Defining the Role of Ubiquitin-interacting Motifs in the Polyglutamine Disease Protein, Ataxin-3*

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Polyglutamine (polyQ) expansions cause neurodegeneration that is associated with protein misfolding and influenced by functional properties of the host protein. The polyQ disease protein, ataxin-3, has predicted ubiquitin-specific protease and ubiquitin-binding domains, which suggest that ataxin-3 functions in ubiquitin-dependent protein surveillance. Here we investigate direct links between the ubiquitin-proteasome pathway and ataxin-3. In neural cells we show that, through its ubiquitin interaction motifs (UIMs), normal or expanded ataxin-3 binds a broad range of ubiquitinated proteins that accumulate when the proteasome is inhibited. The expression of a catalytically inactive ataxin-3 (normal or expanded) causes ubiquitinated proteins to accumulate in cells, even in the absence of proteasome inhibitor. This accumulation of ubiquitinated proteins occurs primarily in the cell nucleus in transfected cells and requires intact UIMs in ataxin-3. We further show that both normal and expanded ataxin-3 can undergo oligoubiquitination. Although this post-translational modification occurs in a UIM-dependent manner, it becomes independent of UIMs when the catalytic cysteine residue of ataxin-3 is mutated, suggesting that ataxin-3 ubiquitination is itself regulated in transfected cells by an ubiquitination activity. Finally, pulse-chase labeling reveals that ataxin-3 is degraded by the proteasome, with expanded ataxin-3 being as efficiently degraded as normal ataxin-3. Mutating the UIMs does not alter degradation, suggesting that UIM-mediated oligoubiquitination of ataxin-3 modulates ataxin-3 function rather than stability. The function of ataxin-3 as a de-ubiquitinating enzyme, its post-translational modification by ubiquitin, and its degradation via the proteasome link this polyQ protein to ubiquitin-dependent pathways already implicated in disease pathogenesis.

Growing evidence suggests that the ubiquitin-proteasome pathway participates in the pathogenesis of neurodegenerative diseases, including those caused by polyglutamine (polyQ)1 expansion (1, 2). The nine known polyQ diseases include spinocerebellar ataxia type 3, also known as Machado-Joseph disease (SCA3/MJD). The disease protein in SCA3/MJD, ataxin-3, binds polyubiquitin chains and is a ubiquitin-specific protease (3–6). These properties link the normal function of ataxin-3 to protein surveillance pathways and suggest that polyQ-induced alterations in disease protein function could compromise protein homeostasis in the cell. As highlighted by recent studies of other polyQ diseases, efforts to define the normal function of neurodegenerative disease proteins can lead to a better understanding of pathogenic mechanisms (7). Here we explore the role that ubiquitin binding by ataxin-3 plays in its normal function, post-translational modification, and degradation.

Ubiquitin binding by ataxin-3 is mediated by three ubiquitin-interacting motifs (UIMs), two of which reside just N-terminal to and C-terminal to the polyQ domain (3, 4, 8). The last of these, present only in an alternative splice form of ataxin-3 (9), is least important to ubiquitin binding (3, 4, 8). Ataxin-3 also contains the catalytic triad of amino acids found in a major class of de-ubiquitinating enzymes, the ubiquitin-specific cysteine proteases (10–13). Thus, ataxin-3 is thought to be a deubiquitinating enzyme. Consistent with this predicted enzyme activity, ataxin-3 cleaves ubiquitin chains from the test substrate lysozyme in vitro, and this activity requires the predicted active site cysteine (3, 14). Moreover, in Drosophila models of polyglutamine disease, normal ataxin-3 suppresses polyglutamine toxicity in a manner that also requires this cysteine (15). Defining the precise links between ataxin-3 and ubiquitin-associated activities of the cell clearly is important. The studies described here begin to address these links.

Conjugation of long polyubiquitin chains to proteins is well known to facilitate their degradation by the proteasome. But the addition of one or a few ubiquitin molecules to proteins is now also recognized to be an important post-translational modification that can regulate protein function and subcellular localization (16). UIM-containing proteins are frequently monoubiquitinated or oligoubiquitinated in this manner. In proteins where this has been well studied, the presence of UIMs facilitates this mono- or oligoubiquitination (17–21). Although many UIM-containing proteins function in endocytosis and vascular protein sorting, they also can participate in numerous other biological pathways. In a given protein, the presence of UIMs influences its function by promoting interactions with

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1 The abbreviations used are: polyQ, polyglutamine; SCA3/MJD, spinocerebellar ataxia type-3/Machado-Joseph disease; UIM, ubiquitin-interacting motif; Alt, alternative; HA, hemagglutinin; IP, immunoprecipitation; CBP, cAMP-responsive element-binding protein-binding protein; mAb, monoclonal antibody.
Ubiquitin-associated Properties of Ataxin-3

Many important questions remain unanswered regarding the role of ataxin-3 in ubiquitin pathways. For example, although it has been suggested that ataxin-3 is ubiquitinated (22), this has not been well demonstrated. The role of UIMs in mediating such ubiquitination has also not been investigated. In addition, the function of ataxin-3 as a de-ubiquitinating enzyme is strongly suggested by cell-free studies (3) but has not been investigated in vivo. Finally, it is unknown whether ubiquitin binding by ataxin-3 or the conjugation of ubiquitin to ataxin-3 influences its subcellular localization or degradation.

Here we investigate how the UIMs, the predicted protease domain, and the polyQ expansion affect ataxin-3 function, post-translational processing, and degradation. Using various ataxin-3 constructs in non-neuronal and neuronal cell lines, we confirm in vivo that ataxin-3 is a ubiquitin-binding protein with apparent protease activity. Mutating the three UIMs reduces ataxin-3 binding to ubiquitinated proteins in vivo, whereas mutating the predicted protease domain markedly increases the cellular level of ubiquitinated proteins. This build-up of ubiquitinated species, which occurs when catalytically inactive ataxin-3 is expressed, requires intact UIMs, consistent with a model of action in which the UIM region of ataxin-3 regulates the N-terminal protease activity. We further show that ataxin-3 is post-translationally modified by the addition of one or more ubiquitins in a manner that is facilitated by the presence of functional UIMs. Finally, studies of ataxin-3 mutated in both its protease domain and its UIMs suggest that ataxin-3 may serve as a de-ubiquitinating enzyme for itself.

EXPERIMENTAL PROCEDURES

Expression Plasmids—In this study a series of constructs were used that encode various ataxin-3 proteins (see Fig. 1C). Wild type and expanded Myc-tagged ataxin-3 expression constructs were described previously (4). pcDNA3-Myc-ataxin-3(Q22)-Alt-C14A and pcDNA3-Myc-ataxin-3(Q80)-Alt-C14A were generated through splice overlap extension PCR with a primer to replace cysteine with alanine at amino acid 14, using pcDNA3-Myc-ataxin-3(Q22)-Alt or pcDNA3-Myc-ataxin-3(Q80)-Alt as templates. The PCR products were digested with NdeI/EcoRI and subcloned into pcDNA3-Myc plasmid. pcDNA3-Myc-ataxin-3(Q22)-Alt-C14A-S→A and pcDNA3-Myc-ataxin-3(Q80)-Alt-C14A-S→A were generated by digesting the respective pcDNA3-Myc-ataxin-3-C14A construct with BamHI/EcoRI and then subcloning the released fragment into the respective pcDNA3-Myc-Alt-S→A construct. The PCR products were verified by sequencing and expression analysis.

Cell Culture and Transfection—HEK-293 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 100 units/ml/500 µg/ml penicillin/streptomycin (Invitrogen). Stably transfected, doxycycline-chem, San Diego, CA) or Me2SO vehicle control. Procedures for co-immunoprecipitation and Western blot were described previously (24).

Pulse-Chase Analysis—The cells were labeled with [35S]methionine (150 µCi/ml; EasyTag; PerkinElmer Life Sciences) for 1 h and then chased in normal serum containing 30 mCi/ml of unlabeled methionine for the time indicated. The cells were solubilized in non-denaturing lysis buffer, denatured with 1% SDS, and then renatured with 4.5% Triton X-100. The lysates were then immunoprecipitated as described above and analyzed by SDS–PAGE and fluorography. When indicated, 10 µM lactacystin was added during the pulse.

RESULTS

Ataxin-3 Contains Functional UIMs—The two major isoforms of ataxin-3 are both expressed in brain; thus one or both could contribute to disease pathogenesis (9, 26). The originally published form lacks the eleventh and final exon of the MJD1 gene (26), whereas the alternative form, here designated ataxin-3(Alt), contains this exon and a different C terminus (9). Both isoforms contain UIM1, UIM2, and the polyQ domain, whereas only ataxin-3(Alt) contains UIM3 (Fig. 1). Here we use both isoforms in experiments, each giving similar results.

To verify that ataxin-3 binds ubiquitinated substrates in a neuronal context, we performed experiments in stably transfected neural cells that inductively express normal ataxin-3 (Gln28) or expanded ataxin-3 (Gln84 or Gln108). Induced neuronal cell lines were treated with the proteasome inhibitor lactacystin to increase the cellular pool of ubiquitinated proteins. After cell lysis, ataxin-3 was immunoprecipitated with ataxin-3 antibody, and the immunoprecipitated material was probed with an ubiquitin antibody that recognizes conjugated ubiquitin (not shown). In Fig. 2A, normal and expanded ataxin-3 both co-precipitate ubiquitinated proteins that electrophorese as a high molecular weight smear on gels.

Proteasome inhibition with lactacystin also increased normal or expanded ataxin-3 levels in all tested cell lines and led to the appearance of a ladder of higher molecular weight ataxin-3 species (for example, see the bands with asterisks in Fig. 2). In later experiments in this report, we show that these higher molecular weight forms are ubiquitinated forms of ataxin-3.

The in vitro pulse chase experiments in Fig. 2A express the originally published ataxin-3 isoform lacking the third UIM. To determine whether ataxin-3(Alt) behaved similarly, we performed experiments in transiently transfected HEK293 cells expressing either splice isoform. First we confirmed that, in HEK293 cells expressing the original isoform with normal or expanded repeat, ataxin-3 co-precipitated ubiquitinated proteins as expected (not shown). Next we performed similar experiments in HEK293 cells expressing normal or expanded ataxin-3(Alt) (Fig. 2B). Like the original ataxin-3 isoform, ataxin-3(Alt) also co-precipitated ubiquitinated proteins. Thus, both brain-expressed ataxin-3 isoforms bind ubiquitinated substrates.

The UIMs in ataxin-3 contain highly conserved serine residues that are essential for polyubiquitin binding in vitro (8, 27)
To determine whether in living cells ataxin-3 binding to ubiquitinated proteins requires UIMs, we expressed ataxin-3(Alt) in which the critical serine residues in each UIM were mutated to alanine (denoted as "S$^3A"). These mutations diminished normal or expanded ataxin-3 binding to ubiquitinated proteins (Fig. 2B). These results indicate both that UIMs are important for polyubiquitin binding in vivo and that expanded ataxin-3 retains the capacity to bind polyubiquitin. Consistent with this view, we recently showed that normal and expanded ataxin-3 bind Lys48-linked tetraubiquitin in vitro with similar affinity (4).

Importantly, when all three UIMs were mutated, ataxin-3 no longer electrophoresed as a ladder of higher molecular species, as illustrated by the absence of the isoforms with ataxin-3-Q22(S$^3A) and ataxin-3-Q80(S$^3A) (Fig. 2B, marked with asterisks). Thus, formation of this ladder of higher molecular weight ataxin-3 isoforms is facilitated by UIMs.

Expression of Catalytically Inactive Ataxin-3 Causes Accumulation of Ubiquitinated Proteins—Ataxin-3 contains a predicted active site cysteine, Cys$^{14}$, essential for ataxin-3 function as a ubiquitin-specific protease (3). Although in vitro studies employing a single test substrate are consistent with de-ubiquitinating activity for ataxin-3 (3, 6), this has not been addressed in living cells. A possible model of ataxin-3 action is that the C terminus recruits ubiquitinated substrates through its UIM domains, subsequently allowing the N-terminal protease domain to cleave the ubiquitin via its catalytic cysteine residue, Cys$^{14}$. This model predicts that mutating Cys$^{14}$ would render ataxin-3 inactive as a protease and lead to an increase in ubiquitinated substrates in the cell because ataxin-3 should still bind, yet not de-ubiquitinate, its target substrates. That is, ataxin-3(C14A) would act as a dominant negative. The degree of accumulation of ubiquitinated proteins would depend on the extent of substrates recognized by ataxin-3. If, for example, ataxin-3 binds only a limited range of substrates in the cell, the build-up could be rather modest.

To test this model, we transfected cells with normal or expanded ataxin-3 constructs in which Cys$^{14}$ was mutated to alanine. These catalytically inactive constructs are designated ataxin-3(Q22)C14A and ataxin-3(Q80)C14A. The C14A mutation markedly increased the level of ubiquitinated proteins in cells, both for normal and expanded ataxin-3 (Fig. 3). The level of increase in ubiquitinated proteins when ataxin-3(C14A) is expressed in cells is roughly equal to what is seen when the proteasome is inhibited pharmacologically. In IP studies, this smear of ubiquitinated pro-
Ubiquitin-associated Properties of Ataxin-3

Ataxin-3 Is Directly Ubiquitinated—Recently, Matsumoto et al. (22) showed that ataxin-3 can be ubiquitinated but did not determine whether ataxin-3 was monoubiquitinated or polyubiquitinated. UIM-containing proteins are frequently post-translationally modified by the covalent attachment of a single ubiquitin moiety; that is, they become monoubiquitinated (17–19). In contrast, proteins that are known targets for proteasome-mediated degradation require the covalent attachment of a polyubiquitin chain containing at least four ubiquitins (28). Because ataxin-3 is a UIM-containing protein, we hypothesized that it might be prone to monoubiquitination or oligoubiquitination.

To test whether ataxin-3 is directly ubiquitinated, we induced PC6–3 neural cell lines expressing normal (Gln28) or expanded (Gln84) ataxin-3 with doxycycline, incubated the cells in the presence or absence of the proteasome inhibitor lactacystin, and then lysed the cells and assessed ubiquitination of ataxin-3 by immunoblot (Fig. 5). For this experiment, because ataxin-3 binds noncovalently to polyubiquitinated proteins (as shown in Fig. 2), it was essential that we separate this noncovalent interaction from true ubiquitination. Therefore, we inactivated the protease domain of ataxin-3 (normal or expanded) with doxycycline, incubated the cells with or without lactacystin before lysis in nondenaturing lysis buffer, immunoprecipitated (IP) with anti-ataxin-3 antibody, and then probed sequentially with anti-ubiquitin antibody. The expressed ataxin-3 proteins, shown above each lane, include: ataxin-3 with inactivated protease domain (C14A), inactivated UIMs (S-A), or both (C14A/S-A), and N-terminal ataxin-3 fragment with or without intact protease domain. Negative controls included empty pcDNA3 vector and pEGFP. HA-ubiquitin was co-expressed in all lysates except those from mock transfected cells.

Fig. 2. Normal and expanded ataxin-3 bind ubiquitinated substrates that accumulate upon proteasome inhibition. Shown are immunoblots of lysates from PC6–3 neural cell lines expressing normal (Q28) or expanded ataxin-3 (Q84 or Q108) (A) and transiently transfected HEK 293 cells expressing normal (Q28) or expanded (Q80) ataxin-3 with doxycycline, incubated the cells in the presence or absence of lactacystin before lysis in nondenaturing lysis buffer, immunoprecipitated (IP) with anti-ataxin-3 (α-MJD) or preimmune serum and then probed sequentially on immunoblot with anti-conjugated ubiquitin antibody (α-Ub Ab) and an ataxin-3-specific mAb (1H9). In the neural cell lines shown in A, lactacystin treatment leads to an accumulation of ubiquitinated proteins and a corresponding increase in ataxin-3 levels (Input lanes) and increased co-precipitation of ubiquitinated proteins with ataxin-3 (IP lanes). Ataxin-3 isoforms include the major ataxin-3 bands (arrows) and several less abundant, higher molecular weight species that increase with lactacystin treatment (asterisks). When all three UIMs are mutated, these higher molecular weight isoforms are no longer seen, and co-precipitation of ubiquitinated substrates is decreased (compare Alt and S-A results in B).

Fig. 3. Inactivating the protease domain of ataxin-3 causes a UIM-dependent accumulation of ubiquitinated proteins. Immunoblot of lysates from HEK 293 cells expressing HA-ubiquitin with wild type or mutated ataxin-3 Alt containing normal (Q28) or expanded repeat (Q80). Expression of protease-inactive ataxin-3 (normal or expanded) causes an accumulation of ubiquitinated proteins that electrophoresed in the gel stacker, detectable with anti-HA or anti-ubiquitin antibody. The expressed ataxin-3 proteins, shown above each lane, include: ataxin-3 with inactivated protease domain (C14A), inactivated UIMs (S-A), or both (C14A/S-A), and N-terminal ataxin-3 fragment with or without intact protease domain. Negative controls included empty pcDNA3 vector and pEGFP. HA-ubiquitin was co-expressed in all lysates except those from mock transfected cells.

Fig. 4 illustrates that catalytically inactive ataxin-3(C14A) causes a build-up in endogenous ubiquitin signal in cells, detected with an antibody that recognizes polyubiquitin. This increase is seen in most but not all transfected cells expressing ataxin-3(C14A), both with normal and expanded polyQ tracts. It is not seen, however, in transfected cells expressing wild type ataxin-3, UIM-mutated ataxin-3, or ataxin-3 mutated in both its protease and its UIM domains (Fig. 4), nor is it seen in cells expressing an unrelated polyQ disease protein, normal or expanded huntingtin fragment (not shown). Interestingly, under the cell conditions tested, this increased polyubiquitin signal primarily localizes to the nucleus.
To further study ataxin-3 ubiquitination, we transiently co-transfected HEK293 cells with constructs encoding ataxin-3 and HA-tagged ubiquitin and then treated cells with or without lactacystin. The resultant lysates were subjected to IP as above, but the immunoblots were probed sequentially with anti-HA to detect HA-ubiquitin and 1H9 to detect ataxin-3. When co-expressed with epitope-tagged ubiquitin, ataxin-3 electrophoresed as a ladder of higher molecular weight, ubiquitinated ataxin-3 isoforms (Fig. 6). Specifically, a ladder of at least three ubiquitinated ataxin-3 species was consistently observed with normal ataxin-3, ataxin-3(Alt), or expanded ataxin-3 (Fig. 6A, bands denoted by arrowheads). Similar results were obtained when the blots were first probed with 1H9 before anti-HA (data not shown). Importantly, these ubiquitinated ataxin-3 species are present under normal cellular conditions and are only modestly increased when the proteasome is inhibited (Fig. 6B).

Ataxin-3 Ubiquitination Depends on Functional UIMs—Research on other UIM-containing proteins suggests that the presence of UIMs mediates mono- or oligoubiquitination (17–21, 27). To determine whether the UIMs of ataxin-3 mediate its ubiquitination, we transfected HEK293 cells with constructs encoding HA-ubiquitin and various forms of ataxin-3 (with intact or mutated UIMs, and with or without the C14A mutation; Fig. 7). After cell lysis followed by a cycle of denaturation and renaturation, the lysates were immunoprecipitated with anti-ataxin-3 antisera, and the immunoblots were probed with anti-HA antiserum or 1H9. Mutating the UIMs of ataxin-3 inhibited its ubiquitination; the two larger ubiquitinated forms were no longer observed. The UIM mutations did not completely abolish ataxin-3 ubiquitination because the smallest ubiquitinated species, which may represent monoubiquitinated ataxin-3, was still present but reduced in intensity. As shown in Fig. 7, similar results were obtained with normal and expanded ataxin-3, whether cells were treated with or without lactacystin (not shown).

In contrast, expression of C14A mutated ataxin-3 led to an increased intensity of the ladder of ubiquitinated ataxin-3 species (Fig. 7). A greater percentage of ataxin-3(C14A) was ubiquitinated, and the pattern of ubiquitination was shifted toward higher molecular forms containing more ubiquitin. In fact, the ubiquitinated species generated from ataxin-3(C14A) without lactacystin treatment are of similar abundance to those generated from wild type ataxin-3 with lactacystin treatment (data not shown).

Interestingly, combining the C14A mutation with the Ser → Ala mutations restored ataxin-3 ubiquitination to that seen under wild type conditions, both for normal and expanded ataxin-3. This is best seen by comparing the ubiquitin ladders...
in the third, sixth, ninth, and twelfth lanes in Fig. 7. This result suggests two possibilities: either the C14A mutation causes ataxin-3 to misfold, making it more susceptible to ubiquitination, or ataxin-3 acts to remove ubiquitin from other ataxin-3 polypeptides via its ubiquitin protease activity. The latter seems more likely because Cys14-mutated ataxin-3 with a normal polyglutamine tract does not form aggregates in our hands, suggesting that it is not grossly misfolded (data not shown).

Taken together, the results in Figs. 5–7 show that the UIMs of ataxin-3 help to mediate its oligoubiquitination and suggest that ataxin-3 is a target for its own deubiquitinating activity.

**Ataxin-3 Is Degraded by the Proteasome**—Neurons in SCA3/MJD disease tissue contain aggregates of ataxin-3 that also contain ubiquitin and proteasomal subunits (25, 29), suggesting that ataxin-3 has become ubiquitinylated in the aggregates and is targeted for degradation by the proteasome. In the preceding experiments, we showed that lactacystin treatment of cells expressing normal or expanded ataxin-3 led to an increase in ataxin-3 protein levels and to the appearance of higher molecular weight, ubiquitinylated forms of ataxin-3 (Figs. 2 and 5). To define this further, we expressed normal ataxin-3(Q28) in HEK293 cells and performed a time course of lactacystin treatment, assessing ataxin-3 protein levels at various time points. Ataxin-3 protein levels increased with longer periods of proteasome inhibition, suggesting that ataxin-3 is normally a target for proteasome-mediated degradation (data not shown).

Using [35S]methionine labeling in transiently transfected 293 cells, we confirmed that ataxin-3 is a target for proteasomal degradation (Fig. 8). Given the interest in autophagic destruction of polyglutamine disease proteins (30, 31), we also assessed ataxin-3 degradation in cells treated with the lysosomal inhibitors leupeptin and bafilomycin A1. Unlike lactacystin, however, these lysosomal inhibitors did not inhibit ataxin-3 degradation (Fig. 8A). Our pulse-chase labeling results suggest ataxin-3 is degraded primarily by the proteasome and is not a target for lysosomal degradation.

One proposed model of polyQ disease pathogenesis is that the protein quality control machinery of the cell “handles” mutant polyQ proteins less well than normal polyQ proteins. Mutant polyQ proteins may, for example, be degraded more slowly than their normal counterparts or be more prone to generate an endoproteolytic fragment resistant to further degradation (22, 32–34). To test this, we used [35S]methionine labeling to study the degradation rates of normal versus expanded ataxin-3. As shown in Fig. 8B, both normal and mutant ataxin-3 have similar rates of degradation, with half-lives of ~6 h in this cell model. In pulse-chase experiments we consistently observed two closely spaced ataxin-3 bands, a more rapidly disappearing, smaller isoform and a more stable, larger isoform (Fig. 8B, asterisk). Further analysis (not shown) confirmed the differential kinetics for these two ataxin-3 species, which reflects either differences in their rate of degradation or a conversion of the smaller form to the larger one through a post-translational modification (apparently not monoubiquitination). Future experiments will be needed to determine what post-translational modification, if any, is responsible for these distinct isoforms and their divergent kinetics.

Finally, because mutating the UIMs of ataxin-3 significantly inhibited ataxin-3 oligoubiquitination, we compared the degradation rates of wild type ataxin-3 with that of UIM-mutated ataxin-3. First, we compared the degradation rates of ataxin-3 containing the original and alternative C termini because they...
differ in total number of UIMs. Although no major differences in protein stability were observed, ataxin-3(Alt) may be slightlymore stable (Fig. 8 C). Likewise, the degradation rates of normal and UIM-mutated ataxin-3 were roughly similar (Fig. 8 D).

**DISCUSSION**

In this study we have identified several links between the SCA3/MJD disease protein ataxin-3 and ubiquitin pathways: 1) both normal and expanded ataxin-3 bind a wide array of polyubiquitinated proteins in neural cells, doing so in a UIM-dependent manner; 2) expression of ataxin-3 mutated in its predicted active site cysteine results in a marked build-up of ubiquitinated proteins in cells, consistent with the predicted function of ataxin-3 as a de-ubiquitinating enzyme; 3) ataxin-3 is a target for oligoubiquitination that occurs in a manner facilitated by its UIMs; 4) ataxin-3 may act in *trans* to de-ubiquitinate ataxin-3 polypeptides; and 5) normal and expanded ataxin-3 are degraded by the proteasome equally efficiently in transfected cells. Taken together, our results establish both that ataxin-3 functions in ubiquitin-dependent pathways and that this protein is itself regulated by ubiquitin-dependent processes.

Our results document that both major splice forms of ataxin-3 bind polyubiquitinated proteins in non-neural andneural cell models. As expected from earlier reports (4, 8), efficient binding requires intact UIMs. Although expanded ataxin-3 may be prone to adopt an altered protein conformation (35–39), we observed no differences in the binding of ubiquitinated proteins to normal and expanded ataxin-3 in transfected cells. This is reminiscent of our recent *in vitro* studies showing that normal and expanded ataxin-3 display essentially identical, quantitative binding to tetraubiquitin chains (4). It is important to stress, however, that our *in vivo* binding analyses here are merely qualitative, global assessments of ubiquitin binding. They do not rule out the possibility that normal and expanded ataxin-3 may bind differentially to particular substrates in the intact cell. To test this, it will be necessary to identify specific cellular substrates for ataxin-3.

The UIMs of ataxin-3 mediate both its binding to ubiquinitated substrates and its tendency to become conjugated to one or a few ubiquitin molecules. Based on our findings, ataxin-3 joins a growing list of UIM-containing proteins known to be targets for mono- or oligoubiquitination. Many UIM-containing proteins become mono- or oligoubiquitinated through a poorly defined process that is facilitated by UIMs in the protein (17–21). Our own data indicate ataxin-3 is oligoubiquitinated because we consistently detect a discrete ladder of ubiquitinated species rather than the smear of ubiquitin-conjugated proteins that typically occurs with longer ubiquitin chains. This is con-
sistent with what Miller et al. (21) recently reported for ataxin-3 in vitro. We do not observe marked differences in the ubiquitination of the two major splice variants of ataxin-3, which differ only in their C termini. This implies that ataxin-3 ubiquitination is facilitated primarily by the two UIMs (UIMs 1 and 2) common to both variants. Even though these UIMs mediate ataxin-3 ubiquitination, inactivating the UIMs does not alter the rate of ataxin-3 degradation, as shown in our pulse-chase experiments. This suggests that the ubiquitinated ataxin-3 species we observe do not contain Lys48-linked polyubiquitin chains of four or greater, which would be expected to target the protein for degradation by the proteasome.

Ubiquitination is increasingly recognized to be a key post-translational modification that can regulate target protein localization and activity (16, 40, 41). We have not seen any obvious differences in the subcellular location of wild type versus UIM-mutated ataxin-3 in transfected cells, suggesting that the oligoubiquitination of ataxin-3 influences protein function rather than subcellular targeting. We propose that oligoubiquitination regulates the de-ubiquitinating activity of ataxin-3. Once neuronal target substrates for ataxin-3 de-ubiquitination are identified, further research can determine whether oligoubiquitination affects ataxin-3 function as a de-ubiquitinating enzyme.

The current studies provide strong, indirect evidence that ataxin-3 is in fact a de-ubiquitinating enzyme. To date, the only compelling data on this question are from in vitro studies showing that recombinant ataxin-3 cleaves, rather inefficiently, ubiquitin chains from two test substrates (3). Here we have shown that expression of ataxin-3 (C14A), a mutant form of ataxin-3 lacking the predicted catalytic cysteine residue, is consistent with what Miller et al. (21) recently reported for ataxin-3 in vitro. Using 35S metabolic labeling, we show that normal and expanded ataxin-3 have similar rates of degradation, with half-lives of roughly 6 h. This contrasts with recently published data suggesting that expanded ataxin-3 is degraded less efficiently (22). Whether polyQ proteins are degraded more slowly in the disease state is an open question. In cell models, many expanded polyQ proteins appear to be degraded more slowly than their full-length counterparts (22, 32–34). However, these data are complicated by the evidence that expanded polyQ proteins readily oligomerize and aggregate (45). Once disease proteins have aggregated, they are probably less efficiently degraded. Still, in vivo evidence indicates that cells are properly equipped with the protein degradation machinery to degrade expanded polyQ proteins (46) and that, if oligomerization is prevented, expanded polyQ proteins are readily degraded (45). Our data in transfected cells suggest that the proteasome efficiently degrades both normal and expanded ataxin-3 proteins and that proteasome inhibition by soluble expanded ataxin-3 likely does not play a major role in SCA3/MJD pathogenesis. Recent studies employing an in vivo reporter of proteasome activity in a mouse model of the polyQ disease SCA7 are consistent with this view (47).

Finally, many neurodegenerative diseases are now known to be due to alterations in protein surveillance machinery and more specifically to alterations in ubiquitination including mutations in E3 ubiquitin ligases (parkin and mahogany), deubiquitinating enzymes (UHC-L1), and ubiquitin itself (1). Ataxin-3, one of nine known polyQ disease proteins, clearly belongs to this list. We propose that ataxin-3 is a ubiquitin-specific protease that binds polyubiquitinated proteins through its C-terminal UIMs and subsequently de-ubiquitates bound substrates through its N-terminal ubiquitin protease domain. Moreover, the mono/oligoubiquitination of ataxin-3 shown here constitutes an intriguing post-translational modification that we suggest regulates the enzymatic activity of the protein. In addition, ataxin-3 may autoregulate its activity by removing in trans ubiquitin from other ataxin-3 molecules. In light of recent

\[ \text{[2] S. J. S. Berke and H. L. Paulson, unpublished observations.} \]
studies showing that ataxin-3 suppresses polyQ-mediated toxicity (15) and the observation that ataxin-3 is present in the aggregates of many neurodegenerative diseases (8, 44, 48, 49), it will now be important to define the precise role of this novel de-ubiquitinating enzyme in neurons and its potential regulation by ubiquitin pathways.

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