Supporting Experimental Procedures

Quantitative mass spectrometry. K48-linked Ub6 (Boston Biochem) was separated by SDS-PAGE and stained with Coomassie Brilliant Blue. The HMW chain complexes were excised from the gel and analyzed by previously established methods (1, 2). Briefly, proteins in gel pieces were digested by trypsin with the addition of eight stable heavy isotope labeled peptides as internal standards, including all seven –GG peptides and one ubiquitin peptide (Thr55-Lys63). The resulting peptide mixtures were analyzed by reverse phase liquid chromatography-tandem mass spectrometry (LC-MS/MS) on an LTQ-Orbitrap hybrid mass spectrometer (Thermo Electron, San Jose, CA). The instrument was operated to monitor the eight stable heavy isoptope labeled peptides and their related native counterparts by selective reaction monitoring (SRM). The quantification analysis was carried out using Xcalibur software (Thermo Finnigan, San Jose, CA).

Size Exclusion Chromatography. For high performance liquid chromatographic analysis of ataxin-3, GST-ataxin-3 was purified and the GST tags removed with Prescission Protease (GE Healthcare) as recommended by the manufacturer. 100 µLs of sample containing 2 µg of purified recombinant ataxin-3-Q22 or ataxin-3-Q80 was separated at 25°C on a Superdex 200 HR 10/30 column (Amersham) in U buffer [50 mM Tris (pH 7.5), 50 mM KCl, 0.2 mM DTT] with a flow rate of 1.0 ml/min and a fraction size of 500 µl. Gel filtration standard (Bio-Rad) was used to predict apparent molecular weight.

For low pressure liquid chromatographic analysis of immunoprecipitated ataxin-3, samples were diluted in sample buffer (0.05 M NaH_2PO_4, 0.15 M NaCl, pH 7.2, filtered through 0.22 µm filter). Cyanocobalamin was added to each sample as an internal control. Samples were run on a calibrated Sephacryl S-300 gel filtration column (Amersham Biosciences) using the BioLogic LP system (BioRad Laboratories). Eluate fractions were precipitated with ammonium sulfate by rotating overnight at 4^o and then centrifuging at 4000 rpm for 30 min. Protein pellets were resuspended in Laemmli buffer.

Supporting References

1. Kirkpatrick, D. S., Hathaway, N. A., Hanna, J., Elsasser, S., Rush, J., Finley, D., King, R. W., & Gygi, S. P. (2006) *Nat Cell Biol* **8,** 700-710.
2. Xu, P., Cheng, D., Duong, D. M., Rush, J., Roelofs, J., Finley, D., & Peng, J. (2006) *Israel Journal of Chemistry* **46,** 171-182.

Supporting Table Legend

SUPPORTING TABLE 1. Mass spectrometry analysis of K63-linked Ub_6 and HMW complexes of K48-linked Ub_6.

Ubiquitin linkages were quantitatively analyzed by reverse phase liquid chromatography/tandem mass spectrometry.

Supporting Figure Legends

SUPPORTING FIGURE 1. Binding of normal and expanded ataxin-3 to K48- and K63-linked ubiquitin chains.

A and B) Ataxin-3 binding to K48- and K63-linked ubiquitin chains. GST or GST-ataxin-3 (Q22 or Q80) (250 nM) prebound to glutathione sepharose beads were incubated with ubiquitin chains (Ub3-7, 250 nM) at 4° C. Unbound supernatant (S) and bound pellet (P) fractions were immunoblotted with anti-ubiquitin antibody.

SUPPORTING FIGURE 2. MALDI-TOF analysis of Ub2 reaction products derived from cleavage of mixed linkage Ub4 chains

A) Protease assays with ataxin-3 and heterotypic ubiquitin chains. 1 µM GST-ataxin-3 was incubated with mixed linkage Ub4 chains (250 nM) for 6 hours and analyzed by silver stain. The arrow indicates the Ub2 reaction product, which when analyzed by mass spectrometry proved to be exclusively K48 linked. Asterisk indicates a protease inhibitor (aprotinin) present in all lanes.

B) Ub2 reaction product was excised and analyzed by trypsin digest and MALDI-TOF. Shown is the peptide spectrum corresponding to the Ub2 reaction product generated from cleavage of Ub4 (48-63-48). Large filled arrow indicates presence of the peptide peak corresponding to K48 isopeptide linkage (m/z=1460.9), while large empty arrowhead indicates the absence of the peak corresponding to K63 isopeptide linkage (m/z=2244.2) is absent. Thin arrow shows a neighboring peak from a trypsin autocleavage fragment.

SUPPORTING FIGURE 3. Size exclusion chromatography of immunopurified ataxin-3. Purified bacterially expressed GST-ataxin-3 (*A*) or immunopurified FLAG-ataxin-3 from 293 FLP-In cells (*B*) were fractionated by size exclusion chromatography and analyzed by UV absorbance (*A*) or anti-ubiquitin immunoblotting (*B*). Both Q22 and Q80 ataxin-3 are soluble and do not form aggregates under the conditions used in our *in vitro* experiments.