Deubiquitinating function of ataxin-3: Insights from the solution structure of the Josephin domain

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Spinocerebellar ataxia type 3 is a human neurodegenerative disease resulting from polyglutamine tract expansion. The affected protein, ataxin-3, which contains an N-terminal Josephin domain followed by tandem ubiquitin (Ub)-interacting motifs (UIMs) and a polyglutamine stretch, has been implicated in the function of the Ub proteasome system. NMR-based structural analysis has now revealed that the Josephin domain binds Ub and has a papain-like fold that is reminiscent of that of other deubiquitinasen, despite primary sequence divergence but consistent with its deubiquitinating activity. Mutation of the catalytic Cys enhances the stability of a complex between ataxin-3 and polubiquestinylated proteins. This effect depends on the integrity of the UIM region, suggesting that the UIMs are bound to the substrate polyubiquitin during catalysis. We propose that ataxin-3 functions as a polubiquestin chain-editing enzyme.

ataxia | polyglutamine | ubiquitin | ubiquitin interaction motif | valosin-containing protein

Spinocerebellar ataxia type 3, also known as Machado–Joseph disease, is one of several hereditary autosomal dominant neurodegenerative disorders caused by expansion of a polyglutamin (polyQ) stretch in the affected gene product (1, 2). The gene responsible for spinocerebellar ataxia type 3 encodes a 42-kDa protein named ataxin-3. Ataxin-3 contains an N-terminal Josephin domain (JD), a conserved module named after the Machado–Joseph disease, two ubiquitin (Ub)-interacting motifs (UIMs), a polyQ stretch, and a short variable tail. In one splice variant, the C-terminal region ends with a third putative UIM (3).

The JD is present in at least 30 predicted proteins, including two human proteins comprising the JD alone. Computations analyses resulted in two different predictions about the JD function. One study noted a distant structural homology to the epsin N-terminal homology/AP180 N-terminal homology domains (4–6) present in adaptor proteins implicated in endocytosis (7). Another study identified signature motifs for Cys residues in Machado–Joseph disease patients (22). Similar expansions of polyQ stretches are responsible for at least nine inherited human neurodegenerative disorders, including Huntington’s disease. Proteins containing such expansions have an increased propensity to misfold and aggregate (23–25), often forming intranuclear inclusions in affected cells. Accordingly, there is evidence that proteins involved in the control of protein folding and aggregation can affect disease progression in animal models (26, 27). The functional link of ataxin-3 to the ubiquitin-proteasome system and to VCP (19, 21) suggests a potential interplay between the properties of these proteins and the pathogenic role of its polyQ expansion. In fact, a very recent study demonstrated that functions of ataxin-3 directly related to its property to bind and process polyubiquitin suppress polyQ-induced degeneration in Drosophila (28).

The proposed role of ataxin-3 as a deubiquitinating enzyme raises the question of a potential coordination between such catalytic activity and polyubiquitin binding by the UIMs. For example, UIMs may help to recruit ataxin-3 to polyubiquitinated substrates, orient such substrates relative to the JD, or mediate a polyubiquitin editing function of ataxin-3. In addition, given the lack of primary sequence similarity of ataxin-3 to other deubiquitinating enzymes, it would be of interest to elucidate the structural basis of its enzymatic activity. To begin addressing these questions, we have performed NMR studies and biochemical analyses. We report that the tertiary structure of the ataxin-3 JD is strikingly similar to other deubiquitinating enzymes, such as UCH-L3. Our results also suggest cooperation between the JD and the UIMs during catalysis and are consistent with an editing function for this enzyme. Collectively, our results define

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Abbreviations: JD, Josephin domain; NOE, nuclear Overhauser effect; polyQ, polyglutamine; Ub, ubiquitin; UIM, Ub-interacting motif; VCP, valosin-containing protein.

Data deposition: The JD chemical shift assignments have been deposited in the BioMagResBank (BMRB accession no. 6742). Coordinates for the ensemble of the JD structures have been deposited in the Protein Data Bank (PDB ID code 2AGA).

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EDTA with anti-FLAG M2 gel for 2 h. After extensive washing, bound 0.1% SDS, pH 7.4. Cleared lysate (1 mg) was then incubated by Western blotting.

After 18–22 h (the reaction occurred very slowly), the reaction containing 1 in 50 mM Hepes, pH 7.4

1–198) and affinity-purified. Monoclonal anti-Ub antibodies were from Sigma. Mao SPARKY (30).

3H2O

ataxin-3 as a critical component of the polyubiquitin-dependent pathways that control protein folding and stability.

Materials and Methods

Antibodies and Reagents. Antibodies against ataxin-3 were generated in rabbits by using recombinant GST-JD (amino acids 1–198) and affinity-purified. Monoclonal anti-Ub antibodies (P4D1) were from Santa Cruz Biotechnology, and monoclonal anti-Flag M2 antibodies were from Sigma.

Cloning, Mutagenesis, and Protein Preparation. A cDNA encoding full-length human ataxin-3 (Machado–Joseph disease 1-1 splice variant carrying a polyQ sequence of 22 residues) was amplified by PCR with Integrated Molecular Analysis of Genomes and Their Expression (I.M.A.G.E.) clone ID 4393766 as a template. The cDNA was subcloned in pGEX-6P-1 and pET43 for expression in bacteria and in pcDNA-FLAG vectors for mammalian expression. The JD domain from the same proteins (amino acids 1–193) was obtained in a similar way. All of the mutants were generated by site-directed mutagenesis, and the constructs were verified by sequencing.

GST and His-6 fusion proteins were produced according to manufacturer’s instructions. For NMR sample preparation, the JD protein was expressed as a GST fusion. Isotopically labeled protein was produced by using the appropriate Spectra-9 bacterial growth medium. The GST tag was removed by PreScission protease from the fusion proteins bound to glutathione beads, and the eluted protein was further purified with size exclusion chromatography. NMR samples of 1.5 mM JD were prepared in a 20 mM potassium phosphate, pH 6.4/0.05% azide/5% 2H2O/10 μM 2,2-dimethyl-2-silapentane-5-sulfonate solution containing 1 μM of the protease inhibitors PMSF, leupeptin, and pepstatin.

Pull-Downs and Deubiquitination Assays. For pull-down experiments, His-tagged ataxin-3 proteins were incubated for 2 h at 4°C with 400 ng of K48-linked polyubiquitin chains and Ni2+ beads in 50 mM Heps, pH 7.4/10% glycerol/1% Triton X-100/1 mM EDTA/1 mM EGTA/10 mM MgCl2/10 mM CaCl2/20 mM imidazole. Bound material was analyzed by Western blotting.

For deubiquitination assays, 300 ng of tetrabubiquitin was incubated with 100 pmol of His-tagged ataxin-3 proteins at 37°C in 50 mM Tris, pH 7.4/5 mM MgCl2/25 mM KCl/1 mM DTT. After 18–22 h (the reaction occurred very slowly), the reaction was stopped with SDS sample buffer and samples were analyzed by Western blotting.

Transfection and in Vivo Binding Experiments. HeLa cells were transfected with ataxin-3 constructs by using Lipofectamine according to the manufacturer’s protocol. Cells were harvested 24 h later and lysed in JS buffer (50 mM Hepes/150 mM NaCl/10% glycerol/1% Triton X-100/1.5 mM MgCl2/5 mM EGTA, pH 7.5) or in radioimmunoprecipitation assay buffer (50 mM Tris/150 mM NaCl/1% Triton X-100/1% deoxycoleate/0.1% SDS, pH 7.4). Cleared lysate (1 mg) was then incubated with anti-FLAG M2 gel for 2 h. After extensive washing, bound proteins were analyzed by Western blotting.

NMR Resonance Assignments and Structure Determination. All NMR spectra were collected at 25°C on a Varian INOVA 600 MHz spectrometer and used pulse sequences available in the Varian BioPack User Library. Sequential and aliphatic side-chain assignments were determined by analysis of 3D-HNCO, HNCA, HN(CB)CA, HCACO, CBCA(CO)NH, (HCA)CO-(CA)NH, CC(CO)NH, HCC(CO)NH, and HCCCH-total correlation spectroscopy NMR experiments. All spectra were processed with NMRpipe (29) and subjected to visual analysis in Sparky (30).

Restrains on the backbone dihedral angles were derived from an analysis of backbone chemical shifts with the TALOS program (31). Hydrogen bonds were identified during the later stages of structure determination based on the consistent proximity of hydrogen bonding partners in the calculated ensembles. Nuclear Overhauser effect (NOE) correlations between nearby protons were identified in 3D 15N-NOEYHQC, 13C-NOESYHQC (aromatic), and 13C-NOESYHQC (aliphatic) NMR spectra.

Structure calculations were ultimately performed with the CYANA software package (32) based on NOEs that were interpreted and calibrated by the program CANDID (33). Manually interpreted NOE restraints were based primarily on the use of symmetry-related 3D NOE crosspeaks during the iterative cycles of NOE interpretation and structure calculation were added to assist structural convergence. The final series of structure calculations used the CANDID-derived NOE restraints and the previously described hydrogen bond and backbone torsion angle restraints. After an initial brief minimization of randomized conformations, simulated annealing began with 5,000 steps of molecular dynamics at high temperature, followed by 35,000 dynamics steps for cooling and a final 10,000 steps of conjugate gradient minimization. Generally, 50 structures were independently calculated, and the 20 structures with the lowest target function values were retained as the final ensemble.

Ub Binding by NMR Chemical Shift Perturbation. 15N- and 13C15N-labeled JD at 100 μM was titrated with unlabeled Ub in concentrations from 33 to 2,400 μM. Two-dimensional 1H15N HSQC spectra were measured for each condition and 3D HNCO NMR spectra were collected for samples at 1:0 and 1:2 molar ratios of Josephin and Ub, respectively. Vice versa, 2D 1H15N HSQC spectra from [15N]Ub alone or [15N]Ub mixed with unlabeled JD were also collected. NMR resonances for the JD were affected in one of three ways: (i) none or minimal change in intensity or chemical shift, (ii) major perturbation of chemical shift with minor intensity reduction, or (iii) major intensity reduction or “missing” crosspeaks due to extreme line broadening. Because of the transient nature of the interaction between Ub and the JD, association–dissociation exchange of Ub is likely to occur on a time scale similar to the NMR chemical shift differences between the two states. For the largest chemical shift differences, NMR line shapes are broadened because of the contribution of the exchange process to NMR relaxation, with the largest chemical shift perturbations resulting in the broadest peaks. Hence, for the purpose of constructing Fig. 3, chemical shift perturbations were scaled from gray to yellow for class II, based on the weighted sum of the chemical shift changes for the amide 1H, 15N, and carbonyl 13C nuclei, and then from yellow to orange for class III, based on the relative decrease in peak heights due to line broadening. Three well-resolved peaks in the 2D 1H13N HSQC spectra experiencing significant chemical shift changes during the titration were selected for simultaneously fitting to a single-site model of ligand-binding using SIGMAFIT to estimate a dissociation constant of 3.4 ± 0.3 mM.

Results

NMR Solution Structure of the JD. The solution structure of the JD (amino acids 1–193 of human ataxin-3) (Fig. 6, which is published as supporting information on the PNAS web site) was determined by NMR spectroscopy. As a first step toward structure determination, the 1H, 13C, and 15N NMR chemical shifts of the JD were assigned to near completion. Chemical shifts for the last 10 aa were observed in freshly prepared samples, but the chemical shifts diminished over time, probably because of proteolysis as the emergence of new NMR signals characteristic of degraded peptides. Thus, the last 10 aa were not included in the final structure calculations. A secondary set of chemical shifts was observed for a contiguous stretch of 22 residues (G51–G72).
The peak intensities of these secondary shifts were significantly smaller than those corresponding to the predominant conformation and had many fewer NOE crosspeaks. Thus, the 22 amino acids were omitted from the final structure calculations. The existence of the secondary set of chemical shifts likely reflects an alternative conformation for these residues, which may be important for protein function (discussed further below). Independent chemical-shift assignments for the ataxin-3 JD were recently published (34). Although chemical shifts for all of the backbone atoms and for the majority of the side chains are identical to ours, there are differences for side-chain atoms in several residues, which are most likely caused by differences in experimental conditions, such as pH.

Tertiary structures were calculated with the CYANA software package (32, 33) based on a total of 2,960 NOE-derived distance restraints, 122 hydrogen bond restraints, and 224 backbone torsion angle restraints. The final ensemble of tertiary structures is presented as a stereo pair in Fig. 1A and structural statistics are listed in Table 1, which is published as supporting information on the PNAS web site. The JD adopts an α/β fold consisting of a six-stranded, antiparallel β-sheet (β1–β6) flanked on both sides by a total of seven α-helices (α1–α7) (Fig. 1). The overall structure has a bi-lobed appearance divided by a groove. One lobe (in red and at the right in Fig. 1B) contains five α-helices (α1–α4 and α7). α1 and α4 are almost parallel to each other and packed by means of strong hydrophobic interactions against the β-strands. The much longer α2 is oriented nearly perpendicular to α1 and α4 at the surface of the domain. The stretch of residues comprising the short α3 and the following loop are associated with two alternative sets of chemical shifts and are therefore likely to undergo dynamic conformational changes. The other lobe (in blue and at the left in Fig. 1B) consists of two α-helices (α5 and α6) and the six-stranded β sheet. The six antiparallel β-strands have an average length of four residues with the following sequence: β1, β6, β2, β3, β4, and β5. The gaps between β1 and β2 and β5 and β6 are filled by α5 and α6, respectively, whereas the connections between β2, β3, β4, and β5 are made by three tight turns.

**The JD Resembles Deubiquitinating Enzymes But Has Unique Structural Features.** A search for homologous structures using the DALI algorithm (35) showed that the JD is structurally similar to proteins in the papain-like Cys–protease family. This family comprises three of the four families of deubiquitinating enzymes whose structures have already been characterized. Among proteins with the highest Z scores (≈5.0) is the Ub C-terminal

![Fig. 1](image1.png)

**Fig. 1.** Tertiary structure of the JD of ataxin-3. (A) Stereoview of the superimposed Cα backbone traces of the 20 lowest-energy calculated structures. Traces are rainbow-colored, with the N terminus (N) in red and the C terminus (C) in blue. Numbers and corresponding small filled circles indicate residue multiples of 20. (B) Ribbon diagram of a representative structure from A shown in the same orientation. The left lobe (blue) comprises a six-stranded β-sheet and two α-helices. The right lobe (red) comprises only α-helices.

![Fig. 2](image2.png)

**Fig. 2.** Structural comparison of the JD with UCH-L3. (A and B) Secondary structure topologies of the JD (A) and UCH-L3 (B) (Protein Data Bank ID code 1UCH). α-Helices are represented by filled circles, and β-strands are represented by triangles. β-Strand direction out from the paper is indicated by left-oriented triangles, and β-strand direction into the paper is indicated by right-oriented triangles. The left lobe of the JD is conserved in UCH-L3 (colored in blue). The main topological differences are observed in the right lobe. The N-terminal α-helices of the JD are “shuffled” as a big insertion between the β2 and β3 strands in UCH-L3. The “substrate-limiting loop,” which crosses over the catalytic site in UCH-L3 (yellow), is missing in the JD. Green letters indicate the positions of the catalytic residues with single-letter amino acid abbreviations. (C and D) Ribbon diagrams of the catalytic sites of the JDs of ataxin-3 (Q and UCH-L3 (D), with the active site residues shown in ball-and-stick models. The catalytic residues have a similar geometric organization in the two proteins, suggesting a similar mechanism of catalysis.
hydrolyase UCH-L3, which has the lowest rms deviation value of 3.2 Å for all Cα atoms. Comparison of the structural topologies of the JD and UCH-L3 reveals that the lobe containing the β-sheet (at left and colored in blue in Figs. 1 and 2) is structurally conserved. In contrast, the other lobe, comprising only α-helices, has a different organization of secondary structure elements relative to UCH-L3. Another major distinction involves the connection between β2 and β3, which, in the JD, is represented by a tight β-turn, whereas, in UCH-L3, it comprises a large insertion including α3, α4, α5, and α6.

Our results demonstrate the validity of a previous bioinformatics analysis that had identified some weak similarities between the regions surrounding the catalytic residues His, Asn/Asp, and Cys of deubiquitinating enzymes and three short segments in ataxin-3 (Fig. 6) (8, 36). In the JD structure, the predicted catalytic residues (C14, H119, and N134) are indeed in close proximity to each other, forming the so-called catalytic triad, which is characteristic of Cys proteases (Fig. 2B). The organization of the catalytic triad residues is in an appropriate geometry for catalytic function. Thus, these structural data are consistent with the reported deubiquitinating activity of ataxin-3 (9, 37).

Based on the conserved topology of the catalytic site residues, a likely mode of Ub binding to the JD can be proposed by analogy with the structures of the deubiquitinating enzyme Yuh1 (the yeast orthologue of UCH-L3) and herpesvirus-associated Ub-specific protease with Ub aldehyde (38, 39). The existence of an alternative conformation or dynamic state for α3 and the α3–α4 loop combined with their chemical shift changes may reflect an inducible structural dynamic for these residues associated with Ub binding. We also collected 2D 1H15N HSQC spectra from [15N]Ub alone or [12]Ub mixed with unlabeled JD. Residues 5–15, 29–36, and 68–75 of Ub displayed the largest chemical-shift perturbations. When mapped onto the Ub surface, these chemical-shift-perturbed residues form a contiguous region (Fig. 3B). A similar surface area of Ub has also been reported by NMR chemical-shift perturbation to be involved in binding to UCH-L3 (40).

The UIMs of Ataxin-3 Are Involved in the Deubiquitinating Reaction. Ataxin-3 has at least two other Ub-binding sites that are the tandem UIMs immediately C-terminal to the JD. A plausible speculation is that these UIMs recognize the polyubiquitinated substrate and help position the polyubiquitin chain close to the catalytic site on the JD, whereas the Ub-binding site on the JD transiently holds the distal Ub during catalysis (see Fig. 5). To test this idea, we performed in vitro and in vivo binding assays.

Pull-down assays of polyubiquitin mixtures on ataxin-3 constructs demonstrated that, in agreement with previous data (16, 17), full-length ataxin-3 interacts with K48-linked polyubiquitin chains with a preference for high-molecular-weight chains (n ≥ 3). As shown by mutation of the Ser critically required for binding in the UIMs, this interaction largely depends on the first and second UIMs (17), although it is independent from the third putative UIM (Fig. 4F). Despite this clear interaction in cell-free assays, no significant coprecipitation between ataxin-3 and polyubiquitinated substrates could be obtained from cell lysates. The binding of ataxin-3 to its substrates may therefore be very transient in vivo. However, if the UIMs of ataxin-3 interact with the polyubiquitin chain during catalysis, mutations that affect the catalytic activity of the JD but not its Ub-binding sites are expected to enhance the stability of the interaction between ataxin-3 and its substrate (see Fig. 5). Under these conditions, the polyubiquitin chain would be held attached to ataxin-3 by two anchorage points (the UIMs and the Ub-binding site of the JD) at both sides of the inactive catalytic site.

We tested this possibility. First, with tetrabiquitin as a substrate, we confirmed that, although purified full-length ataxin-3 and its isolated JD have deubiquitinating activity, their C14A mutants do not (9, 37) (Fig. 4B). Next, we analyzed immunoprecipitates from cells transfected with FLAG-tagged wild-type and mutant ataxin-3 constructs for the presence of coprecipitating ubiquitinated proteins by Western blotting. Immunoprecipitates generated from cells expressing ataxin-3C14A contained much higher levels of ubiquitinated proteins relative to those generated from cells expressing wild-type ataxin-3 (Fig. 4C, lane 2 and 3), and coprecipitation was abolished if cells were lysed in radioimmunoprecipitation assay buffer, i.e., under conditions that disrupt UIM binding to Ub (Fig. 4C, lane 10 and 11). These data strongly suggest the trapping of an enzyme–substrate complex. Furthermore, this effect required portions of ataxin-3 outside the JD, because a corresponding mutant JosephinC14A domain did not retain ubiquitinated proteins (Fig. 4C, lanes 4 and 5). Furthermore, we found that the first and third UIMs are almost dispensable for substrate trapping, because ataxin-3C14A, S265A and ataxin-3C14A, S347A still efficiently pulled down ubiquitinated proteins.
that recognizes its JD. Arrows indicates IgG heavy chains. were analyzed by Western blotting for Ub or for ataxin-3 with an antibody buffer (to disrupt Ub–UIM interactions) (Fig. 4).

Activity mediated by the first two UIMs, for the longest chains. Ctr, control. (B) Deubiquitination assays carried out by incubating tetraubiquitin with recombinant His-tagged ataxin-3 and JD and their mutants. At the end of the reaction, Ub and ataxin-3 proteins were detected by Western blotting. Activity is indicated by the conversion of tetraubiquitin to triubiquitin. (C) In vivo substrate trapping by catalytically inactive ataxin-3. HeLa cells were transduced with FLAG-tagged wild-type ataxin-3 or mutant ataxin-3 constructs or with the empty vector (Ctr lane). FLAG-tagged proteins were then lysed and immunoprecipitated in JS buffer (left) or radioimmunoprecipitation assay buffer (to disrupt Ub–UIM interactions) (Right), and the immunoprecipitates were analyzed by Western blotting for Ub or for ataxin-3 with an antibody that recognizes its JD. Arrows indicates IgG heavy chains. (Fig. 4C, lanes 6 and 9). However, mutations in the second ataxin-3S256A or the first and second UIMs of ataxin-3S236, S256A drastically inhibited substrate trapping (Fig. 4C, lanes 7 and 8), suggesting a critical role of the second UIM in this phenomenon. These “substrate trapping” experiments strongly indicate a tight link between catalytic activity of the JD and polyubiquitin chain binding by the UIM(s).

Discussion

Based on computational modeling, ataxin-3 was predicted to be a distant homolog of endocytic adaptins, such as epsin and API180 (7), or of deubiquitinating enzymes (8). Subsequently, this domain was shown to have deubiquitinating activity (9). With the determination of the solution structure of the JD, ataxin-3 can be conclusively defined as a deubiquitinating enzyme. Ataxin-3 is unusual among the many deubiquitinating enzymes because of the unique array of functional units related to protein quality control and degradation that are contained in its sequence. It is of interest to establish how these various units cooperate. Previous data have shown that the entire portion of ataxin-3 distal to the JD is flexible and nonstructured, with no significant interactions between this region and the N-terminal JD (25). Together with these previous results, our findings support a model in which the functional groups of ataxin-3 are arranged in an extended linear fashion, starting with the Ub-binding site of the JD and followed in order by the catalytic triad, the tandem UIMs, the polyQ stretch, and the variable C terminus. This arrangement could accommodate a polyubiquitin chain with a (distal) Ub bound to the Ub-interacting site on the JD, its C-terminal isopeptide bond juxtaposed to the catalytic site, and proximal Ub(s) bound to the UIMs (Fig. 5). In this fashion, these multiple functional units act in a concerted way in the deubiquitinating reaction. This model allows the proper orientation of the catalytic substrate on the surface of ataxin-3 for the formation of an enzyme–Ub tetrahedral intermediate, as observed in the structures of UCH-L3 and herpesvirus-associated Ub-specific protease complexed with Ub aldehyde (38, 39). The model is also consistent with our in vivo substrate trapping data showing that the UIM region plays an important role in the catalytic activity of ataxin-3. The UIMs may help to recruit polyubiquitinated substrates, to position the polyubiquitin chain relative to the catalytic site, and/or to allow the enzyme to act in trimming polyubiquitin chains in a distal to proximal direction. Accordingly, preliminary results indicate that full-length ataxin-3 prefers to cut the distal Ub of a short Ub chain.

An interaction between ataxin-3 and VCP/p97 was first suggested by the observation that VCP colocalized with expanded polyQ aggregates in cultured cells (18). Subsequently, a direct interaction between VCP/p97 and ataxin-3, which is mediated by the C-terminal region of ataxin-3, including its polyQs stretch, was reported (18, 41). Indeed, not only have we confirmed that the two proteins interact in pull-down experiments, but we have also found that ataxin-3, but not its isolated JD, coprecipitates with VCP/p97 from cotransfected cells (our unpublished observations). VCP/p97 mediates multiple cellular functions, including membrane fusion, endoplasmic reticulum-associated degradation, and other protein quality control activi-
ities (20). Furthermore, recent data suggest that CDC48 (the yeast orthologue of VCP/p97) plays a central role in guiding proteolytic substrates to the proteasome for degradation (42). These findings speak to a role of the deubiquitinating activity of ataxin-3 in a pathway leading to proteasomal degradation, which is centered at VCP/p97.

In conclusion, our findings demonstrate that ataxin-3 is indeed a deubiquitinating enzyme and suggest that it functions as a polyubiquitin chain-editing enzyme that shortens Ub chains. These properties distinguish ataxin-3 from other deubiquitinating enzymes that function in the processing of Ub precursors, the reversal of Ub conjugation, or the recycling of Ub.

Note. Shortly before submission, an independent solution structure of ataxin-3 in a pathway leading to proteasomal degradation, which was supported in part by National Institutes of Health Grants NS36251 (to P.D.C.) and CA108992 (to M.E.H.) and by grants from the Human Frontiers Science Program (to P.D.C. and P.P.D.F.); the Associazione Italiana per la Ricerca sul Cancro (to P.P.D.F. and S.P.); the European Community; the Italian Ministry of Health; the Italian Ministry of Education, University, and Research; the Monzino Foundation (to P.P.D.F.); and the Charlotte Geyer Foundation (to M.E.H.).

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