another example is that the prion-like spread of disease proteins within the brain has now been shown for the HD protein in flies, promising advances into mechanism and biological impact (Goedert *et al.* 2010; Pearce *et al.* 2015). These are some of the ways fly models have been extended. The continued commitment of *Drosophila* scientists to generate new, exciting applications and methods, coupled with new discoveries into disease physiology, assures that the fly will continue as a vital biological complement for studies of human disease.

Such ever-evolving approaches to manipulate the fly genome, including more precise ways in which to induce mutations with the CRISPR/cas9 system to generate humanized mutations *in situ* (Housden *et al.* 2014), and the continuous addition of new tools to manipulate genes (Riabina *et al.* 2015) assures that the fly will continue as a formidable and critical complementary system to reveal important biology. These tools can be coupled with more sophisticated techniques to appreciate the biology and workings of the neural circuits of brain function. These approaches promise to lead to a deeper molecular understanding of basic biological processes, as well as how these processes are impacted in disease, thus unveiling the secrets of brain function, its response to ageing, and the dysfunctional state. Research using *Drosophila* will continue to provide essential groundwork necessary for the development of therapeutics needed to mitigate these devastating diseases of the brain.

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Note added in proof: Two genetic screens of GGGGCC repeat expressing *Drosophila*, an unbiased large-scale screen and a candidate-based screen, identified components of the nuclear

pore complex and related genes that impact nuclear transport as modifiers of GGGGCC-expanded repeat toxicity (Freibaum *et al.* 2015; Zhang *et al.* 2015). These studies converge on the finding that expression of an expanded GGGGCC repeat RNA disrupts nucleocytoplasmic transport. These findings were extended to mammalian cells and neurons derived from human patient tissue. A third screening approach, in the model organism yeast, showed that similar genes modify toxicity of the PR peptide that can be generated from the GGGGCC hexanucleotide repeat (Jovicic *et al.* 2015).

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