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The deubiquitinase ataxin-3 requires Rad23 and DnaJ-1 for its neuroprotective role in *Drosophila melanogaster*

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Abstract

Ataxin-3 is a deubiquitinase and polyglutamine (polyQ) disease protein with a protective role in *Drosophila melanogaster* models of neurodegeneration. In the fruit fly, wild-type ataxin-3 suppresses toxicity from several polyQ disease proteins, including a pathogenic version of itself that causes spinocerebellar ataxia type 3 and pathogenic huntingtin, which causes Huntington's disease. The molecular partners of ataxin-3 in this protective function are unclear. Here, we report that ataxin-3 requires its direct interaction with the ubiquitin-binding and proteasome-associated protein, Rad23 (known as hHR23A/B in mammals) in order to suppress toxicity from polyQ species in *Drosophila*. According to additional studies, ataxin-3 does not rely on autophagy or the proteasome to suppress polyQ-dependent toxicity in fly eyes. Instead this deubiquitinase, through its interaction with Rad23, leads to increased protein levels of the co-chaperone DnaJ-1 and depends on it to protect against degeneration. Through DnaJ-1, our data connect ataxin-3 and Rad23 to protective processes involved with protein folding rather than increased turnover of toxic polyQ species.

Keywords

Ataxin-3; *Drosophila*; Polyglutamine; Machado; Joseph disease; Chaperone; Ubiquitin; Deubiquitinase

Introduction

Abnormal expansion of a CAG repeat in the protein-coding region of various genes causes at least nine inherited neurodegenerative disorders that are collectively known as polyglutamine (polyQ)-dependent diseases. PolyQ diseases include Huntington's disease

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Conflict of interest statement

The authors declare that they have no competing interests.

(HD), dentatorubral-pallidoluysian atrophy, spinobulbar muscular atrophy, and six spinocerebellar ataxias (SCAs types 1, 2, 3, 6, 7 and 17) (Orr and Zoghbi, 2007). SCA3, which is also known as Machado–Joseph disease (MJD), is caused by abnormal polyQ expansion in the deubiquitinase (DUB) ataxin-3 (Costa Mdo and Paulson, 2012; Matos et al., 2011).

DUBs are proteases that regulate the status of protein ubiquitination. Since the post-translational modification of proteins by ubiquitin regulates numerous cellular processes, the ability of DUBs to fine-tune protein modification in this manner means that they control numerous cellular functions. Many DUBs are required during organismal development and in adults; mutations in these proteases are linked to various diseases, including cancers and neurological disorders (Clague et al., 2013; Ristic et al., 2014; Todi and Paulson, 2011; Tsou et al., 2012).

The DUB activities of ataxin-3 have been largely connected to protein quality control (PQC) (Costa Mdo and Paulson, 2012; Matos et al., 2011). PQC is a set of basic cellular pathways that handle unfolded, short-lived, or toxic proteins by refolding or degrading them via the proteasome or autophagy (Buchberger et al., 2010). During some conditions, ataxin-3 leads to increased proteasomal turnover of abnormally folded proteins, while in others it appears to prevent their degradation by the proteasome (Costa Mdo and Paulson, 2012; Scaglione et al., 2011, 2013; Zhong and Pittman, 2006). There is also evidence that ataxin-3 is involved in regulating gene transcription (Reina et al., 2012).

In mice, ataxin-3 appears to be a non-essential protein based on three independent *atxn3* knockout lines (Reina et al., 2010; Schmitt et al., 2007; Switonski et al., 2011), indicating that this DUB is not required during normal physiological conditions. However, ataxin-3 is important for mammalian cell culture viability during heat stress and H_2O_2-induced toxicity (Reina et al., 2010, 2012; Zhou et al., 2013); recently, ataxin-3 was shown to be involved in the repair of DNA strand breaks (Chatterjee et al., 2015). Additionally, studies by the Bonini lab and later work by us indicated that when expressed exogenously in *Drosophila*, ataxin-3 displays a protective role against toxic versions of different polyQ proteins: ataxin-3 (SCA3), ataxin-1 (SCA1) and huntingtin (HD) (Burr et al., 2014; Tsou et al., 2013; Warrick et al., 2005). These data from cultured cells and fruit flies suggest that this DUB is important during conditions of organismal stress. A protective function for ataxin-3 was suggested in mice (Cemal et al., 2002), although there is also evidence that does not fully align with a neuroprotective role for this DUB in milder murine models of polyQ neurodegeneration (Zeng et al., 2013). In *Drosophila*, ataxin-3 clearly has a suppressive effect against several toxic polyQ proteins, and it is this function that we investigate here.

The molecular sequelae that lead to the protective role of ataxin-3 in *Drosophila* are unclear. Here, we investigate ataxin-3 partners and PQC pathways in an effort to understand how ataxin-3 suppresses polyQ-dependent toxicity in flies. We find that ataxin-3 does not require its reported ubiquitin conjugase or ubiquitin ligase partners for its protective role; also dispensable are autophagy and proteasome function. However, ataxin-3 requires its interaction with the ubiquitin-binding protein Rad23 and depends on the heat shock protein DnaJ-1 to suppress degeneration in flies. Altogether, our results propose a model whereby

ataxin-3 increases DnaJ-1 levels in a manner that depends on the catalytic activity of this DUB and on its interaction with Rad23, and that DnaJ-1, acting downstream of the ataxin-3–Rad23 interaction, suppresses degeneration by decreasing polyQ aggregates.

Results

The interaction of ataxin-3 with VCP is not necessary for its protective role in Drosophila

Others and we previously showed that expression of exogenous ataxin-3 (Fig. 1A) suppresses polyQ-dependent degeneration in *Drosophila* (Burr et al., 2014; Tsou et al., 2013; Warrick et al., 2005). When a toxic polyQ species, comprising the isolated polyQ tract of ataxin-3 with 78 repeats and surrounding amino acids (Fig. 1A; polyQ78), is expressed in fly eyes, it leads to depigmentation of the external part of the retina and nearly complete loss of the radial ommatidial array of the internal eye (Figs. 1B, C; the ommatidium is the functional unit of the compound fly eye). Depigmentation, while consistently pervasive, can vary somewhat from fly to fly. When polyQ78 is expressed in the eye, we also observe the presence of densely-staining proteinaceous aggregates, which contain the toxic protein (Warrick et al., 1998, 2005). Also clear is the detachment of the ommatidial array from the lamina, as highlighted by double-bracketed lines in Fig. 1C.

Expression of wild-type ataxin-3 alongside polyQ78 consistently leads to near-complete suppression of the degenerated retinal phenotype. Externally, the retina appears normal and, internally, the ommatidial array seems unperturbed, similar to eyes that do not express the toxic protein (Figs. 1B, C). Aggregates are absent when wild-type ataxin-3 is expressed with the toxic protein species and the laminal-retinal connection is preserved. This protective effect from ataxin-3 requires its deubiquitinase activity, because a version of this DUB that is catalytically inactive fails to suppress polyQ78-dependent toxicity (Figs. 1B, C).

Since ataxin-3 is linked to PQC-dependent processes in mammalian cell culture, one would reason that this DUB suppresses toxicity by discarding the polyQ78 protein. However, as we reported before (Tsou et al., 2013) and as it is again shown in western blots in Fig. 1D, ataxin-3 protects from polyQ78 without eliminating this protein: we observe abundant polyQ78 protein in the presence of ataxin-3 compared to fly eyes that do not express this DUB (Fig. 1D; also see Fig. 1H). These data led us to wonder how ataxin-3 is suppressing polyQ-dependent toxicity in *Drosophila*.

We began our investigations by examining which of the known direct binding partners of ataxin-3 are required for its protective role. Ataxin-3 binds directly to at least two PQC-related proteins, the AAA ATPase VCP (also known as p97) and Rad23 (orthologous to hHR23A and B in mammals; Fig. 1A). VCP and Rad23 function, in part, as proteasome-binding proteins that regulate protein degradation. First, we focused on VCP.

VCP binds ataxin-3 at an arginine-rich region that precedes its polyQ portion (Fig. 1A). Mutating this region from the amino acid sequence RKRR into HNHH has been shown through in vitro biochemical assays and cell-based immunopurifications to abrogate the interaction of ataxin-3 with VCP (Boeddrich et al., 2006; Doss-Pepe et al., 2003; Morreale et al., 2009; Zhong and Pittman, 2006). We generated new transgenic fly lines of ataxin-3

that express a variant of this protein with a mutated VCP-binding site (RKRR → HNHH). Fig. 1E shows external eye photos of flies expressing ataxin-3 that do not bind VCP. These eyes are structurally normal throughout the life of the fly (Fig. 1E and additional data not shown).

As highlighted in Figs. 1F and G, ataxin-3 with its VCP-binding site mutated (mutation HNHH) suppresses toxicity from polyQ78 similarly to the wild-type version of this DUB. Fig. 1H shows that ataxin-3-HNHH is expressed at levels comparable with the wild-type version of this enzyme (supplemental Fig. 1 summarizes the various ataxin-3 transgenic lines that we generated and their protein levels compared with wild-type ataxin-3 in *Drosophila*). Additionally, suppression by ataxin-3-HNHH is not due to clearance of the polyQ78 protein, similar to the effect from wild-type ataxin-3 (Fig. 1H). We conclude that the direct interaction of ataxin-3 with VCP is not required for the protective role of this DUB in flies.

The interaction of ataxin-3 with Rad23 is required for its protective role in flies

We next examined whether the interaction of ataxin-3 with Rad23 is important for the ability of this DUB to suppress polyQ78-dependent degeneration in flies. We generated fly lines that express ataxin-3 with mutated Rad23 binding (Fig. 1E; eyes expressing this version of ataxin-3 are normal throughout their lifetime), by utilizing two different mutations (W87A, W87K) that disrupt this direct interaction by at least 50%, based on co-immunoprecipitation experiments from cell culture and from in vitro studies (Blount et al., 2014; Nicastro et al., 2009, 2010).

Recently, we reported that Rad23 protects ataxin-3 from proteasomal degradation and showed that relatively high expression of transgenes that encode ataxin-3 with the Rad23-binding site mutated is required to approximate levels of wild-type ataxin-3 protein in flies (Blount et al., 2014). Therefore, for the current studies we wanted to ensure that any phenotypic effects observed from disrupting the ataxin-3–Rad23 interaction were not due to simply lower ataxin-3 protein levels, but resulted from the interruption of the binding of these two proteins. Consequently, we selected lines of ataxin-3-W87A or W87K that express the transgene at high enough levels to approximate protein levels of normal ataxin-3 (Fig. 2A and supplemental Fig. 1).

We found that mutating the Rad23 binding site on ataxin-3 markedly diminishes the protective function of this DUB both externally (Fig. 2B) and internally (Fig. 2C) against polyQ78-dependent degeneration. We confirmed that Rad23 is required for the protective role of ataxin-3 through RNA-interference (RNAi): akin to what we see with ataxin-3-W87A and ataxin-3-W87K, knocking down Rad23 reduces the suppressive role of wild-type ataxin-3 (Fig. 2D). We conclude that the ataxin-3–Rad23 interaction is necessary for its protective role.

Previous results showed that ataxin-3 localizes to polyQ inclusions in fly eyes (Warrick et al., 2005). To examine whether the interaction of ataxin-3 with polyQ78 in vivo is perturbed by disrupting its ability to bind Rad23, we conducted co-immunopurification assays from dissected adult *Drosophila* heads expressing polyQ78 in the absence or presence of various

forms of the DUB. As shown in Fig. 2E, mutating the catalytic cysteine of ataxin-3 (mutation C14A) or its Rad23-binding site does not prevent its co-precipitation with polyQ78. Neither the catalytic activity of ataxin-3, nor its ability to bind Rad23 is necessary for this protease to associate with polyQ78 in vivo. Wild-type ataxin-3 and ataxin-3-W87A/W87K co-precipitate similarly with polyQ78. Interestingly, we observe increased interaction of the catalytically inactive form of ataxin-3 with polyQ78. This higher interaction could result from binding of ataxin-3 to ubiquitinated polyQ78 and an inability to disengage because of the lack of deubiquitinase activity. Indeed, when we examine the ubiquitination status of polyQ78 through a stringent, denature-renature immunoprecipitation protocol (Todi et al., 2007, 2009, 2010), we observe a higher extent of ubiquitination of the polyQ78 smear on western blots in the presence of catalytically inactive ataxin-3, compared with wild-type ataxin-3. Ubiquitination of polyQ78 in the presence of normal ataxin-3 is similar to that in the presence of ataxin-3-W87A (Fig. 2F). This is consistent with previous findings that ataxin-3-W87A and ataxin-3-W87K retain catalytic activity in reconstituted systems in vitro (Nicastro et al., 2010; Todi et al., 2010). The results from Fig. 2F suggest that ataxin-3 regulates the ubiquitination status of polyQ78, and that its ability to bind Rad23 is not required for this particular activity. Collectively, the data in Fig. 2 lead us to conclude that the interaction of ataxin-3 with Rad23 is necessary for the ability of this DUB to suppress polyQ-dependent degeneration in fly eyes, although it is not important for ataxin-3 to interact with polyQ78.

The proteasome and autophagy are dispensable for the protective role of ataxin-3 in Drosophila

Several reports link ataxin-3 to PQC through protein ubiquitination and the proteasome (reviewed in (Costa Mdo and Paulson, 2012); also see (Durcan et al., 2011; Scaglione et al., 2011, 2013)). Reasoning that a protective function of this DUB may rely on its functional interaction with the ubiquitin-proteasome system, we next investigated whether perturbing genes that encode ubiquitin conjugases and ligases that have been reported to function with this protease affects its protective role in flies. Briefly, the process of protein ubiquitination depends on the coordinated action of three sets of enzymes: the E1 (ubiquitin activating enzyme) activates ubiquitin and “transfers” it to the E2 (ubiquitin conjugase) which, in the presence of an E3 (ubiquitin ligase), catalyzes the final step of conjugating ubiquitin to a lysine residue of the substrate protein (Pickart, 2000). Ataxin-3 functions with a handful of ubiquitin conjugases and ligases to regulate the ubiquitination status of various proteins (Burnett et al., 2003; Durcan et al., 2011; Scaglione et al., 2011, 2013; Winborn et al., 2008; Zhong and Pittman, 2006).

We tested whether the following E2s and E3s, which have been reported to functionally interact with ataxin-3 (Costa Mdo and Paulson, 2012), are needed for the protective role of ataxin-3: UbcH5 (effete), Ube2W (CG7220), Chip, Ube4b, Parkin and Hrd1. As shown in Fig. 3A, knockdown of these genes in the presence of ataxin-3 does not perturb the suppressive role of this DUB, indicating that none of these conjugases and ligases is singularly required for ataxin-3′s protective function in *Drosophila*. When comparing polyQ78-dependent degeneration in the absence of ataxin-3, knockdown of UbcH5, Parkin, and Ube4b somewhat suppresses retinal degeneration on their own, suggesting roles for

these proteins opposite of neuroprotection. On the other hand, knockdown of Ube2W, Chip and Hrd1 exacerbates toxicity (denoted by further depigmentation and the presence of necrotic spots) when ataxin-3 is not expressed (Fig. 3A).

Next, we examined whether the activity of the proteasome is important for the protective role of ataxin-3. We utilized both RNAi-dependent knockdown of components of the 19S proteasome (PSMD14 and PSMD7), as well as mutations of catalytic 20S subunits of this degradative machinery. As shown in Fig. 3B, knocking down or mutating specific subunits of the proteasome does not abrogate the protective capacity of ataxin-3 in fly eyes. Ataxin-3 even suppresses lethality caused by the expression of polyQ78 in the presence of dominant negative proteasome subunits. This lethality likely results from low level, ubiquitous read-through of polyQ78 that becomes lethal when the proteasome is compromised. It is not uncommon for lines generated through P-element insertion of UAS-transgenes to show "leakage" expression at very low levels in the absence of a Gal4 driver (Brand and Perrimon, 1993; Brand et al., 1994; Groth et al., 2004; Markstein et al., 2008; Ni et al., 2008). The point remains that ataxin-3 does not absolutely require intact proteasome activity to suppress polyQ78-dependent degeneration in *Drosophila*.

Subsequently, we examined whether autophagy is important for the protective role of ataxin-3. Autophagy has been reported to play an important role in toxic protein-dependent degeneration in various models of disease, and increased autophagy can alleviate toxicity in vivo (Williams et al., 2006). We found that knockdown of none of the autophagy genes that we targeted visibly impacts the protective function of ataxin-3 (Fig. 3C).

It is not entirely surprising that the protective role of ataxin-3 in *Drosophila* does not require a fully functional proteasome or intact autophagy as we observe abundant toxic polyQ78 species in the presence of ataxin-3 (Figs. 1D, H), indicative that the toxic protein is not being eliminated. The absence of visible polyQ aggregates in histological sections when ataxin-3 is expressed directed our attention to the protein folding machinery.

DnaJ-1 is critical for ataxin-3′s neuroprotective role in flies

Aggregated structures, a major histological feature of polyQ78, are largely or completely absent when ataxin-3 is co-expressed with the toxic protein in fly eyes. Together with the other findings that we described above, this observation led us to reason that the chaperone network might be important for the ability of ataxin-3 to suppress polyQ78-dependent degeneration.

Through RNAi-dependent knockdown, we found that the protective function of ataxin-3 is reduced or eliminated by the knockdown of DnaJ-1 (which aligns with human DNAJB1, B4 and B5, at values < e−100 and with > 99% coverage, based on BLASTp results), by the knockdown of CG5001 (which also aligns with human DNAJB1, B4 and B5, at values < e −100 and with > 99% coverage, based on BLASTp results), and by the knockdown of Hsf (heat shock factor, which activates the transcription of inducible heat shock proteins during stress (Becker et al., 1990; Clos et al., 1990; Westwood et al., 1991)) (Fig. 4). Knockdown of other heat shock proteins that we tested does not noticeably diminish the ability of ataxin-3 to suppress toxicity in fly eyes caused by polyQ78 (Fig. 4).

DnaJ-1 is a member of the family of J/HSP40 proteins that function to provide client specificity and to stimulate ATPase activity of HSP70 chaperones (Kampinga and Craig, 2010; Koutras and Braun, 2014). DnaJ-1 suppresses retinal toxicity caused by a mutant fragment of the HD protein, huntingtin (Kazemi-Esfarjani and Benzer, 2000). As a result of these earlier findings and the availability of a transgenic line that expresses DnaJ-1 (Kazemi-Esfarjani and Benzer, 2000), we focused on this co-chaperone for the remainder of this work. We confirmed a protective effect from DnaJ-1 on degeneration caused by polyQ78 in *Drosophila* eyes. As shown in Fig. 5A, expression of DnaJ-1 suppresses toxicity from polyQ78: in the external part of the retina, the depigmentation that is characteristic of the toxicity caused by polyQ78 no longer develops. Internally, we no longer observe the aggregated polyQ species when DnaJ-1 is expressed alongside polyQ78 in fly eyes (Fig. 5B). Similarly to ataxin-3, DnaJ-1 co-expression has a protective effect without eliminating the toxic protein, according to western blots (Fig. 5C). Collectively, the results from Figs. 5A–C indicate that DnaJ-1 suppresses toxicity from polyQ78 in fly eyes in a manner similar to ataxin-3 at morphological, histological and biochemical levels.

Based on data from Figs. 4 and 5A–C, DnaJ-1 is required for ataxin-3-dependent protection in *Drosophila*. Additionally, the protective nature of this member of the HSP40 family has molecular and histological characteristics that are reminiscent of protection from ataxin-3. Since ataxin-3-dependent protection requires Rad23, we queried whether DnaJ-1 also needs Rad23 to suppress polyQ-dependent toxicity. According to RNAi experiments, reducing Rad23 does not perturb the ability of DnaJ-1 to protect from polyQ78 in fly eyes (Fig. 5D). Moreover, expression of catalytically inactive ataxin-3 or the versions of the DUB with disrupted Rad23 binding also does not abolish the protective role of DnaJ-1 (Fig. 5E), placing DnaJ-1 downstream of ataxin-3 and Rad23.

The question now arises: how does ataxin-3 suppress polyQ78-toxicity in a manner that relies on DnaJ-1? We wondered whether ataxin-3 affects DnaJ-1 levels. When we conducted quantitative RT-PCR from fly heads that express polyQ78 in their eyes, we found that wild-type ataxin-3 increases the transcription levels of *DnaJ-1* (Fig. 5F). Catalytically inactive ataxin-3 seems to have a dominant negative effect on *DnaJ-1* levels, and the version of this DUB with mutated Rad23 binding does not lead to higher transcription of the gene that encodes DnaJ-1 (Fig. 5F). qRT-PCR results are mirrored by western blots from dissected fly heads that express polyQ78, where we observed that wild-type ataxin-3 leads to higher levels of endogenous DnaJ-1 protein (Fig. 5G). Catalytically inactive ataxin-3 leads to lower levels of DnaJ-1, while the version with mutated Rad23 binding does not significantly alter the protein levels of this co-chaperone (Fig. 5G).

These findings lead us to propose that ataxin-3, through its interaction with Rad23 and in a manner that depends on its deubiquitinase activity, leads to higher DnaJ-1 protein levels by increasing its transcription. When we express exogenous DnaJ-1, the effect of ataxin-3 on the endogenous gene is superseded by exogenous DnaJ-1, rendering ataxin-3 and Rad23 unnecessary, as supported by data in Figs. 5D and E.

Finally, we examined what DnaJ-1 requires for its neuroprotective role in fly eyes. As shown in Fig. 5H, knockdown of proteasome subunits or proteasome mutations do not

perturb the protective role of this co-chaperone, similar to what we observed with ataxin-3 (Fig. 3). However, the protective role of DnaJ-1 requires inducible heat shock proteins, because knockdown of Hsf diminishes the protective role of this HSP40 protein (Fig. 5I), akin to what we observe with ataxin-3 (Fig. 4). Together, the data in Fig. 5 indicate that ataxin-3 functions through DnaJ-1 and stress-inducible chaperones to reduce the toxicity of polyQ78 in *Drosophila*.

Discussion

The functions of ataxin-3 have largely been ascribed to PQC-dependent processes (Costa Mdo and Paulson, 2012; Matos et al., 2011). Here, we presented evidence that the neuroprotective role of this DUB in *Drosophila* indeed depends on members of the PQC family of proteins, Rad23 and DnaJ-1, but that it does not directly involve the proteasome or autophagy. Based on our collective findings, ataxin-3 suppresses polyQ-dependent toxicity not by helping discard toxic protein species, but by ultimately decreasing aggregates. Versions of ataxin-3 that suppress polyQ78-dependent toxicity are invariably associated with largely or fully absent aggregated structures in histological sections, whereas variants that are unable to suppress degeneration are not. Additionally, ataxin-3 suppresses polyQ-dependent degeneration without eliminating the polyQ species, based on western blots, suggesting that the elimination of the disease-causing agent by the proteasome or through autophagy is not part of the protective aspect of ataxin-3 in *Drosophila*; this conclusion is also supported by genetic experiments. These data are consistent with a role for ataxin-3-dependent protection through polyQ78 protein folding, rather than degradation.

What is the function of ataxin-3 during neuroprotection? Our analyses indicate that the ataxin-3–Rad23 interaction is upstream of DnaJ-1. Based on qRT-PCR and western blotting results, exogenous expression of ataxin-3 leads to increased transcription of *DnaJ-1* and higher DnaJ-1 protein levels in a manner that depends on the site that enables the interaction of this DUB with Rad23 and on its catalytic activity. Together, our findings are indicative of a model in which ataxin-3 suppresses polyQ-dependent degeneration by increasing the levels of DnaJ-1, which in turn functions with stress-inducible chaperones to protect neurons by decreasing polyQ aggregates. A role for ataxin-3 in regulating the transcription of heat shock proteins is not entirely novel, as it has also been described in mammalian cells (Reina et al., 2012). However, a function for the ataxin-3–Rad23 interaction in regulating co-chaperone transcription has not been reported to date. Ataxin-3, with Rad23, could modify the transcription of *DnaJ-1* by interacting with DNA regions that regulate the expression of this gene; there is some evidence that this DUB can regulate gene expression in this manner (Evert et al., 2006), and Rad23 is known to function at the DNA and to regulate DNA-binding proteins (Dantuma et al., 2009). Alternatively, the ataxin-3–Rad23 complex could regulate *DnaJ-1* levels by deubiquitinating transcription factors that control the levels of this gene.

The transcription-dependent model that we propose may be supplanted by another, non-mutually exclusive function of ataxin-3. Our biochemical studies suggest that ataxin-3 interacts with polyQ species in vivo and that it regulates their ubiquitination. Perhaps another activity for ataxin-3 during neuroprotection is one where this DUB assists DnaJ-1

with access to the toxic species. An absence of *Drosophila*-specific antibodies for Rad23 hinders our ability to presently explore interactions among ataxin-3, Rad23, DnaJ-1 and polyQ proteins in the fruit fly.

Lastly, there is some conflicting evidence about the protective power of ataxin-3 in mouse models of degeneration, even though work conducted in mammalian cell culture finds a protective role for ataxin-3 against various forms of cell stress (Chatterjee et al., 2015; Reina et al., 2010, 2012; Zhou et al., 2013). On the one hand, one mouse-based study presented some evidence suggesting that wild-type ataxin-3 may ameliorate toxicity from the pathogenic version of this protein (Cemal et al., 2002). Supporting a protective role for ataxin-3 in mice, additional work found that knocking out *atxn3* exacerbates some behavioral anomalies in a mild model of HD, although lack of ataxin-3 does not appear to have a statistically significant effect on other pathological aspects of this murine model (Zeng et al., 2013). On the other hand, over-expression of normal ataxin-3 in mice did not seem to ameliorate toxicity from its pathogenic version in another set of investigations (Hubener and Riess, 2010). Differences in outcomes from mouse studies could be due to the severity of the polyQ models that were utilized. The fly model used here is strongly toxic, although ataxin-3 also suppresses degeneration from a much milder polyQ model in *Drosophila* (as we reported before (Tsou et al., 2013), and other data not shown). Genetic differences in the mouse models among the different studies may also be a factor. Perhaps definitive studies for a protective role for ataxin-3 in mice will require the generation of catalytically inactive ataxin-3 knock-ins or transgenics to capture its significance, as well as the utilization of polyQ models with varying toxic propensities.

To conclude, the work that we presented here identified critical functional partners for ataxin-3 during neuroprotection in *Drosophila*. Our work also opened the door to the consideration of new molecular complexes that work in PQC. These findings, that ataxin-3 requires chaperones (DnaJ-1 and inducible heat shock proteins) to conduct its protective role in *Drosophila*, and that this DUB and Rad23 link toxic proteins to the folding machinery rather than the proteasome, expand the roles of ataxin-3 and its binding partner into different branches of PQC.

Materials and methods

Antibodies

Anti-ataxin-3 (MJD; rabbit polyclonal, 1:15,000 (Paulson et al., 1997)), anti-ataxin-3 monoclonal (clone 1H9, mouse, 1:500–1:1000; Millipore), anti-DnaJ-1 (a generous gift from Dr. Carl Wu, NIH; rabbit polyclonal, 1:1000), anti-HA (rabbit polyclonal Y11, 1:500; Santa Cruz Biotech), anti-tubulin (mouse monoclonal T5168, 1:10,000; Sigma-Aldrich), peroxidase-conjugated secondary antibodies (goat anti-rabbit and goat anti-mouse, 1:5000; Jackson Immunoresearch). Anti-HA mouse monoclonal antibody conjugated to agarose was from Sigma-Aldrich.

Drosophila procedures and stocks

Drosophila husbandry was conducted at 25 °C and ~60% humidity in regulated diurnal environments, except for prosbeta mutants, in which case the crosses were conducted at 29 °C in diurnal environments at ~60% humidity. Offspring were maintained in the same conditions. New fly lines were generated by cloning ataxin-3 with a normal polyQ of 22 repeats into pUASt (Brand and Perrimon, 1993). Injections were conducted by the Duke University Model System Injection service in the w^{1118} background. All lines used here are in the same background (w^{1118}). Ataxin-3 constructs have a HIS^{6} tag at the C terminus. A complete list of fly lines is provided in supplemental table 1.

Histology

Adult flies aged as indicated in the figure legends had their proboscises and wings removed and were fixed overnight in 2% glutaraldehyde/2% paraformaldehyde in Tris-buffered saline with 0.1% Triton X-100. Fixed adults were then dehydrated in a series of 30, 50, 75 and 100% ethanol and propylene oxide, embedded in Poly/Bed812 (Polysciences) and sectioned at 5 μm. Sections were stained with toluidine blue.

Fly head lysis, immunopurification and western blotting

Ten or fifteen fly heads per group were homogenized in boiling SDS lysis buffer (50 mM Tris pH 6.8, 2% SDS, 10% glycerol and 100 mM dithiothreitol). The choice of this specific buffer was determined in an earlier report, where we established that this particular protocol provided us with the highest quantity of SDS-soluble and SDS-resistant species of the polyQ78 protein (Tsou et al., 2013). Heads were mechanically disrupted by using a pestle and then sonicated for 15 s, boiled for 10 min, centrifuged at top speed at room temperature for 10 min, and loaded onto SDS-PAGE gels. Immunopurification of HA-tagged polyQ78 protein from adult fly heads was conducted by using RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholic-acid, 1% NP40, pH 7.4) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Heads were homogenized in RIPA, sonicated, centrifuged and incubated with bead-bound anti-HA antibody (the tag for polyQ78) for 6 h at 4 °C. Beads were rinsed three times with RIPA and bead-bound proteins were eluted with SDS buffer/heat. Denature/renature immunopurification was conducted as previously described (Todi et al., 2007, 2009, 2010), using RIPA lysis buffer supplemented with a complete protease inhibitor cocktail (Sigma-Aldrich). Heads were homogenized in RIPA and then sonicated, centrifuged briefly and supernatants were subsequently denatured for 30 min at room temperature with 1% SDS and then renatured for 30 min at room temperature with 4.5% Triton-X-100. Lysates were incubated with bead-bound anti HA-antibody for 6 h and beads were next rinsed 8 times with RIPA and eluted with SDS/heat.

For immunopurifications and for data in panels 5F and 5G, we utilized a transgenic line that expresses the same polyQ78 peptide, but at levels that lead to milder degeneration (Warrick et al., 1998). This line also develops retinal aggregates in adults as early as day one and retinal cells degenerate progressively over the course of two weeks, but ommatidia have mostly not disintegrated on day one (Tsou et al., 2013; Warrick et al., 1998). We used the milder polyQ78 line in order to have sufficient retinal tissue with which to conduct IPs and

qRT-PCRs. Wild-type and mutant versions of ataxin-3 behave the same with this milder polyQ78 transgenic as with the more toxic one (data not shown and (Tsou et al., 2013)).

Western blots were developed using a CCD-equipped VersaDoc 5000MP system (Bio-Rad) or PXi (Syngene). Quantification of signals from western blots was conducted with Quantity One Software (Bio-Rad) with global noise reduction and using non-saturated blots as described before (Blount et al., 2012, 2014; Winborn et al., 2008).

Quantitative RT-PCR

Total RNA was extracted from newly-eclosed adult flies using TRIzol reagent (Life Technologies). Extracted RNA was treated with TURBO DNAse (Ambion) to eliminate any contaminating DNA. Reverse transcription was performed with the High-Capacity cDNA Reverse Transcription Kit (ABI), and messenger RNA levels were quantified by using the StepOnePlus Real-Time PCR System with Fast SYBR Green Master Mix (ABI). rp49 was used as internal control. For qRT-PCR, we utilized the milder polyQ78 line, described in the preceding section, in order to have sufficient retinal tissue for RNA extraction and comparison. These same flies were also used for Fig. 5G. For DnaJ-1 we used two different sets of primers, both of which gave the same results. Primers used:

rp49-F: 5′-AGATCGTGAAGAAGCGCACCAAG-3′,

rp49-R: 5′-CACCAGGAACTTCTTGAATCCGG-3′;

ataxin-3-F: 5′-GAATGGCAGAAGGAGGAGTTACTA-3′,

ataxin-3-R: 5′-GACCCGTCAAGAGAGAATTCAAGT-3′.

DnaJ-1-F1: 5′-GTACAAGGAGGAGAAGGTGCTG-3′

DnaJ-1-R1: 5′-CAGACTGATCTGGGCTGTATACTT-3′

DnaJ-1-F2: 5′-TGCGAGAAAGAGACAAAACTCA-3′

DnaJ-1-R2: 5′-ATCTTGTAGAAGTCTTTGCCCATC-3′

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplemantary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2015.05.010.

Abbreviations

Chip	carboxyl terminus of Hsc70-interacting protein
DUB	deubiquitinase
HD	Huntington's disease
IP	immunopurification
MJD	Machado–Joseph disease
PBS	phosphate buffered saline
polyQ	polyglutamine
PQC	protein quality control
RNAi	RNA-interference
SCA	spinocerebellar ataxia
RIPA	radioimmune precipitation
qRT-PCR	quantitative real-time PCR
VCP	valosin-containing protein

References

Becker J, et al. Hydrogen peroxide activates immediate binding of a *Drosophila* factor to DNA heat-shock regulatory element in vivo and in vitro. Eur J Biochem. 1990; 189:553–558. [PubMed: 2351135]

Blount JR, et al. Ubiquitin-specific protease 25 functions in endoplasmic reticulum-associated degradation. PLoS One. 2012; 7:e36542. [PubMed: 22590560]

Blount JR, et al. Ubiquitin-binding site 2 of ataxin-3 prevents its proteasomal degradation by interacting with Rad23. Nat Commun. 2014; 5:4638. [PubMed: 25144244]

Boeddrich A, et al. An arginine/lysine-rich motif is crucial for VCP/p97-mediated modulation of ataxin-3 fibrillogenesis. EMBO J. 2006; 25:1547–1558. [PubMed: 16525503]

Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development. 1993; 118:401–415. [PubMed: 8223268]

Brand AH, et al. Ectopic expression in *Drosophila*. Methods Cell Biol. 1994; 44:635–654. [PubMed: 7707973]

Buchberger A, et al. Protein quality control in the cytosol and the endoplasmic reticulum: brothers in arms. Mol Cell. 2010; 40:238–252. [PubMed: 20965419]

Burnett B, et al. The polyglutamine neurodegenerative protein ataxin-3 binds polyubiquitylated proteins and has ubiquitin protease activity. Hum Mol Genet. 2003; 12:3195–3205. [PubMed: 14559776]

Burr AA, et al. Using membrane-targeted green fluorescent protein to monitor neurotoxic protein-dependent degeneration of *Drosophila* eyes. J Neurosci Res. 2014; 92:1100–1109. [PubMed: 24798551]

Cemal CK, et al. YAC transgenic mice carrying pathological alleles of the MJD1 locus exhibit a mild and slowly progressive cerebellar deficit. Hum Mol Genet. 2002; 11:1075–1094. [PubMed: 11978767]

Chatterjee A, et al. The role of the mammalian DNA end-processing enzyme polynucleotide kinase 3′-phosphatase in spinocerebellar ataxia type 3 pathogenesis. PLoS Genet. 2015; 11:e1004749. [PubMed: 25633985]

Clague MJ, et al. Deubiquitylases from genes to organism. Physiol Rev. 2013; 93:1289–1315. [PubMed: 23899565]

Clos J, et al. Molecular cloning and expression of a hexameric *Drosophila* heat shock factor subject to negative regulation. Cell. 1990; 63:1085–1097. [PubMed: 2257625]

Costa Mdo C, Paulson HL. Toward understanding Machado–Joseph disease. Prog Neurobiol. 2012; 97:239–257. [PubMed: 22133674]

Dantuma NP, et al. The ubiquitin receptor Rad23: at the crossroads of nucleotide excision repair and proteasomal degradation. DNA Repair (Amst). 2009; 8:449–460. [PubMed: 19223247]

Doss-Pepe EW, et al. Ataxin-3 interactions with rad23 and valosin-containing protein and its associations with ubiquitin chains and the proteasome are consistent with a role in ubiquitin-mediated proteolysis. Mol Cell Biol. 2003; 23:6469–6483. [PubMed: 12944474]

Durcan TM, et al. The Machado–Joseph disease-associated mutant form of ataxin-3 regulates parkin ubiquitination and stability. Hum Mol Genet. 2011; 20:141–154. [PubMed: 20940148]

Evert BO, et al. Ataxin-3 represses transcription via chromatin binding, interaction with histone deacetylase 3, and histone deacetylation. J Neurosci. 2006; 26:11474–11486. [PubMed: 17079677]

Groth AC, et al. Construction of transgenic *Drosophila* by using the site-specific integrase from phage phiC31. Genetics. 2004; 166:1775–1782. [PubMed: 15126397]

Hubener J, Riess O. Polyglutamine-induced neurodegeneration in SCA3 is not mitigated by non-expanded ataxin-3: conclusions from double-transgenic mouse models. Neurobiol Dis. 2010; 38:116–124. [PubMed: 20079840]

Kampinga HH, Craig EA. The HSP70 chaperone machinery: J proteins as drivers of functional specificity. Nat Rev Mol Cell Biol. 2010; 11:579–592. [PubMed: 20651708]

Kazemi-Esfarjani P, Benzer S. Genetic suppression of polyglutamine toxicity in *Drosophila*. Science. 2000; 287:1837–1840. [PubMed: 10710314]

Koutras C, Braun JE. J protein mutations and resulting proteostasis collapse. Front Cell Neurosci. 2014; 8:191. [PubMed: 25071450]

Markstein M, et al. Exploiting position effects and the gypsy retrovirus insulator to engineer precisely expressed transgenes. Nat Genet. 2008; 40:476–483. [PubMed: 18311141]

Matos CA, et al. Polyglutamine diseases: the special case of ataxin-3 and Machado-Joseph disease. Prog Neurobiol. 2011; 95:26–48. [PubMed: 21740957]

Morreale G, et al. Evolutionary divergence of valosin-containing protein/cell division cycle protein 48 binding interactions among endoplasmic reticulum-associated degradation proteins. FEBS J. 2009; 276:1208–1220. [PubMed: 19175675]

Ni JQ, et al. Vector and parameters for targeted transgenic RNA interference in *Drosophila melanogaster*. Nat Methods. 2008; 5:49–51. [PubMed: 18084299]

Nicastro G, et al. The solution structure of the Josephin domain of ataxin-3: structural determinants for molecular recognition. Proc Natl Acad Sci U S A. 2005; 102:10493–10498. [PubMed: 16020535]

Nicastro G, et al. Josephin domain of ataxin-3 contains two distinct ubiquitin-binding sites. Biopolymers. 2009; 91:1203–1214. [PubMed: 19382171]

Nicastro G, et al. Understanding the role of the Josephin domain in the PolyUb binding and cleavage properties of ataxin-3. PLoS One. 2010; 5:e12430. [PubMed: 20865150]

Orr HT, Zoghbi HY. Trinucleotide repeat disorders. Annu Rev Neurosci. 2007; 30:575–621. [PubMed: 17417937]

Paulson HL, et al. Intranuclear inclusions of expanded polyglutamine protein in spinocerebellar ataxia type 3. Neuron. 1997; 19:333–344. [PubMed: 9292723]

Pickart CM. Ubiquitin in chains. Trends Biochem Sci. 2000; 25:544–548. [PubMed: 11084366]

Reina CP, et al. Proteotoxic stress increases nuclear localization of ataxin-3. Hum Mol Genet. 2010; 19:235–249. [PubMed: 19843543]

Reina CP, et al. Basal and stress-induced Hsp70 are modulated by ataxin-3. Cell Stress Chaperones. 2012; 17(6):729–742. [PubMed: 22777893]

Ristic G, et al. An optimal ubiquitin-proteasome pathway in the nervous system: the role of deubiquitinating enzymes. Front Mol Neurosci. 2014; 7:72. [PubMed: 25191222]

Scaglione KM, et al. Ube2w and ataxin-3 coordinately regulate the ubiquitin ligase CHIP. Mol Cell. 2011; 43:599–612. [PubMed: 21855799]

Scaglione KM, et al. The ubiquitin-conjugating enzyme (E2) Ube2w ubiquitinates the N terminus of substrates. J Biol Chem. 2013; 288:18784–18788. [PubMed: 23696636]

Schmitt I, et al. Inactivation of the mouse Atxn3 (ataxin-3) gene increases protein ubiquitination. Biochem Biophys Res Commun. 2007; 362:734–739. [PubMed: 17764659]

Switonski PM, et al. Mouse ataxin-3 functional knock-out model. Neruomol Med. 2011; 13:54–65.

Todi SV, Paulson HL. Balancing act: deubiquitinating enzymes in the nervous system. Trends Neurosci. 2011; 34:370–382. [PubMed: 21704388]

Todi SV, et al. Cellular turnover of the polyglutamine disease protein ataxin-3 is regulated by its catalytic activity. J Biol Chem. 2007; 282:29348–29358. [PubMed: 17693639]

Todi SV, et al. Ubiquitination directly enhances activity of the deubiquitinating enzyme ataxin-3. EMBO J. 2009; 28:372–382. [PubMed: 19153604]

Todi SV, et al. Activity and cellular functions of the deubiquitinating enzyme and polyglutamine disease protein ataxin-3 are regulated by ubiquitination at lysine 117. J Biol Chem. 2010; 285:39303–39313. [PubMed: 20943656]

Tsou W-L, et al. Systematic analysis of the physiological importance of deubiquitinating enzymes. PLoS One. 2012; 7:e43112. [PubMed: 22937016]

Tsou WL, et al. Ubiquitination regulates the neuroprotective function of the deubiquitinase ataxin-3 in vivo. J Biol Chem. 2013; 288:34460–34469. [PubMed: 24106274]

Warrick JM, et al. Expanded polyglutamine protein forms nuclear inclusions and causes neural degeneration in *Drosophila*. Cell. 1998; 93:939–949. [PubMed: 9635424]

Warrick JM, et al. Ataxin-3 suppresses polyglutamine neurodegeneration in *Drosophila* by a ubiquitin-associated mechanism. Mol Cell. 2005; 18:37–48. [PubMed: 15808507]

Westwood JT, et al. Stress-induced oligomerization and chromosomal relocalization of heat-shock factor. Nature. 1991; 353:822–827. [PubMed: 1944557]

Williams A, et al. Aggregate-prone proteins are cleared from the cytosol by autophagy: therapeutic implications. Curr Top Dev Biol. 2006; 76:89–101. [PubMed: 17118264]

Winborn BJ, et al. The deubiquitinating enzyme ataxin-3, a polyglutamine disease protein, edits Lys63 linkages in mixed linkage ubiquitin chains. J Biol Chem. 2008; 283:26436–26443. [PubMed: 18599482]

Zeng L, et al. The de-ubiquitinating enzyme ataxin-3 does not modulate disease progression in a knock-in mouse model of Huntington disease. J Huntingtons Dis. 2013; 2:201–215. [PubMed: 24683430]

Zhong X, Pittman RN. Ataxin-3 binds VCP/p97 and regulates retrotranslocation of ERAD substrates. Hum Mol Genet. 2006; 15:2409–2420. [PubMed: 16822850]

Zhou L, et al. Ataxin-3 protects cells against H2O2-induced oxidative stress by enhancing the interaction between Bcl-X(L) and Bax. Neuroscience. 2013; 243:14–21. [PubMed: 23562578]

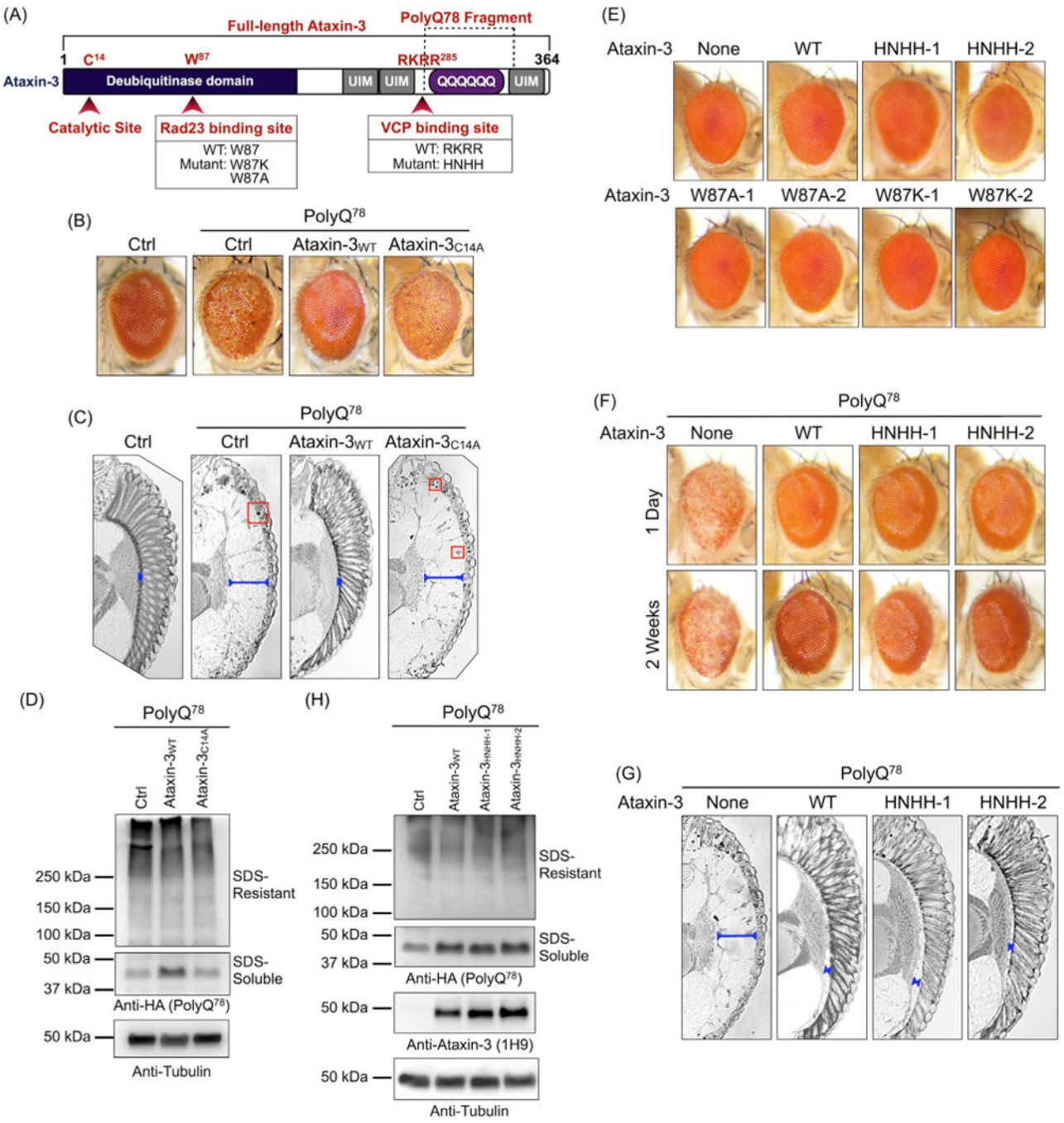

Fig. 1.

The VCP-ataxin-3 interaction is not necessary for protection against polyQ78 in *Drosophila* eyes. A) Diagram of the domain composition of the wild-type ataxin-3 protein. The N-terminal portion contains the catalytic domain. The catalytic cysteine is at position 14. At position 87 is a tryptophan residue that is important for the direct binding of ataxin-3 to Rad23 (Nicastro et al., 2005, 2009, 2010). Following the catalytic domain are ubiquitin-interacting motifs (UIMs) and the polyglutamine (polyQ) region, which causes SCA3 when expanded. Preceding the polyQ tract is the VCP-binding site. Highlighted with dashed lines

is the part of ataxin-3 that was utilized to make the polyQ78 model of degeneration in *Drosophila* using an isoform of ataxin-3 that does not have the third UIM (Warrick et al., 1998). This construct comprises the polyQ fragment of ataxin-3 with a few amino acids preceding it without a functional VCP-binding area (Boeddrich et al., 2006; Warrick et al., 1998) and also includes part of the region following the polyQ tract without the third UIM. The specific mutations used for the Rad23-binding site and the VCP-binding site are noted below the diagram. B, C) External photos (B) and histological sections (C) of fly eyes expressing UAS-polyQ78 through the gmr-Gal4 driver in the absence (Ctrl) or presence of UAS-ataxin-3 wild-type (WT) or catalytically inactive (C14A). All flies were heterozygous for all transgenes (driver and UAS-constructs). Flies were one day old. Boxes: aggregated structures that contain polyQ78 (Warrick et al., 1998, 2005). Bracketed blue lines denote the separation of the lamina from the retina, two structures that are normally juxtaposed. D) Western blots from fifteen adult fly heads per genotype homogenized in boiling SDS lysis buffer. Group denotations are as in panels B and C. Two different species are observed with our homogenization protocol: SDS-soluble and SDS-resistant, as shown in the panel and described before (Tsou et al., 2013). Flies were one day old. E) External eye photos of flies expressing no ataxin-3 (None) or expressing the specified versions of UAS-ataxin-3, driven by gmr-Gal4. Flies were heterozygous for the noted ataxin-3 transgenes and for gmr-Gal4. No polyQ78 was expressed in these flies. Adults were one day old. Numbers: each line was from a different founder. F, G) External eye photos (F) and histological sections (G) from flies expressing UAS-polyQ78 in the absence (None) or presence of the specified versions of UAS-ataxin-3. HNHH-1 and HNHH-2 denote two independent transformant lines of this version of ataxin-3. Flies were heterozygous for gmr-Gal4 and the UAS-transgenes. Flies in histological sections were one day old. H) Western blots from 15 dissected adult fly heads per group homogenized in boiling SDS lysis buffer. Flies were heterozygous for all transgenes and were one day old. Group denotations are as in panels F and G.

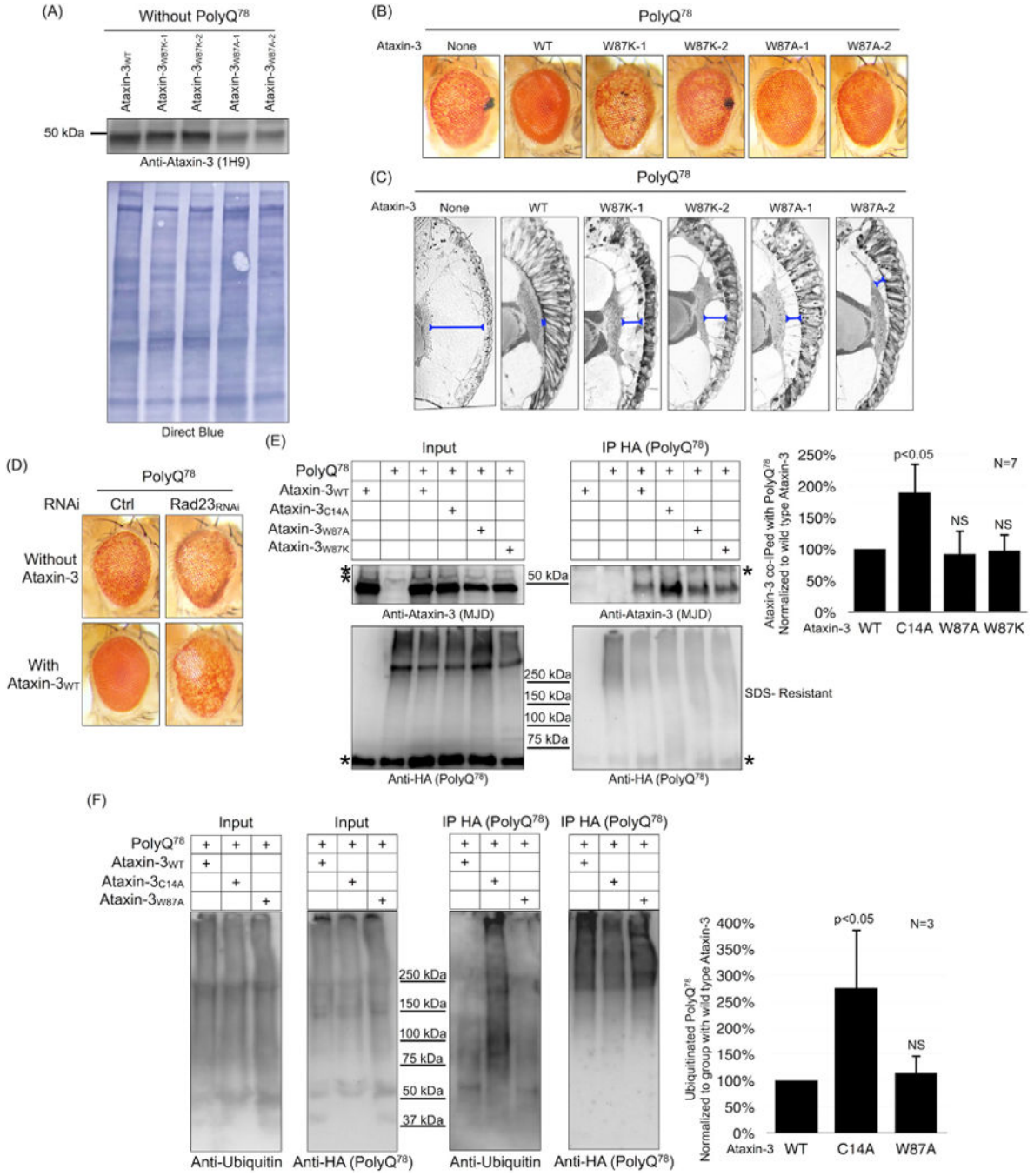

Fig. 2.

The Rad23–ataxin-3 interaction is necessary for protection against polyQ78-dependent degeneration. A) Western blots from 15 dissected adult fly heads per genotype from flies expressing the noted versions of UAS-ataxin-3 through the gmr-Gal4 driver. No polyQ78 was expressed in any of these flies. Direct blue stain shows the total protein transferred onto the PVDF membrane. Flies were one day old and were heterozygous for all transgenes. B, C) External eye photos (B) and histological sections (C) of adult flies expressing UAS-polyQ78 in the absence (None) or presence of the specified versions of UAS-ataxin-3.

Numbers in W87A and W87K denote independent transformant lines. Flies were one day old and heterozygous for all transgenes. Bracketed blue lines denote separation of retinal structures. D) External eye photos of flies expressing polyQ78 in the absence of ataxin-3 without or with UAS-Rad23-RNAi knockdown, and in the presence of wild-type UAS-ataxin-3 without or with Rad23 knockdown. Flies were one day old and heterozygous for all transgenes. Ctrl RNAi: Background for the UAS-Rad23-RNAi line. E) Left: Western blots from immunoprecipitation of polyQ78 from dissected heads of one day old adult flies. Twenty dissected fly heads per group were utilized. Note that homogenization in RIPA buffer does not allow us to visualize the monomeric, SDS-soluble form of the polyQ78 protein shown in previous panels, where heads were homogenized in boiling SDS lysis buffer. With RIPA-based homogenization, we can normally visualize only the SDS-resistant species. Asterisks: non-specific bands. Right: quantification of ataxin-3 signal from blots on the left and other, independent experiments. Loading control for ataxin-3 was the HA-polyQ78 signal from the corresponding IP blots. Signal from ataxin-3 wild-type was set to 100%. P values are from ANOVA with Tukey's post-hoc correction and compare the amount of wild-type ataxin-3 co-precipitated by polyQ78 protein with other versions of the DUB co-precipitated by polyQ78. NS: non-statistically significant. Flies were heterozygous for all transgenes. Inputs and immunopurifications were run on different gels. Please see Materials and methods for additional experimental details. F) Left: Western blots from stringent immunopurification of HA-tagged polyQ78 from dissected fly heads expressing UAS-polyQ78 in the presence of wild-type, catalytically inactive, or Rad23-site mutated UAS-ataxin-3. Driver was gmr-Gal4. Twenty heads per group were used. Right: quantification of signal from ubiquitin smears from IP blots from the left and other, independent experiments. Loading control was HA-polyQ78 signal from the corresponding IP blots. Ubiquitin signal was normalized to that from heads expressing wild-type ataxin-3. P values are from ANOVA with Tukey's post-hoc correction and compare ubiquitin signal in the presence of wild-type ataxin-3 to ubiquitin signal in the presence of the other versions of the DUB. NS: non-statistically significant. Flies were one day old and were heterozygous for all transgenes. Inputs and immunopurifications were run on the same gel; membrane was cropped for organization. Please see Materials and methods for additional experimental details.

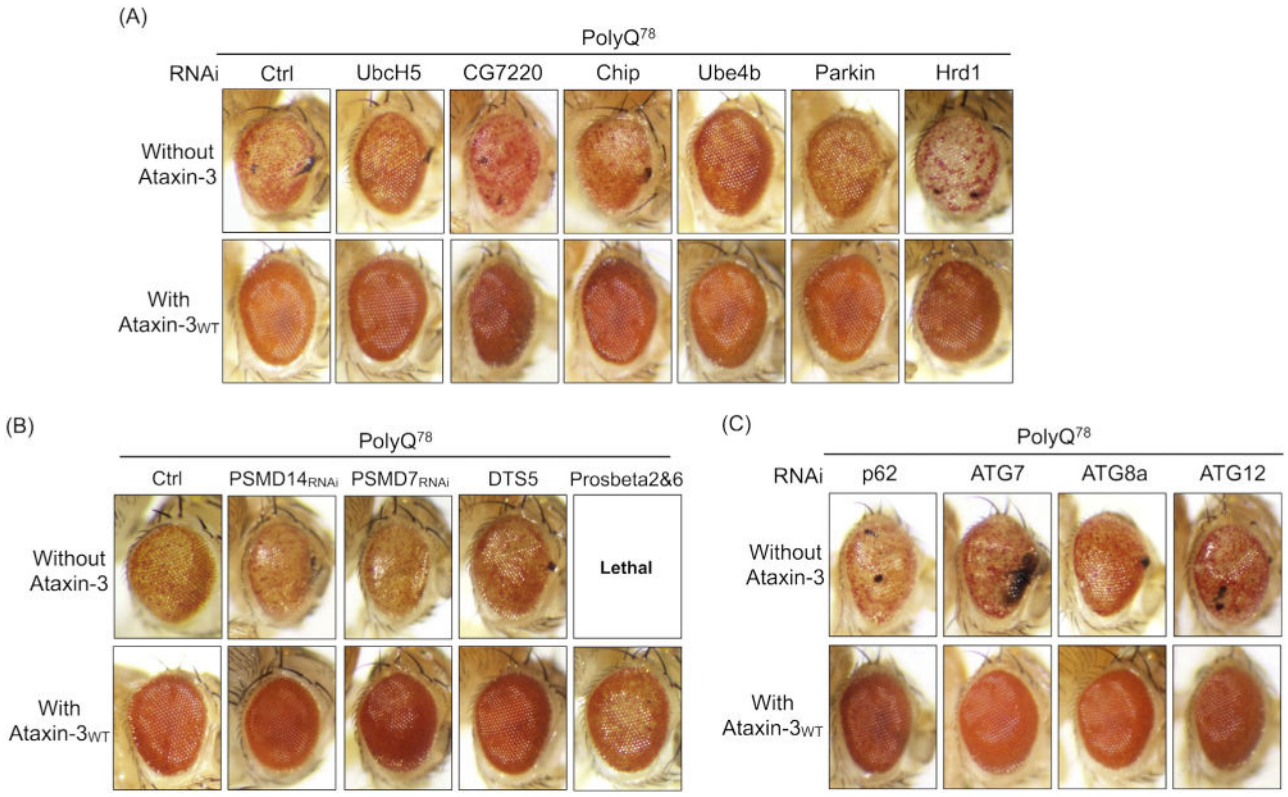

Fig. 3.

Ataxin-3 does not require the proteasome or autophagy to suppress toxicity from polyQ78. A–C) External eye photos from adult flies aged for fourteen days. All fly eyes expressed UAS-polyQ78 driven by gmr-Gal4 either in the absence of UAS-ataxin-3, or in the presence of the wild-type (WT) version of this DUB. All flies were heterozygous for all transgenes. We used two or more UAS-RNAi lines, with similar results. These lines are listed in supplemental Table 1. In panel B, DTS5 is a 20Sβ6 subunit mutation, whereas Prosbeta2&6 is a UAS-driven dominant negative line that expresses mutant proteasome β2 and β6 subunits. Ctrl: isogenic background of modifier lines.

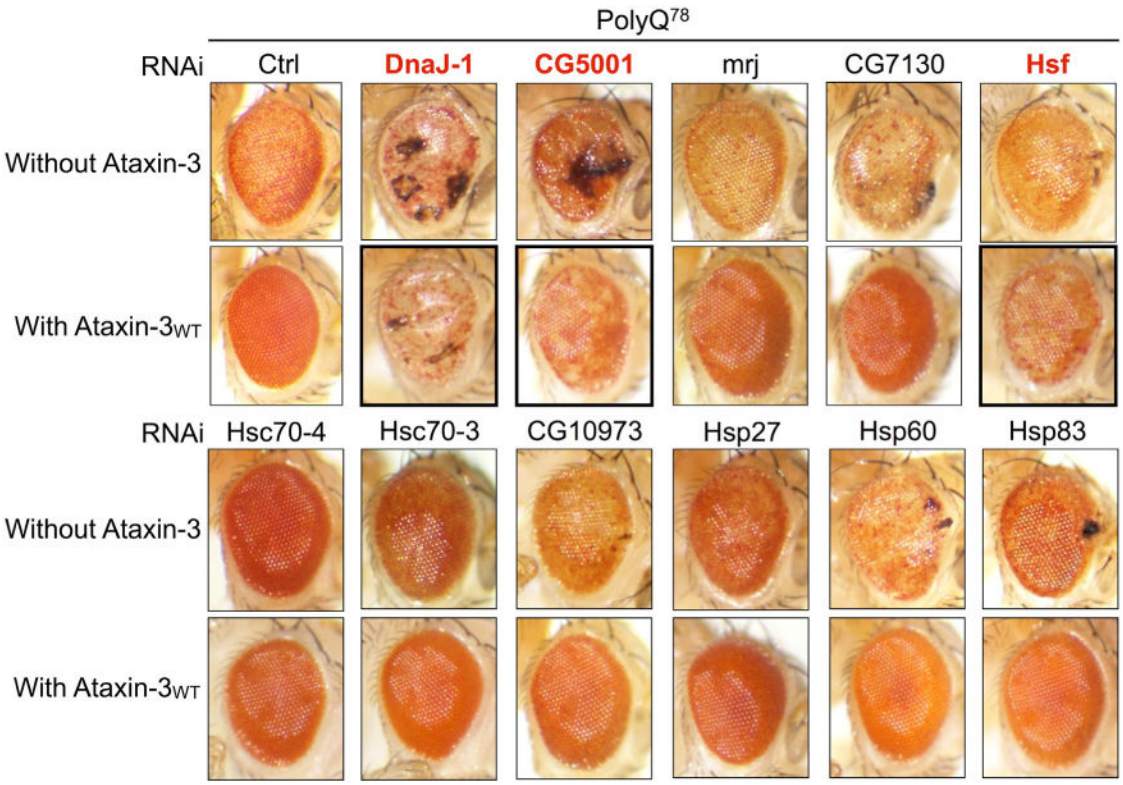

Fig. 4.

DnaJ-1 is required for the protective role of ataxin-3. External eye photos of fourteen-day old adult flies expressing UAS-polyQ78 in the absence or presence of wild-type (WT) UAS-ataxin-3 alongside the noted UAS-RNAi lines or their background control. We used two or more UAS-RNAi lines, with similar results (supplemental Table 1). All flies were heterozygous for all transgenes. Red font and thicker frames highlight factors important for ataxin-3-dependent rescue.

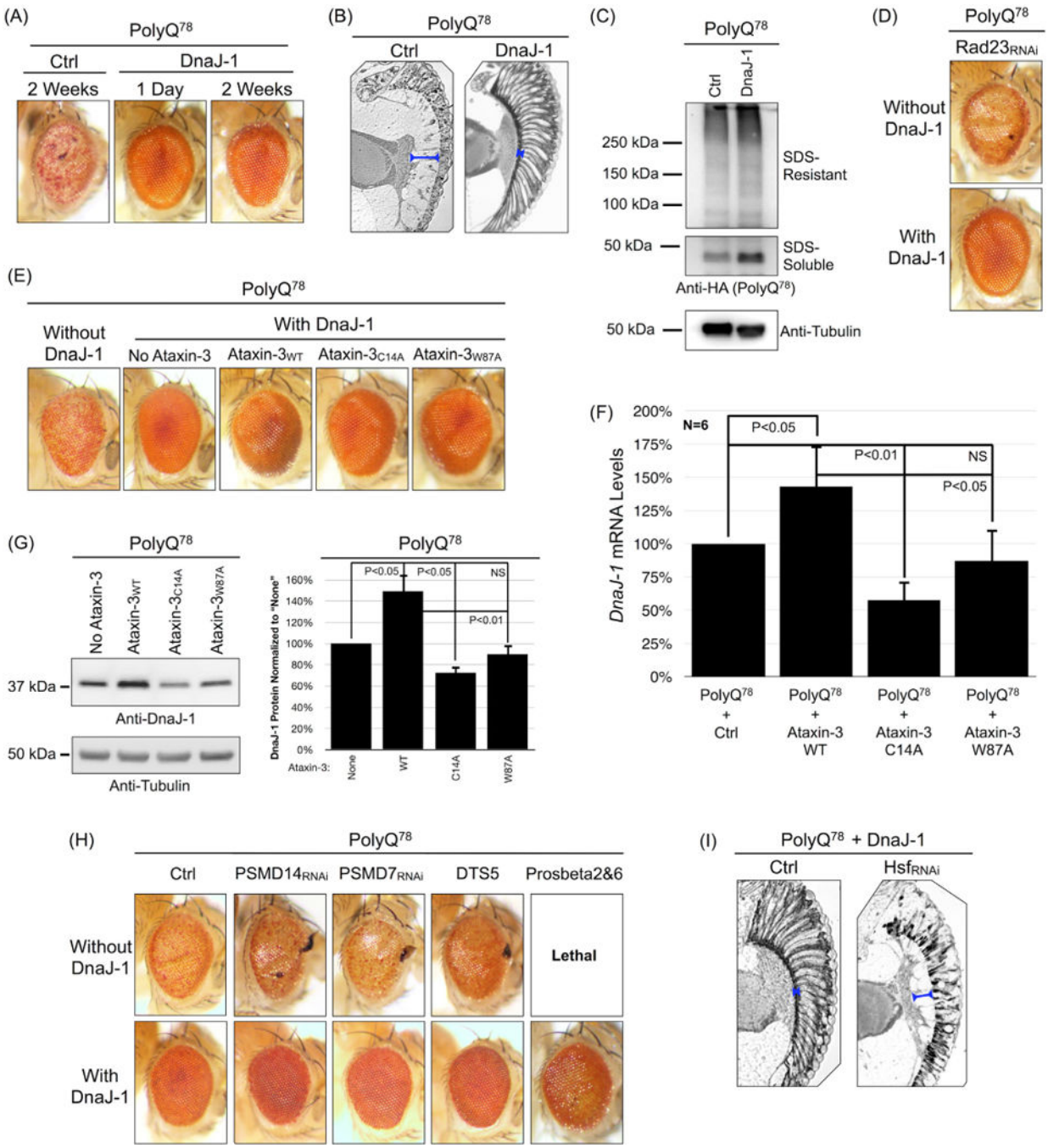

Fig. 5.

DnaJ-1 suppresses polyQ78-dependent degeneration in *Drosophila*. External eye photos (A, D, E, H) and histological sections (B, I) of flies expressing UAS-polyQ78 in the absence or presence of UAS-DnaJ-1. Flies were heterozygous for the gmr-Gal4 driver and UAS-transgenes or chromosomal mutations. Bracketed blue lines denote separation of retinal structures. Ctrl: isogenic background of modifier lines. Flies in panels B and E were one day old, the ones for panels D, H and I were fourteen days old. C) Western blots from 15 dissected fly heads per genotype. Flies were heterozygous for the gmr-Gal4 driver and UAS-

transgenes and were one day old. Ctrl: isogenic background of the modifier. F) qRT-PCR from dissected one day old fly heads expressing the indicated transgenes. Driver: gmr-Gal4. Ctrl: trans heterozygous for polyQ78 and gmr-Gal4 in the isogenic background of ataxin-3 lines. All flies were heterozygous for all transgenes. Shown in histograms are means −/+ standard deviations. Significance results are from ANOVA with Tukey's post-hoc correction. NS: non-statistically significant. Please see Materials and methods for additional experimental details. G) Left: Western blots from dissected fly heads with the same genotype as in panel F, homogenized in boiling SDS lysis buffer. Right: Quantification of DnaJ-1 signal from the left and other independent experiments. Signal from DnaJ-1 antibody was normalized to its own tubulin loading control lane, and normalized DnaJ-1 signal from the "None" lane was set to 100%. P values are from ANOVA with Tukey's post-hoc correction comparing the other lanes to the "None" lane. NS: non-statistically significant. N = 3 independent repeats. For panels F and G, this is most likely an underestimation of changes in endogenous DnaJ-1 levels in fly eyes. Ataxin-3 and polyQ78 expression was restricted to the eyes, but qRT-PCR assays and western blots were conducted using the entire fly head, which includes non-eye tissues where DnaJ-1 levels are not expected to be changed since expression of UAS-transgenes was not targeted to those tissues. For additional details, please see Materials and methods.