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Studying Polyglutamine Diseases in *Drosophila*

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Abstract

Polyglutamine (polyQ) diseases are a family of dominantly transmitted neurodegenerative disorders caused by an abnormal expansion of CAG trinucleotide repeats in the protein-coding regions of the respective disease-causing genes. Despite their simple genetic basis, the etiology of these diseases is far from clear. Over the past two decades, *Drosophila* has proven to be successful in modeling this family of neurodegenerative disorders, including the faithful recapitulation of pathological features such as polyQ length-dependent formation of protein aggregates and progressive neuronal degeneration. Additionally, it has been valuable in probing the pathogenic mechanisms, in identifying and evaluating disease modifiers, and in helping elucidate the normal functions of disease-causing genes. Knowledge learned from this simple invertebrate organism has had a large impact on our understanding of these devastating brain diseases.

Common neurodegenerative diseases such as Alzheimer's disease (**AD**) and Parkinson's disease (**PD**) have complicated etiologies. Although environmental factors have been increasingly suspected to play a role in these diseases, the causes for the majority of cases are unclear, and only a small proportion is linked to specific genetic factors. In this regard, the family of polyglutamine (**polyQ**) diseases, also called glutamine repeat diseases**,** stands out for their relatively simple genetic basis, thus providing a model for more complex neurodegenerative diseases.

I. Polyglutamine diseases

I-1. The family of polyglutamine diseases

Currently, there are nine known polyQ diseases (Table 1), including Huntington's disease (HD), Dentatorubral-pallidoluysian atrophy (DRPLA), spinobulbar muscular atrophy

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(SBMA), spinocerebellar ataxia type 1 (SCA1), type 2 (SCA2), type 3 (SCA3 or Machado-Joseph disease or MJD1), type 6 (SCA6), type 7 (SCA7) and type 17 (SCA17)[1-14]. All these diseases are caused by an abnormal expansion of a CAG repeat encoding a glutamine (Q) track in the protein-coding region of the mutated alleles of the respective disease genes (Table-1). For example, HD, the best-known polyQ disease, is caused by an abnormal expansion of CAG repeats in the exon 1 (HTTex1) of Huntingtin (**HTT,** Figure 1). In healthy individuals, the number of CAG repeats in HTT varies from 6 to 34. In contrast, in HD patients, the mutated allele is always expanded to more than 35 repeats[1, 15, 16]. DRPLA is caused by an unstable expansion of CAG repeats to a range of 49 to 88 in the middle of the **Atrophin-1** gene, which normally has 6 to 35 CAG repeats[17-21].

Notably, all these disorders are dominantly transmitted and share several characteristic genetic as well as clinical features that are linked to the variation in the number of CAG repeats, such as phenotypic heterogeneity, an inverse relationship between the repeat length and the age of disease onset, and the phenomenon of genetic anticipation. The variable length of CAG repeats can only be partially responsible for the phenotypic heterogeneity, as patients with the same length of CAG repeats often show different phenotypic manifestations[14, 19, 22-24]. In the case of HD, although people with CAG repeats of 40 or longer invariably develop the disease, those carrying the CAG repeats in the range of 36-39 have reduced penetrance[14, 22-28]. Together, these observations suggest that in addition to CAG repeats, additional environmental factors and/or genetic modifiers exist that also affect disease pathogenesis.

I-2. Expanded polyQ tract causes dominant neuronal toxicity

It is generally believed that the expanded polyQ tract confers on their host proteins a dominant gain-of-function effect that is toxic to neurons[14, 22-24, 26-28]. First, all of these diseases are dominantly inherited, which is usually associated with a gain of function. Second, patients carrying loss of function mutations in polyQ disease genes show phenotypes that are different from neurodegeneration. For example, although patients with SBMA caused by polyQ expansion in the androgen receptor (AR) show some signs of loss of receptor activity[29], SBMA cannot be solely due to loss of AR function, as patients with inactivation mutations in AR have a different phenotype (testicular feminization or androgen insensitivity syndrome) that does not include neuronal degeneration[30, 31]. Third, CAG expansion usually does not interfere with the normal expression of disease-causing genes. Finally, numerous studies in different animal models all support the gain-of-toxicity hypothesis in polyQ diseases[32, 33] (see below).

I-3. Protein aggregates, a unifying pathological feature with an unclear pathogenic role

Abnormal protein aggregates (i.e., compact protein deposits) are a shared pathological hallmark of almost all neurodegenerative disorders, including extracellular plaques and intracellular tangles observed in AD and Lewy bodies in PD[27, 34]. Similarly, nuclear and cytoplasmic aggregates have been found in brain tissues of human patients in the majority of polyQ diseases and in corresponding established animal models[1, 25, 27, 28, 34-39]. This unifying pathological feature implicates a potential common pathogenic mechanism involving aggregates.

It is believed that once polyQ length exceeds the pathogenic threshold, mutated disease proteins become prone to misfold and adopt abnormal conformations that resist degradation by cellular clearance machineries such as the ubiquitin-proteasome system (UPS) and autophagy[22, 27, 28, 34, 40]. This idea is also supported by the observation that these protein aggregates contain 20S proteasome and molecular chaperones and are typically ubiquitinated[41-43]. It is proposed that the expanded polyQ tracts can be organized into polar zipper-like β-sheet structures held together by hydrogen bonding between the main chain and the side chain amides, with longer glutamine repeats leading to increasing stability of this association[44].

Consistently, the propensity of the mutant proteins to form aggregates is tightly linked to the length of the polyQ tract[27, 28, 45]. For example, in cultured striatal cells, expressed HTTex1 proteins with normal polyQ lengths (<34) remained soluble while those with pathogenic polyQ lengths (>36) formed aggregates in a time- and polyQ length-dependent manner, with longer polyQ lengths enabling faster aggregation[45].

Given the tight link between polyQ length and both the pathogenesis of these diseases and with aggregate formation, it is natural to hypothesize that the aggregates themselves are the toxic agents that kill neurons. However, despite being a common pathogenic feature, the role of aggregates in neuronal degeneration remains highly controversial. On the basis of different studies, aggregates have been assigned diverse roles such as neurotoxic agents, beneficial factors, or simply by-products of the diseases[22, 27, 34, 40, 45]. For example, the regional distribution of aggregates in tissues from polyQ disease patients does not always correspond to the sites of degeneration[46-52]. In addition, in HD mice, aggregates either appear in large quantities in cells that are spared in HD, or are detected with very low frequency (<1%) in the striatum where neuronal loss is prominent[42, 53-55]. Moreover, SCA1 transgenic mice that express mutant Ataxin 1 (ATXN1), the disease-causing gene, with 77 glutamines, but lacking the self-association region, develop ataxia and Purkinje cell pathology but without apparent nuclear aggregates[56].

To date, it is generally believed that formation of aggregates is a dynamic process involving many smaller oligomeric species that are likely to be more toxic, whereas the large aggregates might be inert or even protective[22, 27, 34, 40, 45]. However, the nature of the toxic aggregate species and the exact role of aggregates in the pathogenesis of polyQ diseases remain to be determined.

I-4. Selective neurodegeneration in polyQ diseases

Another intriguing feature of polyQ diseases is the selective neuronal degeneration in the brain. All the identified disease genes are expressed ubiquitously. However, each disease more or less affects a specific subset of neurons[13, 14, 23, 24, 34]. For example, Purkinje cells are the primary target of degeneration in SCA1[57], whereas dentate neurons are the primary site of cerebellar pathology in SCA3[4]. DRPLA mainly causes a combined degeneration of the dentatofugal and pallidofugal regions in the central nervous system[20, 24, 58, 59], whereas in HD, the medium spiny projection neurons in the caudate and putamen are most notably affected[60]. This observation suggests that besides the expanded polyQ tract, other regional-, cell type- and protein-specific factors also account for pathogenesis.

I-5. Unclear pathogenic mechanisms underlying polyQ diseases

Over the past two decades, studies in different model systems have led to a growing number of hypotheses on the pathogenic mechanisms underlying polyQ diseases, from aggregates and apoptosis to transcriptional dysregulation and mitochondrial dysfunction, to malfunctioned cellular clearance machineries, among many others[61-73]. In addition, increasing evidence, supported by the observation of selective neuronal degeneration in polyQ diseases, have led to the hypothesis that the alteration of normal cellular functions of disease genes also plays a role in disease pathogeneses[24, 38, 61, 62, 70]. These diverse mechanisms might not be mutually exclusive. Rather, given the complexity of the diseases, it is likely that in each disease, multiple mechanisms contribute to different stages of disease initiation and progression. Nevertheless, to date, despite extensive studies, except for the consensus that the expanded polyQ tract is the culprit behind all the polyQ diseases, it is still not clear which of these molecular mechanisms plays an initiating role, which are secondary, and how they collectively contribute to the selective neuronal degeneration in each of the diseases.

II. *Drosophila melanogaster*, an excellent model organism

II-1. The fruit fly: small insect, big promise

Drosophila has been an excellent animal model to uncover the function of many evolutionarily conserved proteins[74-80]. Many genes essential for development are well conserved between the fly and human. As an experimental organism, *Drosophila* has been subjected to thorough genetic analysis for over a century, and its developmental biology is very well understood[75]. Many powerful experimental tools and *in vivo* assays have been perfected in the fly, such as easy and convenient methods to generate transgenic flies and manipulate its genome and the UAS/GAL4 binary expression system for targeted overexpression or knockdown of any gene in selected tissues[75, 80-85], all of which allow convenient genetic manipulation in whole animals or in specific tissues. Furthermore, the ease of raising flies in large quantities and their short life cycle make *Drosophila* amenable to large-scale genetic screens, allowing the identification of essential genes and the isolation of novel components in signaling pathways. In fact, the functions of many important genes, including entire signaling pathways, such as the Wingless/Wnt and Notch signaling pathways, were first elucidated in the fly[74-80]. As such, this small insect has evolved into a favorite model organism for the functional analyses of many basic biological questions.

Drosophila has proven to be a valuable system to uncover the function of human neurodegenerative disease genes[80]. For example, fly homologs for many human neurodegenerative disease genes exist, including the AD genes Presenilin and amyloid precursor protein (APP)[86-89]. Loss of fly APP causes behavioral defects that can be compensated for by a functional human APP transgene, demonstrating the evolutionarily conserved function of fly APP [86]. Analyses of the fly presenilin homolog have provided the first evidence that Presenilin is involved in the Notch signaling pathway and required for its proper processing[87, 88, 90].

Conversely, earlier mutagenesis screens in the fly have led to the isolation of many novel mutants [91], such as *swiss cheese*[92], *bubblegum*[93], *spongecake* and *eggroll*[94], which show late-onset progressive degeneration of the adult nervous system resembling various human diseases. For example, mutants for *bubblegum*, which encodes a fly homolog of human very long chain fatty acids (VLCFAs) acyl CoA synthetase[93], show elevated levels of VLCFAs, as seen in the human disease adrenoleukodystrophy (ALD) [93]. Unsaturated fatty acids have been shown to lower the excessive VLCFAs in ALD. Feeding the *bubblegum* mutant flies with glyceryl trioleate oil, an unsaturated fatty acid, can block the accumulation of excess VLCFAs [93]. Recent screens in *Drosophila* have led to the identification of many additional neurodegenerative disease genes such as *nmnat*[80, 95]. As summarized in many reviews, *Drosophila* has becoming an important model organism for studying neurodegenerative diseases[22, 96-105].

II-2. *Drosophila* eye, an excellent model tissue

Over the years, the *Drosophila* eye has emerged as a favorite experimental system to elucidate biological questions and model human diseases. First, the adult eye is not essential for the viability or fertility of the animal, allowing manipulations that severely disrupt eye development. Second, the adult eye phenotype can be easily examined under a dissecting microscope. Third, the structure of the *Drosophila* eye has been well characterized, and its developmental process extensively analyzed, thus a particular eye phenotype can be linked to a specific developmental process[106-110]. Fourth, eye-specific tools, such as the *eyeless* and *glass* promoters, enable eye-specific genetic manipulations, including targeted knockdown and ectopic expression (See Table II). Furthermore, a large percentage of essential genes and almost all the important signaling pathways in the fly are required for proper eye patterning. Finally, as a neuronal tissue, the *Drosophila* eye is well suited to model neurodegenerative diseases.

The wild-type adult fly eye is a beautifully organized lattice structure consisting of about 800 ommatidia (Figure 2)[106-110]. Within each ommatidium, there are eight neuronal photoreceptor cells surrounded by other non-neuronal accessory cells, including pigment cells and cone cells (Figure 2B and 2C). These cells can be easily recognized, which allows easy detection of even minor developmental defects (Figure 2B and 2C and Figure 3). The integrity of the internal photoreceptor cells can be easily visualized and quantified using the corneal pseudopupil technique without further dissection[111], greatly facilitating phenotype evaluation in large-scale screens (Figure 3D-G).

The adult eye is derived from a sac of single layered epithelial cells called the eye imaginal disc. The eye differentiation process, visible as an indented morphogenetic furrow (MF), is initiated during 3rd instar larval stage from the posterior end of the eye disc and gradually moves within several days to the anterior end to specify the neuronal photoreceptor cells and other accessory cells, a process that lasts several days[106-110, 112, 113].

III. *Drosophila* models of polyQ diseases

III-1. Experimental designs for modeling polyQ diseases in *Drosophila*

In *Drosophila*, one highly successful approach to model human diseases is to generate transgenic flies for a wildtype or mutant human disease gene and characterize the resulting phenotypes in the eye and other tissues. This is usually followed by phenotype-based modifier screens to uncover perturbed signaling pathways and novel pathogenic factors. The targeted overexpression or knockdown of selected genes is mostly achieved using the UAS/Gal4 binary expression system[81], directed by the established tissue-specific Gal4 lines (Table II). For example, the promoter region from *eyeless* directs gene expression in all cells anterior to the MF, where cells are proliferating and no differentiation occurs[114, 115]. Promoters from *elav* and *appl* target gene expression to all specified neurons, including photoreceptor cells. GMR- (Glass-Multiple-Repeats) promoter is expressed in all cells within and posterior to the MF and continues to be expressed at high levels in these cells into adulthood[116-118]. Because of this, GMR-Gal4 is one of the most commonly used drivers to direct the continuous expression of a transgene in the eye. Importantly, the temporal expression pattern of GMR-Gal4 mirrors the highly ordered differentiation process of the eye during development, and cells at the posterior end of the eye disc mature roughly 2 days earlier than those in the anterior end.

III-2. *Drosophila* models recapitulate the pathological features of polyQ diseases

Two landmark studies, one on an **SCA3** model by Warrick et al. from Nancy Bonini's group and the other on **HD** by Jackson et al., convincingly show that the main pathogenic features of polyQ diseases can be faithfully recapitulated in *Drosophila*, thus establishing this tiny insect as an excellent organism to model these debilitating diseases[119, 120].

Using the UAS/Gal4 system, Warrick et al. generated transgenic fly lines expressing a truncated human SCA3-causative gene Ataxin 3 (ATXN3) with a normal repeat of 27 glutamines (SCA3tr-Q27) or a pathogenic repeat of 78 glutamines (SCA3tr-Q78)[120]. Almost simultaneously, Jackson et al. reported the first fly HD model based on an N-terminal human HTT fragment containing the first 142 amino acids (a.a.) with a polyQ tract of normal (Q2) or pathogenic lengths directly under the control of the eye-specific GMR promoter (GMR-HTT$^{1\text{-}142}$-Q75 (HD-Q75) or GMR-HTT$^{1\text{-}142}$-Q120 (HDQ120))[119]. In both models, only the mutant proteins, but not controls of normal polyQ lengths, induce toxicity that is clearly illustrated in the eye. For SCA3 flies, when driven by GMR-Gal4, progeny from strong SCA3tr-Q78 lines show abnormally thin and severely de-pigmented eyes that are especially fragile and easily collapse, primarily due to the severe loss of underlying eye cells. However, during earlier development, the stereotypic differentiation and patterning of the pigment and neuronal photoreceptor cells all proceed normally, and the abnormality becomes detectable only after the completion of eye development, suggesting a late-onset loss of eye cells in these flies. Consistent with this conclusion, progeny from weak SCA3tr-Q78 lines show morphologically normal eyes at eclosion, but over time gradually lose eye pigmentation. More tellingly, the depigmentation starts from the posterior end of the eye and progressively spreads to the anterior end, nicely mirroring the temporal expression pattern of the GMR-Gal4 driver and providing a vivid illustration of progressive cellular degeneration,

a feature that has been reproduced in other fly models of polyQ diseases (Figure 3B shows a HD model as an example).

Similar, although milder, eye degeneration also develops in the HD model: both HD-Q75 and HD-Q120 flies show normal eye morphology and intact ommatidial structure at eclosion (day 0), but by day 10, a significant subset of rhabdomeres are disrupted, with more severe disruption in HD-Q120 than in HD-Q75 flies.

Taking advantage of the flexibility of the UAS/Gal4 binary system, Warrick et al. further targeted the expression of truncated ATXN3 proteins to several other tissues and cell types using a battery of tissue-specific Gal4 lines. In all the tested Gal4 lines, SCA3tr-Q27 does not induce any obvious effect, whereas SCA3tr-Q78 show robust cell type-specific and dosage-sensitive toxicity. For example, when directed to differentiated neurons (by the pan-neuronal driver *elav*-Gal4), strong SCAtr-Q78 lines do not produce viable adult offspring. Progeny from the weak lines, corresponding to lower level of transgene expression, do survive to adulthood with normal external morphology, but they have significantly shortened lifespan, and over time their brains shrink in size, and the photoreceptors in the eye progressively degenerate.

The toxicity of SCA3tr-Q78 is not just restricted to neurons, as its expression in muscle (targeted by 24B Gal4), even from weak transgenic lines, causes animal lethality. In contrast, no toxicity is observed when SCA3tr-Q78 is expressed in epithelial cells (by dpp-Gal4), supporting cell type-specific toxicity of the SCA3tr-Q78 protein.

Importantly, both SCA3tr and HD flies also develop aggregates in an age-dependent manner, another pathologic feature of polyQ diseases. For example, at the subcellular level, while SCA3tr-Q27 maintains exclusive cytoplasmic distribution, SCA3tr-Q78 gradually translocates from the cytoplasm to the nucleus and forms prominent aggregates that grow larger over time. At the biochemical level, in addition to mutant SCA3tr-Q78, these aggregates also contain chaperones and other ubiquitinated proteins and can form highly compact structures that resist harsh treatments such as boiling in strong detergents solutions[121, 122]. Further, aggregates can develop in almost all cells that express SCA3tr-Q78, not only in the neurons and pigment cells that are vulnerable to SCA3tr-Q78 protein, but also in cells, such as epithelial cells, in which it is not toxic, suggesting that the aggregates alone do not necessarily cause degeneration[120].

Following these studies, additional models of polyQ diseases, including **SCA1**, **SCA7**, **SCA17**, **DRPLA** and **SBMA,** have been subsequently established in *Drosophila*[102, 123-130]. These fly models largely recapitulate the main pathological features of the polyQ diseases, including progressive neuronal degeneration, cell type-specific toxicity of the mutant proteins, and aggregate formation. Detailed characterizations of these fly models have provided new insights into the complicated mechanisms of these diseases.

III-3. Lessons from *Drosophila* models of polyQ diseases

The polyQ track alone is toxic—To examine whether the polyQ tract alone, in the absence of any disease protein context, is sufficient to induce neurodegeneration, Marsh and

Thompson's group generated transgenic flies expressing a peptide with either 22 (Q22) or 108 (Q108) glutamines flanked by only a few amino acids at both ends[131]. Tested with a similar set of Gal4 lines used in the SCA3tr study[120], only the Q108 peptide can elicit strong deleterious effects. A similar study by Kazemi-Esfarjani and Benzer, who generated flies expressing peptides with either 20 (Q20-HA) or 127 (Q127-HA) glutamines tagged with a short HA epitope, produced similar results[132].

PolyQ expansion might affect but does not abolish the normal function of the disease proteins—A question in polyQ diseases is whether polyQ expansion affects the normal function of its host protein. To explore this question, Marsh et al. used *disheveled* (*dsh*), a *Drosophila* gene of the Wingless/Wnt signaling pathway with well-characterized mutant phenotypes whose gene product is a ubiquitously expressed protein with a native 28-glutamine tract[133-135]. For the engineered *dsh* transgenes that express Dsh with no (Dsh-Q0), 27 (Dsh-Q27), or 108 (Dsh-Q108) glutamines under its own native promoter, Dsh-Q27 can fully rescue the phenotypes of flies with a null *dsh* mutation, while both Dsh-Q0 and Dsh-108Q have only partial rescue efficiency, suggesting that the polyQ tract is not critical for the normal function of Dsh but is necessary for its full biological activity *in vivo*[131]. Similarly, in both *Drosophila* and mammalian systems, pathogenic ATXN1 (SCA1-Q82) shows conserved functional interactions with the same group of binding partners (e.g., scaffold protein 14-3-3, transcription regulators Capicua and Senseless/Gfi-1) as wildtype ATXN1 (SCA1-Q2), although with altered affinity, to regulate transcription and neurogenesis[136-138]. In addition, human Atrophin-1 with 118Q (Atrophin-1-Q118) still functions as a transcriptional co-repressor *in vivo*, similar to the fly Atrophin-1 homolog (*atro*) and wildtype Atrophin-1, but with reduced activity[139]. Moreover, pathogenic ATXN3 in SCA3 flies (SCA3-Q78 or Q84) retains a neuronal protective function through a proteasome-mediated mechanism, resembling that observed in normal ATXN3[130]. Lastly, in full-length AR-based fly SBMA model, AR with expanded polyQ can translocate into the nucleus and activate transcription in an androgen-dependent manner[125, 127]. Similar observations have also been documented for other polyQ disease genes. For example, in mouse and human samples, the polyQ tract in HTT is not essential for HTT's function, but both the complete removal of the polyQ or its expansion partially affects HTT's full activity[139-146], post-translational modifications (PTMs) and/or stability, such as the inflammatory kinase IKK-mediated phosphorylation of HTT, which in turn regulates additional PTMs and fate of HTT protein[147]. Together, they suggest that in most cases, the polyQ does not abolish the normal function of host protein but might play a modulatory role for its full functionality.

Protein context determines the aggregation dynamics and toxicity of polyQ proteins—Examination of *Drosophila* polyQ models reveals that although the length of the polyQ tract is the main determining pathogenic factor, with the longer polyQ being associated with faster aggregation and stronger neurodegeneration, protein context plays a prominent role in the aggregation property and toxicity of the disease proteins. This is clearly exemplified in the SBMA and the extensively studied HD models. In full-length AR-based SBMA model, the toxicity of pathogenic AR requires its binding with its ligand androgen, which induces AR translocation into the nucleus to activate transcription[125-127],

whereas the toxicity elicited by a truncated AR (ARtr-Q112) is not androgen-dependent[123]. In HD, the polyQ tract, encoded in HTT's exon 1, is near the very N-terminus of the encoded large HTT protein (~3,144 a.a.). Multiple naturally occurring N-terminal HTT fragments, potentially arising from proteolytic processing or aberrant splicing, have been documented in patient samples and in animal models[148-160]. Because of this, in addition to full-length HTT (Htt^{FL}), large numbers of HD models based on truncated HTT with various lengths of polyQ have been established[161-166]. In all these HD models, HTT variants with normal lengths of polyQ mainly localize to the cytoplasm, do not form aggregates and do not induce toxicity. Although majority of HTT variants with pathogenic polyQ lengths cause toxicity, their effects vary significantly[161-166]. For example, Htt^{FL}-Q128, which induces mild late-onset neurodegeneration, remains in the cytoplasm and does not form aggregates even in neurons of older flies[164]. However HTTex1-Q93 forms prominent cytoplasmic aggregates and causes severe degeneration (Figure 5 as an example), while Htt^{1-336}-Q128 forms large aggregates primarily in the nucleus[162, 164]. In a carefully controlled study to compare the pathogenic potential and biophysical properties of the 7 naturally occurring N-terminal HTT fragments all carrying a Q120 tract, it has been shown that the shortest N-terminal HTTex1 fragment (90 a.a. plus 120 glutamines, HTT^{1-90}-Q120) is the most toxic, most aggregation-prone, exhibiting unique biochemical properties and having the most potent amyloid seeding ability. On the other hand, the longer HTTex1 fragments either developed only lower levels of aggregates at a slower pace (e.g., HTT^{1-108}-Q120) or remained diffused in the cytoplasm, hardly forming any visible aggregates at all (e.g., HTT^{1-469}-Q120)[161].

Native functions of the disease genes in pathogenesis—Increasing evidence from fly- and mammalian-based studies suggest that the native functions of the disease genes directly affect pathogenesis. For example, in both SCA1 and SBMA models that express full-length human ATXN1 or AR, although the expanded proteins (SCA1-82Q or AR-Q52) cause stronger toxicity, overexpression of wildtype ATXN1or AR (SCA1-30Q or AR-Q12) also leads to neurodegeneration[124, 125]. In addition, the toxicity of ATXN1 in both flies and mice relies on its conserved AXH domain and requires its interaction with its endogenous binding partners such as Capicua, Senseless and 14-3-3, which control neurogenesis and ATXN1 stability[136-138]. Studies of SCA1 mouse models led to similar conclusions[136, 137, 167]. In the fly SBMA model, the AR-induced toxicity is ligand-dependent, requiring the presence of androgen or other known agonists[125, 127]. Furthermore, the native functions of AR, including its ability to bind target DNA sequences and recruit transcriptional coregulators, are essential for its toxicity[125]. In contrast, in SCA3 flies, overexpression of full-length wildtype ATXN3 (SCA3-Q27), encoding a protein with both ubiquitin binding motifs and ubiquitin protease activity, shows no deleterious effects and instead can potently suppress polyQ-induced neurodegeneration[130]. Because the pathogenic ATXN3 (SCA3-Q84) still retains this intrinsic neuroprotective function[130], it raises the question as to how two opposite activities in the same protein counteract each other during disease pathogenesis. Nevertheless, these findings reveal the importance of the disease genes' normal function in pathogenesis.

Axonal trafficking defect in polyQ diseases—When examining different fly models of polyQ diseases, both the Goldstein and Littleton groups have observed strong axonal trafficking defects in flies expressing polyQ-expanded proteins, but not in controls[163, 168]. For example, in larval motor neurons of wild type and control flies, cargoes such as synaptic proteins, vesicles, and mitochondria are effectively delivered to the neuromuscular junctions (NMJs) through the axon. In contrast, in polyQ flies (e.g., Htt$^{1-548}$-Q128) that develop cytoplasmic aggregates, but not in lines (e.g., SCA3tr-Q78 and Q127-HA) that form exclusively nuclear aggregates, diminished delivery efficiency of the cargoes are observed, accompanied by prominent accumulation of aggregates with trapped synaptic organelles and mutant proteins along the swollen axon track. These animals also display sluggish movement, indicating the substantially compromised function of motor neurons. Further, reducing the dosage of key components of dynein- and kinesin-based motor complexes can strongly enhance this “axonal jamming” phenotype[168]. Given that neuronal cells have a particularly high reliance on axonal transport for long-distance delivery of essential constituents that maintain the survival and functionality of axonal projections, these findings implicate compromised axonal trafficking as one underlying contributing factor for polyQ diseases.

Transcriptional dysregulation in polyQ diseases—Notably, several polyQ disease proteins function either as transcription factors (e.g., TBP and AR) or transcription coregulators (e.g., Atxain-7 and Atrophin-1), or have been implicated extensively in transcriptional regulation (e.g., ATXN1 and HTT)[136-138, 169-182]. Such a convergence on transcription implicates a role of transcriptional dysregulation in disease pathogenesis, a hypothesis that has been supported by many studies from both fly and mouse models, as exemplified by the Capicua- and Senseless-mediated ATXN1 toxicity discussed earlier[138-140]. In addition, in the fly SBMA model, misappropriation of target gene expression by pathogenic AR (AR-Q52) is suspected to play a major role in its toxicity[125]. As another example, in mammalian cells, HTTex1 can inhibit histone acetyltransferases (HAT) activities of the transcriptional co-activator CREB-binding protein (CBP) and p300/CBP-associated factor (P/CAF), which modulate the accessibility of chromatin to sequence-specific transcription factors, resulting in reduced levels of acetylated histone H3 and H4 in mammalian cells[176, 183, 184]. In *Drosophila*, the robust neurodegeneration of HTTex1-Q48 and HTTex1-Q93 flies can be arrested by manipulating the cellular levels of histone acetylation, either pharmacologically (i.e., feeding with histone deacetyltransferase (HDAC) inhibitors SAHA and butyrate) or genetically. This result not only underscores the *in vivo* importance of transcriptional dysregulation in HD pathogenesis, but also nicely demonstrates the feasibility of using fly models to screen for potential bioactive compounds against polyQ diseases.

Aberrant neurotransmission and calcium homeostasis in HD pathogenesis—Both the Littleton and Botas groups have observed abnormal neurotransmission defects in HD flies. For example, in the giant fiber neuronal circuit, which controls escape response and flight initiation of the adult animals, Htt$^{1-548}$-Q128 but not control Htt$^{1-548}$-Q0 flies show increased neuronal activity[163]. However, given the severe degeneration and axonal blockade phenotypes in Htt$^{1-548}$-Q128 flies, it is not clear whether the observed

electrophysiological defects are a source of toxicity or just a secondary effect downstream of other cellular abnormalities. HTT^{FL}-128Q flies, which develop mild late-onset neurodegeneration, also show abnormal electrophysiologic responses as early as in the third-instar larval NMJ before any apparent neuronal degeneration, showing an aberrantly higher level of resting presynaptic Ca^{2+} levels accompanied with increased neurotransmission release. As these animals do not develop detectable aggregates, evidence of axonal blockade, or translocation of HTT^{FL}-128Q protein to the nucleus even in older animals, these data imply a cytoplasm-derived toxicity distinct from that induced by aggregate formation or impairments in axonal trafficking or transcription[164]. Further, genetic manipulations that blunt synaptic transmission or lower presynaptic Ca^{2+} levels can suppress the electrophysiological defects and neurodegeneration in HTT^{FL}-128Q flies[164]. As aberrant calcium signaling has been observed in HD patients and mouse models[53, 149, 153, 185-187], these findings suggest that abnormal Ca^{2+} homeostasis and Ca^{2+}-dependent neurotransmission release may be early pathogenic events in HD, preceding aggregate formation, axonal blockade, and transcriptional dysregulation.

IV. Identification of genetic modifiers of polyQ diseases using *Drosophila*

With the successful generation of polyQ disease models in *Drosophila*, a wave of studies has followed to identify potential genetic modifiers of polyQ-associated toxicity and aggregate formation. The robust phenotypes manifested in fly polyQ models, especially the prominent eye degeneration and animal lethality, offer a convenient functional readout to test whether the toxicity can be influenced by specific genetic manipulations. In this regard, the easily accessible and assessable adult eye is frequently the tissue-of-choice in modifier screens. For a given fly polyQ model, eye color and external eye morphology can be directly evaluated under a dissection microscope, while the integrity of internal photoreceptor cells can be easily quantified using the corneal pseudopupil technique[111], thus allowing for quick assessment of a large number of candidate modifier genes (examples in Figure 3 and Figure 5).

IV-1. Diverse molecular pathways modulate polyQ toxicity and aggregate formation

Through candidate-based approaches or unbiased forward genetic screens, a large number of genes involved in diverse molecular pathways have been isolated as genetic modifiers of polyQ diseases[122-124, 132, 188-192]. For example, Steffan et al. showed that different posttranslational modifications on HTTex1, such as ubiquitination or small ubiquitin-like modifier (SUMO)ylation, resulted in opposite effects on the pathogenic and biochemical properties of the HTTex1-Q97 protein[193]. These effects could be reversed genetically by manipulating the genes involved in SUMOylation or ubiquitination[193]. Separately, through large-scale mutagenesis screens, Kazemi-Esfarjani and Benzer isolated the chaperone DNAJ1(dHDj1/Hsp40) and other genes as modifiers of Q127 toxicity[132, 189]. Similarly Fernandez-Funez et al. identified SCA1 modifiers with roles in protein folding (DNAJ1), UPS, transcriptional regulation, and RNA processing, among others[124], while Bilen and Bonini isolated 18 modifier genes for SCA3tr-Q78 with functions that converge on protein misfolding[188]. Separately, using an image-based genome-wide RNA interference (RNAi) screen in *Drosophila* S2 cells that stably express eGFP-tagged HTTex1-Q46 (Figure 5A),

Zhang et al. isolated over 100 genes whose depletion modulates aggregate formation by HTTex1-Q46. Functionally, these aggregation modulators are associated with diverse cellular processes such as protein folding (e.g., Hsp110), transcriptional regulation (e.g., Rpd3), signal transduction (e.g., Tor), and others. Among them, several (e.g., DNAJ1, Sin3A, Sec61a) have been isolated previously as toxicity modifiers, suggesting that although aggregates are not directly responsible for toxicity, the misfolding process is intimately associated with toxicity, probably due to the production of intermediate oligomer species. Thus, aggregation of polyQ proteins such as HTTex1 is not only determined by polyQ length and protein context, but also by other cellular and genetic factors. Identification of these factors could allow systematic dissection of the molecular networks governing the formation and toxicity of aggregates.

IV-2. Common and disease-specific disease modifiers

Among the large number of genetic modifiers identified from different fly screens, except for a few common hits (e.g., chaperones, see below), most modifiers of different polyQ diseases do not overlap. For example, one study noted that none of the tested genetic suppressors isolated from other fly polyQ models rescue the lethality of HTT^{1-548}-Q128 flies[163]. To examine whether these modifiers exert similar effects across different polyQ disease models, Branco et al. performed a comparative analysis on SCA1-Q82 and HTT^{1-336}-Q128 models[162]. While many genetic modifiers for SCA1-82Q similarly affect HTT^{1-336}-128Q flies, others show no effect and a few of them even behave in a contradictory manner. As an example, the serine/threonine kinase Akt1 has been shown to act as an enhancer of SCA1-Q82 toxicity, but with HTT^{1-336}-128Q, it behaves as a suppressor. These findings highlight the importance of protein context in polyQ diseases, and also indicate that both common and distinct mechanisms affect their origin and progression.

IV-3. Protein folding machinery in polyQ toxicity

An emerging theme from multiple modifier screens is the convergence on molecular chaperones, which are an important cellular protection mechanism against cellular stress and protein misfolding[194, 195]. Powered by its ATPase activity, Hsp70 chaperones operate through ATP-dependent iterative cycles of substrate binding and release, thereby preventing aggregation of misfolded proteins and promoting their folding to the stable, functional state. Hsf1, a conserved stress-responsive master transcriptional regulator[195], controls the expression of these chaperones. Optimal functionality of Hsp70 depends on its co-factors, Hsp40 and Hsp110, which stimulate Hsp70's ATPase activity and accelerate the exchange of ADP for ATP in Hsp70, respectively, thereby facilitating the chaperone cycle of binding and refolding of sequestered clients[196-198]. Recently, the Hsp40/Hsp70/Hsp110 chaperone triad has been shown to also act as the long-speculated metazoan disaggregase, with the capacity to extract and refold substrates from protein aggregates[199, 200].

In support of the importance of proper protein folding in polyQ toxicity, these chaperones have been independently identified as strong suppressors of polyQ diseases. DNAJ1, the fly homolog of human Hsp40, has been isolated multiple times as a strong suppressor of toxicity caused by different polyQ proteins, and DNAJ1 together with Hsp110 and Hsf1 are

also among the top suppressors of aggregate formation by HTTex1-Q46[122, 124, 132, 166]. Moreover, manipulation of endogenous Hsp110 level, either alone or together with DNAJ1, can significantly affect the neurodegenerative phenotypes of HD flies (Figure 3A-C)[166, 201]. Additionally, over-expression of HspA1L, a human Hsp70 protein, potently rescues the eye degeneration and lethality of SCA3 flies, demonstrating the conserved role of Hsp70 in preventing protein misfolding diseases[121, 130]. Moreover, both HspA1L and endogenous Hsp70 and DNAJ1 proteins are highly enriched in SCA3tr-Q78-positive nuclear aggregates. However, overexpression of these chaperones, either alone or together, cannot alter the onset, size or number of nuclear aggregates, but instead significantly increase the soluble monomeric portion of SCA3tr-Q78, suggesting that these chaperones modulate toxicity by altering the biochemical properties of SCA3tr-Q78[122]. Consistently, a comparative analysis of the reported genetic modifiers of SCA1 and HD models shows that whereas some genetic modifiers can alter the formation of nuclear aggregates, their effect on aggregation does not correlate with their effect on the toxicity of the proteins[162]. Collectively, these results support protein misfolding as the molecular basis of polyQ diseases, although aggregates *per se* might not be the specific agent responsible for the toxicity. Importantly, mammalian-based studies have confirmed the protective effects of Hsp70 and Hsp40 chaperones against polyQ-induced toxicity[202-204]. Exploiting chaperone machineries might be a potentially effective therapeutic strategy against these protein-misfolding diseases.

V. Drosophila facilitates drug discovery and mammalian-based studies

Currently, there are no effective preventive therapies or drugs against polyQ diseases. Increasingly, the established *Drosophila* polyQ models are being employed as convenient *in vivo* tools to facilitate drug design and to prioritize candidate modifiers from mammalian-based screens.

V-1. *Drosophila* in drug discovery

By testing in the established *Drosophila* polyQ models, a growing list of bioactive compounds, from HDAC inhibitors to Lithium, have been shown to be effective in alleviating the toxicity of polyQ-expanded proteins[205-215]. For example, directly feeding Congo red and cystamine, two compounds that are effective in reducing aggregation of mutant HTT in neuronal PC12 cells, to HTTex1-Q48 flies can significantly suppress the eye degeneration and animal lethality[206]. Separately, overexpression of designed suppressor peptides, which can inhibit aggregation of mutant HTT in mammalian COS-1 cells, also inhibits the aggregation and rescues the neurodegenerative phenotypes of Q48 and Q108 flies [216]. Further, in both the fly and mammalian cells, pharmacological stimulation of Hsp70 chaperone promotes the clearance of pathogenic AR and mitigates its toxicity[204].

One promising family of drug candidates are inhibitors of the mechanistic target of rapamycin (mTOR), a master regulator of cellular metabolic pathways and a strong inhibitor of autophagy. Autophagy is a key cellular clearance mechanism against protein aggregates[217-220]. In *Drosophila*, activation of autophagy by inhibiting mTOR genetically or pharmacologically (e.g., mTOR inhibitors rapamycin) markedly suppresses HTTex1-Q120 neurodegeneration[221], a protective effect that has been subsequently confirmed in mouse models of HD, SCA3 and other protein misfolding diseases[221-227]. These results

demonstrate the potential of exploiting autophagy in treating polyQ diseases and validate the fly polyQ models as a convenient *in vivo* tool for drug selection.

V-2. Using *Drosophila* to facilitate mammalian-based studies

A growing number of candidate disease modifiers are being identified from mammalian-based genetic and proteomic studies. For example, HTT alone already has more than 1,000 reported HTT interacting proteins (HIPs)[62, 228-233]. Thus, one pressing challenge is to evaluate the *in vivo* relevance of these candidate modifiers to disease pathogenesis. *Drosophila* polyQ models have proven to be effective for such studies, especially in helping to assess the large number of binding partners and downstream targets of the disease proteins isolated from large-scale screens. For example, fly SCA1 models have provided important *in vivo* evidence in determining the functional importance of several ATXN1 interactors (e.g., Capicua, Senseless and 14-3-3) and AKT signaling in mediating SCA1 neurodegeneration[136-138]. In two recent proteomic studies, fly HD models have been applied to test a selected group of high-confidence mammalian HIPs for their effectiveness in modulating the neuronal dysfunction induced by mutant HTT[230, 232].

VI. Using *Drosophila* to dissect the normal functions of polyQ disease genes

As more evidence link the normal functions of disease genes directly to pathogenesis, a better understanding of these genes' endogenous functions become highly relevant for disease studies[61, 62, 124, 125, 136, 167, 181, 182, 229, 234-238]. Most of the polyQ disease genes are conserved in *Drosophila* (Table 1). Among them, SBMA (AR), SCA6 (CACNA1A) and SCA17 (TBP) genes have well-defined functions[239-242]. Characterization of the fly homologs of other less-understood genes, such as ATXN1, Atrophin-1 and HTT, as briefly summarized below, have helped our understanding of these human diseases.

VI-1. ATXN1 regulates transcription and controls neuronal development and survival

ATXN1 represents an excellent example of using the fly to uncover functional roles for human disease genes and underlying pathogenic mechanisms[136-138]. As descried earlier, both the physical and functional interactions between ATXN1 and its binding partners (e.g., Senseless, Capicua and 14-3-3) and the signaling pathways involved (e.g., 14-3-3 and AKT) are conserved in *Drosophila.* For example, in flies, overexpression of ATXN1 leads to very similar neuronal defects as that induced by its *Drosophila* homolog (dAtx-1), including eye abnormality and bristle loss. Further, dAtx-1 physically interacts with and down-regulates Senseless, a transcription factor required for the development of sensory organs (e.g., bristles) in flies[136]. Importantly, a similar functional interaction is conserved between ATXN1 and vertebrate Senseless homolog Gfi-1, a gene important for the survival of Purkinje cells. In these cases, the functional importance of these interactions in SCA1 pathogenesis have almost always been revealed first using fly model and subsequently validated in mouse SCA1 models or human patient samples, for example decreased expression of Gfi-1 is shown to exacerbate the pathogenesis of SCA1 mice[136]. Thus, fly-based studies, coupled with validation in mammalian systems, have demonstrated that

ATXN1, through its interaction with multiple partners, regulate transcription and neurogenesis that are critical for SCA1 pathogenesis[136-138].

VI-2. Atrophin-1 is a versatile transcriptional co-repressor

Flies carrying mutations for *atro*, the Atrophin-1 homolog, show diverse developmental abnormalities, including excessive neurogenesis, polarity defects, and split thorax. These phenotypes are characteristic of defects in multiple signaling pathways such as Notch, Frizzled/PCP and JNK[139, 243, 244]. During embryogenesis, *atro* mutants show a plethora of patterning phenotypes that reveal Atro's critical role in restricting the boundary of embryo segmentation, a regulation mainly achieved through transcriptional regulation. Most tellingly, *atro* mutants show strong genetic interaction with a bona fide transcription repressor, *even-skipped*, and Atro is essential for its repressive activity. Moreover, when directly tethered to DNA using a reporter assay in *Drosophila* embryos, both Atro as well as wildtype and polyQ-expanded human Atrophin-1 repress transcription *in vivo*[139]. Together, they demonstrate that Atrophin-1 functions as a versatile transcriptional co-repressor in multiple signaling pathways and diverse cellular processes. Subsequent studies in mammalian systems have since validated this finding, showing that Atrophin-1 is a corepressor for nuclear receptors (NRs)[245-247].

VI-3. Characterization of *Drosophila* HTT homolog

HTT, an enigmatic protein—Both human HTT and its *Drosophila* homologue (*dhtt*) encode large proteins (HTT: 3,144a.a.; dHtt: 3,583 a.a.) that contain no obvious functional domains to offer clues of their normal cellular functions[1,248, 249]. Structurally, both are composed of a string of HEAT (Huntingtin, Elongation factor 3, A subunit of protein phosphatase 2A and TOR1) repeats, a 40-a. a. long, anti-parallel helical structural motif of unknown function (Figure 1)[249-252]. Extensive studies since its identification in 1993 have led to many proposed roles for HTT, including endocytosis, transcriptional regulation, trafficking, and cell death, among others. It is now generally believed that HTT acts as a scaffold to integrate inputs from many cellular signals and coordinate cellular responses, although its exact physiological functions remain controversial[61-63, 67, 228, 229, 252].

Mice lacking HTT die in early embryonic stages **(**day E7.5)[253-255]. Surprisingly, flies carrying the null *dhtt* allele *(dhtt-ko)* are homozygous viable and develop normally into adults with no apparent developmental defects. Because flies develop *ex utero*, this phenotypic discrepancy could be attributed partially to the difference in embryogenic processes between flies and mice, in particular their divergent reliance on extraembryonic tissues (*e.g.*, placenta). An elegant study by the Zeitlin group has demonstrated that the early lethality of HTT knockout (KO) mice is largely due to HTT's essential role in extraembryonic tissues, not in the embryo *per se*[256].

Expression of dHtt can rescue a spindle orientation defect observed in HTT-depleted mammalian cells. A similar although milder spindle phenotype has also been observed in *dhtt-ko* flies[257]. Furthermore, resonating with observations from early mammalian studies[258], analysis of *dhtt* null flies has revealed a potential involvement of *dhtt* in epigenetic control, as *dhtt* shows genetic interactions with heterochromatin genes and

components of chromatin remodeling complex and can facilitate the global demethylation of histone H3K4[259]. These findings suggest that HTT has conserved functions in regulating mitotic spindle orientation and epigenetic regulation.

In mice, targeted KO of HTT in postnatal brain or reduced levels of HTT expression both lead to prominent apoptosis, severe brain degeneration and rapid loss of brain volume[142, 260]. Although *dhtt-ko* flies are viable as adults, they have shortened lifespan and an accelerated decline of mobility as they age, accompanied by mild axonal defects in the brain, indicating a role for *dhtt* in neuronal function[249]. Furthermore, *dhttko* flies are vulnerable to additional stresses, showing greatly exacerbated mobility decline, neuronal loss and early death when challenged by the ectopically expressed HTTex1-Q93[249]. Together, these findings demonstrate that HTT has a conserved neuronal protective role and its function is critical for maintaining neuronal viability in higher species. However, little is known exactly how HTT carries out its essential neuroprotective role, and how polyQ expansion affects HTT's normal functions and contributes to disease development.

HTT is a scaffold protein for selective macroautophagy—Using both *Drosophila* and mammalian systems, our group in collaboration with the Cuervo group and independently, the Steffan group recently showed that HTT plays an important role in *selective* autophagy, an important cellular protective mechanism[146, 261]. Macroautophagy is a cellular catabolic process that involves the formation of double-membrane structures called autophagosomes to enclose cytosolic constituents and deliver them into the lysosome for their eventual degradation[262, 263]. Initiation of the autophagy cascade is controlled by a serine/threonine kinase ULK1, and the formation of the autophagosome requires a critical structural component LC3. During starvation stress, a strong autophagic response leads to *nonselective* bulk engulfment of nonessential cellular materials for recycling, although autophagy during starvation may also be selective. Under nutrient-rich and starvation conditions, *selective* autophagy mainly targets specific substrates such as protein aggregates (also called aggrephagy), lipid droplets (lipophagy) and damaged organelles such as mitochondria (mitophagy) and peroxisomes (pexophagy)[262, 263]. Moreover, selective autophagy often involves receptors such as p62/SQSTM1, which bind to both LC3 and ubiquitinated substrates, thereby facilitating their sequestration into the autophagosome for eventual degradation[264-267].

Starting with genetic analyses in *Drosophila* and further characterized biochemically in mammalian systems, we showed that HTT is required for stress-induced selective autophagy. Furthermore, HTT regulates the functions of the kinase ULK1 to control the activation of autophagy in response to stresses, and also modulates the interaction between the autophagy receptor p62 and K63-ubiquitinated substrates. Thus, by acting as a scaffold, HTT orchestrates both autophagic activation and effective sequestration of specific cargos into autophagosomes, thereby achieving efficient autophagic response against stresses[146]. The Steffan group also reported autophagic defects in *dhtt-ko* flies with accumulation of Ref(2)p, the *Drosophila* p62 homolog, in addition to a build-up of p62 in the striatum of HTT-KO mouse brains with aging, extending HTT's selective autophagic relevance to mammals *in vivo*. They also demonstrated physical interaction of HTT with multiple autophagy proteins including ULK1 complex proteins ULK1, FIP200, and mATG13,

mammalian Atg8s GABARAPL1 and LC3B, and mitophagy receptors p62, BNIP3 and NIX, suggesting a role for HTT in mitophagy[261]. Together, these findings demonstrate a conserved role of HTT in an important cellular protective mechanism as a selective autophagic scaffold. Interestingly, more recent studies support the role of HTT in regulating additional aspects of the autophagy pathway, and reciprocally, implicate autophagy in HD pathogenesis[268]. In particular, samples from HD patients and mouse HD models show an "empty autophagosome" phenotype arising from defective cargo recognition[145]. Furthermore, in mice, complete removal of the polyQ stretch from endogenous HTT enhances neuronal autophagy and animal longevity[140]. Combined with our finding that HTT regulates selective autophagy, these observations raise an intriguing possibility that the polyQ expansion in HTT compromises its own cellular protective role, which in turn contributes to HD pathogenesis. A detailed understanding of HTT's roles in coordinating autophagy and other cellular processes will not only provide a comprehensive functional atlas of this large, enigmatic protein, but also help dissect the pathogenic mechanisms underlying HD.

VII. Challenges and Promises

Over the past two decades, *Drosophila* has proven to be a valuable system to model various human neurodegenerative diseases[22, 96-105], as illustrated by the successful creation of these polyQ disease models and by the significant role of the fruit fly in the functional dissection of other brain disease genes such as PD genes *Parkin* and *Pink1*[269-272].

Despite its many successes, there are limitations in utilizing *Drosophila* for studying polyQ and other brain diseases, especially considering the vast differences between flies and humans, including differences in the complexity of the regulatory elements and proteins encoded in their genomes, their developmental process and physiology, brain structures and neurotransmitters utilized, among others. In addition, homologs for some disease genes (e.g., Ataxin-3 and PD gene αSynuclein) are missing in *Drosophila*. Also, because most fly models rely on overexpression of human disease genes, the physiological relevance of the findings from such overexpression studies need to be validated. Thus, it is important to consider these limiting factors when integrating the many lessons learned from the fly for human diseases.

By evaluating the large numbers of fly polyQ models, it also becomes clear that the assay conditions, the tissues targeted, and the protein context of the studied mutations (i.e., polyQ tract) all have major influences on the phenotypic outcomes and the conclusions deduced. This is clearly exemplified in the very different toxicity and aggregating behaviors observed in HTTex1- and HTT^{FL}-based fly HD models[161-166], and between ARtr- and AR^{FL}-based SBMA models[123, 125]. Importantly, studies from other model organisms reach very similar conclusions. For example, the fast-progressing mouse R6/2 model for HD, which is based on overexpression of expanded HTTex1, exhibits rapid neuronal loss but no apparent degeneration of the striatum, the region most affected in HD[273], whereas several genomic HTT^{FL}-based HD models (e.g., YAC72, BACHD and Q175 knockin) show slow but selective degeneration of the striatum accompanied by progressive motor and physiological phenotypes that recapitulate the human disease more closely[53, 274-278]. Considering this, and

given the increasingly appreciated role of these disease genes' native functions in the pathogenic process, it might be preferable to focus the studies on disease models derived from full-length proteins, as such models likely better recapitulate the whole series of pathogenic events that lead to disease. Moreover, in studying animal models of human diseases, it is important to consider the potential influence of different genetic backgrounds, such as the presence of the disease gene homologs (e.g., dHtt and dAtx-1 for HD and SCA1 studies in the fly) and their associated signaling pathways (e.g., the conserved nuclear receptor pathway for SBMA studies using *Drosophila*[125, 279]). For example, HTTex1-Q93 induces stronger toxicity in *dhtt-ko* flies than in wildtype control background[249].

Among future challenges in fly-based studies, one is to evaluate existing disease models and identify the ones most closely resembling the pathogenic events in humans, perhaps by analyzing and comparing the alterations of gene expression profiles, so as to optimize the models to more closely parallel the human disease conditions. It is equally important to take advantage of the power of *Drosophila* genetics and carry out detailed characterization of the endogenous functions of the disease genes. Another challenge is to integrate the findings from fly models with the large number of data sets from mammalian-based studies, so as to pin down the early molecular events most relevant to disease pathogenesis, and to identify the most promising pathways for therapeutic intervention. Given that *Drosophila* is highly amenable to genetic manipulation, aided with a great number of sophisticated experimental tools available in this model organism, these challenges might also become opportunities to make more effective *Drosophila* models for human disease studies in the future.

In perspective, although the genetic cause for polyQ diseases is simple, our understanding of these disorders is still far from complete and their pathogenesis has proven to be far more complicated, as revealed by findings from different model systems including *Drosophila*. Yet, questions compounding the studies on polyQ disorders, including the role of aggregates and the involvement of disease genes' normal cellular functions, are important concerns similarly confronting other genetically complicated diseases such as AD and PD. Currently, there are no disease-modifying therapies available for these brain degenerative disorders. The remarkably faithful recapitulation of pathological features of human brain diseases in *Drosophila* as well as the valuable knowledge learned about the normal functions of disease genes have and will continue to help our pursuit of a clear understanding of the molecular mechanisms underlying these devastating brain diseases, and will ultimately aid our search for targeted and effective therapeutic approaches.

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Highlights

. *Drosophila* models can recapitulate main pathological features of polyQ diseases

. Study on fly disease models reveals important principles regarding pathogenesis

. In addition to polyQ tract, protein context also determines the disease outcome

. Altered native functions of polyQ disease genes can be important pathogenic factor

. *Drosophila* is valuable for dissecting disease genes' native functions

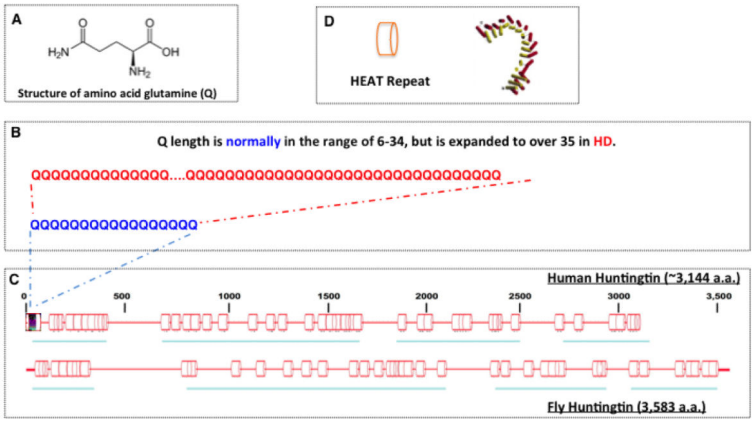

Figure 1. HTT proteins and HD mutation

(**A**) Schematic illustration of the structure of amino acid glutamine (Q), which is encoded by the tri-nucleotide CAG.

(**B**) HD is caused by an abnormal expansion of the glutamine tract (polyQ) located near the N-terminus of HTT protein.

(**C**) Schematics of predicted secondary structures of human and *Drosophila* HTT proteins. Both are composed mainly of HEAT repeat (represented as cylinder boxes in the diagram, also see D).

(**D**) Illustration of the proposed structure of the HEAT repeat, a ~40 amino acid long hairpin-like protein motif.

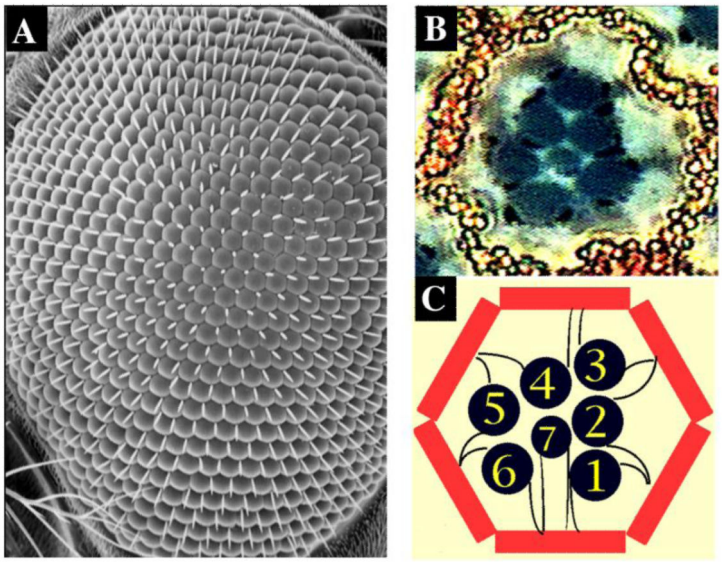

Figure 2. The wildtype *Drosophila* eye structure

(**A**) Scanning electron micrograph of a wild-type adult eye.

(**B**) Tangential section of one ommatidium unit. High-magnification view. Neuronal photoreceptor cells (black) are surrounded by pigment cells (red).

(**C**) Illustration of an ommatidium structure. The identity of each photoreceptor cell (black) is labeled. Pigment cells are painted in red.

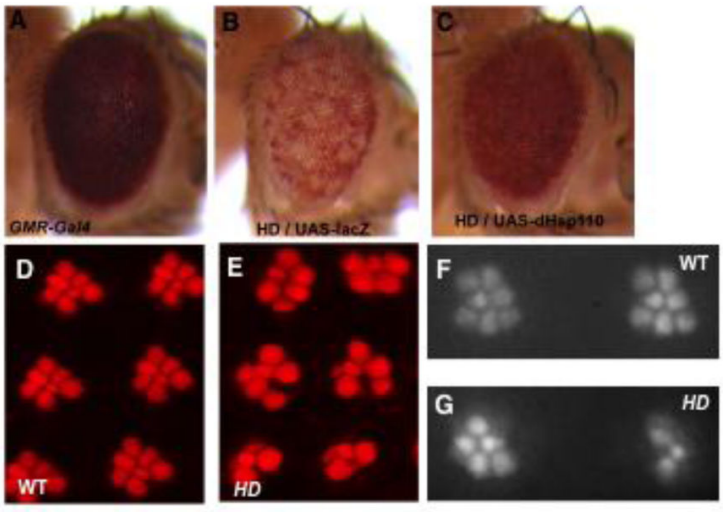

Figure 3. Progressive neurodegeneration in a *Drosophila* HD model and its suppression by a modifier gene

(**A-C**) 30-day-old adult fly eyes. (**A**) GMR-Gal4 control. (**B** and **C**) HD model that expresses HTT exon 1 (HTTex1)-Q93 together with (B) a LacZ control or (C) wildtype dHsp110 protein. Note the dramatic de-pigmentation of the eye in (B), indicating the significant loss of underlying eye tissues, which is clearly suppressed by the co-expression of dHsp110 (C) but not LacZ (B).

(**D-G**) Examination of photoreceptor cells in 7-day-old adult eyes, (**D** and **E**) visualized after dissection and immunofluorescent staining for F-Actin, or (**F** and **G**) visualized directly using pseudopupil technique without dissection. The seven well-organized photoreceptors in (D, F) wildtype (WT) were partially lost in (E, G) HTTex1-Q93 (HD) flies. Note that the pseudopupil method (**F** and **G**) produces comparable resolution for photoreceptor cells in the eye as that obtained by the more tedious dissection and staining approach (**D** and **E**).

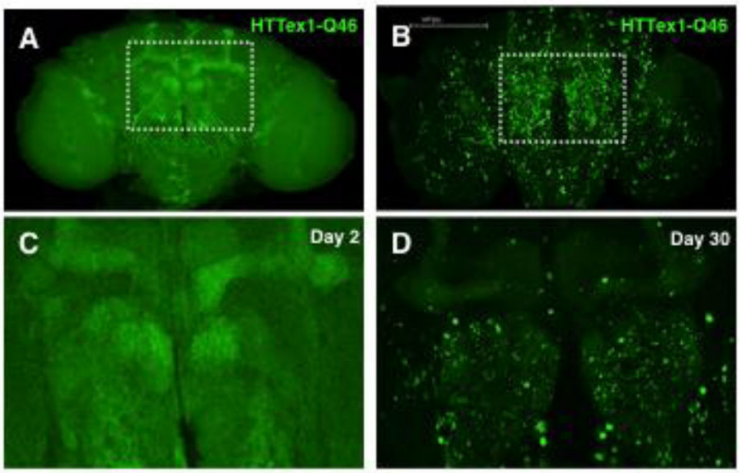

Figure 4. Mutant HTT protein forms age-dependent aggregates in the fly brain

Confocal images of adult brains expressing HTTex1 with a 46 glutamine tract (HTTex1-Q46) at (**A**) day 2 and (**B**) day 30. (**C** and **D**) High-magnification views of the regions highlighted above. HTTex1-Q46 protein is evenly dispersed in mushroom bodies and other structures in young brains but forms prominent aggregates by day 30.

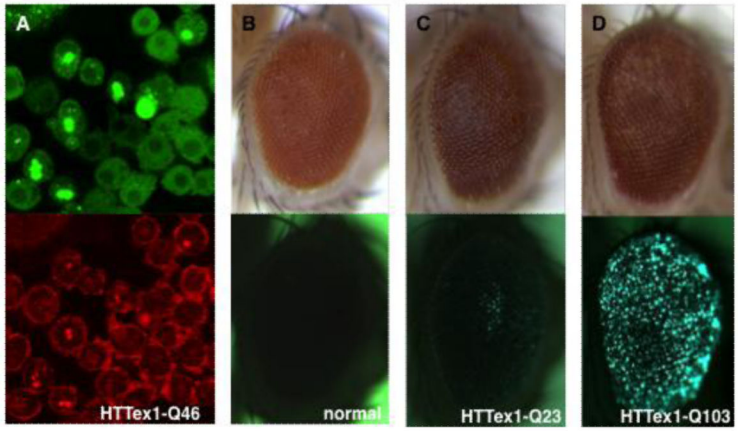

Figure 5. Aggregate formation by mutant HTT in *Drosophila*

Formation of aggregates by mutant HTT protein can be modeled and studied in (A) cultured *Drosophila* cells and (B-D) adult fly eyes. In these studies, mutant HTT exon 1 fragment is revealed by eGFP tag fused in frame at its C-terminus.

(A) A double-labeling image of cultured *Drosophila* cells that express HTTex1-Q46. Aggregates (bright dots in top picture) are evident in some of the cells. The overall morphology of these cells are marked by staining for cytoskeletal protein F-actin (bottom picture in red), which reveals the sequestration of F-actin in these aggregates (bright dots in bottom picture).

(**B-D**) Images of same adult fly eyes illuminated by (top panels) bright light to show the overall eye morphology and by (bottom panels) fluorescent light to reveal the presence of eGFP-label HTTex1 aggregates, respectively.

(B) No fluorescent signal in the eye of a wildtype control fly (normal) that did not express human HTT protein.

(C) No clear aggregates in the eye of a transgenic fly that expressed HTTex1 with 23 glutamine (HTTex1-Q23).

(D) Numerous aggregates (bright dots) in the eye of a transgenic fly that expressed mutant HTTex1 with 103 glutamine (HTTex1-Q103).

Table I

Polyglutamine Diseases

Disease	Gene	*Drosophila* homolog (Annotation symbol)	Transmission	Glutamine repeat size	
				Normal	*Patients*
Dentatorubral-pallidoluysian atrophy (DRPLA)	Atrophin-1 (ATN1)	Atro (also called Grunge)(CG17108)	Autosomal dominant	6-35	49-88
Huntington's disease (HD)	Huntingtin (HTT)	dHtt (CG9995)	Autosomal dominant	6-35	36-121
Spinobulbar muscular atrophy (SBMA, Kennedy disease)	Androgen receptor (AR)	Estrogen-related receptor (CG7404)	X-linked, Dominant	9-36	38-62
Spinocerebellar ataxia type 1 (SCA1)	Ataxin-1 (ATXN1)	Ataxin 1 (CG4547)	Autosomal dominant	6-44	39-82
Spinocerebellar ataxia type 2 (SCA2)	Ataxin-2 (ATXN2)	Ataxin-2 (CG5166)	Autosomal dominant	15-31	36-63
Spinocerebellar ataxia type 3 (SCA3), Machado-Joseph disease, (MJD1)	Ataxin-3 (ATXN3)	-	Autosomal dominant	12-40	55-84
Spinocerebellar ataxia type 6 (SCA6)	a_{1A}-voltage-dependent calcium channel subunit (CACNA1A)	Cacophony (CG43368)	Autosomal dominant	4-18	21-33
Spinocerebellar ataxia type 7 (SCA7)	Ataxin-7 (ATXN7)	-	Autosomal dominant	4-35	37-306
Spinocerebellar ataxia type 17 (SCA17)	TATA-binding protein (TBP)	Tbp (CG9874)	Autosomal dominant	29-42	45-63

Table II

Tissue-specific Gal4 drivers

Promoter (Gal4 driver)	Promoter specificity
GMR-Gal4 (*glass)*	All cells in and posterior to the MF [116]
sevenless-Gal4	R1, R3, R4, R6, R7 and cone cells [280, 281]
eyeless-Gal4	All cells in the eye imagincal disc, beginning early in development, prominently anterior to the MF [114]
elav-Gal4	All central and peripheral nervous system [282-284]
scabrous-Gal4	All central and peripheral nervous system [285]
24B (Gal4)	Presumptive mesoderm and muscle cells [81]
dpp-Gal4	Epithelial cells, along the anterior/posterior border of imaginal discs [286, 287]

* MF, morphogenetic furrow.