Supplemental Material to:

Preventing Ataxin-3 protein cleavage mitigates degeneration in a Drosophila model of SCA-3

Joonil Jung¹#, Kexiang Xu¹, Derek Lessing¹,² and Nancy M. Bonini¹,²,*

¹Department of Biology
University of Pennsylvania
²Howard Hughes Medical Institute
Philadelphila, PA 19104-6018

# Present address: Broad Institute, 7 Cambridge Center, Cambridge, MA 02142.

*To whom correspondence should be addressed: Dr. Nancy M Bonini, Department of Biology, University of Pennsylvania, Howard Hughes Medical Institute, Philadelphia, PA 19104-6018, Phone: 1-215-573-9267, FAX: 1-215-573-5754, Email: nbonini@sas.upenn.edu
Supplemental Fig. 1: Ataxin-3 protein cleavage occurs to a greater extent when expression is directed to the brain compared to the eye. Western immunoblot, probed with anti-MJD polyclonal antibody. 7d heads from w control, or flies expressing the Myc-Atx3Q78 protein in all neurons of the eye and brain with elav-gal4 or only in the eye with gmr-gal4. There was more cleavage fragment with expression in neurons compared to the eye, which has neurons but also is predominantly pigment and other accessory cells. Both drivers are expressed from the same stage of development. Previous studies also indicate that the full-length protein is more selectively toxic to neurons compared to pigment cells of the eye (S1). Genotypes: w^{1118} elav-gal4/UAS-Myc-Atx3Q78. gmr-gal4/UAS-Myc-Atx3Q78.

Reference:
S1. Warrick, J.M., Morabito, L.M., Bilen, J., Gordesky-Gold, B., Faust, L.Z., Paulson, H.L. and Bonini, N.M. (2005) Ataxin-3 suppresses polyglutamine neurodegeneration in Drosophila by a ubiquitin-associated mechanism. Mol. Cell, 18, 37-48.
Supplemental Fig. 2: Ataxin-3 protein cleavage pattern is similar in SL2 cells and the fly *in vivo*.

Western immunoblot probed with 1H9 antibody. Samples from SL2 cells expressing the pathogenic protein with heat shock, as detailed in Fig. 2A. Fly head samples are from flies expressing the protein in the brain using the *elav-gal4* driver line. The expression is generally much greater in SL2 cells than in the fly samples; sample amounts were adjusted to load similar levels of the full length Ataxin-3 protein. Top arrow, and the red bars mark the ~37 kDa fragments; note that the fragments run slightly higher in the protein tagged at the C-terminus with Flag. The lower arrow and red arrowhead mark the fragment of the normal, non-pathogenic Ataxin-3 protein. SL2 samples: untransfected cells, and cells transfected with *hs-gal4 UAS-myc-Atx3Q84-Flag*. Fly genotypes: *w*\(^{1118}\), and *elav-gal4* in trans to *UAS-Myc-Atx3Q84-Flag* transgenic lines 2 or 7, *UAS-Myc-Atx3-Q84* line 18.1 or *UAS-Myc-AtxQ27* line 46.1.
Supplemental Fig 3. Proteasome and calpain inhibitors do not significantly modify Ataxin-3 protein cleavage in SL2 cells.
Ataxin-3 protein cleavage was carried out following the protocol detailed in Fig 2A. The constructs used were pUAST-Myc-Atx3Q84 driven by heat shock (pHS-gal4). Cleavage products of a ~ 50 kDa (**) and ~37 kDa (****) were observed as in Fig 2B. As in previous experiments, zVAD-fmk reduced the level of ~37 kDa fragment (****), and a slight build up of higher molecular weight products including ~50 kDa fragment (**) relative to the full length protein (*) was observed. zFA-fmk treatment had no effect. MG132 (a proteasome inhibitor) did not significantly alter the amount of ~37 kDa fragment in SL2 cells. However, the relative intensity of minor bands (***) between ~ 50 kDa and ~37 kDa fragments consistently appeared weaker, suggesting the involvement of the proteasome for the appearance of certain Ataxin-3 fragments in SL2 cells. ALLN (a caspase inhibitor) did not alter the cleavage pattern significantly. Combined treatment of zVAD and MG132 did not elicit a synergistic effect, suggesting they are independent processes.
Supplemental Fig. 4: The caspase inhibitor zVAD-fmk mitigates proteolysis of the ~37kDa fragment in SL2 cells.
Western blot of cell culture extracts, probed with 1H9. Sensitivity of Ataxin-3 proteolysis to zVAD-fmk is apparent with 1H9 antibody. A representative blot is shown at left, from an experiment performed as in Fig. 2. The fragment bands are indicated by the arrow. Equivalent amounts of protein were loaded in each lane. Graph at right shows quantification from three independent experiments. Intensity of the fragment bands with 100µM drug treatment is shown relative to the 0µM control treatment ±SEM. \( P=0.0165 \) (Student's \( t \)-test).
Supplemental Fig 5. Elimination of all six caspase-sites suppresses most of Ataxin-3 protein cleavage.

Western immunoblots of various transgenes expressed in SL2 cells.

A. The cleavage pattern of wild type protein with the polyclonal anti-MJD antibody was compared with that of triple (3M; D171/208/217N) and sextuplet (6M; D171/208/217/223/225/228N) mutant proteins. See Fig. 3A for schematic with the location of putative caspase sites. Cleavage of the 6M mutant protein was dramatically reduced relative that of the WT protein, while the 3M protein showed an intermediate effect. To the right, a longer exposure of the western immunoblot with the 6M sample is shown to highlight the difference in the amount of ~37 kDa fragment (**) relative to full length protein (<). A non-specific band is indicated (*).

B. Detection of the N-terminal tag with an anti-Myc antibody did not detect the ~37 kDa product from the fly brain. This antibody was used to compare and match protein expression levels of the full length Ataxin-3 protein (<).

C. Effect of caspase site mutations on the cleavage of the double tagged Myc-Atx3Q84-Flag protein. The 6M protein showed a nearly complete suppression of cleavage. To the right is a longer exposure of the 6M part of the western blot to highlight the lack of cleavage of the 6M protein. <, Full length protein; **, ~37 kDa fragment; *, non-specific band.