Ubiquitination regulates the neuroprotective function of the deubiquitinase ataxin-3 in vivo

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ABSTRACT
Deubiquitinases (DUBs) are proteases that regulate various cellular processes by controlling protein ubiquitination. Cell-based studies indicate that the regulation of DUB activity is important for homeostasis and is achieved by multiple mechanisms, including through their own ubiquitination. However, the physiological significance of DUB ubiquitination to their functions in vivo in intact organisms is unclear. Here, we report that ubiquitination of the DUB ataxin-3 at lysine residue (K) 117, which markedly enhances its protease activity in vitro, is critical for its ability to suppress toxic protein-dependent degeneration in vivo in Drosophila melanogaster. Compared to ataxin-3 with only K117 present, ataxin-3 that does not become ubiquitinated performs significantly less efficiently in suppressing or delaying the onset of toxic protein-dependent degeneration in flies.

INTRODUCTION
Diverse cellular pathways are regulated by the post-translational modification of proteins with ubiquitin (Ub), which can control substrate protein localization, interaction with protein partners, or turnover. Conjugation of Ub to specific substrates depends on the coordinated action of three proteins: the Ub-activating enzyme (E1) transfers Ub to an E2 Ub-conjugating enzyme which then, in the presence of an E3 (Ub-ligase), completes the transfer of Ub to a lysine residue of a substrate protein through an isopeptide bond. A substrate can be mono- or poly-ubiquitinated and different types of Ub-Ub linkages can be formed on proteins with various outcomes. For example, mono-Ub of a membrane receptor can signal its internalization, while certain poly-Ub chains signal the degradation of a protein by the proteasome (Reviewed in (1)).

Protein ubiquitination can be reversed by deubiquitinating enzymes (DUBs). DUBs are key players in various cellular processes and several of them are linked to malignancies and neurological diseases (1-5). In fact, the regulation of DUB activity is being considered as an entry point into therapeutics for several human diseases (2,4,6-8).
Despite progress in understanding the functions of DUBs at the structural and cellular level, general understanding of DUB physiology, particularly \textit{in vivo}, is limited.

As with other classes of enzymes, the functions of DUBs can be regulated at several levels, such as gene transcription, protein turnover, phosphorylation, as well as by their own ubiquitination (3,9). A growing number of DUBs are known to become ubiquitinated, including ataxin-3, JosD1, USP4, USP6, USP7, USP25, USP28, USP37 and UchL1 ((9-12) and our unpublished observations). Of these, ubiquitination of ataxin-3 and JosD1 enhances their catalytic activity \textit{in vitro} (10,11,13), while ubiquitination of UchL1 and USP25 is proposed to decrease (UchL1) or increase (USP25) their cellular activities (14,15). In light of increasing evidence of E3 ligase/DUB interactions (12), regulation of DUB functions by ubiquitination likely applies broadly to DUBs.

Although \textit{in vitro} biochemical and cell biological studies provide compelling evidence for the control of DUB activities by their own ubiquitination (9), the biological relevance of this type of regulation \textit{in vivo} in intact organisms is presently uncertain. To begin to understand the physiological importance of DUB ubiquitination for their \textit{in vivo} functions, we set a defined goal: to determine whether ubiquitination-dependent regulation of the catalytic activity of the DUB ataxin-3 is important for its protective function \textit{in vivo}.

Ataxin-3 (Figure 1A) has been implicated in regulating protein stability in quality control pathways and has also been reported to regulate transcriptional events (16); still, its exact physiological functions are not well understood. An important feature of ataxin-3 is its polyglutamine (polyQ) region that, when abnormally expanded, causes age-related neurodegeneration in the polyQ-repeat disorder Spinocerebellar Ataxia Type 3 (SCA3, also known as Machado-Joseph Disease) through a mechanism that remains to be elucidated (16).

Elegant studies conducted by the Bonini lab showed that ataxin-3 serves a neuroprotective role in \textit{Drosophila melanogaster} by suppressing degeneration caused by several polyQ disease proteins, including its own self (SCA3), huntingtin (Huntington’s Disease) and ataxin-1 (SCA1) (17). PolyQ-repeat diseases are neurodegenerative disorders caused by abnormal expansion of the polyQ tract in otherwise unrelated genes (18,19). Understanding how ataxin-3 operates and is regulated \textit{in vivo} is therefore important for both neurodegenerative and neuroprotective processes. Here, we present evidence that ubiquitination of this DUB is critical for its neuroprotective role \textit{in vivo}.

\textbf{EXPERIMENTAL PROCEDURES}

\textit{Constructs} — All of the ataxin-3 constructs used in this report are the full-length human version of the protein and contain a normal polyQ stretch of 22 repeats. Ataxin-3 used in recombinant assays is in pGEX6P1 and was cleaved from GST by using PreScission Protease (GE Healthcare). Ataxin-3 that was cloned into pUAST and was used to generate transgenic fly lines has a 6HIS tag appended at the C-terminal end of the protein, immediately succeeding the last native amino acid. Where indicated, lysine residues were mutated into the similar, but non-ubiquitinatable, amino acid arginine by using the QuickChange Mutagenesis kit (Stratagene) (13). CHIP and Ubch5C constructs have been described before (11,13,20).

\textit{In vitro reagents and reactions} — Recombinant protein purification from BL21 bacterial cultures (Ubch5C, CHIP, ataxin-3) was conducted using standard techniques that have been detailed previously (11,13,21). Recombinant E1 was purchased from Boston Biochem. \textit{In vitro} ubiquitination of ataxin-3 was performed with recombinant proteins as described before (11,13,20). Briefly, GST-tagged recombinant ataxin-3 was combined with E1, Ubch5C (E2), CHIP (E3), Ub and ATP/MgCl\textsubscript{2} for 90 minutes at 37°C in kinase reaction buffer (50mM Tris, pH 7.5, 50mM KCl, 0.2mM DTT). GST-ataxin-3 was then isolated from the other reaction components by using Glutathione Sepharose beads, and ataxin-3 was eluted from beads by cleaving its GST tag with PreScission Protease. Unmodified ataxin-3 used in DUB reactions underwent the same procedure, but in the absence of ATP/MgCl\textsubscript{2}. DUB reactions have also been described before (11,13,20), and were conducted with untagged ataxin-3 (200 nM) that was either unmodified or had been ubiquitinated by CHIP as summarized above. Ataxin-3 was combined with penta-Ub K63-linked chains (1µM; Boston Biochem) at 37°C in DUB reaction buffer (50mM HEPES, 0.5mM EDTA, 1mM DTT, 0.1mg/ml ovalbumin, pH 7.5) and fractions were collected at predetermined time points in boiling 2% SDS buffer.
supplemented with 100mM DTT. For semi-quantification analyses of DUB reactions, Ub products were quantitated as follows: (Ub4 + Ub3 + Ub2 + Ub1)/(Ub5 + Ub4 + Ub3 + Ub2 + Ub1). SDS-PAGE electrophoresis using 4%-20% gradient gels or 15% gels, western blotting, CCD imaging of western blots and quantification of sub-saturated western blots (VersaDoc 5000MP and Quantity One software (Bio-Rad)) were conducted using standard techniques and were detailed before (11,13,21-23).

_Drosophila_ lines and procedures — _Drosophila_ maintenance and husbandry was conducted in diurnally-controlled environmental chambers at 25˚C (24-26). _Drosophila_ transgenics were created using the Gal4-UAS system. Ataxin-3 constructs were cloned into the pUAST vector (27). Injection of pUAS constructs into the parental w1118 line was performed by Duke University Model Systems. For histology, whole flies with removed proboscises were fixed in 2% glutaraldehyde/2% paraformaldehyde in Tris-Buffered Saline overnight. Fixed flies were then dehydrated in a series of 30%, 50%, 75% and 100% ethanol and propylene oxide, embedded in Poly/Bed812 (Polysciences) and sectioned at 5µm. Sections were stained with toluidine blue, as described previously (17,28). UAS-PolyQ78Severe (originally MJD.tr-Q78-c211.2), UAS-PolyQ78Mild (originally MJD.tr-Q78-c37.3), UAS-CHIP-RNAi lines, gmr-Gal4 and other common stocks were from the Bloomington _Drosophila_ Resource Center. The genotypes of all of the flies shown in figures are listed in table 1. For western blotting, whole flies or dissected fly heads that had been frozen were homogenized with a dounce homogenizer in boiling 2% SDS lysis buffer (50mM Tris pH 6.8, 2% SDS, 10% glycerol, 100mM DTT), sonicated briefly, boiled for 10 minutes, centrifuged (or not) at 13,000XG for 10 minutes at room temperature, and supernatants were loaded into SDS PAGE gels (26). We used 50µL lysis buffer per whole fly or 15µL buffer per dissected head. For extraction using Triton-X-100, the procedure was based on reference (29). Triton-X-100 buffer: 50mM Tris pH 7.5, 1% triton-X-100, 1mM EDTA, complete protease inhibitor cocktail (Sigma-Aldrich). Ten dissected fly heads per sample were homogenized, sonicated, and then centrifuged at 75,000XG (4˚C) for 30 minutes. Supernatant and pellet were separated. Pellet was dissolved in 2% SDS/100mM DTT and boiled for 10 minutes. Urea/SDS extraction and procedure was based on reference (30). Extraction buffer: 8M Urea, 200mM Tris pH 7.5, 1mM EDTA, 5% SDS. Ten dissected fly heads per sample were homogenized, sonicated and heated at 65˚C for 10 minutes. One sample was centrifuged (13,000XG at room temperature for 30 minutes), the other sample was not; supernatants were loaded into SDS PAGE gels. Photos of external fly eyes were taken with an Olympus SZ61 microscope equipped with a DP21 digital camera. Photos of histological sections were acquired with an Olympus BX53 microscope equipped with a DP72 digital camera, and retinal depth was quantified using cellSens Dimension software (Olympus).

_Antibodies_ — Anti-ataxin-3 antibody was mouse monoclonal 1H9 (Millipore; used at 1:1,000 dilution), anti-HA was rabbit polyclonal Y11 (Santa Cruz Biotech; used at 1:500 dilution), anti-Ub was rabbit polyclonal (Dako; used at 1:500 dilution), anti-rhodopsin was monoclonal Y11 (Santa Cruz Biotech; used at 1:500 dilution), anti-tubulin was mouse monoclonal (Sigma-Aldrich; used at 1:20,000 dilution), anti-GAPDH was mouse monoclonal (Millipore; used at 1:500 dilution). Peroxidase-conjugated secondary antibodies were from Jackson Immunoresearch (used at 1:10,000 dilution).

_Mouse brain lysate preparation_ — Flash-frozen brains from CHIP knockout mice or littermate controls (20) were homogenized in RIPA buffer with protease inhibitors (Sigma-Aldrich), sonicated, and centrifuged at 75,000XG (4˚C for 30 minutes). Supernatants were supplemented with 1% final SDS and 100mM DTT, boiled, and loaded into SDS–PAGE gels.

**RESULTS**

Ubiquitination of ataxin-3 is important for its ability to suppress severe toxic protein-dependent degeneration in vivo — We recently reported that the catalytic activity of the DUB ataxin-3 (Figure 1A) is controlled by its ubiquitination at lysine (K) at position 117 (13). This modification markedly enhances the DUB activity of ataxin-3 in vitro (11,13). Normal ataxin-3 (WT) and a version that contains only lysine at position 117 (K117), with all of the other lysines mutated into the similar but non-ubiquitinatable amino acid arginine, are equally potentiated by their ubiquitination (Figure 1B). However, it is unclear whether ubiquitination
of ataxin-3 is important for its function in intact organisms.

One function of ataxin-3 in vivo is its neuroprotective role in Drosophila melanogaster. In the fruit fly, ataxin-3 suppresses degeneration caused by various polyQ proteins in a manner that depends on its catalytic activity (17). To test the hypothesis that ubiquitination of ataxin-3 is important for this in vivo function, we generated new transgenic Drosophila lines that express one of the following versions of ataxin-3 through the Gal4-UAS system: wild type (WT), catalytically inactive (C14A), lysine-less non-ubiquitinatable (K-null), or containing only K117 (Figure 1C). All of the lysine residues in K-null are mutated into arginine.

The rationale to generate flies that express these specific forms of ataxin-3 was informed by our previous work (11,13,22). Expression of exogenous WT ataxin-3 dramatically suppresses degeneration from polyQ78, recapitulating in our hands the effect demonstrated by Bonini and colleagues (17). In the presence of normal ataxin-3, degeneration of the external eye is essentially undetectable, the ommatidia are intact and there are no visible inclusions (Figure 2A, middle panel). Unlike WT ataxin-3, its catalytically inactive version does not detectably suppress degeneration (Figure 2A, right panel), demonstrating that the DUB activity of this protease is at the core of its protective role. Therefore, we asked whether ubiquitination of ataxin-3, which enhances its catalytic activity (11,13), is important for its protective role in Drosophila.

Western blotting shows several ataxin-3-positive bands above unmodified ataxin-3, which, based on our previous work (11,13,22), are ubiquitinated forms of this protein. These slower-migrating bands are absent in the K-null non-ubiquitinatable version of ataxin-3 (Figure 1C). Because the rest of our studies in this report focus on the Drosophila compound eye, we confirmed that expression of none of the ataxin-3 forms used causes detectable anomalies to the external or internal structures of this organ (Figure 1D and data not shown).

We consistently observe several ubiquitinated ataxin-3 bands with WT, C14A and K117 in purified systems (Figure 1B; (11,13)), in mammalian cell preparations, in mouse brain (11,13) and in vivo in Drosophila (Figure 1C). It is unclear whether the DUB activity of these different ubiquitinated species of ataxin-3 is enhanced to different extents. However, based on our previously published work mono-ubiquitination is sufficient for activation (11,13).

Expression of an expanded polyQ repeat peptide (polyQ78severe) selectively in fly eyes causes massive degeneration of external and internal structures (Figure 2A; this line was originally described in ref. (28)). PolyQ78severe is the isolated polyQ region of pathogenic ataxin-3, comprising a stretch of 78 glutamines and immediately adjoining amino acids (28). This peptide is devoid of any of the other domains of ataxin-3 (Figure 1A) and probably serves best as a generic model of polyQ-dependent degeneration rather than solely of SCA3 (31,32).

Degeneration caused by polyQ78severe is marked by loss of pigmentation of the external eye, loss of the functional units (the ommatidia; internal eye sections), a dramatic reduction of retinal depth, and the presence of aggregated structures/inclusions (Figure 2A, left panel). The inclusions were previously shown to contain polyQ78severe, and are reminiscent of neuronal inclusions found in human post-mortem brains afflicted by various polyQ-diseases (17-19,28,31).

Expression of exogenous WT ataxin-3 somewhat suppresses polyQ78severe-dependent degeneration of Drosophila eyes early in the life of adults (Figure 2B). However, by two weeks of age the potency of K-null ataxin-3 to suppress degeneration is markedly lower than that of WT or K117 ataxin-3 (Figure 2B, day 14; figure 2C). Compared to eyes that co-express ubiquitinatable ataxin-3 and the disease protein, those that express K-null ataxin-3 alongside polyQ78severe show more severe degeneration and reduced retinal depth (Figure 2C).

To further understand the time course of protection from polyQ78severe by the different forms of ataxin-3, we examined depigmentation...
and necrosis of the external eye for 30 days (Figure 2D). We categorized eyes using a scale from 0-5, where 0 denotes no visible signs of degeneration and 5 means pervasive depigmentation and necrosis of the external fly eye. From these observations, it is clear that K-null ataxin-3 does not suppress degeneration as well as K117 ataxin-3 (Statistical significance at P<0.01), although K-null ataxin-3 performs better than the catalytically inactive version (Figure 2D; statistical significance at P<0.01). We should note that eyes co-expressing K117 and the toxic protein trend slightly higher in quantification of degeneration compared to eyes expressing WT ataxin-3 and polyQ78Severe, but these differences do not reach statistical significance. Based on these collective results (Figure 2), we conclude that ataxin-3 ubiquitination at K117 is critical for its neuroprotective role in Drosophila against severe forms of polyQ-dependent degeneration.

Ataxin-3 suppresses degeneration in Drosophila without affecting levels of the toxic polyQ protein — It is unclear how ataxin-3 performs its neuroprotective role in Drosophila (17). A major histological hallmark of polyQ-dependent degeneration is the presence of aggregated bodies/inclusions in various models of polyQ-repeat diseases and in human post-mortem brain tissue (17-19,28,31). PolyQ78Severe, as highlighted by boxes in figure 2A, leads to numerous inclusions in fly eyes. These structures are completely or nearly completely absent in the presence of catalytically active ataxin-3 in young flies, although they do accumulate with age (Figure 2B, compare day 1 to day 14 in histological preparations). Therefore, we used western blotting to examine whether ataxin-3 suppresses polyQ78Severe-dependent degeneration by decreasing its protein levels, which could explain the reduced presence of inclusions. We tested different buffers to extract soluble and aggregated species of polyQ78Severe from dissected fly heads (Triton-X-100, SDS, or urea and SDS; figure 3A). We proceeded with an SDS-containing buffer that provided the best differentiation between SDS-soluble and SDS-resistant forms of polyQ78Severe.

Western blots from one-day-old adult flies indicate that toxic protein levels are not lowered by the presence of WT ataxin-3 compared to degenerating eyes that do not express any ataxin-3 (Figure 3B). In fact, we sometimes observe less polyQ78Severe protein in the absence of any ataxin-3 compared to when the active DUB is present (Figure 3C, right panel). We detect two types of polyQ species in western blots: SDS soluble and SDS-resistant, as also described by previously-published work from other investigators (e.g. in refs. (33,34)). Neither type of polyQ species is reduced in the presence of ataxin-3 (Figure 3).

PolyQ78Severe protein levels are similar among WT, K-null and K-117 ataxin-3-expressing eyes (Figure 3C). However, expression of C14A ataxin-3 sometimes is associated with lower levels of polyQ78Severe compared to other versions of the DUB (Figure 3C, compare polyQ78Severe levels between the two different experiments).

By two weeks of age, there is consistently less toxic protein detectable in fly eyes expressing C14A or K-null ataxin-3 compared to those that express ubiquitinatable versions of this DUB (Figure 3D). The lower levels of polyQ78Severe represent an artifact of eye degeneration. This artifact reflects a loss of the overall eye caused by polyQ78Severe in the presence of C14A or K-null ataxin-3, but not so much in the presence of WT or K117 ataxin-3. Retinal loss is highlighted by blotting for the eye pigment rhodopsin (Figure 3). Note how rhodopsin is nearly undetectable in eyes whose degeneration is not suppressed. The loading control – tubulin – indicates the total amount of lysate from dissected whole heads, which also includes non-eye tissue such as the brain.

Based on these results, ataxin-3 does not seem to protect from degeneration by decreasing the protein levels of polyQ78Severe. Instead, the data in figure 3 (and also in figure 4, discussed in the next section) suggest that this DUB’s protective role lies elsewhere. As we observe few to no inclusions in polyQ78Severe expresses fly eyes in the presence of catalytically active ataxin-3 (Figure 2), perhaps its protective function stems from an ability to reduce inclusion formation without altering overall protein levels.

Ataxin-3 ubiquitination is important for its ability to suppress mild degeneration — To further understand the importance of ataxin-3 ubiquitination to its protective role in vivo, we examined whether this modification is also required for milder forms of polyQ-dependent degeneration. Perhaps ubiquitination of ataxin-3 is important to suppress severe degeneration, but dispensable in milder cases of toxicity. This type of detail would provide a better understanding of the role of ataxin-3 and its ubiquitination-
dependent regulation in polyQ toxicity in Drosophila.

To investigate this possibility, we used a fly line containing a different chromosomal insertion of polyQ78 that expresses the same polyQ78 construct at levels that cause milder degeneration (28), here denoted as polyQ78Mild. As shown in figure 4A, expression of this toxic protein by itself in fly eyes leads to inclusions and mild ommatidial loss in newly-eclosed adult flies (compare to the extensive degeneration caused by polyQ78Severe in figure 2A). By two weeks of age, most of the ommatidia have disappeared.

Expression of WT, K-null and K117 ataxin-3 each has a similar suppressive effect in this milder form of polyQ-dependent toxicity for the first two weeks of life (Figure 4A). By one month of age, however, it is again clear that ubiquitination of ataxin-3 is important for its suppressive role. Eyes that co-express K-null ataxin-3 alongside polyQ78Mild show loss of internal eye structure, while normal and K117 ataxin-3 continue to suppress toxicity from this polyQ protein (Figure 4B). As with polyQ78Severe (Figure 3), none of the ataxin-3 versions lowers the levels of polyQ78Mild protein (Figure 4C), supporting the notion that ataxin-3 suppresses degeneration without decreasing levels of the toxic protein.

Together with the data from above, these results indicate that ataxin-3 ubiquitination at K117 regulates the function of this DUB in vivo and is especially important in later stages or more severe forms of polyQ-dependent degeneration.

**CHIP is dispensable for ataxin-3 ubiquitination and its neuroprotective function in Drosophila**

Ataxin-3 is ubiquitinated by the E3 ligase CHIP in reconstituted systems in vitro (11,13,20) and cooperates with this ubiquitin ligase to regulate the turnover of select CHIP substrates in cultured mammalian cells (20). CHIP functions in protein quality control pathways by targeting various misfolded proteins for proteasomal degradation through its interaction with heat shock proteins (35,36). Consequently, we investigated whether CHIP is important for the ubiquitination and neuroprotective function of ataxin-3 in vivo.

We used two different UAS-RNAi lines that target Drosophila CHIP. The extent of knockdown that we achieved with each line, according to quantitative real time PCR (qRT-PCR) is shown in figure 5A. The qRT-PCR results underestimate the actual extent of CHIP knockdown in fly eyes. While expression of UAS-CHIP-RNAi is restricted to the eyes, RNA is isolated from whole dissected heads, which include non-eye tissue. CHIP knockdown in fly eyes is therefore expected to be considerably higher than what qRT-PCR reports.

We examined the ubiquitination pattern of ataxin-3 in the presence or absence of CHIP RNAi through western blotting. As shown in figure 5B, we do not observe differences in the ubiquitination pattern of ataxin-3 from dissected fly heads. The overall levels of ataxin-3 protein are also not affected by CHIP knockdown.

We also examined ataxin-3 protein in CHIP knockout mouse brains. We again did not notice a difference in the overall levels of ataxin-3 in the absence of CHIP (Figure 5C). Importantly in the absence of CHIP, ataxin-3 is more heavily ubiquitinated than when CHIP is present (Figure 5C). Together, these data from flies and mice argue against CHIP being required for ataxin-3 ubiquitination in vivo.

Lastly, we examined whether CHIP plays a role in ataxin-3-dependent protection. CHIP knockdown by itself exacerbates external eye degeneration that is caused by polyQ78Severe, highlighted by the presence of necrotic spots (Figure 5D). Expression of ataxin-3 suppresses polyQ78Severe-dependent degeneration, as also seen in figure 2. However, this protective function does not appear to be perturbed by the knockdown of CHIP (Figure 5D). According to these results, the neuroprotective function of ataxin-3 does not depend on Drosophila CHIP.

**DISCUSSION**

Various regulatory processes control DUBs’ catalytic activities and functions. Through in vitro reconstituted assays, we recently demonstrated a dramatic upregulation of the catalytic activity of the DUBs ataxin-3 and JosD1 by their own conjugation to ubiquitin (10,11,13). Here, our main objective was to determine whether ubiquitination can regulate DUB roles in vivo.

Ubiquitination at K117 dramatically enhances the ubiquitin protease activity of ataxin-3 in vitro (Figure 1), but it does not alter this protein’s subcellular localization in cells (13). Now, we found that ubiquitination of ataxin-3 at K117 is important for this DUB’s ability to suppress polyQ-dependent degeneration in Drosophila (Figures 2, 4). These findings lead us to conclude that direct upregulation of enzymatic
activity by ubiquitination refines the protective function of ataxin-3 in vivo.

Our work provides new information on the neuroprotective role of ataxin-3, about which little is known. Since this DUB has been reported to regulate the stability of some misfolded proteins in cultured mammalian cells (16), one would hypothesize that ataxin-3 protects from polyQ-dependent degeneration by regulating the turnover of the toxic peptide. In Drosophila, however, ataxin-3 does not decrease overall levels of the polyQ protein (Figures 3, 4). These results suggest that ataxin-3 suppresses polyQ-dependent degeneration by functioning at steps other than the overall stability of the toxic protein.

One of the described functions of ataxin-3 in cultured cells is its ability to enhance the presence of aggresomes/inclusions by aggregation-prone proteins (13,37). In fly eyes, ataxin-3 also affects inclusions that contain misfolded proteins, but in this instance by reducing – rather than enhancing – their presence, and without altering total protein levels of the toxic species (Figures 2-4). These findings suggest that ataxin-3 might regulate the equilibrium between inclusion-prone polyQ species and more soluble forms or conformations. The difference in aggregation “handling” by ataxin-3 in cells compared to flies might stem from the use of cultured cells vs. intact organisms. Together, however, these findings strengthen the connection of ataxin-3 to protein quality control, particularly in protein solubility/aggregation. Future studies are needed to determine how precisely the DUB activity of ataxin-3 protects from degeneration.

Because ataxin-3 cooperates with the E3 ubiquitin ligase CHIP in vitro and in cultured cells to help target CHIP substrates for degradation (20), we tested whether CHIP is important for this DUB’s neuroprotective role in flies. We found that Drosophila CHIP is not required for the protective function of ataxin-3 in fly eyes (Figure 5), suggesting that the functional cooperation between ataxin-3 and CHIP is restricted to specific substrates. As we also did not observe reduced ataxin-3 ubiquitination when CHIP was knocked down in flies or knocked out in mice, we further conclude that CHIP is likely not the only E3 ubiquitin ligase that ubiquitinates ataxin-3 in vivo.

We do not commonly observe robust bands consistent with ubiquitinated ataxin-3 in control mice (Figure 5, ref. (11) and data not shown), but we consistently observe ubiquitinated ataxin-3 in flies (this report). This variation could be due to inherent differences in ataxin-3 ubiquitination in mice compared to Drosophila. Another possibility might be the longer process of isolating protein from mouse brain compared to flies, which may lead to rapid disappearance of small amounts of ubiquitinated proteins. Regardless of the exact reason for the difference in basal ataxin-3 ubiquitination between these two organisms, our results indicate that CHIP is not singularly required for ataxin-3 ubiquitination in vivo.

Finally, we comment on an apparent inconsistency with findings from Drosophila, mouse models of degeneration and cultured cells about the protective function of ataxin-3. As we demonstrated in this work and as had been published previously (17), catalytically active ataxin-3 protects against polyQ-dependent degeneration in flies. In cell culture, murine Atxn3 knockout cells do not tolerate well heat stress compared to cells that have endogenous ataxin-3 (38). A study conducted in mice also suggested that co-expression of pathogenic and normal ataxin-3 transgenes led to a milder phenotype than expression of pathogenic ataxin-3 alone (39). That investigation, however, did not include all of the necessary controls to make a definitive claim about protection from normal ataxin-3, as it was not its primary focus. Overall, these results could explain why SCA3 patients with two copies of the disease allele have a more severe phenotype than those with one normal and one pathogenic copy of ATXN3 (40), and why age of onset for SCA3 patients is slightly improved by the presence of the normal ATXN3 allele (41). An inconsistency about the protective role of ataxin-3 arises, however, from another mouse study: co-expression of pathogenic ataxin-3 alone (39). That report did not include a catalytically inactive version of ataxin-3 as a control. Perhaps future mouse studies should include forms of this DUB that are enzymatically deficient and/or utilize Atxn3 null lines to control for the normal functions of this enzyme in vivo. The apparent discrepancy notwithstanding, data from various models collectively support a protective role for ataxin-3.

In summary, our findings establish a physiological importance for ubiquitination-dependent regulation of ataxin-3’s catalytic activity in Drosophila. This work leads us to suggest that the ubiquitination of other DUBs also...
dictates their functions \textit{in vivo} by modulating their protease activities in perhaps unexpected ways.

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2 The abbreviations used are: CHIP, C-terminus of Hsc70 Interacting Protein; DUB, Deubiquitinating enzyme, deubiquitinase; E1, Ubiquitin activating enzyme; E2, Ubiquitin conjugating enzyme; E3, Ubiquitin ligase; PolyQ, polyglutamine; RIPA, radioimmunoprecipitation assay buffer; RNAi, RNA-interference; SCA, Spinocerebellar Ataxia; UAS, upstream-activating sequence; Ub, ubiquitin; UIM, ubiquitin-interacting motif.

COMPETING INTERESTS
The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS
Designed experiments: WLT, KMS, SVT.
Prepared reagents: WLT, AAB, MO, JRB, KMS, SVT.
Conducted experiments and analyzed the data: WLT, AAB, MO, JRB, KMS, SVT.
Prepared the figures and wrote the manuscript: WLT, AAB, KMS, SVT.

TABLE LEGEND
TABLE 1: Genotype of flies used in each figure.

FIGURE LEGENDS
FIGURE 1: New ataxin-3-expressing *Drosophila* lines. (a) Top: Schematic of the ataxin-3 protein. C14: Catalytic cysteine (21). K117: Lysine residue that is primarily ubiquitinated in cells (13). Triangles: relative position of lysine residues other than K117. UIMs: Ub-interacting motifs that bind poly-Ub chains at least four residues long (21). QQQ: polyglutamine (polyQ) region that, when abnormally expanded, causes SCA3 (16). Bottom: Abbreviations that are used here for the different ataxin-3 constructs. All of the ataxin-3 versions used in this report contain a normal polyQ repeat of 22 residues. (b) Top: Unmodified or ubiquitinated recombinant WT or K117 ataxin-3 (each at [200nM]) were mixed with penta-Ub K63-linked chains (a preferred *in vitro* substrate of ataxin-3 (21); [1µM]). Ataxin-3 proteins were untagged. Bottom: Semi-quantification of results from the top and other similar independent experiments. Shown are means +/- standard deviations. P values are from Student T-tests.
using data from the 6hr time point. (c) New transgenic fly lines that express ataxin-3 through the Gal4-UAS system. Expression of UAS-ataxin-3 for these blots was driven by the ubiquitous sqh-Gal4 driver (24,43). Western blots are from 20 whole flies per genotype, probed with anti-ataxin-3 or anti-tubulin antibodies, as indicated. Flies were heterozygous for the Gal4 driver and ataxin-3. (d) Photos and sections of fly eyes expressing ataxin-3. “None”: flies contained only gmr-Gal4, in the absence of ataxin-3. The other groups were heterozygous for driver and UAS-ataxin-3. Flies were one day old. Genotypes for the flies shown in panels C and D are listed in table 1.

**FIGURE 2**: Ataxin-3 ubiquitination is important for its protective role in vivo. (a) Photos and sections of fly eyes expressing a toxic UAS-polyQ repeat peptide (polyQ$^{78 \text{Severe}}$) driven by gmr-Gal4. Double-headed arrows: retinal depth. Boxes: inclusions. All flies were heterozygous for all constructs. Images are representative of at least 5 flies per genotype, with similar results. Flies were one day old. (b) External and internal structures of fly eyes expressing the indicated UAS constructs driven by gmr-Gal4. Flies were heterozygous for all constructs. Double-headed arrows: retinal depth. Dashed lines: another area used to measure thickness for (2c). Bracketed lines: ommatidial boundaries, which disintegrate as degeneration progresses. Boxes: inclusions. Retinal depth shown at the bottom of panels is from measuring along the double-headed arrows. (c) Quantification of retinal thickness from 14 day-old flies shown in panel (b) and other flies, normalized to measurements from WT ataxin-3 flies. Shown are means +/- standard deviations. Asterisks indicate P<0.01 from Student T-tests. NS: not significantly different. Retinal depth was measured at two separate areas for each section, indicated by the double-headed arrows and the dashed lines in (2b). A total of ten measurements were taken from equivalent retinal sections and equivalent measurement areas from five flies per genotype. (d) Quantification of eye degeneration caused by polyQ$^{78 \text{Severe}}$ in the absence or presence of different ataxin-3 constructs. Flies were heterozygous for the driver gmr-Gal4 and UAS-constructs (ataxin-3 and polyQ$^{78 \text{Severe}}$). At least 20 flies per genotype per insertion were monitored for the entire length of observation, from at least three different insertion lines per version of ataxin-3. Shown in the graph are means +/- standard deviations. Asterisks denote P values of less than 0.01 from Student T-tests comparing K-null and K-117 ataxin-3. No statistically significant differences were found between WT and K117. All P values from Student T-tests comparing K-null to C14A were <0.01. Degeneration scale: 0: no signs of depigmentation; 1: minimal depigmentation; 2: substantial depigmentation, but >50% of the external eye appears normal; 3: >50% of the eye shows depigmentation; 4: necrotic spots; 5: pervasive necrosis. Genotypes for flies in all panels are listed in table 1.

**FIGURE 3**: Ataxin-3 does not reduce levels of polyQ$^{78 \text{Severe}}$ protein in vivo. (a) Testing different buffers to extract polyQ$^{78 \text{Severe}}$ protein from Drosophila heads. See main text and experimental procedures for details. TX-100: Triton-X-100. (b-d) Western blots from 15-20 dissected fly heads per genotype. Heads were homogenized in 2% SDS lysis buffer and lysates were centrifuged after boiling; see experimental procedures for details. Driver: gmr-Gal4. All flies were heterozygous for all constructs. For (c), blots from two independent experiments are shown to highlight that sometimes we observe less polyQ$^{78 \text{Severe}}$ protein in the absence of any ataxin-3 or in the presence of C14A ataxin-3, while in other instances the levels are similar to polyQ$^{78 \text{Severe}}$ in the presence of other types of ataxin-3. For (d), the results are consistently similar to the ones shown. Genotypes are listed in table 1.

**FIGURE 4**: Ataxin-3 ubiquitination is important for suppression of mild degeneration. (a, b) Sections of fly eyes expressing the indicated constructs through the gmr-Gal4 driver. All flies were heterozygous for all constructs. Boxes: inclusions. Double-headed arrows: retinal depth. Images are representative of at least 5 flies per genotype, with similar results. Histograms in (b) summarize retinal depth measurements from sections shown in the panels above and other flies. Measurements were taken as indicated in (2b). A total of fourteen measurements were obtained from equivalent retinal sections and equivalent measurement areas from seven flies per genotype. Shown are means +/- standard deviations. Asterisks denote P<0.01 from Student T-tests. NS: not significantly different. (c) Protein levels of polyQ$^{78 \text{Mild}}$ in the absence or presence of the noted ataxin-3 constructs. At least 15 heads were homogenized per genotype. Genotypes for flies in all panels are listed in table 1.
FIGURE 5: CHIP is dispensable for ataxin-3 ubiquitination and protective function. (a) Histograms show mRNA levels of Drosophila CHIP when it is knocked down using two different UAS-RNAi lines. At least 15 dissected fly heads were analyzed per group. Experiment was conducted twice. Note that this is an underestimate of actual knockdown in fly eyes: knockdown was restricted to the fly eyes, but qRT-PCR assays were conducted using the entire fly head that includes tissues where CHIP levels were not reduced. (b) Ubiquitination pattern of ataxin-3 when Drosophila CHIP is knocked down in fly eyes. Western blots from 15 fly heads per genotype. Driver, gmr-Gal4. Arrows: ubiquitinated ataxin-3. Asterisk: non-specific band that is sometimes observable with anti-ataxin-3 antibody. (c) Ataxin-3 ubiquitination in CHIP knockout mouse brains. Brain lysates from mice of the indicated ages were probed in western blots with an anti-ataxin-3 antibody. Arrows: ubiquitinated ataxin-3. (d) Left: external eye photos, right: quantification of degeneration from flies expressing the indicated UAS constructs through the gmr-Gal4 driver. All flies were heterozygous for all constructs. Images are representative of at least 5 flies per genotype, with similar results. Shown in histograms are means +/- standard deviations from at least five flies per genotype from two crosses per genotype. P values are from Student T-tests. Genotypes for flies are listed in table 1.
| Figure | Fly Genotype |
|--------|--------------|
| C | UAS-ataxin-3(WT)/w ; sqh-Gal4/+ ; + w ; + ; gmr-Gal4/+ (1 or 2 different insertion lines for each ataxin-3 version) |
| | w ; UAS-ataxin-3(3C14A)/+/+ ; gmr-Gal4/+ |
| | w ; UAS-ataxin-3(K-null)/w ; sqh-Gal4/+ ; + w ; UAS-ataxin-3(K-null)/sqh-Gal4/+ |
| | w ; UAS-ataxin-3(K117)/w ; sqh-Gal4/+ ; + w ; UAS-ataxin-3(K117)/sqh-Gal4/+ |
| | w ; UAS-ataxin-3(3C14A)/sqh-Gal4 ; + |
| | w ; UAS-ataxin-3(K-null)/sqh-Gal4 ; + |
| | w ; UAS-ataxin-3(K117)/sqh-Gal4 ; + |
| A | w ; UAS-polyQ78<sup>b</sup>/+ ; gmr-Gal4/+ |
| | w ; UAS-polyQ78<sup>b</sup>/UAS-ataxin-3(3WT) ; gmr-Gal4/+ |
| | w ; UAS-polyQ78<sup>b</sup>/UAS-ataxin-3(3C14A) ; gmr-Gal4/+ |
| | w ; UAS-polyQ78<sup>b</sup>/UAS-ataxin-3(3WT) ; gmr-Gal4/+ |
| B | w ; UAS-polyQ78<sup>b</sup>/UAS-ataxin-3(3WT) ; gmr-Gal4/+ |
| | w ; UAS-polyQ78<sup>b</sup>/UAS-ataxin-3(3C14A) ; gmr-Gal4/+ |
| | w ; UAS-polyQ78<sup>b</sup>/UAS-ataxin-3(3K-null) ; gmr-Gal4/+ |
| | w ; UAS-polyQ78<sup>b</sup>/UAS-ataxin-3(3K117) ; gmr-Gal4/+ |
| C | w ; UAS-polyQ78<sup>b</sup>/UAS-ataxin-3(3WT) ; gmr-Gal4/+ |
| | w ; UAS-polyQ78<sup>b</sup>/UAS-ataxin-3(3C14A) ; gmr-Gal4/+ |
| | w ; UAS-polyQ78<sup>b</sup>/UAS-ataxin-3(3K-null) ; gmr-Gal4/+ |
| | w ; UAS-polyQ78<sup>b</sup>/UAS-ataxin-3(3K117) ; gmr-Gal4/+ |
| D | w ; UAS-polyQ78<sup>b</sup>/UAS-ataxin-3(3WT) ; gmr-Gal4/+ |
| | w ; UAS-polyQ78<sup>b</sup>/UAS-ataxin-3(3C14A) ; gmr-Gal4/+ |
| | w ; UAS-polyQ78<sup>b</sup>/UAS-ataxin-3(3K-null) ; gmr-Gal4/+ |
| | w ; UAS-polyQ78<sup>b</sup>/UAS-ataxin-3(3K117) ; gmr-Gal4/+ |
| A | w ; gmr-Gal4/+ ; UAS-polyQ78<sup>Mid</sup>/+ |
| | w ; UAS-ataxin-3(3WT)/gmr-Gal4 ; UAS-polyQ78<sup>Mid</sup>/+ |
| | w ; UAS-ataxin-3(3C14A)/gmr-Gal4 ; UAS-polyQ78<sup>Mid</sup>/+ |
| | w ; UAS-ataxin-3(3K-null)/gmr-Gal4 ; UAS-polyQ78<sup>Mid</sup>/+ |
| | w ; UAS-ataxin-3(3K117)/gmr-Gal4 ; UAS-polyQ78<sup>Mid</sup>/+ |
| B | w ; UAS-ataxin-3(3WT)/gmr-Gal4 ; UAS-polyQ78<sup>Mid</sup>/+ |
| | w ; UAS-ataxin-3(3C14A)/gmr-Gal4 ; UAS-polyQ78<sup>Mid</sup>/+ |
| | w ; UAS-ataxin-3(3K-null)/gmr-Gal4 ; UAS-polyQ78<sup>Mid</sup>/+ |
| | w ; UAS-ataxin-3(3K117)/gmr-Gal4 ; UAS-polyQ78<sup>Mid</sup>/+ |
| C | w ; gmr-Gal4/+ ; UAS-polyQ78<sup>Mid</sup>/+ |
| | w ; UAS-ataxin-3(3WT)/gmr-Gal4 ; UAS-polyQ78<sup>Mid</sup>/+ |
| | w ; UAS-ataxin-3(3C14A)/gmr-Gal4 ; UAS-polyQ78<sup>Mid</sup>/+ |
| | w ; UAS-ataxin-3(3K-null)/gmr-Gal4 ; UAS-polyQ78<sup>Mid</sup>/+ |
| | w ; UAS-ataxin-3(3K117)/gmr-Gal4 ; UAS-polyQ78<sup>Mid</sup>/+ |
| D | w ; gmr-Gal4/+ ; UAS-polyQ78<sup>Mid</sup>/+ |
| | w ; UAS-ataxin-3(3WT)/gmr-Gal4 ; UAS-polyQ78<sup>Mid</sup>/+ |
| | w ; UAS-ataxin-3(3C14A)/gmr-Gal4 ; UAS-polyQ78<sup>Mid</sup>/+ |
| | w ; UAS-ataxin-3(3K-null)/gmr-Gal4 ; UAS-polyQ78<sup>Mid</sup>/+ |
| | w ; UAS-ataxin-3(3K117)/gmr-Gal4 ; UAS-polyQ78<sup>Mid</sup>/+ |

**Table 1**

| Figure | Fly Genotype |
|--------|--------------|
| A | w ; + ; P{y[+t7.7]=CaryP}attP2/gmr-Gal4 |
| | w ; + ; P{y[+t7.7]=CaryP}attP2/gmr-Gal4 |
| B | w ; UAS-ataxin-3(3WT)+ ; P{y[+t7.7]=CaryP}attP2/gmr-Gal4 |
| | w ; UAS-ataxin-3(3WT)+ ; P{y[+t7.7]=CaryP}attP2/gmr-Gal4 |
| D | UAS-ataxin-3(3WT)/w ; UAS-polyQ78<sup>b</sup>/+ ; P{y[+t7.7]=CaryP}attP2/gmr-Gal4 |
| | UAS-ataxin-3(3WT)/w ; UAS-polyQ78<sup>b</sup>/+ ; P{y[+t7.7]=CaryP}attP2/gmr-Gal4 |

**Fly Genotype**

- UAS-ataxin-3(WT)/w ; sqh-Gal4/+ ; +
- UAS-ataxin-3(3C14A)/+/+ ; gmr-Gal4/+ (1 or 2 different insertion lines for each ataxin-3 version)
- UAS-ataxin-3(K-null)/w ; sqh-Gal4/+ ; +
- UAS-ataxin-3(K-null)/sqh-Gal4/+ (1 or 2 different insertion lines for each ataxin-3 version)
- UAS-ataxin-3(K117)/w ; sqh-Gal4/+ ; +
- UAS-ataxin-3(K117)/sqh-Gal4/+ (1 or 2 different insertion lines for each ataxin-3 version)
Figure 1

A. C14 and K117: Primary site of ataxin-3 ubiquitination

Normal Ataxin-3: WT
Catalytically inactive Ataxin-3: C14A
Lysine-less Ataxin-3: K-null
Ataxin-3 with only K117 present: K117

B. Ub5-K63

| Time (h) | WT | WT-Ub | K117 | K117-Ub |
|---------|----|-------|------|---------|
| 0       | 0  | 0     | 0    | 0       |
| 2       | 2  | 2     | 2    | 2       |
| 6       | 6  | 6     | 6    | 6       |

C. Ataxin-3-Ub(n)

Ataxin-3-Ub(2)
Ataxin-3-Ub(1)
Ataxin-3

X Chromosome Inserts
Chromosome 2 Inserts
Chromosome 3 Inserts

Tubulin

D. UAS-Ataxin-3

None
WT
C14A
K-null
K117

External Morphology
Internal Cross-sections

% Ub Reaction Products

WT vs. K117: P=0.39
WT vs. WT-Ub: P=1.7X10^-7
K117 vs. K117-Ub: P=1.1X10^-7
WT-Ub vs. K117-Ub: P=0.72
Figure 2

A. UAS-polyQ78<sup>Severe</sup> + UAS-Ataxin-3

| Day | UAS-Ataxin-3 | None | WT | C14A |
|-----|--------------|------|----|------|
| 1   | 1            | 1    | 1  | 1    |

B. UAS-polyQ78<sup>Severe</sup> + UAS-Ataxin-3

| Day | UAS-Ataxin-3 | WT | K-null | K117 |
|-----|--------------|----|--------|------|
| 1   | 1            | 14 | 1      | 14   |

Depth: 69 µM

Depth: 38 µM

Depth: 65 µM

C. 14 Day-old Flies

Retinal Depth Normalized to WT

Retinal Depth: 0% Ataxin-3 WT K-null K117

D. * P <0.01, K-null vs. K117

Average Severity of Degeneration

Day 1 5 10 15 20 25 30

* C14A

* No Ataxin-3

* K-null

NS

120%
100%
80%
60%
40%
20%
0%

5 4 3 2 1 0

NS

Average Severity of Degeneration
Figure 3
Figure 4

A

|         | UAS-polyQ78<sup>mis</sup> |         |         |         |
|---------|---------------------------|---------|---------|---------|
| UAS-Ataxin-3 | None                     | WT      | C14A    | K-null  | K117    |
| Day 1   | ![Image](image1.png)      | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) |
| Day 14  | ![Image](image6.png)      | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) | ![Image](image10.png) |

B

|         | UAS-polyQ78<sup>mis</sup> |         |         |         |
|---------|---------------------------|---------|---------|---------|
| UAS-Ataxin-3 | WT                       | K-null  | K117    |         |
| Day 28  | ![Image](image11.png)      | ![Image](image12.png) | ![Image](image13.png) | ![Image](image14.png) |

C

|         | UAS-polyQ78<sup>mis</sup> |         |         |         |
|---------|---------------------------|---------|---------|---------|
| UAS-Ataxin-3 | None                     | WT      | K-null  | K117    |
| Day 1   | ![Image](image15.png)      | ![Image](image16.png) | ![Image](image17.png) | ![Image](image18.png) |
| Day 14  | ![Image](image19.png)      | ![Image](image20.png) | ![Image](image21.png) | ![Image](image22.png) |

Retinal Depth Normalized to WT

0% 20% 40% 60% 80% 100% 120%

NS

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Ubiquitination regulates the neuroprotective function of the deubiquitinase ataxin-3 in vivo

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