Genetic modulation of polyglutamine toxicity by protein conjugation pathways in Drosophila

H.Y. Edwin Chan1,*, John M. Warrick1,*, Isabella Andriola2, Diane Merry2 and Nancy M. Bonini1,*

1Howard Hughes Medical Institute, Department of Biology, University of Pennsylvania, Philadelphia, PA 19104, USA and 2Department of Biochemistry and Molecular Pharmacology, Thomas Jefferson University, 208 Bluemle Life Sciences Building, 233 South 10th Street, Philadelphia, PA 19073, USA

Received July 3, 2002; Revised and Accepted August 16, 2002

Spinal and bulbar muscular atrophy (SBMA) is a heritable neurodegenerative disease caused by the expansion of a polyglutamine [poly(Q)] repeat within the androgen receptor (AR) protein. We studied SBMA in Drosophila using an N-terminal fragment of the human AR protein. Expression of a pathogenic AR protein with an expanded poly(Q) repeat in Drosophila results in nuclear and cytoplasmic inclusion formation, and cellular degeneration, preferentially in neuronal tissues. We have studied the influence of ubiquitin-dependent modification and the proteasome pathway on neural degeneration and AR protein fragment solubility. Compromising the ubiquitin/proteasome pathway enhances degeneration and decreases poly(Q) protein solubility. Our data further suggest that Hsp70 and the proteasome act in an additive manner to modulate neurodegeneration. Through the over-expression of a mutant of the SUMO-1 activating enzyme Uba2, we further show that poly(Q)-induced degeneration is intensified when the cellular SUMO-1 protein conjugation pathway is altered. These data suggest that post-translational protein modification, including the ubiquitin/proteasome and the SUMO-1 pathways, modulate poly(Q) pathogenesis.

INTRODUCTION

Spinal and bulbar muscular atrophy (SBMA) is an inherited, adult-onset neurodegenerative disease caused by expansion of a polyglutamine [poly(Q)] repeat within the androgen receptor (AR) protein (1). Huntington’s disease (HD), dentatorubral–pallidoluysian atrophy (DRPLA), spinocerebellar ataxias 1, 2, 6 and 7 (SCA1, 2, 6 and 7), as well as Machado–Joseph disease (MJD/SCA3) are caused by similar poly(Q) expansions in the respective disease proteins (2–9). These diseases are characterized by the formation of inclusions, typically nuclear inclusions (NIs), of the expanded poly(Q) protein within degenerating neurons. Despite the fact that each of these diseases is associated with poly(Q) expansion, different populations of neurons are affected in each situation (10). Since poly(Q) expansion is the underlying cause of all the diseases, neurotoxicity in each case likely proceeds through partially common molecular pathways.

SBMA has been modeled in transfected cells and mice by expression of an N-terminal fragment of the AR protein with an expanded poly(Q) repeat (11,12). In human cell culture, mouse models, and human patients, the AR protein undergoes proteolysis to smaller fragments (13–15). In patients, the protein, which is normally found within the cytoplasm of cells, now forms inclusions within nuclei (16). Molecular chaperones have been identified in the protein inclusions formed by AR and other poly(Q) proteins (12,17,18), suggesting that protein misfolding contributes to poly(Q) disease. Overexpression of the molecular chaperones Hsp70 and Hsp40 has been shown to suppress AR inclusion formation and apoptosis in transfected cells (17–19). In vivo in Drosophila, the chaperones Hsp70 and Hsp40 have been shown to slow or prevent poly(Q)-induced neurodegeneration (20–23).

NIs in SBMA patients are immunoreactive for ubiquitin (16), a molecular tag for protein degradation, suggesting that targeted protein turnover also plays a role in disease pathogenesis. Consistent with this, components of the proteasome macromolecular protein degradation machinery have been detected in NIs of mammalian cells in culture (17,18,24,25). Also, when expanded poly(Q) proteins are expressed in cultured cells, proteasome activity is affected (26,27). Moreover, inhibition of proteasome activity enhances both AR protein aggregation and toxicity (16), and the proteasome also appears to be required for the degradation of expanded AR protein (18).

In addition to the ubiquitin protein conjugation pathway, other ubiquitin-like (ubl) molecules have been identified.
Although ubl molecules share significant sequence homology to ubiquitin and conjugate through similar enzymatic mechanisms, their cellular functions can be distinct from protein degradation (28,29). For example, one role of the small-ubiquitin-related modifier 1 (SUMO-1) is in subcellular localization of proteins. Similar to ubiquitin, SUMO-1 is processed through, firstly, the E1 Aos1/Ub2 heterodimeric SUMO-1 activating enzyme complex and, secondly, the E2 SUMO-1 conjugating enzyme Ubc9 (30). SUMO-1 modifying enzymes have been shown to localize in the nucleus in Drosophila (31), and SUMO-1 modification has been implicated in nuclear import of protein in flies (32). Additionally, recent work has shown an increase in SUMOylated proteins in human brains affected by poly(Q) disease, as well as a transgenic mouse model expressing mutant ataxin-1 (33). These data suggest that the SUMO-1 system may be involved in poly(Q) disease pathology (33).

Using the truncated form of the androgen receptor protein (ARtr) with a normal or expanded poly(Q) repeat (11,12), we have examined interactions between ubiquitin-dependent proteolysis and Hsp70 in poly(Q) protein toxicity. Our results further suggest a role of a SUMO-1-regulated pathway, in addition to ubiquitin conjugation pathways, in poly(Q) toxicity.

RESULTS

The human AR disease protein induces neurodegeneration in Drosophila

Constructs encoding a truncated form of the AR protein with a normal (Q16) and expanded (Q112) poly(Q) repeat were subcloned into the fly pUAST transformation vector. We then generated transgenic lines bearing the control UAS–ARtrQ16 transgene, and the disease protein UAS–ARtrQ112 transgene. Expression of the transgene was directed to the Drosophila eye with the gmr–GAL4 line, which drives transgene expression in the pigment cells and photoreceptor neurons. The Drosophila eye is a highly regular structure, composed of some 800 individual units, such that any disruption of the structure is markedly apparent. We observed no phenotype upon expression of the control ARtrQ16 protein, such that the eye remained normal externally and internally (Fig. 1A and E). However, expression of the ARtrQ112 protein induced mild degeneration of the external eye and more severe degeneration of the internal eye structure (Fig. 1B, C, F and G). Re-mobilization of UAS–ARtrQ112 transgene yielded a strong expressing transgenic line such that, when outcrossed to gmr–GAL4, a severely degenerate eye phenotype was seen (see Materials and Methods; Fig. 1D and H).

We also directed expression of the AR protein to the entire nervous system using elav–GAL4. When expressed strongly in all cells of the central and peripheral nervous system, the ARtrQ112 protein conferred embryonic lethality. Moderate expression of the ARtrQ112 protein, using the same elav–GAL4 line, yielded adults that displayed an early-death phenotype, dying within 2–5 days after eclosion with poor motility and an irregular tremor. Flies expressing the non-pathogenic ARtrQ16 protein displayed normal viability with a normal lifespan (96% of the flies were still alive after 4 weeks).

These studies indicate that the pathogenic ARtrQ112 protein is toxic when expressed selectively in the eye as well as broadly within the nervous system. Since motor dysfunction is observed in SBMA patients, we also expressed the ARtrQ112 protein in motor neurons using the D42–GAL4 driver (34). Moderate expression of the ARtrQ112 protein selectively in motor neurons conferred a less severe phenotype when compared with pan-neuronal expression with elav–GAL4, with flies starting to die 2 days after eclosion from the pupal case. All flies died within 18 days. In addition to the early-death phenotype, these flies became less active and exhibited an irregular tremor starting at 10 days.

The pathogenic ARtr protein forms inclusions in Drosophila

By western blot analysis, the expanded poly(Q) protein formed SDS-insoluble complexes that remained within the stacking gel, as is typical of expression of the expanded AR protein in transfected cells and transgenic mice (Fig. 2A: lanes 4 and 5). In contrast, the control protein ran only as a monomer (Fig. 2A: lanes 2 and 3). The molecular weights of the control protein and the pathogenic protein of the moderate ARtrQ112 lines corresponded to those of the proteins expressed in COS cells (see Fig. 4: lane 8). The protein expressed in the strong ARtrQ112 transgenic line was slightly lower in molecular weight than in the parental line from which the ARtrQ112 insertion was mobilized, indicating that the poly(Q) repeat underwent a slight contraction in that line. The more severe phenotype observed indicates that the new genomic location of the insertion allowed higher levels of transcript expression. This appears as less protein by western immunoblot, due to greater aggregation of the protein.

By immunocytochemistry, the ARtrQ16 protein showed a diffuse cytoplasmic expression pattern, whereas the protein with an expanded poly(Q) domain formed NIs in eye imaginal discs (Fig. 2B and C). Moreover, by using an antibody that selectively detects the stress-induced form of Drosophila Hsp70 (35), we found that Hsp70 co-localized to the NIs by immunostaining (Fig. 2D and E). These studies suggest that flies bearing the ARtrQ112 protein have upregulated the stress pathway in response to the pathogenic protein. Similar to human disease tissue, NIs formed in flies were also decorated by anti-ubiquitin antibody (Fig. 2F and G), suggesting that the protein in inclusions is misfolded and targeted for degradation by the proteasome.

Effects of molecular chaperones on ARtrQ112-induced neurodegeneration

Hsp70 has been shown to be a potent suppressor of poly(Q) toxicity in several disease models, including SCA3/MJD and HD (20,36). We addressed whether Hsp70 modulated the phenotype caused by the ARtrQ112 poly(Q) disease protein. Upon co-expression of Hsp70 with the ARtrQ112 protein, the external and internal eye phenotypes were restored toward normal (Fig. 3A and F). We also addressed whether inhibition of endogenous levels of the Hsp70 chaperones would affect the phenotype by co-expressing a dominant-negative form of Hsc70, Hsc4.K71S. In this situation, the phenotype was...
enhanced such that the flies showed a severely degenerate eye, whereas in the background of ARtrQ112 alone the eye was only mildly affected (Fig. 3B and G; compare with Fig. 1B and F). These studies indicate that the SBMA phenotype was modulated by the levels of Hsp70-class chaperones, such that overexpression of Hsp70 suppressed degeneration, whereas dominant-negative inhibition of Hsc70 enhanced degeneration. Taken together, these data indicate that Hsp70-like chaperones appear to be universal suppressors of poly(Q) toxicity in vivo.

We also addressed the effect of the chaperones on the solubility properties of the ARtr protein. Normally, upon extraction from fly heads, the protein ran primarily as an SDS-insoluble complex within the stacking gel, with very little protein soluble and running as a monomer (Fig. 4: lane 2). Upon co-expression of Hsp70, an increased amount of the protein was detected as an SDS-soluble monomer (Fig. 4: lane 3). Consistent with soluble monomer levels being inversely correlated with toxicity of the protein, co-expression of the dominant-negative Hsc4.K71S, which enhanced degeneration, resulted in less SDS-soluble monomer (Fig. 4: lane 6).

In SBMA patients, AR immunoreactive inclusions are found in the nucleus (16). Whereas in imaginal eye tissue, the protein formed NIs, consistent with the finding in SBMA patients, ARtrQ112 immunoreactive inclusions were found in both the cytoplasm and the nucleus of adult flies (Fig. 5A), although they were predominantly nuclear. Interestingly, upon Hsp70 co-expression, the frequency of cytoplasmic inclusions appeared to be lower, while nuclear inclusions appeared unchanged (Fig. 5D).

Modulation of degeneration by proteasome activity

We then addressed whether inhibition of proteasome activity would modulate degeneration in vivo. A proteasome mutant, DTS5, has been defined in Drosophila that encodes a dominant-negative protein of the β subunit of the proteasome complex (37). A transgenic line UAS–DTS5 was made encoding this mutant subunit under control of the GAL4–UAS transcriptional system (38). Expression of DTS5 alone in the eye with gmr–GAL4 caused a mild rough eye phenotype and occasional loss of pigmentation in ~20% of the flies, suggesting that there is a modest effect of proteasome inhibition on aspects of eye development.

We then co-expressed DTS5 with the ARtrQ112 protein. Upon co-expression, we found that degeneration induced by the ARtrQ112 protein was enhanced (Fig. 3D and I). This finding indicates that limiting the activity of the proteasome increases toxicity of the disease protein. Analysis of protein extracts revealed that the enhancement of degeneration was associated with a change in the solubility properties of the ARtrQ112 protein: DTS5 co-expression resulted in a reduction in protein monomer levels (Fig. 4: lane 4), presumably reflecting enhanced aggregation of the pathogenic protein.
These results suggest that the interaction is not just an additive effect, but rather a specific enhancement of the toxic properties of the ARtrQ112 protein.

**Interactions between chaperones and the proteasome in poly(Q) pathogenesis**

Given that upregulation of Hsp70 mitigates neurodegeneration, and inhibition of the proteasome enhanced neurodegeneration, we were interested in elucidating the relationship between these two pathways in disease progression. Although Hsp70 is well known biochemically to modulate protein folding, Hsp70 could also be affecting protein turnover by shunting protein through the proteasome-mediated protein degradation pathway (18,39–41). If Hsp70 functioned by increasing protein degradation, then inhibition of proteasome activity would prevent the suppression of toxicity conferred by Hsp70.

We co-expressed both Hsp70 and DTS5 together with the disease protein and analyzed the phenotype. In this situation, the eye phenotype was suppressed (Fig. 3C and H), suggesting that upregulation of molecular chaperone activity is either dominant to or additive with enhancement of toxicity by proteasome inhibition. Analysis of these flies for protein solubility indicated that ARtrQ112 protein monomer was present, although the levels corresponded to an intermediate level between that of Hsp70 expressed alone and Hsp70 co-expressed with DTS5 (Fig. 4: lane 5). These data suggest that the chaperone and proteasome appear to function in an additive manner to modulate degeneration, with both affecting solubility properties of the protein. Co-expression of the dominant-negative Hsc4.K71S with the proteasome mutant DTS5 also showed an additive phenotype, in this case of enhanced toxicity (Fig. 3E and J).

**Effect of SUMO-1, a ubiquitin-like conjugating protein, on poly(Q) toxicity**

Ubiquitin has been found in protein inclusions of most, if not all, poly(Q) diseases (10). Further, we (this report) and others (23) have also demonstrated the involvement of the
We also observed that the effect of Uba2.C175S phenotype, sometimes with loss of pigmentation (Fig. 6A) was variable, affecting the eye of a slightly glossy, rough eye some is mitigated upon co-expression of Hsp70. Flies of genotype w; gmr–GAL4/UAS–HspA1L/UAS–ARtrQ112(M). (B and G) Co-expression with a dominant-negative form of Hsc70 resulted in increased loss of pigmentation and disruption of internal retinal morphology. Flies of genotype w; gmr–GAL4/UAS–HSC4.K71S/UAS–ARtrQ112(M)/+. (C and H) Co-expression of both the proteasome mutant and Hsp70 with ARtrQ112 protein. The loss of pigmentation and disruption of internal eye structure caused by the mutant proteasome is mitigated upon co-expression of Hsp70. Flies of genotype w; gmr–GAL4/UAS–HspA1L; UAS–ARtrQ112(M)/UAS–DTS5–11. (D and I) Co-expression of a mutant proteasome component with the ARtrQ112 protein increased (D) pigmentation loss and (I) disruption of internal eye structure. Fly of genotype w; gmr–GAL4/++; UAS–ArrQ112(M)/UAS–DTS5–11. (E and J) Co-expression of the mutant proteasome and the Hsc70 dominant-negative caused severe pigmentation loss and greatly disrupted internal retinal morphology. Flies of genotype w; gmr–GAL4/UAS–HSC4.K71S; UAS–ARtrQ112(M)/UAS–DTS5–11.

Figure 3. Modification of degeneration induced by the ARtr protein upon co-expression of chaperone and proteasome components. (A and F) Co-expression of Hsp70 suppressed degeneration induced by the ARtrQ112 protein as seen by the restored external (A) and internal (F) eye structures of 1-day-old flies. Flies of genotype w; gmr–GAL4/UAS–HspA1L/UAS–ARtrQ112(M). (B and G) Co-expression with a dominant-negative form of Hsc70 resulted in increased loss of pigmentation and disruption of internal retinal morphology. Flies of genotype w; gmr–GAL4/UAS–HSC4.K71S/UAS–ARtrQ112(M)/+. (C and H) Co-expression of both the proteasome mutant and Hsp70 with ARtrQ112 protein. The loss of pigmentation and disruption of internal eye structure caused by the mutant proteasome is mitigated upon co-expression of Hsp70. Flies of genotype w; gmr–GAL4/UAS–HspA1L; UAS–ARtrQ112(M)/UAS–DTS5–11. (D and I) Co-expression of a mutant proteasome component with the ARtrQ112 protein increased (D) pigmentation loss and (I) disruption of internal eye structure. Fly of genotype w; gmr–GAL4/++; UAS–ArrQ112(M)/UAS–DTS5–11. (E and J) Co-expression of the mutant proteasome and the Hsc70 dominant-negative caused severe pigmentation loss and greatly disrupted internal retinal morphology. Flies of genotype w; gmr–GAL4/UAS–HSC4.K71S; UAS–ARtrQ112(M)/UAS–DTS5–11.

Ubiquitin/proteasome pathway in poly(Q) toxicity in fly models. Ubiquitination typically targets proteins for degradation through the proteasome complex. In addition to ubiquitin, at least four ubiquitin-like (ubl) molecules, including SUMO-1, RUB, Apg12 and Urm1, have been identified and are involved in a larger variety of cellular processes, including nuclear transport (28) and regulation of the heat-shock response (42,43). For example, one characterized substrate of SUMO-1 is Ran–GAP, a GTPase activating protein involved in protein nuclear transport. Mutants in the E2 SUMO-1 conjugating enzyme ubc9 have defects in protein nuclear import in Drosophila (32). Most recently it has been found that there is an increase in SUMOylated proteins in human brain affected by poly(Q) disease and a transgenic mouse model for SCA1 (33). To address whether there was a role of SUMO conjugation in our Drosophila model of a poly(Q) disease, we co-expressed a mutant form of Uba2 with the disease protein. Uba2 is a SUMO-1 activating enzyme (28). An analogous mutation (C177S) in this particular cysteine residue of the yeast Uba2 protein has been demonstrated to be essential for its function (44). Expression of Uba2.C177S by the gmr–GAL4 driver results in pigment cell degeneration at high temperatures (45).

Overexpression of high levels of Uba2.C175S (~4–5-fold of endogenous Uba2 levels) (45) by gmr–GAL4 resulted in a variable effect on the eye of a slightly glossy, rough eye phenotype, sometimes with loss of pigmentation (Fig. 6A) (see also 45). We also observed that the effect of Uba2.C175S is temperature-dependent (data not shown). When Uba2.C175S was co-expressed with ARtrQ112, degeneration was consistently enhanced, as illustrated by severe loss of pigmentation (Fig. 6C), similar to the phenotype induced by the dominant-negative forms of the proteasome α subunit (DTS5) and Hsc70 (Hsc4.K71S). Enhancement of degeneration was only observed when the disease protein with a pathogenic poly(Q) stretch was co-expressed, and not with the control protein with a poly(Q) stretch within the normal range (data not shown). We also addressed the versatility of these findings by confirming similar results with the MJDtr model of neurodegeneration using the ataxin-3 protein (46; and data not shown).

It has been reported that the DNA-binding affinity of heat-shock transcription factors is affected by SUMO-1 modification (42,43). In order to address the relationship between the heat-shock response pathway and SUMO-1 modification, co-expression of Hsp70 and Uba2.C175S was performed in the presence of ARtrQ112. Unlike in the case of Hsp70 and DTS5, Uba2.C175S enhancement was poorly rescued by Hsp70 (Fig. 6D). This suggests that interference in the SUMO-1 pathway appears epistatic to the suppression effects of Hsp70.

DISCUSSION

In this report, we provide genetic evidence for interactions with distinctive suppression pathways of poly(Q) toxicity. Using an
N-terminal fragment of the androgen receptor disease protein (ARtrQ112), we saw late-onset progressive neurodegeneration, as well as locomotor defects conferred by the disease ARtr protein. Further, we demonstrate a role for the proteasome in modulation of the disease phenotype, and this provides in vivo evidence that inhibition of proteasome activity increases protein aggregation and enhances toxicity (17). Our studies further reveal an additive relationship between the Hsp70 chaperone and the proteasome pathways, indicating that compromise of these pathways may represent at least two distinct pathogenetic pathways of poly(Q) toxicity. We also suggest a role for SUMO-1 conjugation in pathogenesis induced by toxic poly(Q) protein.

Subunits of the ubiquitin/proteasome complex have been detected in NIs of several disease proteins (12,17,23,25). In addition, specific inhibition of proteasome activity in cultured cells leads to increased inclusion formation and cell death (17,18). In contrast, in a transgenic mouse model of SCA1, absence of the E6-AP ubiquitin ligase (an enzyme that transfers ubiquitin molecules to proteins that are targeted for degradation through the proteasome pathway) results in formation of fewer nuclear inclusions in Purkinje cells, but a more severe pathology (47). Mutations in a number of different proteins in the ubiquitin and proteasome pathway enhance the rough-eye phenotype of ataxin-1 when expressed in Drosophila (23). In SCA7 patients, immunoreactivity of select proteasome subunits is altered in brain regions affected (48). This suggests that proteasome activity is involved in the metabolism of mutant ataxin-7 protein. We addressed the involvement of the proteasome pathway in poly(Q)-induced neurodegeneration by using a transgene of a dominant-negative mutant form of the J6 proteasome subunit DTS-5 (37). Upon co-expression of DTSS with the pathogenic ARtrQ112 protein, degeneration was strongly enhanced. This suggests that endogenous proteasome activity is normally involved in clearance of the toxic form of pathogenic poly(Q) protein. Analysis of the inclusions has revealed that the molecular chaperones Hsp70 and Hsp40 suppress toxicity in the absence of a large effect on the inclusions (20,21). Here we have found that the poly(Q) protein also forms some cytoplasmic inclusions, visible in the lamina of adult flies where the photoreceptor axon terminals are found. These are lessened upon chaperone co-expression (see Fig. 5).

Interestingly, cytoplasmic inclusions have been noted to correlate more closely with pathology in human disease tissue (49). Our studies have the limitation that only a fragment of the AR protein was used. Studies with the full-length protein may reveal interesting aspects of normal AR protein function, in ligand- and DNA-binding activities, that become modulated by poly(Q) expansion and are specific to SBMA. Such a model may also reveal different and additional modifier pathways.

To address potential crosstalk between pathways regulated by chaperones and the proteasome, we co-expressed Hsp70 and the dominant-negative proteasome subunit transgenes. In this situation, we determined whether Hsp70 could still effect suppression when proteasome activity was compromised. We anticipated that if the effects of Hsp70 were fully dependent upon a functional proteasome pathway, then Hsp70 would not be able to suppress the enhanced degeneration observed upon DTSS co-expression. However, the effect of the proteasome mutant was largely suppressed by Hsp70 (see Fig. 3C and H).

Moreover, we observed an additive, rather than synergistic, effect when both dominant-negative Hsc70 and DTSS mutants were co-expressed with the ARtrQ112 disease protein. Taken together, these results suggest that the Hsp70 pathway and the proteasome pathways interact in an additive manner to modulate poly(Q) protein toxicity. Recently a chaperone-interacting protein, CHIP, has been demonstrated to play a pivotal role in the interactions between the chaperone and ubiquitin/proteasome pathways (40,41). Although this particular kind of crosstalk is only observed for specific proteasome substrates (40), detailed characterization of CHIP and/or other related proteins will be required to determine if similar interactions exist between these pathways in poly(Q) pathogenesis.

Protein conjugation pathways include not only the ubiquitin–proteasomal pathway but also cellular chemistries involving ubiquitin-like molecules. These pathways share similar conjugation mechanisms but have diverse cellular functions (29). We tested the effect of an Uba2 mutant protein in order to address the potential role of the SUMO-1 protein conjugation system in poly(Q) pathogenesis. A number of lines of evidence indicate that Uba2 is specific for SUMO modification. In yeast, overexpression of Uba2 cannot rescue the growth arrest...
phenotype of Uba1 [Uba1 is the ubiquitin activating enzyme in yeast (44)]. In addition, a number of biochemical assays have been performed, but have failed to demonstrate that Uba2 forms thioester linkage with ubiquitin, while under the same reaction conditions Uba1–ubiquitin thioesters were detected (44). We found that interference of the SUMO-1 protein conjugation system appears to enhance poly(Q) degeneration. In the semushi mutant of the E2 SUMO-1 conjugating enzyme Ubc9 in Drosophila (32), nuclear import of bicoid protein is blocked (32). We tested, but could not find, a change in nuclear/cytoplasmic partitioning of the ARtrQ112 protein (H.Y.E. Chan and N.M. Bonini, unpublished observations). SUMO-1 has also been implicated in the regulation of DNA-binding affinity of heat-shock transcription factors, and it is possible that deregulation of global heat-shock response (42,43) as well as other cellular pathways (50,51) contribute to pathogenesis. We were not able to see a consistent effect of the Uba2 mutant protein on Hsp70 levels in the presence of the ARtrQ112 protein (M. Watson and N. Bonini, unpublished observations). By genetic analysis, we revealed an epistatic relationship between SUMO-1 and Hsp70 pathways (see Fig. 6). Although our data suggest a role of SUMO-1 in poly(Q) pathogenesis, it is also possible that the effects that we see are additive, since interference with Uba2 has effects on its own (44). Further investigation is needed to elucidate the relationship of SUMO-1 modification and poly(Q) diseases fully. In addition to Ran–GAP, proteins that undergo SUMO-1 modification have been identified and a number of them reside in the nucleus (28). Consistent with this, SUMO modification enzymes are found in the nucleus (31). As poly(Q) inclusions are also found in the nucleus, it is possible that SUMO modification is affected in poly(Q) diseases (see (32)).

The promyelocytic leukemia protein (PML), a nuclear matrix-associated protein, is modified by SUMO-1 at three major lysine residues in the protein (52). Although SUMO modification of PML has recently been reported to be important for its interaction with a dual-function nuclear protein, Daxx, the subnuclear localization of PML itself does not seem to be affected by SUMO modification (53). Daxx has been shown to be able to mediate Fas-mediated apoptosis (54) and transcriptional repression (53). Daxx interacts with histone deacetylase (HDAC), and administration of an HDAC inhibitor reversed its transcription repression (53). This suggests that histone deacetylation is involved in Daxx repression. In a recently described fly model of Huntington’s disease, progressive degeneration of photoreceptor neurons, as revealed by the optical neutralization technique, was arrested upon administration of HDAC inhibitors (55). It is possible that Daxx may play a role in transcription repression in poly(Q) diseases through SUMO modification of PML. The ataxin-1 protein has already been shown to reside in the nuclear matrix and co-localize with PML in a cell model of SCA1 (56). As SUMO appears to be involved in the regulation of subcellular localization and activities of proteins, it is possible that the phenotypic enhancement of Uba2.C175S that we observed is the result of a number of cellular pathways that have gone awry.

Chaperone-mediated suppression of neural degeneration is associated with an increase in the solubility properties of

Figure 5. ARtrQ112 protein forms both cytoplasmic and nuclear aggregates, and the effect of molecular chaperones in different subcellular compartments. (A–F) Expression of the ARtrQ112 protein in the adult eye. Immunostained adult head section labeled for HA to detect the ARtrQ112 protein in green (A, C, D and F) and nuclei in blue (B, C, E and F). The protein aggregates were detected in both the cytoplasm (arrows) and the nucleus (A and C). Fly of genotype w; gmr–GAL4/+; UAS–ARtrQ112(M)/+. (D–F) Co-expression of the ARtrQ112 protein and Hsp70 in the adult eye. Protein aggregates were detected predominately in the nucleus (D and F). Fly of genotype w; gmr–GALAUAS–HspA1L/UAS–ARtrQ112(M).
poly(Q) disease protein (22). In the present study, the solubility properties of ARtrQ112 also increased under conditions when degeneration was suppressed, indicating that this feature may be general to chaperone suppression of poly(Q) toxicity. Moreover, we also observed that protein solubility decreased when degeneration was enhanced, both upon inhibition of chaperone as well as proteasome activities. Phenotypic rescue of the DTS5 enhancement by Hsp70 also showed a net increase in solubility of the pathogenic ARtrQ112 protein. The amount of monomer in the latter situation was only comparable to the amount of the DTS5 enhancement by Hsp70 also showed a net increase in solubility of the pathogenic ARtrQ112 protein. The amount of monomer in the latter situation was only comparable to the

Our data indicate that both the chaperones and the proteasome have modulatory effects on disease phenotype that are associated with alteration of protein solubility properties. The chaperones presumably modulate the protein structure or protein interactions, potentially restoring or maintaining the protein in a native conformation, which results in a lessening of the toxicity. Although morphological changes of NIs were not observed, partial solubilization of poly(Q) protein, and possibly other recruited cellular proteins, from NIs may be sufficient for attenuation of the CAG repeat domain (ARtrF: 5'-CGA GTG GAT CCA GAA CCC GG-3'; ARtrR: 5'-TGC TGT TCC TCA TCC AGG AC-3'). We remobilized a UAS–ARtrQ112 transgenic insertion by crossing the flies to those bearing P-element transposase activity, and obtained several new transgenic lines that, when outcrossed to gmr–GAL4, showed a severely degenerate eye phenotype [UAS–ARtrQ112(S)]. This line had a poly(Q) contraction of two poly(Q) repeats. The constructs have an HA tag at the N terminus (11).

Microscopy
Fly stocks were aged appropriately, then prepared for sectioning by fixing heads in 2% paraformaldehyde/1% glutaraldehyde, embedded in Epon, then sectioned (~1 μm thick), as previously described (20).

Cytoplasmic and nuclear fractionation
Fractionation was performed using Pierce NE-PER Nuclear and Cytoplasmic Extraction Reagents, and the manufacturer’s instructions were followed. In summary, 26 heads were homogenized in 100 μl of CERI buffer, followed by subsequent addition of 5.5 μl CERI and 50 μl of NER buffers.

Immunocytochemistry and immunoblot analysis
Immunocytochemistry and western blot analysis were performed as described previously (11,20,46). The antibodies

MATERIALS AND METHODS
Fly stocks and transgenic lines
All crosses were carried out at 25°C on standard medium according to standard protocols. Fly lines used include UAS–HspA1L, Hsp70 (20), UAS–Hsc4.K71S (67), UAS–DTS5–11 (38) and UAS–Uba2.C175S(92E) (45). Transgenic flies were made bearing constructs of the AR. The AR constructs (11) were subcloned into the pUAST vector (68). Transformant lines were generated according to standard procedures using w1118 as the parental line. DNA sequencing was performed on PCR products that had been amplified from single fly genomic DNA templates of UAS–ARtrQ112(M) and UAS–ARtrQ112(S) lines. Primers ARtrF and ARtrR were used anneal to amplify and sequence the CAG repeat domain (ARtrF: 5'-CGA GTG GAT CCA GAA CCC GG-3'; ARtrR: 5'-TGC TGT TCC TCA TCC AGG AC-3'). We remobilized a UAS–ARtrQ112 transgenic insertion by crossing the flies to those bearing P-element transposase activity, and obtained several new transgenic lines that, when outcrossed to gmr–GAL4, showed a severely degenerate eye phenotype [UAS–ARtrQ112(S)]. This line had a poly(Q) contraction of two poly(Q) repeats. The constructs have an HA tag at the N terminus (11).

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Adult head cryosections
Fly heads to be cryosectioned were saturated in OCT for 10–15 min, then frozen by immersion in liquid nitrogen. Ten-micrometer sections were cut at −20°C, dried on a slide warmer at 37°C for 30–90 mins, then fixed in 0.5% paraformaldehyde at room temperature for 30 min. The slides were rinsed in PBS, blocked in PBSTG (1% goat serum, 0.2% BSA and 0.01% saponin in PBS) for 1 h at room temperature, and stained with antibodies. The primary antibody was anti-HA (Y11, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:50 concentration in blocking solution overnight at 4°C. The secondary antibody was conjugated to fluorescein (FITC) (Jackson ImmunoResearch), also used at 1:50 concentration in blocking solution for 2 h at room temperature.

Cytoplasmic and nuclear fractionation
Fractionation was performed using Pierce NE-PER Nuclear and Cytoplasmic Extraction Reagents, and the manufacturer’s instructions were followed. In summary, 26 heads were homogenized in 100 μl of CERI buffer, followed by subsequent addition of 5.5 μl CERI and 50 μl of NER buffers.

Immunocytochemistry and immunoblot analysis
Immunocytochemistry and western blot analysis were performed as described previously (11,20,46). The antibodies
used were anti-HA (Y-11, 1:300, Santa Cruz Biotechnology); anti-Hsp70 (35); N20 (anti-AR; 1:1000; Santa Cruz Biotechnology), fluorescein-conjugated anti-rat and cyanine-3-conjugated anti-rabbit secondary antibodies (1:2500; Jackson ImmunoResearch Laboratories, West Grove, PA), and horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2500; Santa Cruz Biotechnology). Images were analyzed by NIH Image version 1.61.

NOTE ADDED IN PROOF
A Drosophila model for SBMA using the full length AR protein has been published, which reveals interesting features of the full length protein (Takeyama et al. (2002) Neuron 35, 855–864).

ACKNOWLEDGEMENTS
We thank Susan Lindquist for Hsp70 antibodies, and Karen Palter, Francois Schweisguth, Leslie Griffith and the Bloomington Stock Center for fly lines. This research was funded, in part, by the Wellcome Trust (H.Y.E.C.), the Wills Foundation and the Age Related Neurodegenerative Diseases Training Program Grant T32AG00255 (J.M.W.), and the NEI Bloomington Stock Center for Drosophila

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