Antibody Characterization Report for Ataxin-3

YCharOS Antibody Characterization Report

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Target:

Recommended protein name: Ataxin-3

Alternative protein names: Machado-Joseph disease protein 1, Spinocerebellar ataxia type 3 protein

Gene name: *ATXN3*

Uniprot: P54252

This report guides researchers to select the most appropriate antibodies for Ataxin-3. We used an antibody characterization pipeline [1] based on knockout (KO) cells to perform head-to-head comparisons of commercial antibodies for Ataxin-3 by immunoblot (Western blot), immunoprecipitation and immunofluorescence. HEK293T and U2OS were selected based on evidence of appropriate Ataxin-3 protein expression determined through public proteomics databases, namely PaxDB [2] and DepMap [3]. Both HEK293T and U2OS were modified with CRISPR/Cas9 to knockout [4] the corresponding *ATXN3* gene. Moreover, human induced pluripotent stem cells (iPSCs) were modified to knockout [5] the *ATXN3* gene and both WT and KO lines were further differentiated into neural progenitor cells (NPCs) [6].

The authors do not provide an assessment of the quality of the tested antibodies as their respective performances are limited to our finite experimental conditions. The readers should interpret the present findings based on their own scientific expertise. The authors acknowledge that an antibody that demonstrates specificity in the stated test conditions can be suboptimal in a different experimental format or in cell lines that differ from those directly tested here.

1. Laflamme, C., et al., *Implementation of an antibody characterization procedure and application to the major ALS/FTD disease gene C9ORF72.* Elife, 2019. **8** DOI: 10.7554/eLife.48363.
2. Wang, M., et al., *Version 4.0 of PaxDb: Protein abundance data, integrated across model organisms, tissues, and cell-lines.* Proteomics, 2015. **15**(18): p. 3163-8 DOI: 10.1002/pmic.201400441.
3. Nusinow, D.P., et al., *Quantitative Proteomics of the Cancer Cell Line Encyclopedia.* Cell, 2020. **180**(2): p. 387-402 e16 DOI: 10.1016/j.cell.2019.12.023.
4. Shlaifer, I., et al. *Generation of Knockout Cell Lines Using CRISPR-Cas9 Technology*. February 24, 2020; Available from: https://zenodo.org/record/3738361#.YIyeDu2SlaR.
5. Nicouleau, M., et al., *Generation of Knockout Cell Lines Using CRISPR-Cas9 and ddPCR Technology*. 2020.
6. Bell, S., et al., *Disruption of GRIN2B Impairs Differentiation in Human Neurons.* Stem Cell Reports, 2018. **11**(1): p. 183-196 DOI: 10.1016/j.stemcr.2018.05.018.

Table 1: Summary of the Ataxin-3 antibodies tested

Company	Catalog number	Lot number	RRID (Antibody Registry)	Clonality	Clone ID	Host	Concentration (µg/µl)	Vendors recommended applications
Thermo	702788	2240061	AB_2716894	recombinant-mono	13H9L9	rabbit	0.50	Wb, IF
Proteintech	13505-1-AP	93879	AB_2061192	polyclonal	-	rabbit	0.50	Wb
Abcam	ab96316	GR3251517-4	AB_10680570	polyclonal	-	rabbit	0.35	Wb, IF
Aviva Sys. Bio.	OAAB05835	-	AB_2892707	polyclonal	-	rabbit	0.50	Wb
Aviva Sys. Bio.	OABB01906	21BP16A29	AB_2892708	polyclonal	-	rabbit	0.45	Wb, IF
GeneTex	GTX101343	39694	AB_1240486	polyclonal	-	rabbit	0.55	Wb, IF
GeneTex	GTX115032	43817	AB_10621230	polyclonal	-	rabbit	0.35	Wb, IF

Wb=Western blot; IP= immunoprecipitation; IF=immunofluorescence

Table 2: Summary of the cell lines used

Institution	Catalog number	RRID (Cellosaurus)	Cell line	genotype
ATCC	CRL-3216	CVCL_0063	HEK293T	WT
Montreal Neurological Institute	-	CVCL_A0SP	HEK293T	*ATXN3* KO
ATCC	HTB-96	CVCL_0042	U2OS	WT
Montreal Neurological Institute	-	CVCL_A0SQ	U2OS	*ATXN3* KO
Montreal Neurological Institute	-	-	iPSC	WT
Montreal Neurological Institute	-	-	iPSC	*ATXN3* KO
Horizon Discovery	C631	CVCL_Y019	HAP1	WT

Figure 1: Ataxin-3 antibody screening by immunoblot.

A) Lysates of HEK293T (WT and *ATXN3* KO) were prepared and 30 µg of protein were processed for immunoblot with the indicated Ataxin-3 antibodies. The Ponceau stained transfers of each blot are shown. Antibody dilution used: 702788 at 1/50, 13505-1-AP at 1/1000, ab96316 at 1/500, OABB01906 at 1/1000, OAAB05835 at 1/1000, GTX101343 at 1/5000 GTX115032 at 1/3000. Expected band size: ~42 kDa.

B) Lysates of HEK293T, U2OS, NPC (WT and *ATXN3* KO) and HAP1 WT were prepared and processed as in A). Antibody 13505-1-AP was used at 1/1000.

Figure 2: Ataxin-3 antibody screening by immunoprecipitation.

HEK293T lysates were prepared and immunoprecipitation was performed using 1.0 µg of the indicated Ataxin-3 antibodies pre-coupled to protein A Sepharose beads. Samples were washed and processed for immunoblot with the indicated Ataxin-3 antibody. For immunoblot, 13505-1-AP was used at 1/1000. The Ponceau stained transfers of each blot are shown. SM=10% starting material; UB=10% unbound fraction; IP=immunoprecipitate.

Figure 3: Ataxin-3 antibody screening by immunofluorescence.

U2OS WT and *ATXN3* KO cells were labelled with a green or a far-red fluorescent dye, respectively. WT and KO cells were mixed and plated to a 1:1 ratio on coverslips. Cells were stained with the indicated Ataxin-3 antibodies and with the corresponding Alexa-fluor 555 coupled secondary antibody. Acquisition of the green (identification of WT cells), red (antibody staining) and far-red (identification of KO cells) channels was performed. Representative images of the red (grayscale) channels are shown. WT and KO cells are outlined with yellow and magenta dashed line, respectively. Antibody dilution used: 702788 at 1/500, 13505-1-AP at 1/500, ab96316 at 1/400, OABB01906 at 1/500, OAAB05835 at 1/500, GTX101343 at 1/500, GTX115032 at 1/350. Bars = 10 µm.

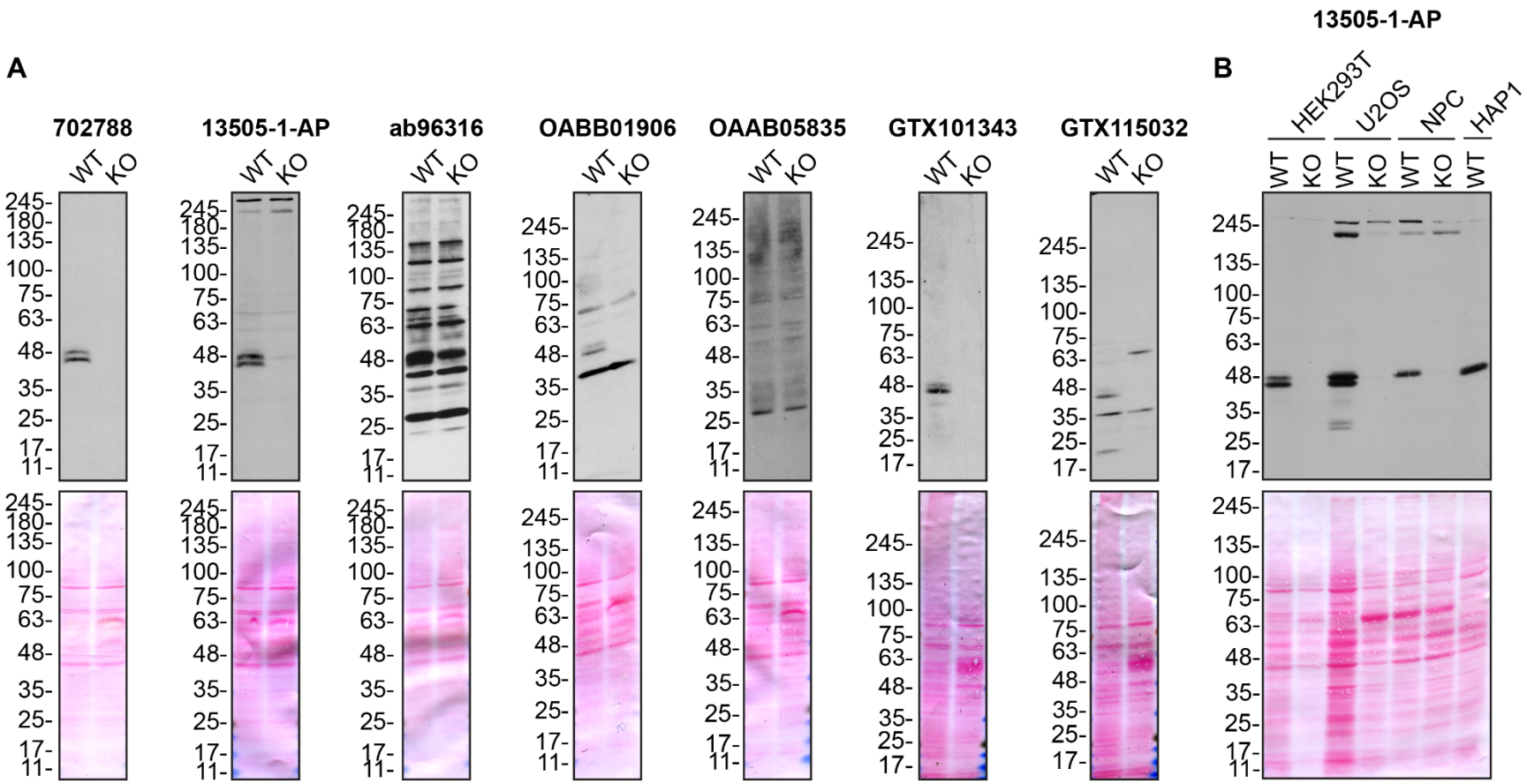

Figure 1: Ataxin-3 antibody screening by immunoblot

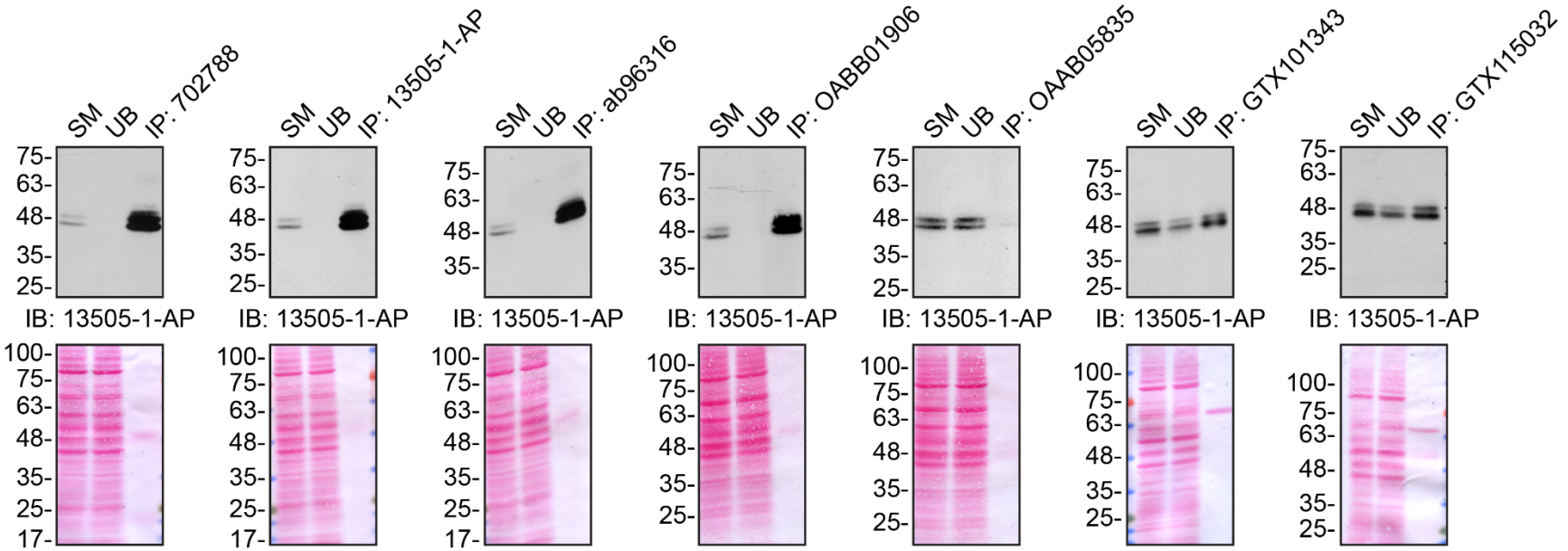

Figure 2: Ataxin-3 antibody screening by immunoprecipitation

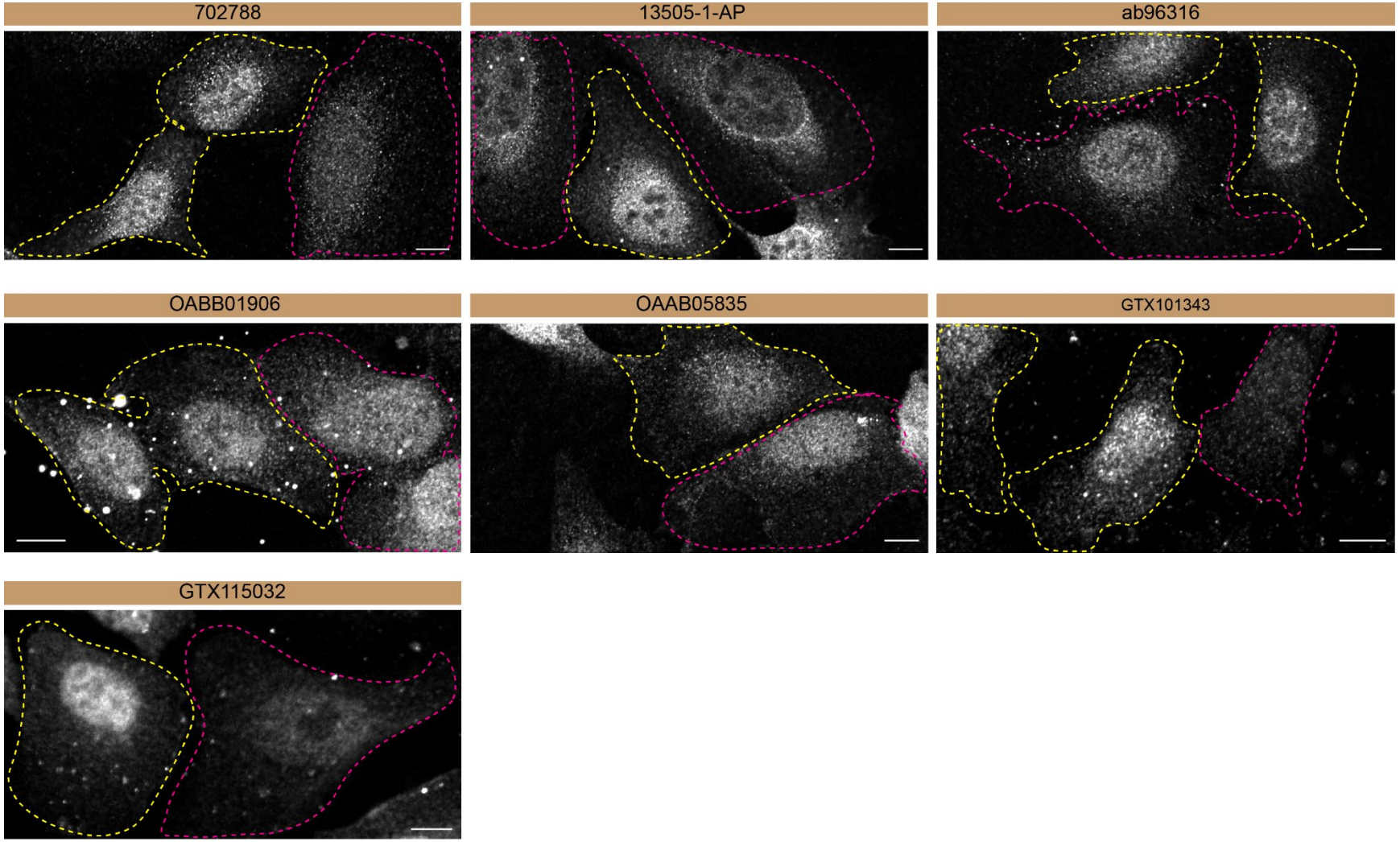

Figure 3 : Ataxin-3 antibody screening by immunofluorescence

Materials and methods

Antibodies

All Ataxin-3 antibodies are listed in Table 1. Peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies are from Thermo Fisher Scientific (cat. number 65-6120 and 62-6520). Alexa-555-conjugated goat anti-mouse and anti-rabbit secondary antibodies are from Thermo Fisher Scientific (cat. number A21424 and A21429).

CRISPR/Cas9 genome editing

Cell lines used are listed in Table 2. Two guide RNAs were used to introduce a STOP codon in the *ATXN3* gene in HEK293T, U2OS and iPSC (sequence guide 1: TGCCTGAATAACTTATTGCA, sequence guide 2: AGGATGAGAATGGCAGAAGG).

Cell culture

HEK293T and U2OS were cultured in DMEM high-glucose (GE Healthcare cat. number SH30081.01) containing 10% fetal bovine serum (Wisent, cat. number 080450), 2 mM L-glutamate (Wisent cat. number 609065, 100 IU penicillin and 100 µg/ml streptomycin (Wisent cat. number 450201). iPSCs were culture and differentiated as described in [5].

Antibody screening by immunoblot

HEK293T, U2OS, HAP1 and NPC were collected in RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1.0 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor. Lysates were sonicated briefly and incubated 30 min on ice. Lysates were spun at ~110,000xg for 15 min at 4°C and equal protein aliquots of the supernatants were analyzed by SDS-PAGE and immunoblot. BLUelf prestained protein ladder from GeneDireX (cat. number PM008-0500) was used.

Immunoblots were performed with large 5-16% gradient polyacrylamide gels and transferred on nitrocellulose membranes. Proteins on the blots were visualized with Ponceau staining which is scanned to show together with individual immunoblot. Blots were blocked with 5% milk for 1 hr, and antibodies were incubated O/N at 4°C with 5% bovine serum albumin in TBS with 0,1% Tween 20 (TBST). Following three washes with TBST, the peroxidase conjugated secondary antibody was incubated at a dilution of ~0.2 µg/ml in TBST with 5% milk for 1 hr at room temperature followed by three washes with TBST. Membranes are incubated with ECL from

Pierce (cat. number 32106) prior to detection with HyBlot CL autoradiography films from Denville (cat. number 1159T41).

Antibody screening by immunoprecipitation

Antibody-bead conjugates were prepared by adding 1.0 µg of antibody to 500 ul of PBS with 0,01% triton X-100 in a microcentrifuge tube, together with 30µl of protein A- (for rabbit antibodies) or protein G- (for mouse antibodies) Sepharose beads. Tubes were rocked O/N at 4°C followed by several washes to remove unbound antibodies.

HEK293T WT were collected in HEPES buffer (20 mM HEPES, 100 mM sodium chloride, 1 mM EDTA, 1% Triton X-100, pH 7.4) supplemented with protease inhibitor. Lysates are rocked 30 min at 4°C and spun at 110,000xg for 15 min at 4°C. One ml aliquots at 1.0 mg/ml of lysate were incubated with an antibody-bead conjugate for ~2 hrs at 4°C. Following centrifugation, the unbound fractions were collected, and beads were subsequently washed three times with 1.0 ml of HEPES lysis buffer and processed for SDS-PAGE and immunoblot on 5-16% polyacrylamide gels. Prot-A:HRP was used as a secondary detection system (MilliporeSigma, cat. number P8651) at a dilution of 0.1 µg/ml.

Antibody screening by immunofluorescence

U2OS WT and *ATXN3* KO were labelled with a green and a far-red fluorescence dye, respectively. The fluorescent dyes used are from Thermo Fisher Scientific (cat. number C2925 and C34565). WT and KO cells were plated on glass coverslips as a mosaic and incubated for 24 hrs in a cell culture incubator. Cells were fixed in 4% PFA (in PBS) for 15 min at room temperature and then washed 3 times with PBS. Cells were permeabilized in PBS with 0,1% Triton X-100 for 10 min at room temperature and blocked with PBS with 5% BSA, 5% goat serum and 0.01% Triton X-100 for 30 min at room temperature. Cells were incubated with IF buffer (PBS, 5% BSA, 0,01% Triton X-100) containing the primary Ataxin-3 antibodies O/N at 4°C. Cells were then washed 3 × 10 min with IF buffer and incubated with corresponding Alexa Fluor 555-conjugated secondary antibodies in IF buffer at a dilution of 1.0 µg/ml for 1 hr at room temperature. Cells were washed 3 × 10 min with IF buffer and once with PBS. Coverslips were mounted on a microscopic slide using fluorescence mounting media (DAKO).

Imaging was performed using a Zeiss LSM 880 laser scanning confocal microscope equipped with a Plan-Apo 40x oil objective (NA = 1.40). Analysis was done using the Zen navigation software (Zeiss). All cell images represent a single focal plane. Figures were assembled with Adobe Illustrator.