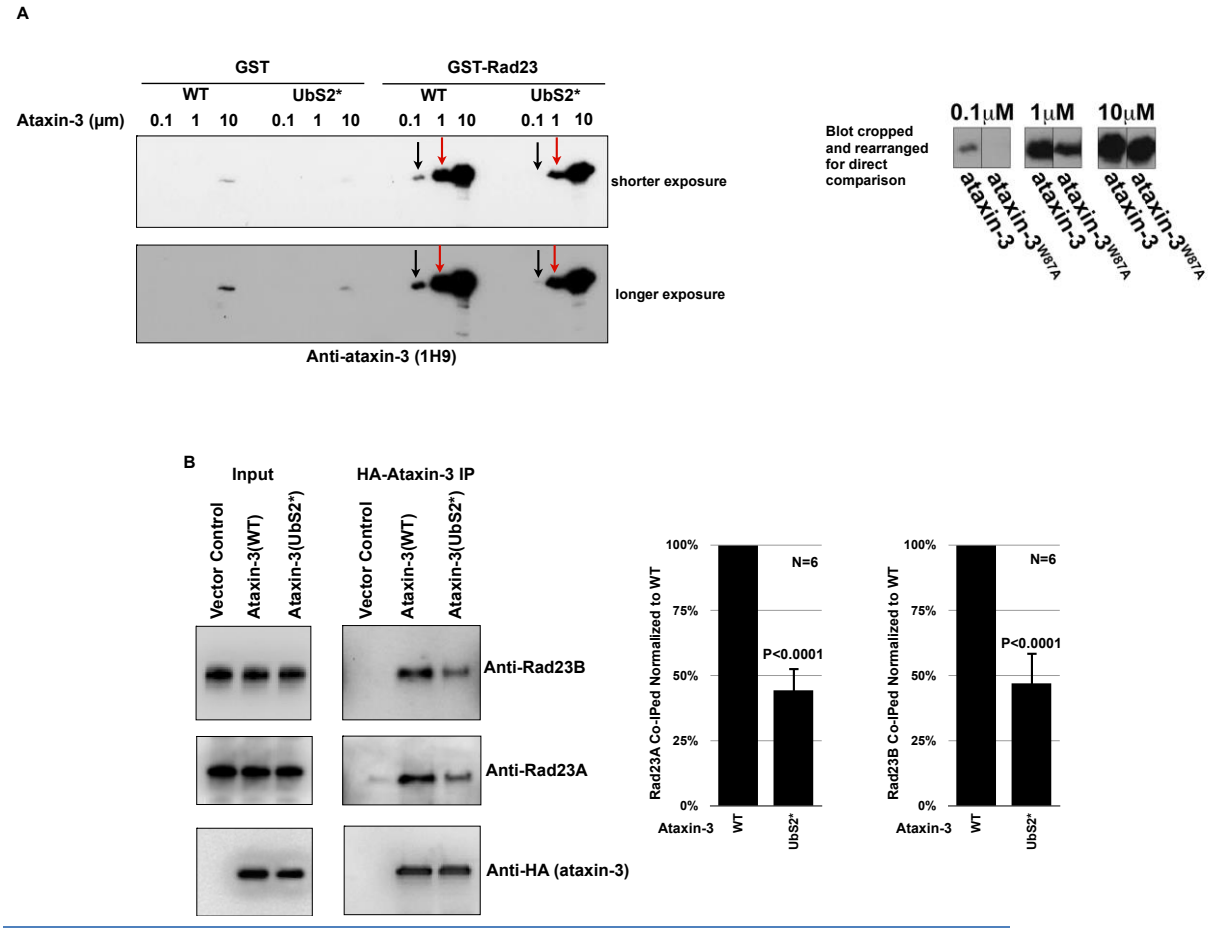


Supplementary figure 1

Reduced binding of ataxin-3 with mutated UbS2 to Rad23A and Rad23B.

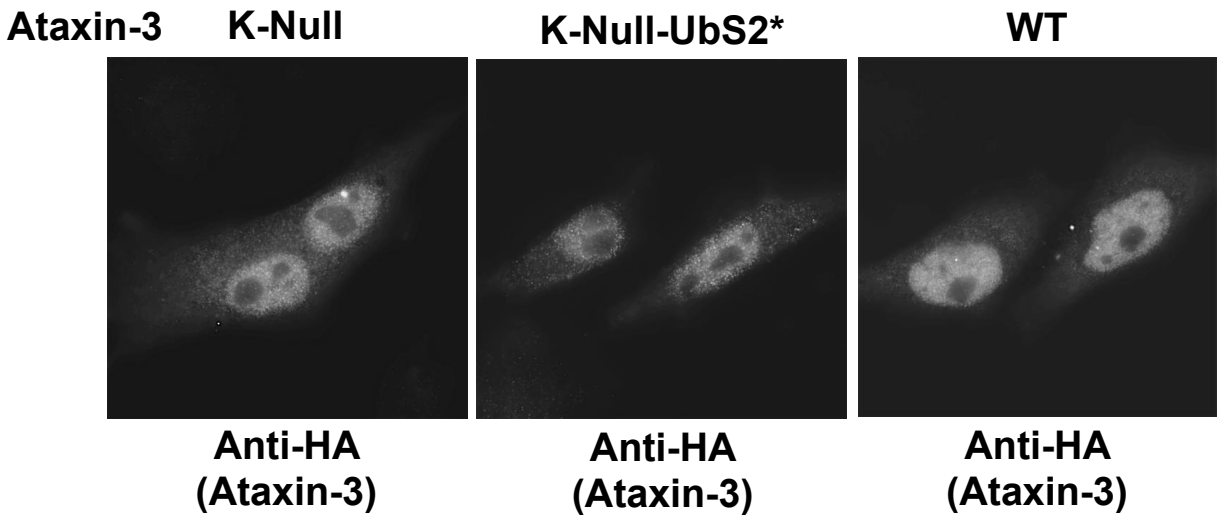
A) Left: *In vitro* binding assay demonstrates reduced capacity of recombinant, untagged, full-length ataxin-3 with mutated UbS2 to interact with bead-immobilized recombinant, full-length GST-tagged Rad23A. UbS2 mutation was W87A. Black and red arrows indicate reduction in binding at lower concentrations. Right: Blot from left cropped and rearranged to facilitate comparison of ataxin-3 that was pulled down by GST-Rad23A. Experiment was performed independently at least 5 times with similar results.

Pull-down assay: GST or GST-Rad23 were pulled down on glutathione agarose beads (Gold Biotechnology) and washed three times with NETN (50mM Tris pH 7.5,

150 mM NaCl, and 0.5% IPEGAL ca-630). 20µL of protein-coated beads were then incubated with a 20µL volume of ataxin-3(WT) or ataxin-3(UbS2*) (concentration indicated) for one hour on ice. Samples were then washed 3 times with 1mL of NETN and all buffer was removed after the final wash. 20µL of 1X laemli buffer was then added to the beads and samples were analyzed by SDS-PAGE/Western Blot.

B) HeLa cells were transiently transfected with the indicated HA-tagged ataxin-3 constructs. To obtain approximately equal levels of ataxin-3 protein in the different groups, 2X more ataxin-3(UbS2*) construct was transfected compared to ataxin-3(WT), which was supplemented with empty vector. 48 hours later cells were harvested and HA-tagged ataxin-3 was immunoprecipitated with agarose-bound anti-HA antibody. Shown are blots of endogenous Rad23A, endogenous Rad23B and transfected ataxin-3. UbS2 mutation was W87A. UbS2-mutated ataxin-3 was otherwise normal, and contained all of its lysine residues. Quantifications are from blots on the left and other similar experiments. P values are from Student T-tests. Means +/- standard deviation. N=6 independently conducted experiments.

For co-IPs, HeLa cells were harvested in PBS and lysed in NETN lysis buffer supplemented with complete protease inhibitor cocktail (Sigma Aldrich). Lysates were spun for 20 minutes at 14,000XG at 4˚C, and subsequently incubated with anti-HA agarose (Sigma Aldrich), tumbling for 4 hours at 4˚C. Beads were rinsed gently three times with PBS, and bead-bound complexes were eluted using 2% SDS buffer supplemented with 100µM DTT at 95˚C for 5 minutes.

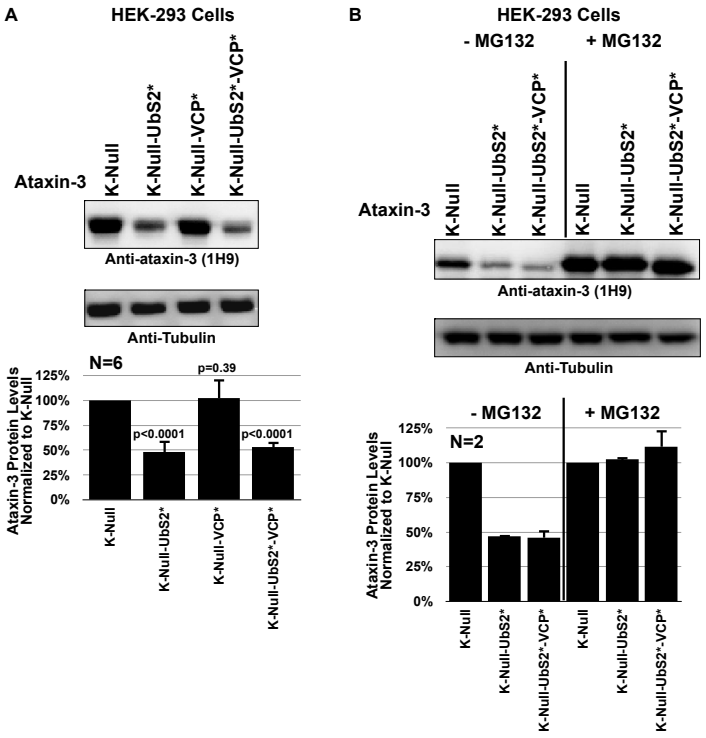


Supplementary figure 2

Mutating UbS2 does not alter ataxin-3 distribution in cultured cells.

Images of HeLa cells transfected with the indicated constructs, fixed 24 hours later and probed with an anti-HA antibody to detect transfected ataxin-3 while excluding signal from the endogenous protein. For imaging we selected cells with low intensity of fluorescence. The more densely staining structures are the nuclei (confirmed with DAPI staining).

For images, HeLa cells were plated on 12-well plates with coverslips coated with collagen, transfected with the indicated constructs and fixed in 4% paraformaldehyde 24 hours post-transfection. Fixed cells were permeabilized with 0.1% TritonX 100, blocked with 3% BSA, incubated overnight with primary antibody (anti-HA, Y11, Santa Cruz Biotech; 1:500), rinsed three times with PBS, and incubated for two hours with AlexaFluor 488 (Invitrogen) goat anti-rabbit secondary antibody (1:2,000). Cells were then rinsed three times with PBS, mounted with glycerol/DAPI medium, and imaged with an Olympus BX53 fluorescent microscope.

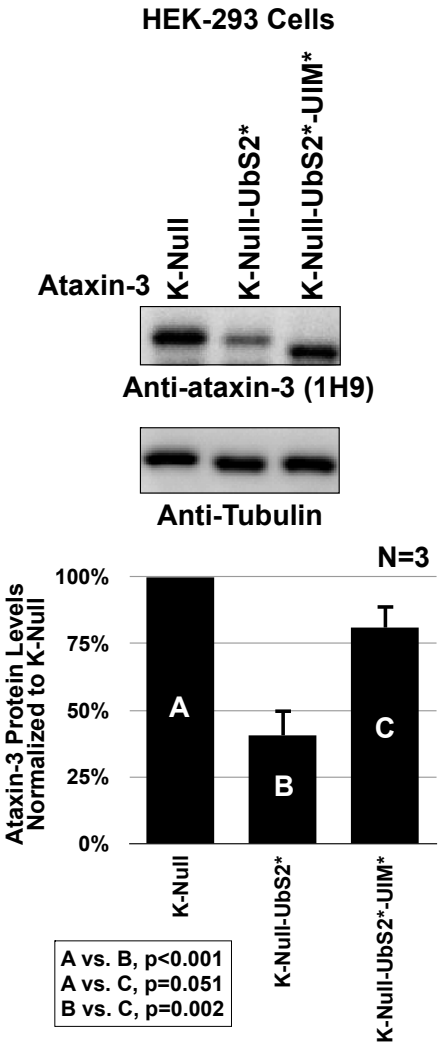


Supplementary figure 3

Mutating UbS2 leads to lower protein levels of ataxin-3 in HEK-293 cells.

A) Top: Western blots of whole cell lysates of HEK-293 cells transfected with the indicated constructs and harvested 48 hours later. Bottom: Quantification of ataxin-3 signal from blots from the top and other similar independent experiments. Error bars: standard deviations. P values are from ANOVA with Tukey post-hoc correction comparing the levels of ataxin-3 variants with mutated domains to K-Null ataxin-3 with all domains intact. N=6 independently conducted experiments.

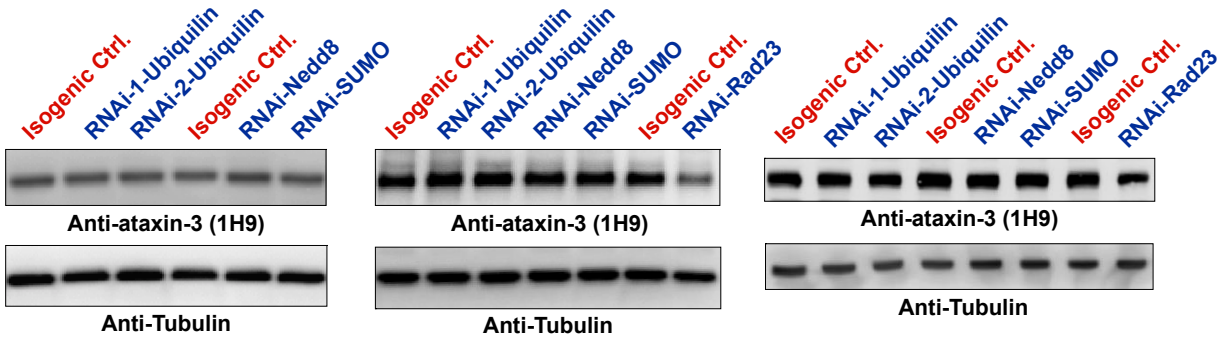
B) Top: Western blots of whole cell lysates of HEK-293 cells transfected as indicated and treated or not with the proteasome inhibitor MG132 (15µM) for 6 hours 24 hours after transfections. Bottom: Quantification of ataxin-3 signal from blots from the top and other similar independent experiments. Error bars: standard deviations. N=2 independently conducted experiments.



Supplementary figure 4

Mutating the UIMs increases levels of UbS2-mutated ataxin-3 in HEK-293 cells.

Top: Western blots of whole cell lysates of HEK-293 cells transiently transfected with the indicated constructs and harvested 48 hours later. Bottom: Quantification of ataxin-3 signal from blots from the top and other similar independent experiments. Error bars: standard deviations. P values are from ANOVA with Tukey post-hoc correction comparing the levels of ataxin-3 variants with mutated domains to K-Null ataxin-3 with all domains intact. N=3 independently conducted experiments.

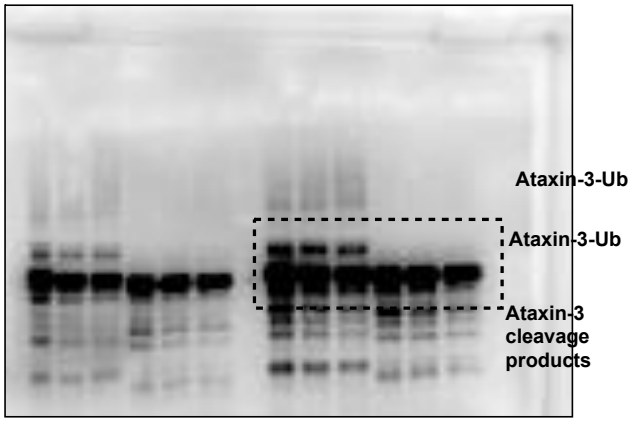


Supplementary figure 5

Knockdown of genes that encode SUMO, Nedd8 or Ubiquilin does not lead to lower levels of ataxin-3 in flies.

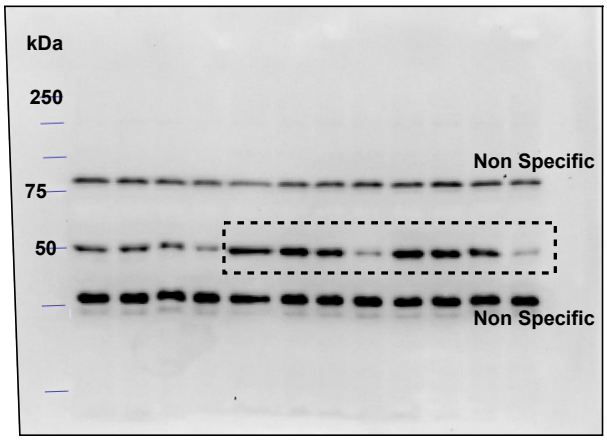
Western blots from dissected fly heads expressing ataxin-3 with all of its domains intact, while simultaneously knocking down through RNAi the fly genes indicated in the panels. Blots are from three independent experimental repeats. Fifteen fly heads per genotype were homogenized. Driver was gmr-Gal4 and all flies were heterozygous for driver, UAS-ataxin-3 and the specified UAS-RNAi line. Isogenic controls are to the left of the respective RNAi lines. RNAi lines for ubiquilin (HMS00949, HMS00860), SUMO (JF02869) and Nedd8 (HMS00818) were generated by the TRiP project at Harvard Medical School, and were purchased from the Bloomington *Drosophila* Stock Center. Flies were 1-3 days old.

Figure 1B

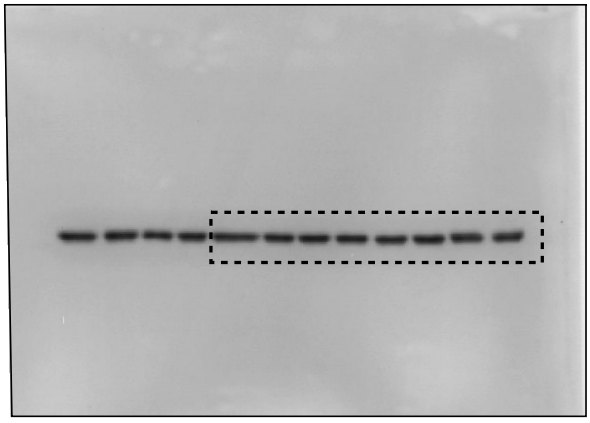


Anti-ataxin-3 (1H9)

Figure 1C

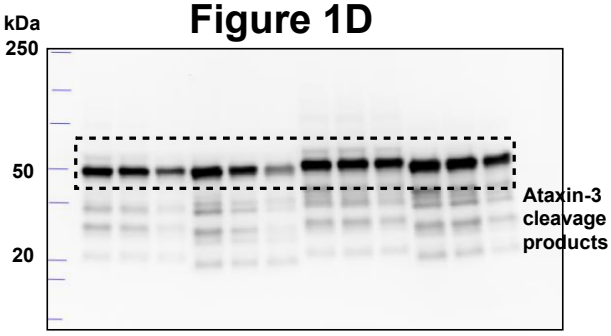


Anti-HA (Ataxin-3)

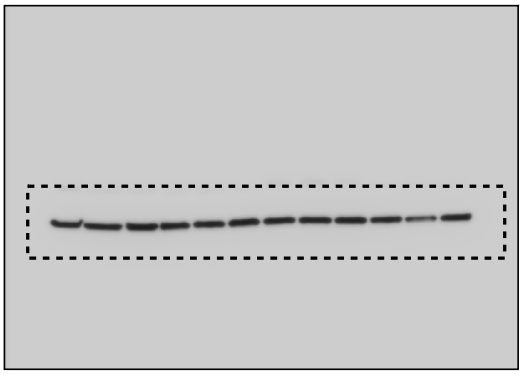


Anti-Tubulin

Figure 1D

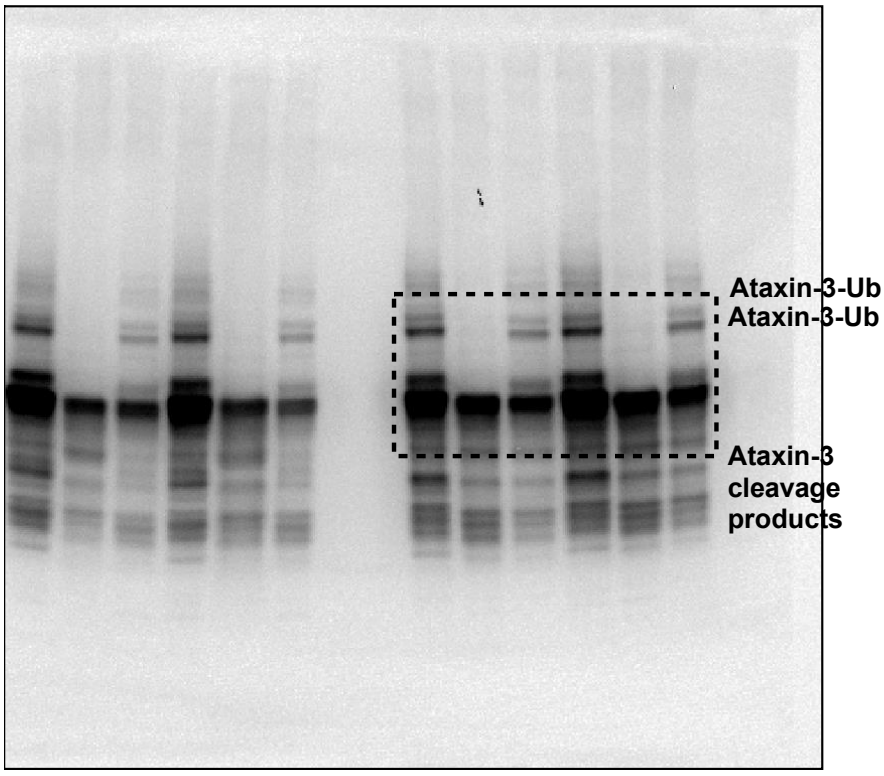


Anti-ataxin-3 (1H9)

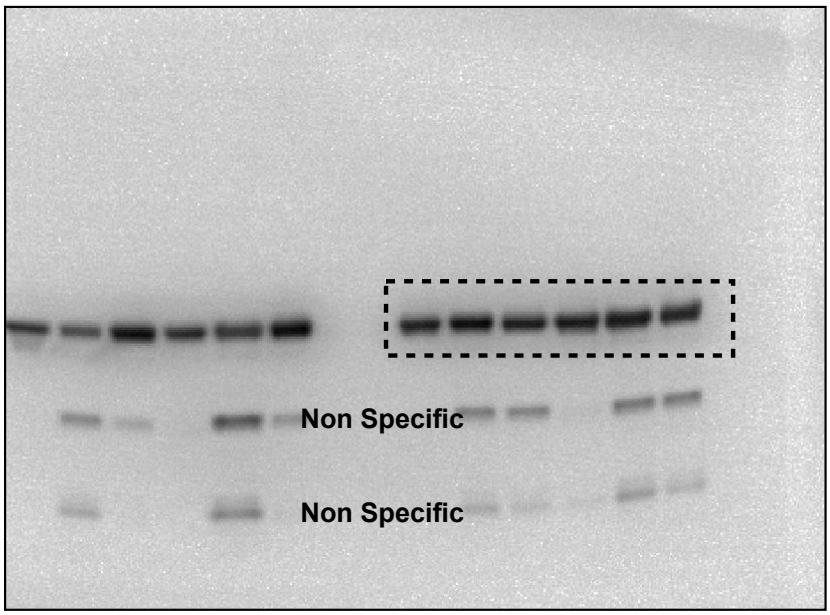


Anti-Tubulin

Figure 2B



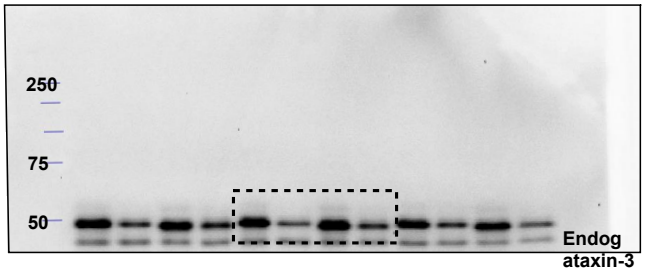
Anti-ataxin-3 (1H9)



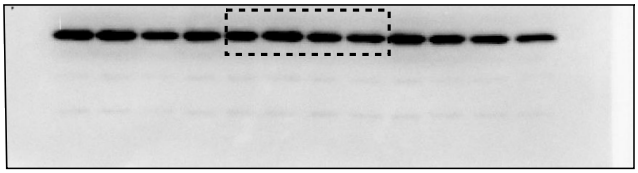
Anti-Tubulin

Figure 3A

Membrane was cut to probe with two different antibodies



Anti-ataxin-3 (1H9)



Anti-GAPDH

Figure 3C

Membrane was incubated with both ataxin-3 and GAPDH antibodies at the same time

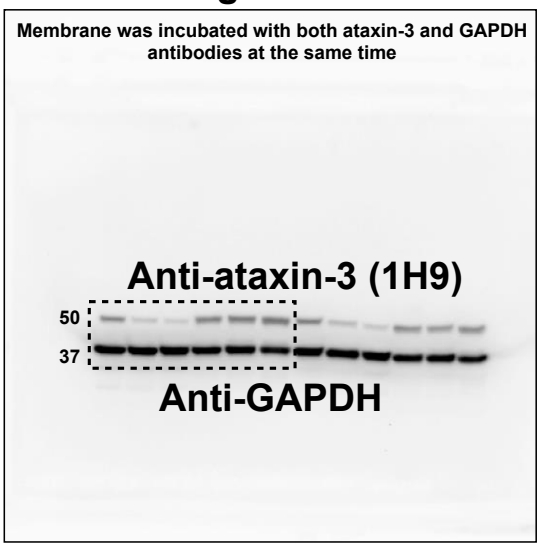
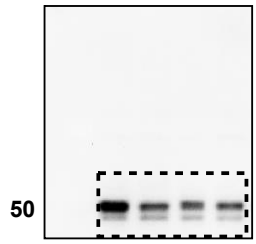
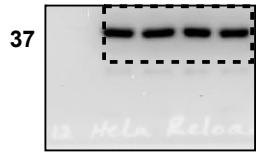


Figure 3D

Membrane was cut for use with other experiments, and to probe with two different antibodies

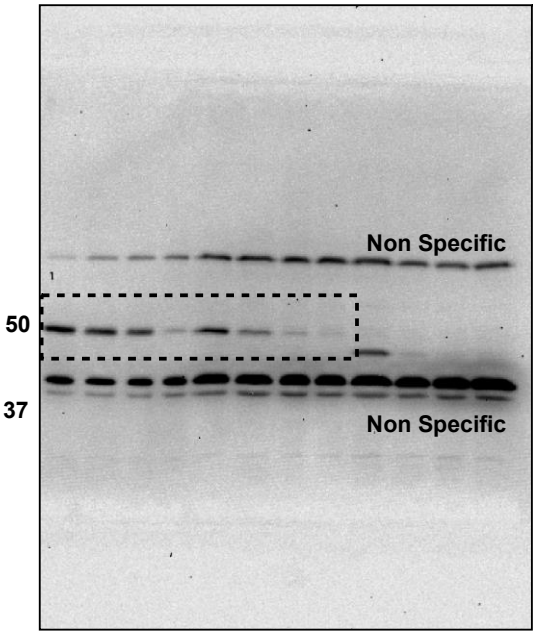


Anti-ataxin-3 (1H9)

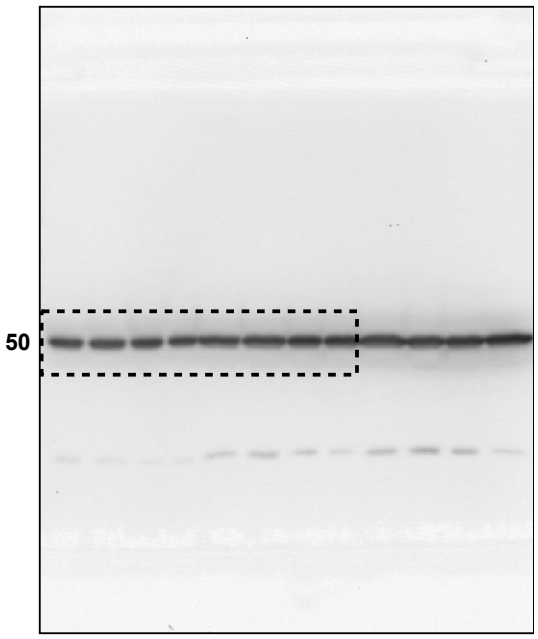


Anti-GAPDH

Figure 3E

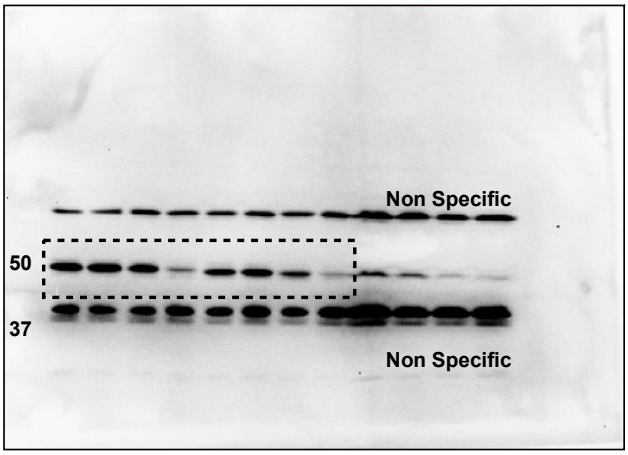


Anti-HA (Ataxin-3)

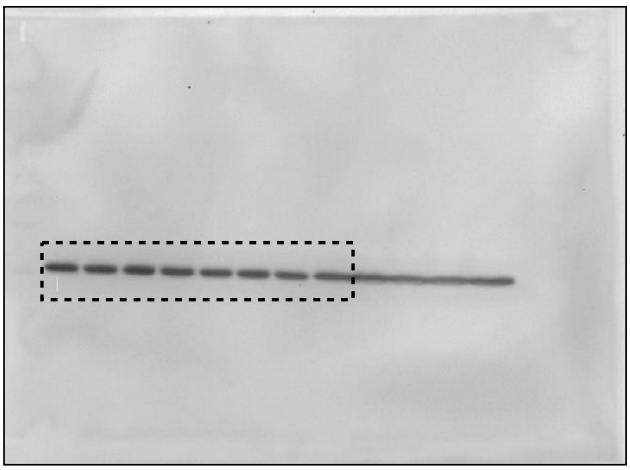


Anti-Tubulin

Figure 3F

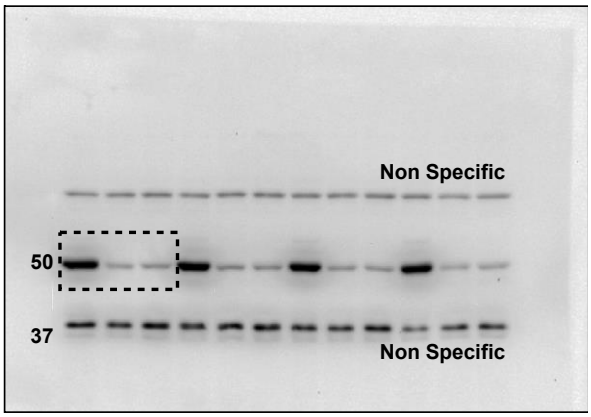


Anti-HA (Ataxin-3)

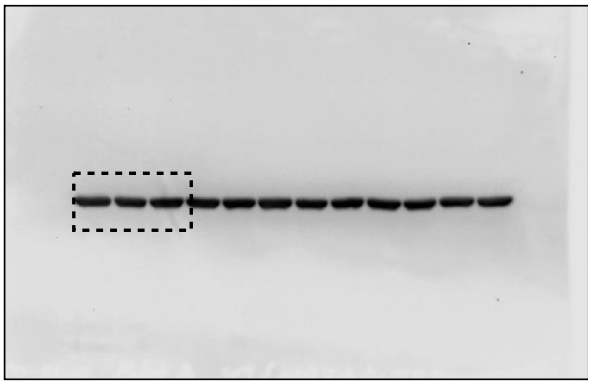


Anti-Tubulin

Figure 3G

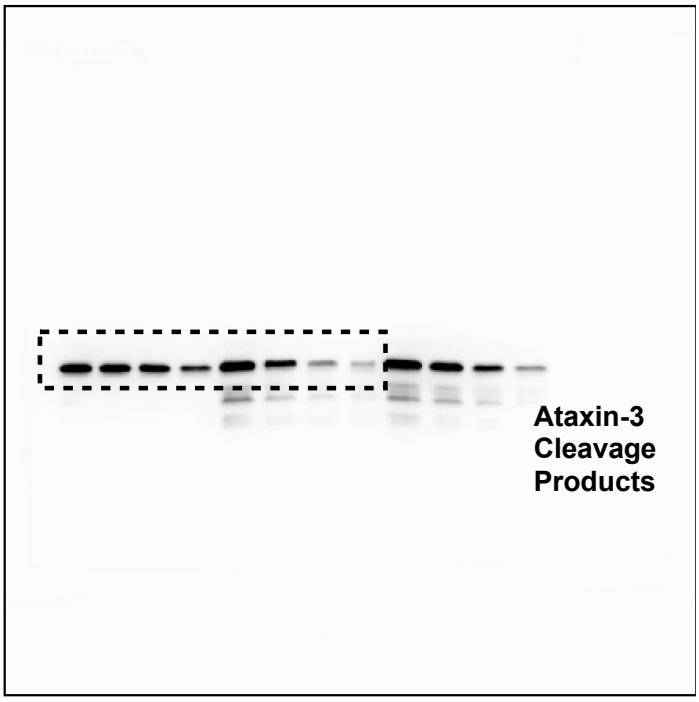


Anti-HA (Ataxin-3)

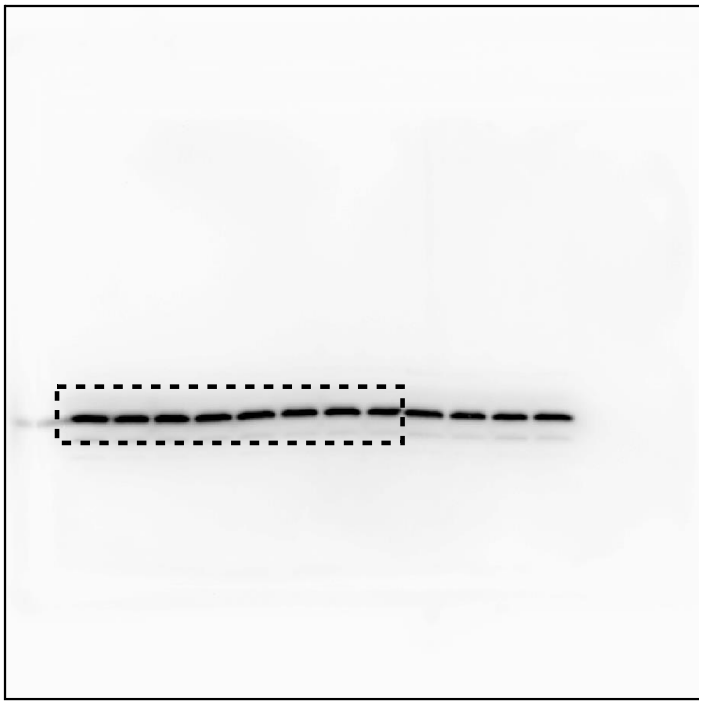


Anti-Tubulin

Figure 3H



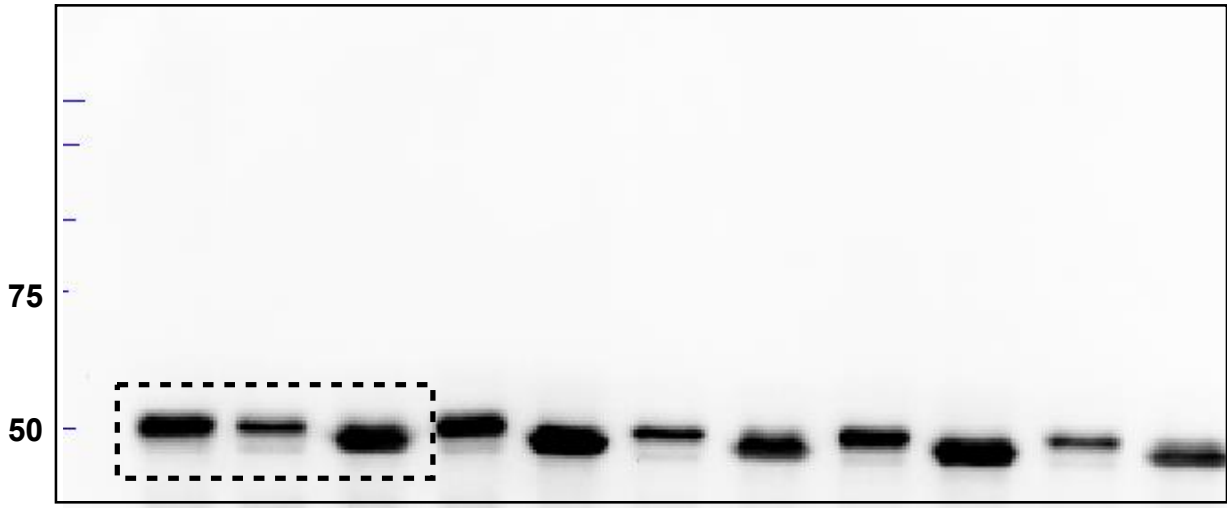
Anti-ataxin-3 (1H9)



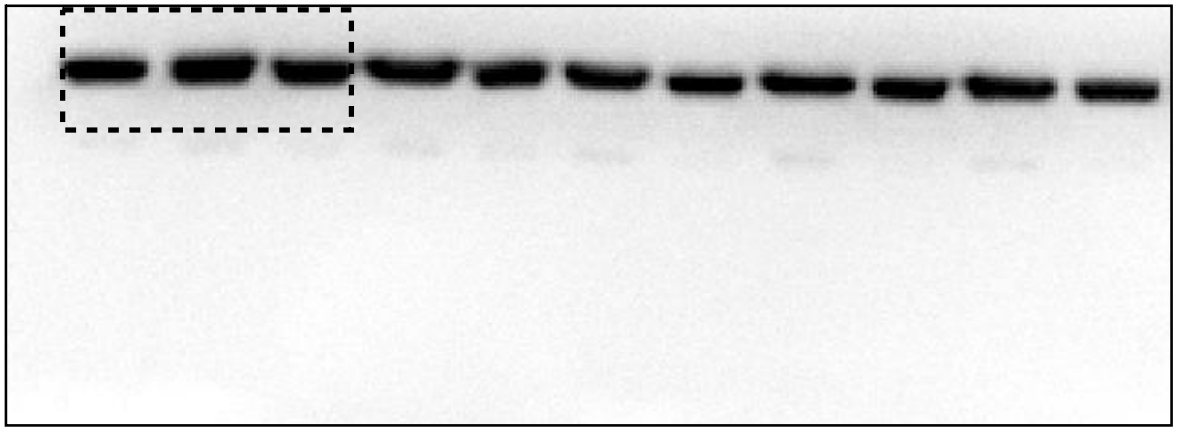
Anti-Tubulin

Figure 4A

Membrane was cut to probe with two different antibodies

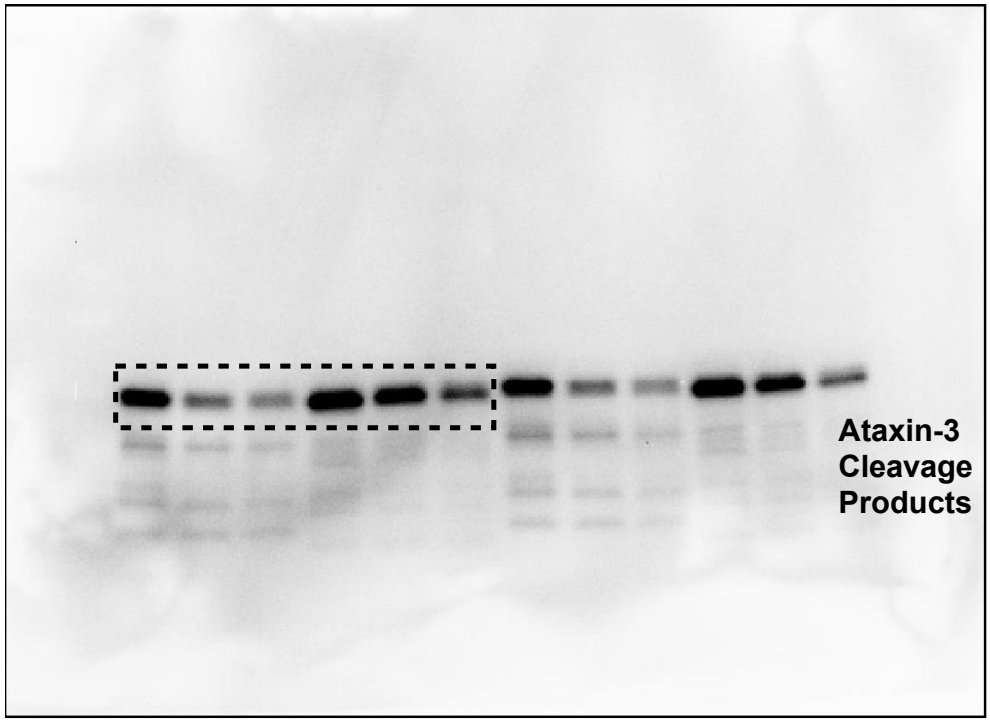


Anti-ataxin-3 (1H9)

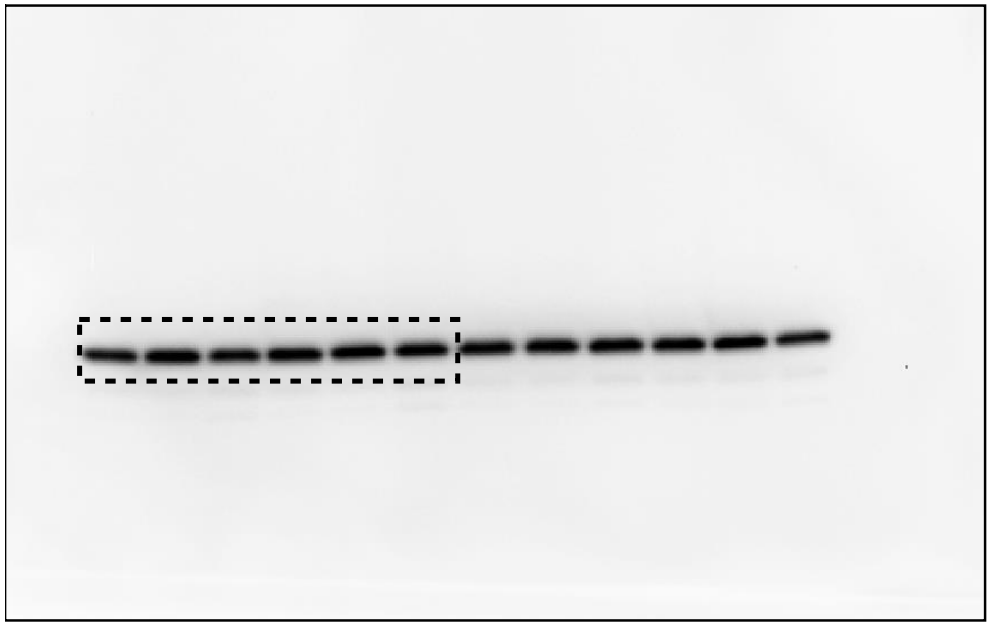


Anti-GAPDH

Figure 4B

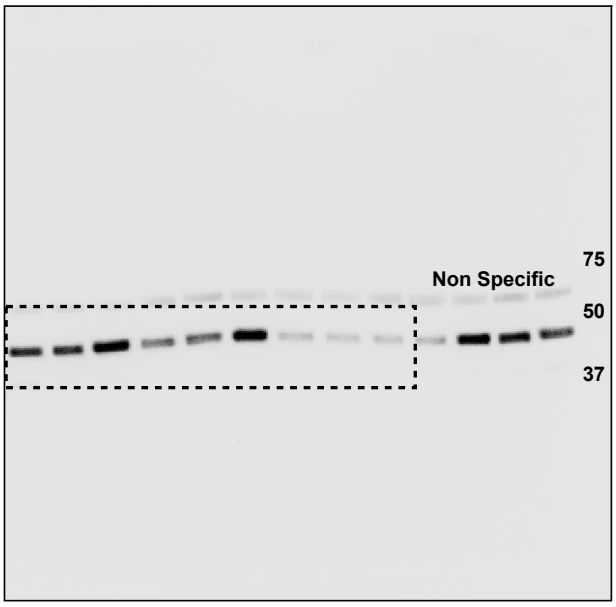


Anti-ataxin-3 (1H9)

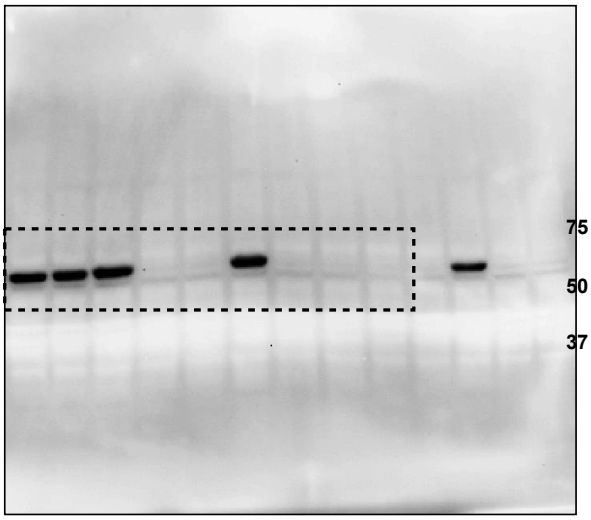


Anti-Tubulin

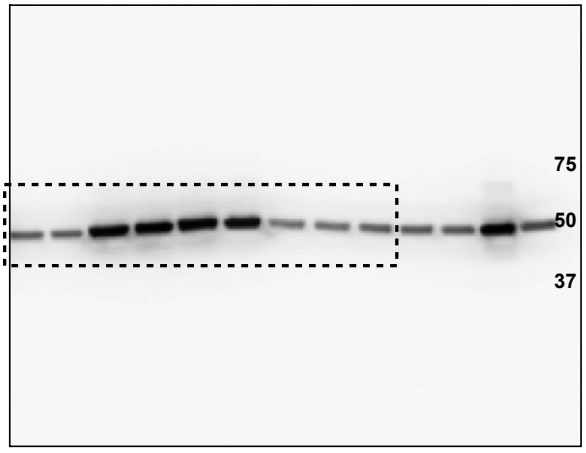
Figure 4C



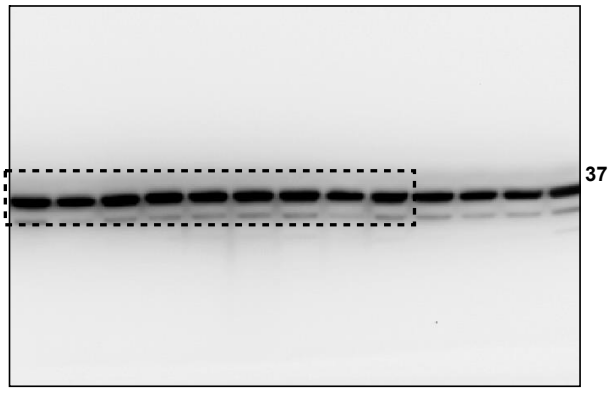
Anti-ataxin-3 (1H9)



Anti-Rad23B

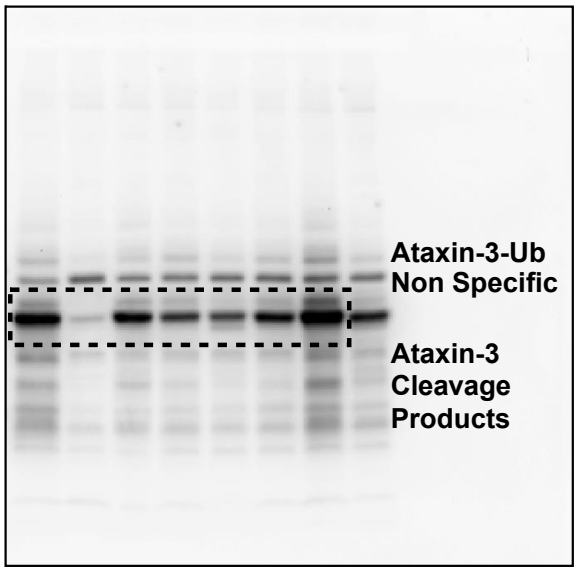


Anti-Rad23A

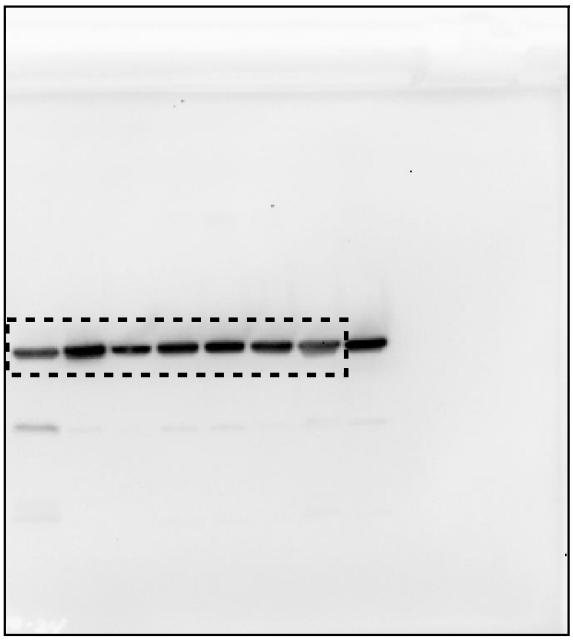


Anti-GAPDH

Figure 5B

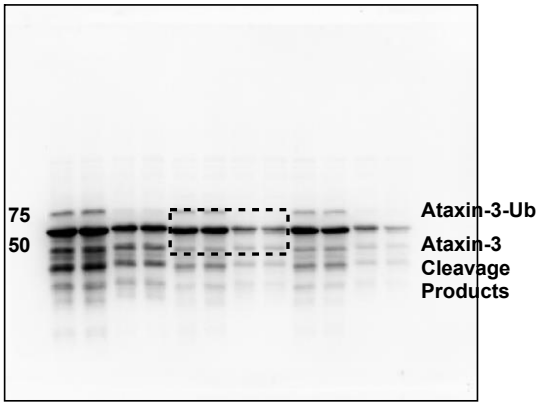


Anti-ataxin-3 (1H9)

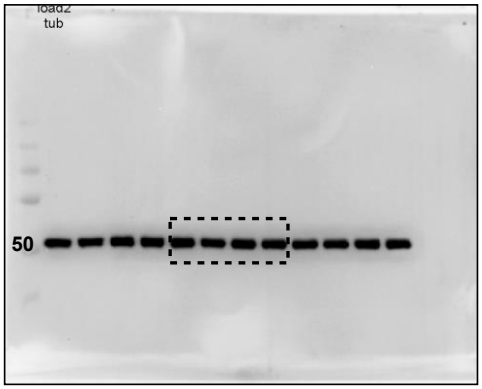


Anti-Tubulin

Figure 6A

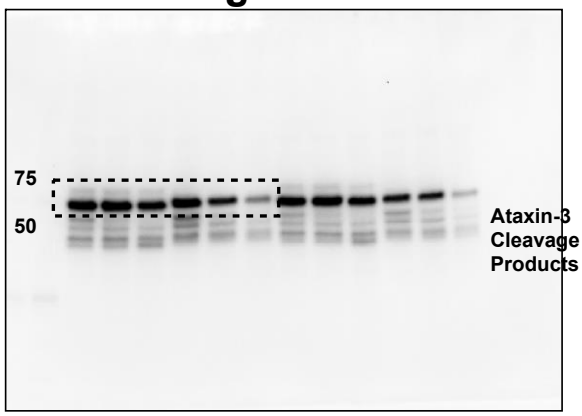


Anti-ataxin-3 (MJD)

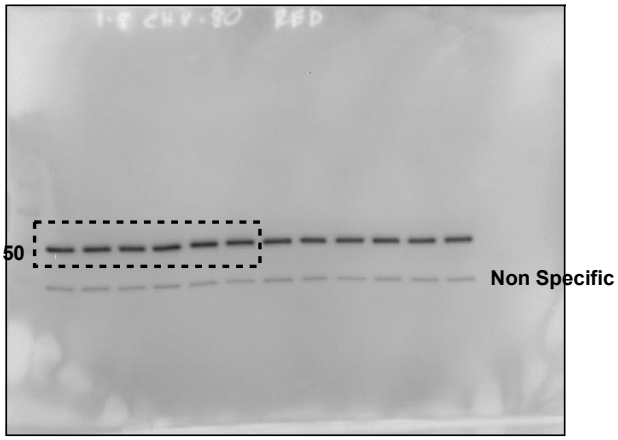


Anti-Tubulin

Figure 6B



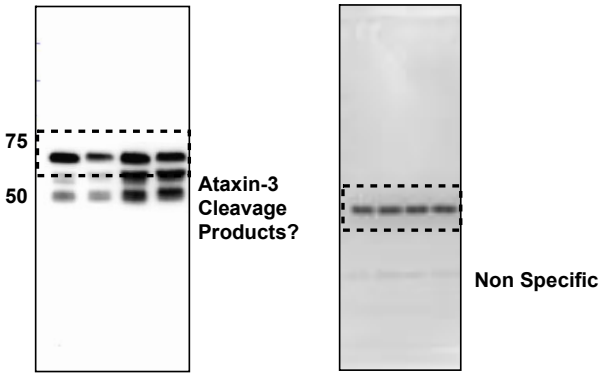
Anti-ataxin-3 (MJD)



Anti-Tubulin

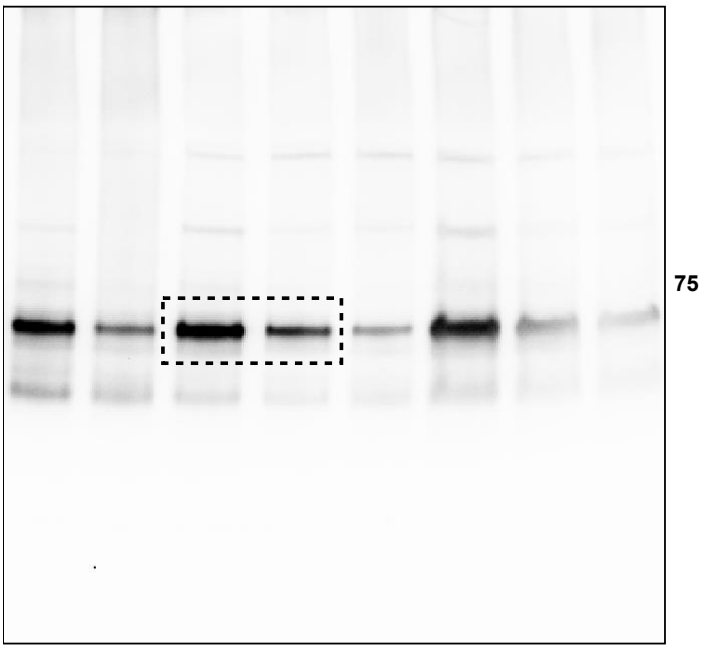
Figure 6C

Membrane was cut for use with other experiments



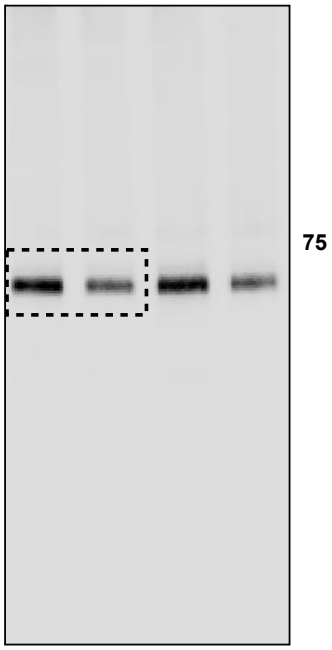
Anti-ataxin-3 (MJD) Anti-Tubulin

Figure 6D

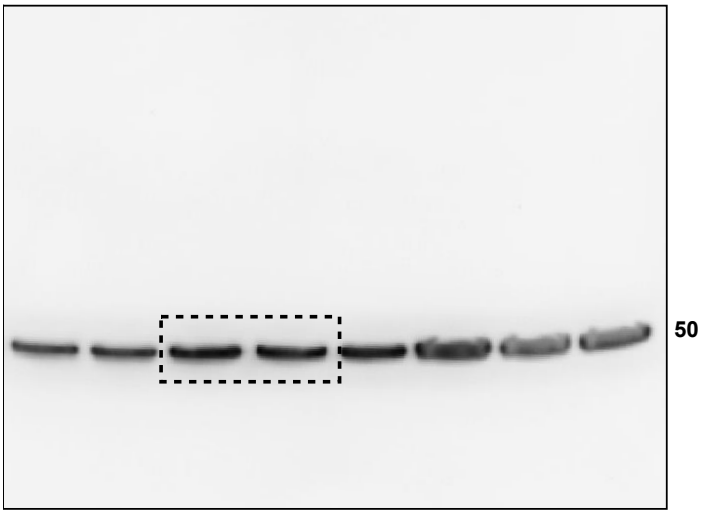


Anti-myc (Ataxin-3)

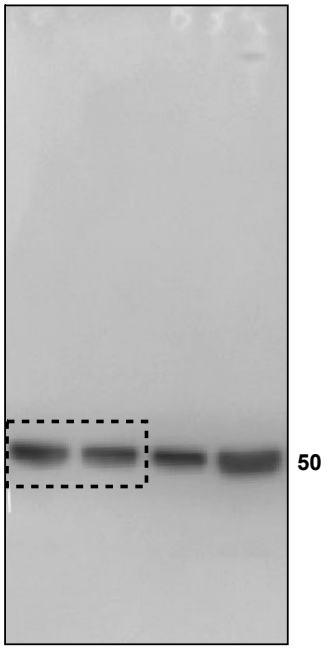
Membrane was cut
for use in other experiments



Anti-myc (Ataxin-3)



Anti-Tubulin



Anti-Tubulin

Supplementary figure 6.

Full blots for cropped images shown in main figures 1-6.

Notes on full blots that follow:

1- Bands that are ubiquitinated species of ataxin-3 are highlighted when detected. These bands are not always detectable by the ataxin-3 antibodies that we use.

2- There are some instances of ataxin-3 antibody-positive bands that are consistent with caspase- and calpain-cleaved ataxin-3 species [1]. Blotting with the ataxin-3 antibodies that we use, MJD and 1H9, can show some such species, whereas the anti-HA antibody does not. The presence of these smaller, more quickly migrating ataxin-3-positive bands does not affect the interpretation of our studies on the stability and degradation of the full-length ataxin-3 protein.

3- Anti-Tubulin antibody sometimes detects non-specific bands in fruit fly lysates.

SUPPLEMENTARY REFERENCES

1. Costa Mdo C, Paulson HL. Toward understanding Machado-Joseph disease. *Progress in Neurobioly* **97**, 239-257 (2012).