RNA association and nucleocytoplasmic shuttling by ataxin-1

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Summary

Spinocerebellar ataxia type 1 (SCA1) is a dominant neurodegenerative disease caused by the expression of mutant ataxin-1 containing an expanded polyglutamine tract. Ataxin-1 is a nuclear protein that localizes to punctate inclusions similar to neuronal nuclear inclusions seen in many polyglutamine expansion disease proteins. We demonstrate that ataxin-1 localization to inclusions and inclusion dynamics within the nucleus are RNA and transcription dependent, but not dependent on the polyglutamine tract. Ataxin-1 nuclear inclusions are distinct from other described nuclear bodies but recruit the mRNA export factor, TAP/NXF1, in a manner that is enhanced by cell heat shock. By FRAP protein dynamic studies in live cells, we found that wild-type, but not mutant, ataxin-1 was capable of nuclear export. These results suggest that the normal role of ataxin-1 may be in RNA processing, perhaps nuclear RNA export. Thus, nuclear retention of mutant ataxin-1 may be an important toxic gain of function in SCA1 disease.

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Key words: Ataxin-1, Spinocerebellar ataxia type 1, RNA processing, Nuclear transport

Introduction

Spinocerebellar ataxia type 1 (SCA1) is an autosomal dominant, progressive neurodegenerative disease. Primary sites of neurodegeneration in the brain are the Purkinje cells of the cerebellar cortex and specific neuronal populations in the brain stem. Neural dysfunction and cell death at these sites lead to bulbar dysfunction and characteristic ataxia in SCA1 patients. The disease is caused by a CAG repeat expansion in the *SCA1* gene, resulting in an expanded polyglutamine tract in ataxin-1, the *SCA1* gene product (Orr et al., 1993; Orr and Zoghbi, 2001). Thus, SCA1 is a member of a family of neurodegenerative diseases characterized by polyglutamine expansion that falls into a larger class of neurological disorders caused by triplet nucleotide repeat expansions. In addition to SCA1, this group of polyglutamine expressing disorders includes Huntington's disease (HD), spinobulbar muscle atrophy (SBMA), dentatorubropallidoluysian atrophy (DRPLA) and the spinocerebellar ataxias (SCA2, SCA3/Machado-Joseph Disease, SCA6, SCA7 and SCA17) (Cummings and Zoghbi, 2000; Nakamura et al., 2001). A pathological hallmark of most of these polyglutamine diseases is the presence of nuclear and cytoplasmic inclusions containing the mutant polyglutamine protein.

Ataxin-1 is a nuclear protein whose cellular function is not entirely known. It has been shown to interact with the SMRT transcription co-repressor, suggesting a role in transcriptional regulation (Tsai et al., 2004). Mutant ataxin-1 must enter the nucleus in order for the polyglutamine-induced neurodegenerative disease to develop (Klement et al., 1998), and ataxin-1 has been shown to have an in vitro RNA-binding activity (Yue et al., 2001). In order to assess the potential functions of ataxin-1 in the nucleus, we studied the properties of nuclear ataxin-1 and its co-localization with some known nuclear proteins.

We show that ataxin-1 nuclear inclusions were distinct from several known nuclear bodies including speckles (Misteli et al., 1997), Nup98 bodies (Griffis et al., 2002) and hnRNP proteins (Burd and Dreyfuss, 1994; Siomi and Dreyfuss, 1995). However, ataxin-1 did co-localize in nuclear bodies with the mRNA export factor TAP/NXF1 (Gruter et al., 1998; Segref et al., 1997), in a manner that was enhanced by cell heat shock. Importantly, ataxin-1 localization to nuclear bodies was dependent on the presence of RNA. Using a live-cell nucleocytoplasmic transport assay that employs laser confocal microscopy and fluorescence recovery after photobleaching, or FRAP (Howell and Truant, 2002), we demonstrated that wild-type, but not polyglutamine expanded mutant ataxin-1, has the ability to export from the nucleus. By removing the polyglutamine tract in human ataxin-1, leaving only two glutamine residues, we noted that inclusion formation, inclusion movement and TAP/NXF1 recruitment were not affected, indicating that these characteristics are not related to the polyglutamine tract in ataxin-1. The role of active nuclear transport and mRNA in ataxin-1 inclusion formation suggests that lack of export of polyglutamine expanded ataxin-1 may be an important gain of function that leads to SCA1.

Materials and Methods

Nucleocytoplasmic transport assay and plasmid constructs

Cell growth, media, GFP plasmid construction and FRAP shuttle assays were performed as described elsewhere in detail (Howell and Truant, 2002). PmRFP-C1, a monomeric red fluorescent protein (Campbell et al., 2002) fusion vector was created by PCR amplifying mRFP cDNA (a generous gift from Roger Tsien, UC San Diego, CA) with oligonucleotides adding *Age*I and *Bsp*EI restriction endonuclease sites to the 5′ and 3′ ends of mRFP cDNA and ligating into *Age*I-*Bsp*EI cleaved peGFP-C1 (Clontech). eGFP-ataxin-1[Q26] (wild type) and eGFP-ataxin-1[Q84] (mutant) expression plasmids were constructed by PCR amplification of wild-type or mutant human ataxin-1 cDNA with DNA oligonucleotides including 5′ and 3′ *Bam*HI restriction sites and ligating the digested fragment into *Bam*HI-digested plasmid peGFP-C2 (Clontech). eGFP-ataxin-1[Q2] was created by inverse PCR on the eGFP-ataxin-1[Q26] (wild-type) template using oligos with a single CAG DNA oligonucleotide in opposite directions and blunt-end ligation. PmRFP-TAP/NXF1 was created by PCR amplification of human TAP/NXF1 cDNA (generous gift from Bryan Cullen, Duke University, Durham, NC) intoducing *Eco*RI and *Bam*HI restriction enzyme ends and ligating into *Eco*RI- and *Bam*HI-cleaved pmRFP-C1. All PCR and DNA manipulation enzymes were purchased from New England Biolabs. All plasmid constructs were verified by PCR sequencing by the McMaster Mobix facility. All plasmids were purified by the PEG/lithium chloride method and transfected into human HeLa cells or mouse NIH3T3 cells (ATCC) as described previously (Howell and Truant, 2002). Nuclear FRAP assays were performed 18 hours post-transfection in live cells with the addition of 50 μg/ml cycloheximide (Calbiochem) to the medium. Results presented are representative of six out of eight experiments performed.

Live-cell time course imaging of ataxin-1 and RNA disruption

For RNAse treatment experiments, HeLa cells expressing Q26 or Q84 ataxin-1 in 25 mm live-cell culture dishes with #1 glass coverslips were permeabilized with 40 μg/ml digitonin (Calbiochem) 24 hours post-transfection. Cells were then washed five times with phosphate-buffered saline (PBS) and then incubated with PBS with 5 mm $MgCl_2$ with or without 100 μg/ml RNase A on ice for 1 hour. Cells were finally washed twice in PBS and fixed with 2% formaldehyde in PBS for 20 minutes before observation.

For transcription inhibition experiments, HeLa or NIH3T3 cells, grown in live-cell glass bottomed dishes (Howell and Truant, 2002), expressing Q26 or Q84 GFP-ataxin-1 were incubated with either 0.05 μg/ml actinomycin D or 25 μg/ml α-amanatin for 3 hours at 37°C (Misteli et al., 1997). For actinomycin D experiments, cells were also incubated with 0.01% DMSO as a solvent control for 2 hours at 37°C with no effect noted. Cells were treated with 2-10% DMSO alone for 2-4 hours with inclusion effects seen at 5% DMSO. eGFP-ataxin-1 expressing HeLa or NIH3T3 cells were also treated with 25 μM carbobenzoxy-L-leucyl-leucyl-L-leucinal (MG132), 0.5 μM colchicine or 50 μg/ml cycloheximide as controls. All compounds were supplied by Calbiochem.

For total RNA visualization, eGFP-ataxin-1[Q84] transfected NIH3T3 cells in live-cell 25 mm culture dishes were stained in PBS for 30 minutes at 37°C with STYO Green RNA select dye (Molecular Probes) dissolved in water according to supplier's instructions.

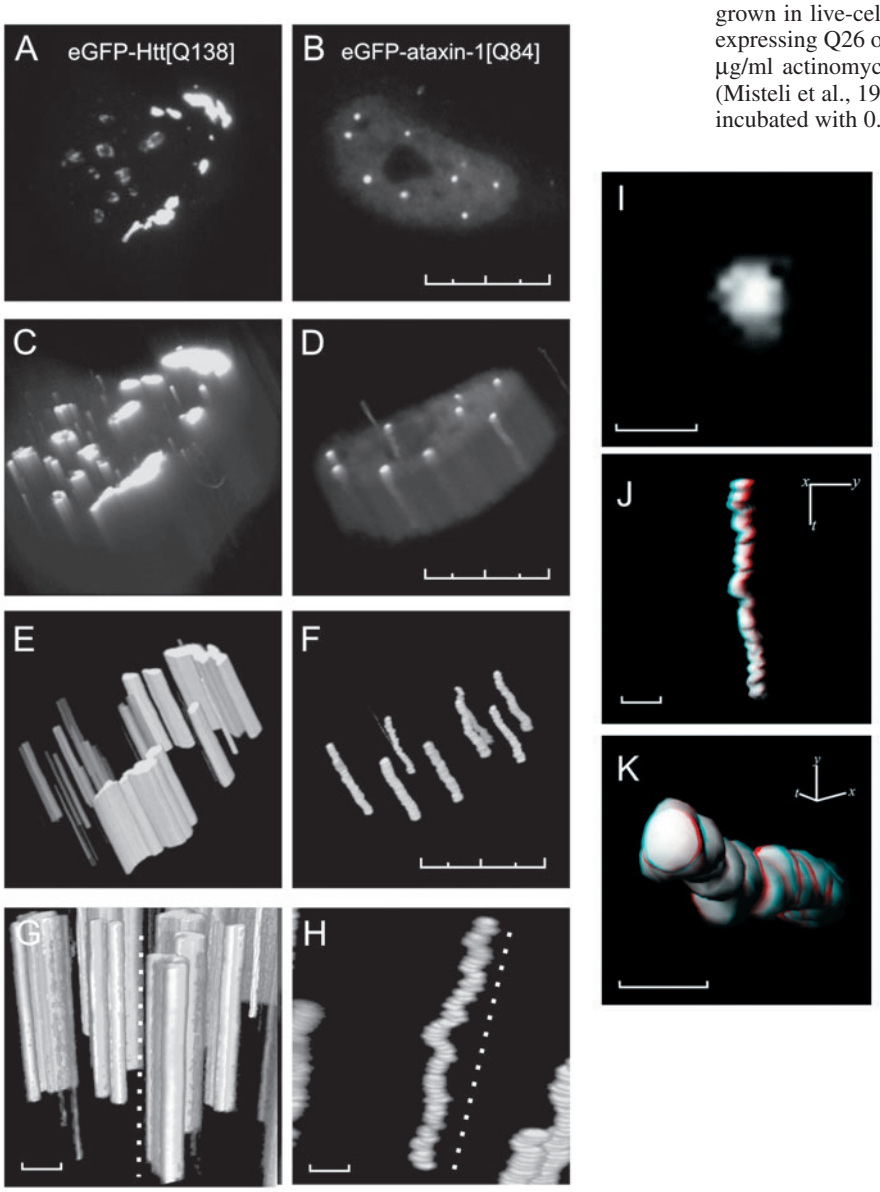

Fig. 1. Ataxin-1 nuclear inclusions are dynamic and distinct from huntingtin exon1 polyglutamine aggregates. Three-dimensional (x,y,time) analysis of huntingtin and ataxin-1 nuclear inclusions by live-cell video microscopy. (A,B) Fluorescence images of eGFP-huntingtin exon1[Q138] and eGFP-ataxin-1[Q84] video sequences captured at 0.5-second intervals for 30 seconds at 37°C. (C,D,E,F) x,y,t voxel volume images of time sequences in orthogonal view; intensities thresholded to inclusions only in E and F. (G,H) Three-dimensional single representative inclusions of huntingtin and ataxin-1 with a dotted line as a straight reference. (I) A single ataxin-1 nuclear inclusion movement (see also Movie 1 in supplementary material). (J,K) Voxel volumes were rendered with an isometric surface projection to reveal a spiral structure indicating a spinning motion. Bars: (A-F) 10 μm, (G-K) 1 μm. J, K are presented as red (left eye) and cyan (right eye) stereo projections. Three dimensional x,y,t axis added for orientation reference.

All results presented are representative of four repeated experiments and 150-200 transfected cells observed.

Fluorescence microscopy co-localization studies

CFP-Q26Ataxin-1 and YFP-Asf/SF2 (a kind gift from D. Spector, Cold Spring Harbor, NY) or GFP-Nup98 (a kind gift from Maureen Powers, Emory University, Atlanta, GA) and mRFP-Q2 ataxin, or HnRNPA1-dsRed1 (Howell and Truant, 2002) and CFP-Q26Ataxin-1, or mRFP-TAP/NXF1 and GFP-Q26 ataxin-1, were transfected into human HeLa cells (2 μg each) by the polyethylimine method (Exgen500, Fermentas) and expressed for 10-18 hours in 25 mm live-cell culture dishes. Expression times were kept as low as possible and optimized for the limit of signal detection on a protein-optimized fluorescence microscope imaging system to avoid over expression artifacts. All live-cell images were captured without prior fixation. eGFP-PML was a generous gift from T. Sata (NID, Japan).

For TAP/NXF1 immunofluorescence, cells expressing eGFP-ataxin-1 moieties were fixed with 2% paraformaldehyde for 20 minutes and permeabilized with 0.5% Triton X-100 for 30 minutes 24 hours post-transfection, in 25 mm glass-bottomed culture dishes. Cells were then washed with PBS and incubated with a 1:500 dilution of anti-TAP (NXF1) antibody overnight (Santa Cruz Biotechnology), then washed with PBS and incubated with 1:3000 Alexa 594 anti-goat secondary antibody (Molecular Probes) for 4 hours before washing with PBS. Cells were observed directly in the glass-bottomed dishes in PBS solution without mounting.

For TAP/NXF1 heat shock, eGFP-ataxin-1[Q84] and mRFP-TAP/NXF1 were co-transfected in HeLa cells and allowed to express for 18 hours. Cells were then cooled to room temperature, heated for 5 minutes to 42°C, then allowed to cool for 20 minutes to 37°C before image capturing started. Controls of eGFP-ataxin-1[Q84] and mRFP alone or mRFP-TAP/NXF1 alone were treated in an identical manner. Results were similar in six out of six repeat experiments.

Fluorescence and laser confocal microscopy

Description and construction of glass bottomed live-cell tissue culture plates is described elsewhere (Howell and Truant, 2002). Confocal and photobleaching images were captured with a Zeiss LSM 510 laser confocal microscope using 488 nm argon and 543 nm helium-neon lasers and a 63× water objective (McMaster Imaging Facility). Red, green and differential interference contrast (DIC) channels were digitally pseudo-colored and merged using the LSM510 scanning control program and Image Browser v2.0311 (Zeiss, EMBL). Live-cell time course and co-localization images were captured on a Nikon Te200 inverted fluorescence microscope with a 175 W xenon lamp source (Sutter Instruments) 63× plan apochromat oil objective (Nikon) and computer-controlled shutters and filter wheels (Sutter Instruments). Z-stack co-localization images were captured with the addition of a z-stepping motorized stage (Prior) and SimplePCI v5.2 (Compix) software. Some images were digitally deconvolved using Autodeblur v9.1 (Autoquant) deconvolution software.

Three-dimensional voxel analysis of nuclear inclusions

Time course movies were made of live HeLa cells expressing either eGFP-ataxin-1[Q2], [Q26] or [Q84] or eGFP-huntingtin-exon1[Q138] were captured at 0.5-second intervals (2 frames/second, 100 millisecond exposure) at 63× objective magnification for a total of 60 frames. Three dimensional (x,y,t) image stacks were imported into Imaris 4.0 software (Bitplane AG, Zurich, Switzerland) using time in the z-plane to generate three-dimensional (3D) voxel images. Voxel objects of the nuclei were thresholded to the relative intensity of the nuclear inclusions to visualize the movement path of the inclusions only. Voxel objects of ataxin-1 and huntingtin proteins were then compared on an orthogonal axis for x,y movement over the 30-second time period. Stereo images were rendered in Imaris as red-cyan stereo.

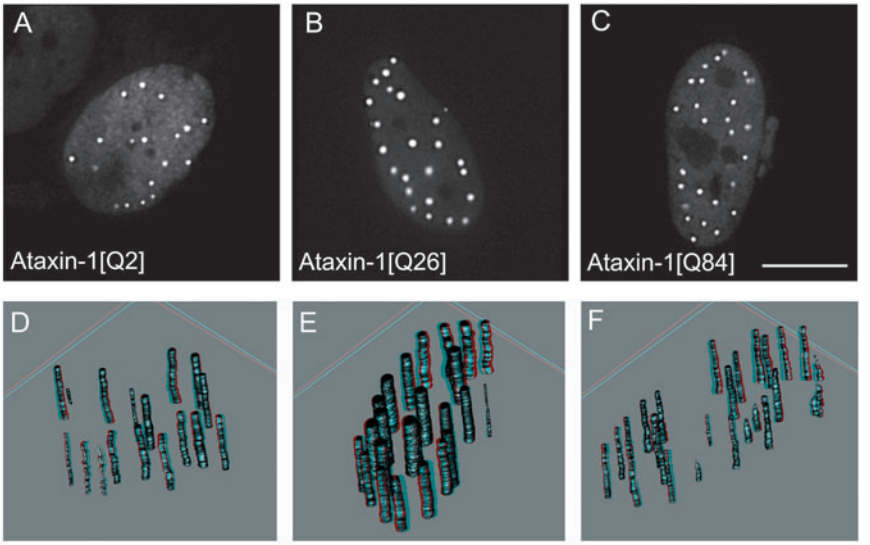

Fig. 2. Ataxin-1 nuclear inclusion formation and movement are not dependent on polyglutamine. (A-C) Fluorescence microscopy images of eGFP ataxin-1[Q2] (A), [Q26] (B) or [Q84] (C) from live-cell video observations at 37°C in HeLa cells. (D-F) Three dimensional x,y,t voxel and isosurface representation of ataxin-1 body movement in either Q2 (D), Q26 (E), or Q84 (F) context showing similar body movement. Bar: 10 μm. (See also Movies 3-5 in supplementary material.) D-F are presented as red-cyan stereo images.

Results

Ataxin-1 nuclear inclusions are dynamic bodies

Several polyglutamine disease proteins as well as ataxin-1 are seen to form neuronal nuclear inclusions, or NNI (Ross, 1997). A well-described example is huntingtin protein (DiFiglia et al., 1997). To address any similarities between ataxin-1 and huntingtin nuclear inclusions, we undertook experiments to observe these inclusions in live cells by the use of green fluorescent protein (GFP) fusion technology. We expressed eGFP-ataxin-1[Q84] in HeLa cells and visually detected dynamics of ataxin-1 inclusions at 37°C. To qualitatively compare the movement of ataxin-1 nuclear inclusions with those of eGFP-huntingtin exon1[Q138] protein (see Fig. 1), time course images were taken at the identical rate of 2 frame per second for 30 seconds (Fig. 1A,B and see Movies 1, 2 in supplementary material), and three-dimensional voxel images were generated using time in the z plane (Fig. 1C,D). The voxel images were then thresholded to the fluorescence intensity of the inclusions only, for both ataxin-1 and huntingtin protein (Fig. 1E,F). By comparing movement of individual inclusions of ataxin-1 and huntingtin both larger and smaller in size that the ataxin-1 in Fig. 1G,H, we observed that ataxin-1 inclusions

oscillated 1-2 μm, whereas huntingtin inclusions, both cytoplasmic and nuclear, remained static. Three dimensional analysis of a single ataxin-1 inclusion over time revealed a tumbling and spinning motion within the nucleus (Fig. 1I-J). Translation, tumbling and spinning of ataxin-1 inclusions revealed that these nuclear bodies behaved differently from amino-terminal fragment huntingtin protein polyglutamine aggregates, even if compared to longer term observations of huntingtin aggregate movement (Xia et al., 2003). The dynamics of ataxin-1 inclusions were greatly reduced when cells were allowed to cool to room temperature.

To determine if nuclear inclusions and movements of ataxin-1 protein were polyglutamine dependent, we used inverse PCR to mutate and remove all but two polyglutamine residues from human ataxin-1 protein. As seen in Fig. 2, the formation of nuclear inclusions of ataxin-1 was not dependent on the presence of polyglutamine, as has been seen by others in Q2 and Q0 contexts (Tsai et al., 2004; Stenoien et al., 2002). We assayed for inclusion dynamics and found that ataxin-1[Q2] also displayed similar inclusion dynamics to ataxin-1[Q26] or [Q84] at 37°C (Fig. 2D-F) (see Movies 3-5 in supplementary material).

Ataxin-1 sub-nuclear localization is dependent on the presence of RNA

Previously, it was shown that ataxin-1 has RNA-binding activity in vitro (Yue et al., 2001). Some RNA-binding proteins can have their localization in the nucleus affected by transcription inhibitors (Misteli et al., 1997). Therefore, we asked whether the presence of characteristic ataxin-1 nuclear inclusions was affected by treatments that alter RNA in vivo. Ataxin-1-expressing cells were treated with the transcription inhibitors actinomycin D or α-amanatin (Misteli et al., 1997). In both instances, in cells expressing wild-type or mutant ataxin-1, ataxin-1 nuclear inclusions dispersed over 2-3 hours at 37°C (mutant ataxin-1[Q84], Fig. 3B panels A-D and see Movie 6 in supplementary material). In addition, we treated ataxin-1-expressing cells with RNAse

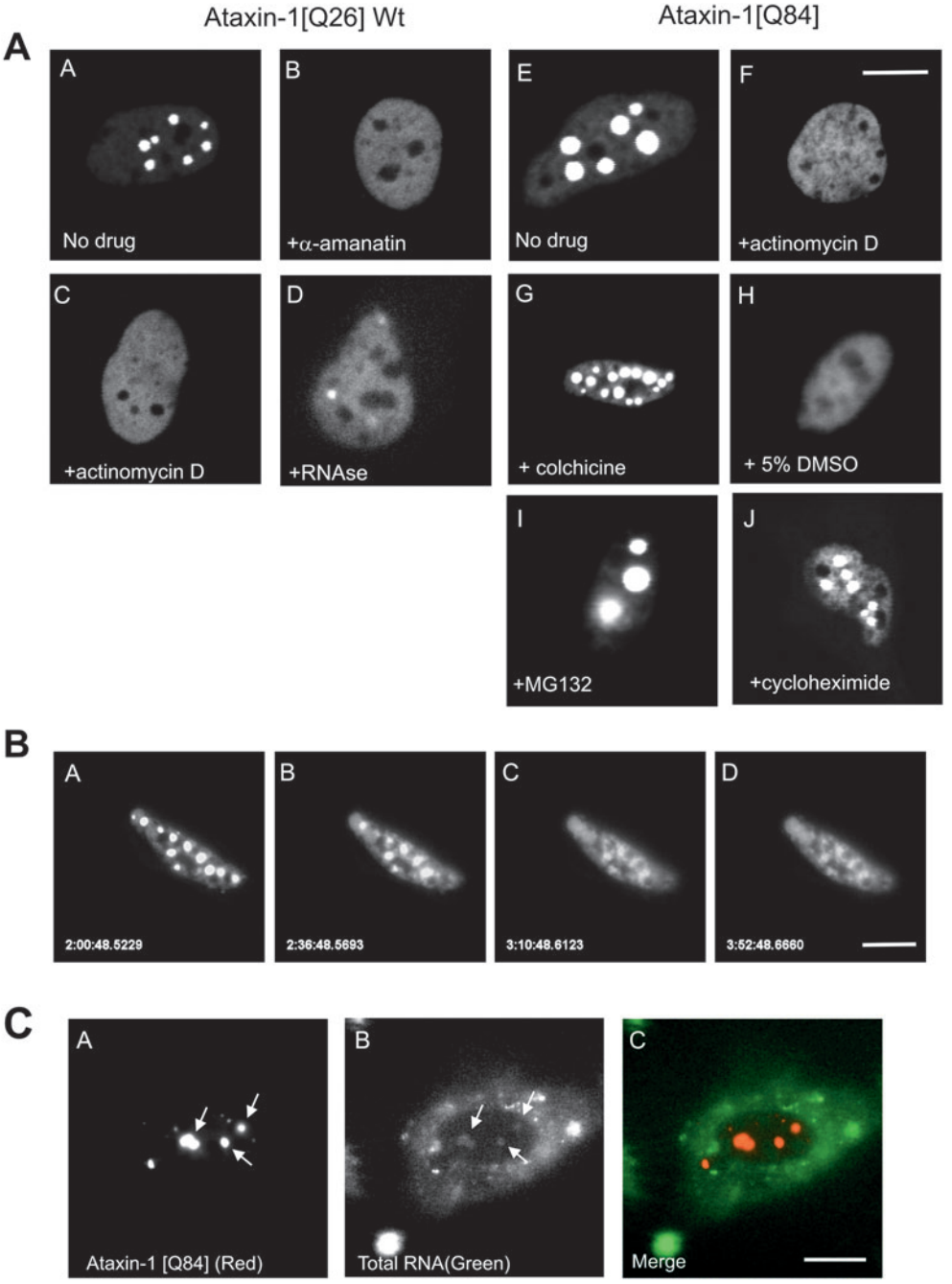

Fig. 3. Ataxin-1 sub-nuclear localization is dependent on RNA and transcription. Fluorescence micrographs of eGFP-ataxin-1[Q26]- or [Q84]-expressing NIH 3T3 cells. (A) panels A,E: no drug added; all other panels show the effects of the indicated drugs under the following conditions: panel B: 25 μg/ml α-amanatin for 3 hours at 37°C; panels C,F: a 0.05 μg/ml actinomycin D at 37°C for 3 hours; panel B: 25 μg/ml α-amanatin for 3 hours at 37°C; panel D: RNase for 1 hour; panel G: 0.5 μm colchicine for 3 hours; panel H, 5% DMSO for 1 hour; panels I, J, MG132 and cycloheximide respectively, for 1 hour. (B) panels A-D: live-cell microscopy of ataxin-1[Q84]-expressing cell over a time course of 2-4 hours of treatment with 25 μg/ml α-amanatin, showing inclusion dispersal. (See also Movie 6 in supplementary material.) (C) SYTO Green total RNA staining of cells expressing mRFP-ataxin-1[Q84] (panels A-C). Arrows indicate the presence of RNA in nuclear ataxin-1 inclusions in panels A and B. Bar: ~10 μm.

and noted a similar dispersion of ataxin-1 inclusions in the nucleus (Fig. 3A, panel D). However, no effect over the same time period was found with the microtubule polymerization inhibitor colchicine (Fig. 3A, panel G) or with the translation inhibitor cycloheximide (Fig. 3A, panel J). The apparent fusion of nuclear inclusions and size increase was seen with the proteosome inhibitor MG132 (Fig. 3A, panel I). Addition of small amounts (0.05%) of DMSO as a solvent control for actinomycin D had no effect on ataxin-1 inclusions (data not shown), but the addition of high amounts of DMSO (5%) caused the disruption of ataxin-1 nuclear inclusions (Fig. 3A, panel H). These results indicated that over-expressed mutant ataxin-1[Q84] was capable of dispersing from formed inclusions in the nucleus upon inhibition of RNA transcription or removal of RNA. To assess the presence of RNA in ataxin-1 nuclear inclusions, live mRFP-ataxin-1[Q84] expressing cells were stained with a RNA-specific SYTO green fluorescent RNA dye to visualize total cell RNA (Fig. 3C). RNA signal could be detected in nuclear inclusions of ataxin-1 (Fig. 3C, panels B,C). RNA could not be detected in very small ataxin-1 inclusions or in the rare case where cytoplasmic inclusions of ataxin-1 were seen (data not shown). These data indicate that ataxin-1 inclusions are transcription dependent and contain RNA.

Ataxin-1 inclusions recruit a messenger RNA export factor

The observations correlating RNA and active transcription to ataxin-1 localization within the nucleus led us to examine whether ataxin-1 co-localized with known RNA processing, splicing or transport factors. Previously, we showed by immunofluorescence that ataxin-1 did not co-localize with the coilin coiled body component p80, the spliceosome component SC35 or the transcription factor Bcl-6, (Skinner et al., 1997). These studies were extended in live cells with aequoria fluorescent protein (AFP) fusions minimally expressed by transient transfection (typically 0.5-1 μg total plasmid in a 35 mm dish, for 10-18 hours), observed at the detection limit of a fluorescence microscope with optimized filters for AFP detection. In cells expressing ataxin-1 and ASF/SF2 mRNA splicing factor (Misteli et al., 1997), no co-localization of ASF with ataxin-1 was seen (Fig. 4A-C). In addition, no recruitment to inclusions was seen between ataxin-1 and the pre mRNA/mRNA binding protein, hnRNPA1 (Siomi and Dreyfuss, 1995) (Fig. 4D-F) and no co-localization in inclusions with nup98, a dynamic nuclear pore complex protein observed to form nuclear inclusions (Griffis et al., 2002) (Fig. 4G-I). To test if our eGFP-ataxin-1 inclusions were still able to make similar protein-protein interactions as untagged ataxin-1, we co-expressed eGFP-ataxin-1 with a known ataxin-1-interacting protein, 14-3-3 zeta (mRFP fused), and observed co-localization in live cells (Fig. 4J-L), similar to that seen by immunofluorescence (Chen et al., 2003). Co-localization was also observed between wild-type ataxin-1 and TAP/NXF1 protein, a mRNA export factor (Segref et al., 1997). When expressed alone in live cells, TAP/NXF1 localized entirely to the nucleus, but remained evenly distributed in the nucleoplasm, exclusive of nucleoli (Fig. 5M-O). This co-localization was also seen with ataxin-1 with an expanded polyglutamine tract (Fig. 5A-D). In addition, ataxin-1 could be seen by fixed cell immunofluorescence to recruit endogenous TAP/NXF1 protein (Fig. 5I-L). TAP/NXF1 was not recruited to huntingtin exon1 [Q138] inclusions in live cells (Fig. 5E-H). Therefore, TAP/NXF1 protein could be specifically localized to ataxin-1 nuclear inclusions.

While TAP/NXF1 co-localized with ataxin-1 was consistently seen in all fixed cells, co-localization of mRFP-TAP/NXF1 with eGFP-ataxin-1 in live cells was variable. Co-localization of TAP/NXF1 and ataxin-1 in live cells was enhanced when the cells were subjected to either heat or cold-

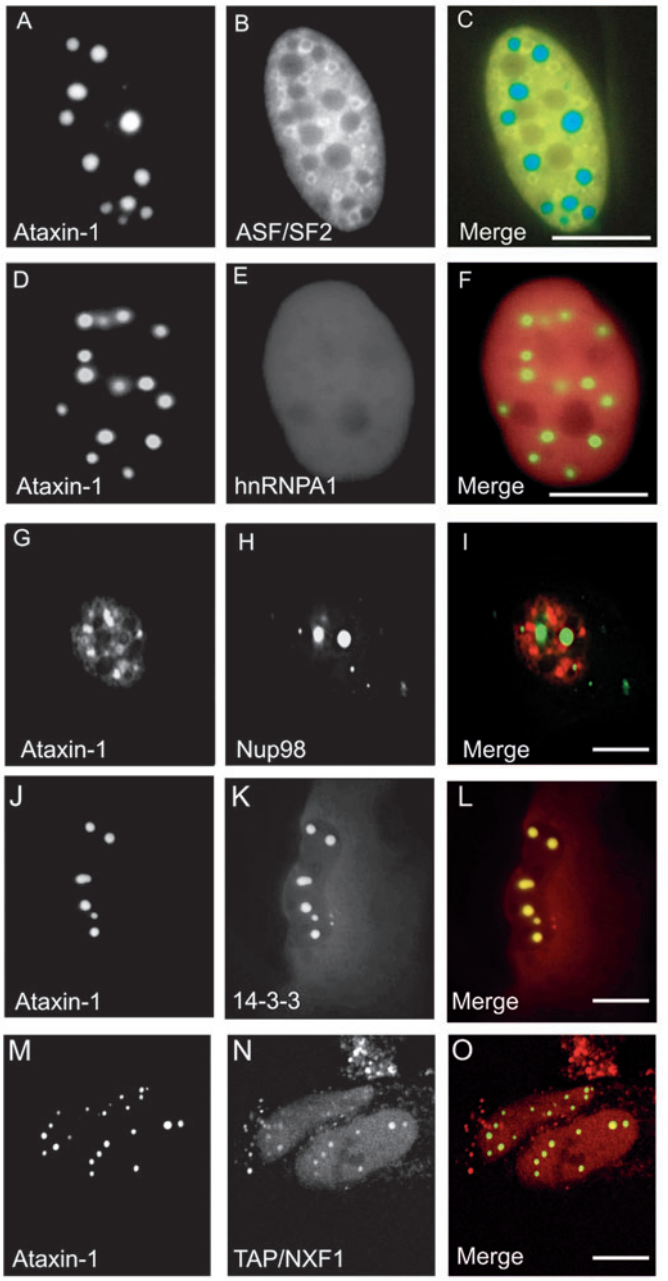

Fig. 4. Ataxin-1 nuclear inclusions are distinct from other nuclear bodies. Live-cell fluorescent patterns of ataxin-1 (A,D,G,J) and ASF/SF2 (B), hnRNPA1 (E), Nup98 (H), or 14-3-3 ζ (K) proteins fused to fluorescent protein variants. (M-O) Co-expression of eGFP-ataxin-1[Q2] (M) and mRFP-TAP/NXF1 (N) is shown by the merge image in O. Bar: ~10 μm.

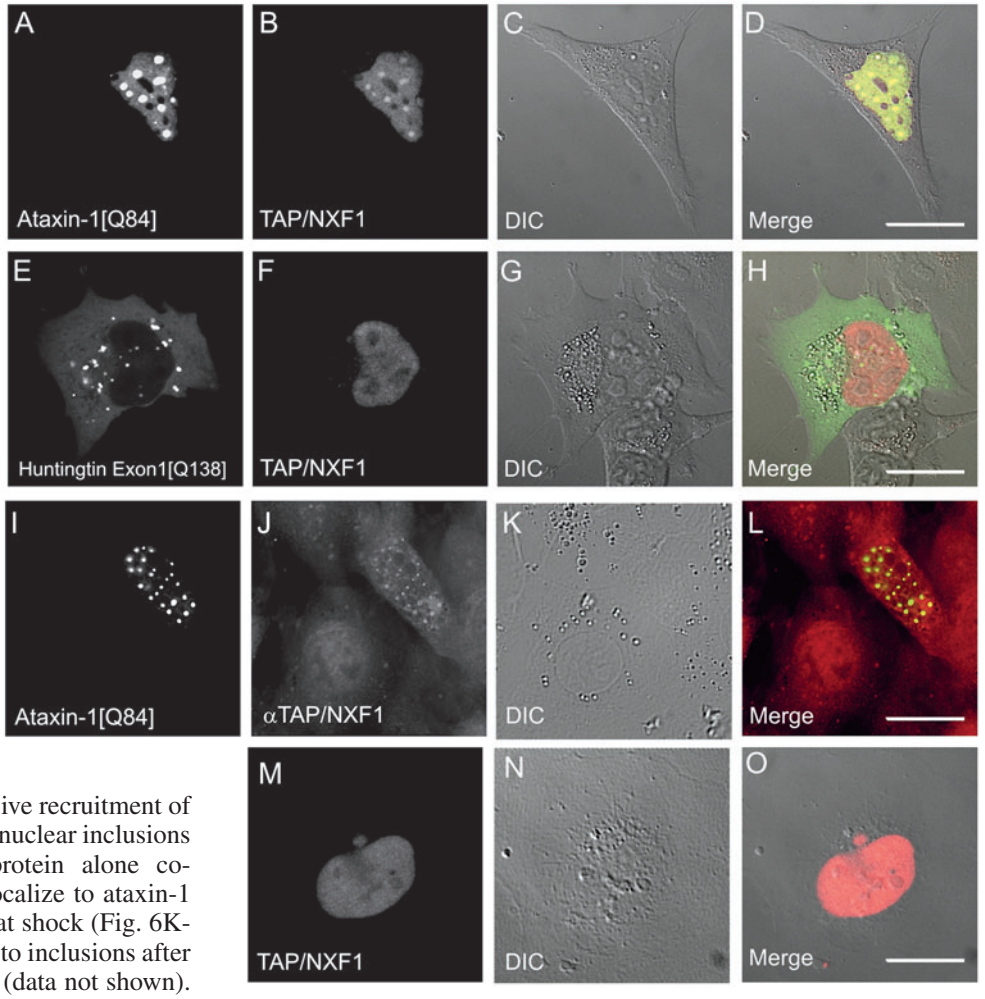

Fig. 5. Ataxin-1 nuclear inclusions co-localize with the TAP/NXF1 mRNA export factor. Laser confocal microscopy images of eGFP-ataxin-1 and mRFP-TAP/NXF1 (A-D), huntingtin exon-1[Q138] and mRFP-TAP/NXF1 (E-H) proteins. (I-L) Immunofluorescence with anti-TAP primary antibody and Alexa 594 secondary antibody on fixed NIH3T3 cell transfected with eGFP-ataxin-1[Q84] to detect endogenous TAP protein. (M-O) Cells transfected with mRFP-TAP/NXF1 alone, displaying no punctate localization (as in F and J). Bar: ~10 μm.

shock (i.e. cooling on ice prior to fixation). To better assess whether cellular stress would affect the localization of TAP/NXF1 to ataxin-1 inclusions, cells co-transfected with mRFP-TAP/NXF1 and eGFP-ataxin-1 were heat shocked at 42°C for 5 minutes followed by two-channel fluorescence microscopy at 37°C at 10-minute intervals. As seen in Fig. 6, heat-shock caused the progressive recruitment of mRFP-TAP protein to eGFP-ataxin-1 nuclear inclusions (Fig. 6C-H). In contrast, mRFP protein alone co-expressed with ataxin-1 did not co-localize to ataxin-1 inclusions up to one hour after the heat shock (Fig. 6K-M). TAP/NXF1 also failed to localize to inclusions after heat shock in the absence of ataxin-1 (data not shown). Additionally, as a specificity control for other nuclear bodies, we subjected cells expressing eGFP-PML (promyelocytic leukemia protein) and mRFP-TAP/NXF1 to heat shock, as with ataxin-1, but observed no re-distribution of TAP/NXF1 to typical nuclear PML bodies, even when PML was massively over-expressed (Fig. 6N-P). Thus, ataxin-1 RNA-containing nuclear bodies were able to recruit a general mRNA nuclear export factor.

Ataxin-1 shuttles between the nucleus and the cytoplasm

The co-localization of ataxin-1 with RNA and the TAP/NXF1 mRNA nuclear export factor prompted an examination of whether ataxin-1 could be exported from the nucleus. Transfected HeLa cells were examined by fluorescence recovery after photobleaching (FRAP) analysis using a qualitative live cell nuclear shuttling assay (Howell and Truant, 2002). 'Bikaryons' were formed from cells transfected with eGFP-ataxin-1[26Q] (Fig. 7A-E), and eGFP-ataxin-1[84Q] (Fig. 7F-J). To prevent recovery of the bleached nucleus directly from protein in the cytoplasm, the entire area of the bikaryon was bleached except for one of the nuclei (Fig. 7B,G). Additionally, all FRAP was done in the presence of cycloheximide to prevent new protein synthesis. Images of the bikaryons were captured immediately post-bleaching (Fig. 7C,H) and at 10-minute intervals (Fig. 7D,E,I,J). From these experiments, we saw the recovery of the bleached nucleus in cells expressing wild-type ataxin-1 (Fig. 7D,E), but not in those expressing mutant ataxin-1 (Fig. 7I,J). Recovery of mutant ataxin-1 could not be detected even 1 hour after photobleaching (data not shown). Interestingly, we noted that wild-type ataxin-1 appeared in the identical sub-nuclear locations as prior to photobleaching (Fig. 7A,E). Thus wild-type, but not mutant, ataxin-1 has the ability to shuttle to and from the nucleus.

Discussion

Except for the androgen receptor mutated in SBMA (Fischbeck et al., 1999), the general transcription factor TATA-box binding protein TBP, mutated in SCA17 (Nakamura et al., 2001) and a calcium channel associated with SCA6 (Zhuchenko et al., 1997), identification of the normal functions for the other six polyglutamine disease proteins have been illusive. In the case of SCA1, evidence has accumulated indicating that the nucleus is the primary subcellular site of mutant ataxin-1-induced pathogenesis and, thus, might also be a critical site in terms of normal function (Klement et al., 1998). Ataxin-1 has RNA-binding activity in vitro (Yue et al., 2001). Ataxin-1 probably binds RNA via an AXH domain, as also seen in the apparently unrelated HBP-1 transcription factor (de Chiara et al., 2003). The AXH domain structure in ataxin-1 contains an OB-fold, a

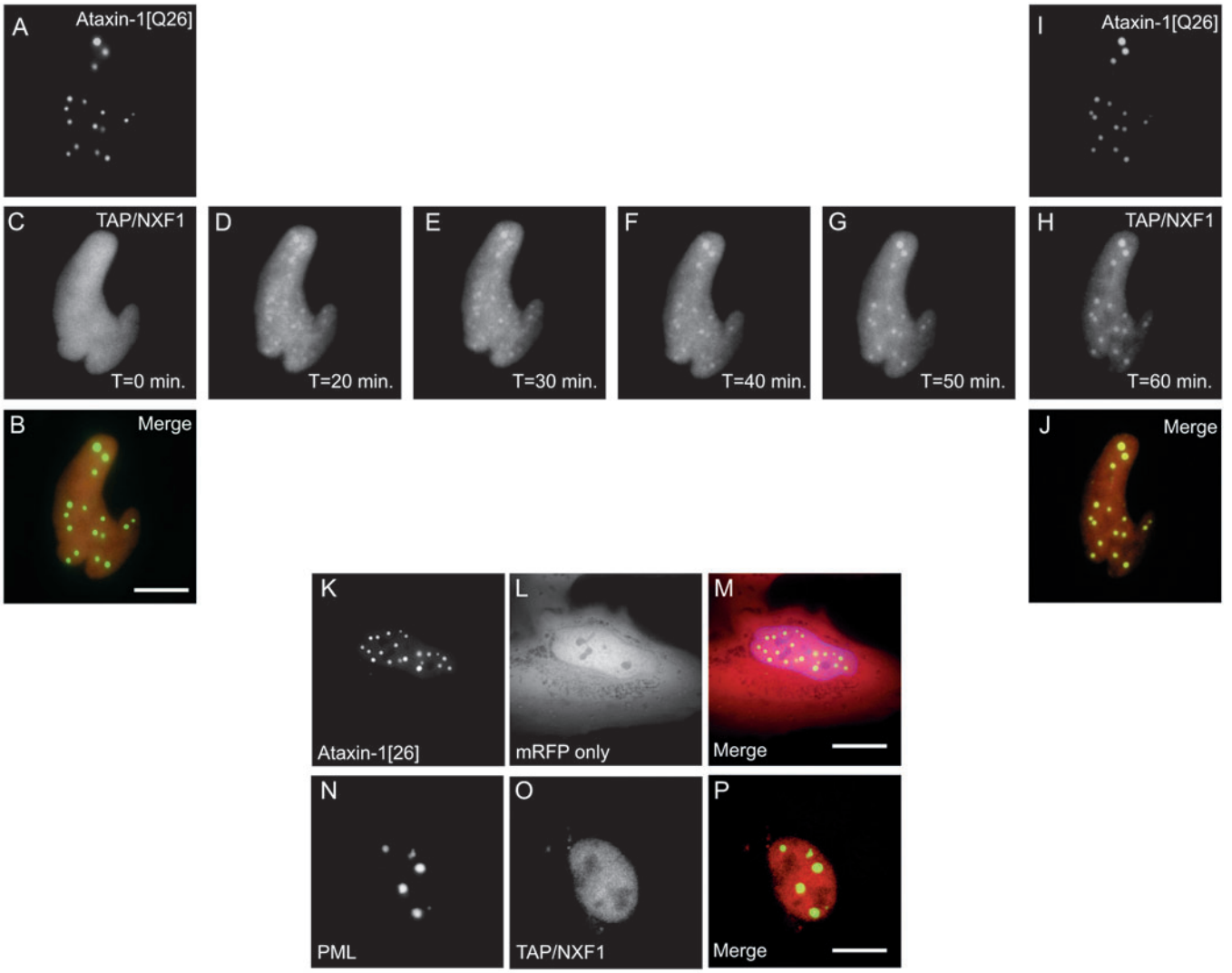

Fig. 6. Ataxin-1 recruits TAP/NXF1 protein to nuclear inclusions as the result of cell heat-shock. Fluorescence micrographs of eGFP-ataxin-1[Q26] and mRFP-TAP/NXF1 in live cells after heat shock at 42°C for 5 minutes (A-C), followed by incubation for 1 hour at 37°C (I-J), with time course of TAP/NXF1 localization to ataxin-1 nuclear inclusions over 60 minutes shown in C-H. (K-M) Control of mRFP alone or expressed with eGFP-ataxin-1[Q26] after similar heat shock treatment. (N-P) Control of eGFP-PML protein co-expressed with TAP/NXF1-mRFP after heat shock as in panels A-J. Bar: ~10 μm.

structural motif found in many oligonucleotide-binding proteins (Chen et al., 2004).

Here we provided data that ataxin-1 inclusion formation is RNA dependent and that wild-type ataxin-1 can shuttle between the nucleus and cytoplasm of transfected HeLa cells. Nucleocytoplasmic transport ability is also seen in other polyglutamine disease proteins. The androgen receptor (Saporita et al., 2003) and atrophin-1 (Nucifora et al., 2003) have defined nuclear import and export signals, and lack of nuclear export has been linked to DRPLA and SBMA disease. Ataxin-1 localized to characteristic nuclear inclusions that contained TAP/NXF1 (Gruter et al., 1998; Herold et al., 2000; Weis, 2002), or Mex67p in yeast (Segref et al., 1997), which are mRNA nuclear export factors. Ataxin-1 inclusion movement as observed by live-cell video microscopy appeared similar to promyelocytic leukemia (PML) body movement (Muratani et al., 2002). However, in our short and long term observations of ataxin-1 inclusions, we did not detect other characteristics of PML body movement with ataxin-1 inclusions, such as fission of PML inclusions, as recently described by others (Eskiw et al., 2003). These observations were purposely done at low transient expression levels to focus on potential functions of normal ataxin-1 protein and to distinguish them from effects caused by long polyglutamine tract protein over expression.

By sequence analysis, ataxin-1 does not appear to contain a typical leucine-type nuclear export signal sequence. Moreover, nuclear export of ataxin-1 was not sensitive to leptomycin B (data not shown). Thus, ataxin-1 probably complexes with another protein or RNA to exit the nucleus or can be exported by a CRM-1/exportin-independent mechanism.

Ataxin-1 localization to nuclear inclusions was dependent upon RNA and active transcription. This suggests that ataxin-1 inclusions in the nucleus, even with expanded polyglutamine at 84 residues, are not static, aggregated or misfolded protein, but probably sites of high local concentration related to the

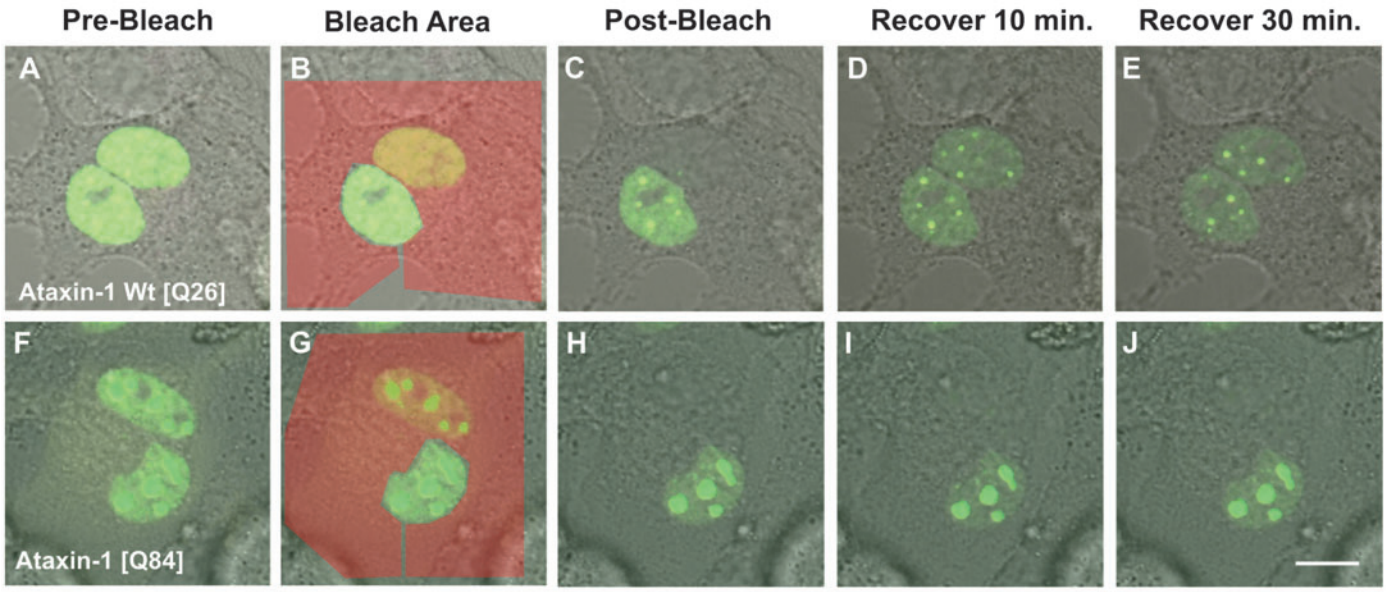

Fig. 7. Wild-type ataxin-1 can shuttle to and from the nucleus. FRAP live cell shuttle assay. Laser confocal fluorescence over DIC overlay of either GFP-wild-type or -mutant ataxin-1 proteins in bikaryons treated with cycloheximide before (panels A,F) and after (panels C,H) photo bleaching the entire area of the bikaryon except one nucleus (mask area, panels B, G). Recovery of wild-type ataxin-1 is seen by 10 minutes (D,E), but no recovery of mutant ataxin-1 is seen even after 30 minutes (I,J). Bar: ~10 μm.

function of ataxin-1. Sites of ataxin-1 accumulation have also been seen to co-localize with the SMRT transcription co-repressor (Tsai et al., 2004), indicating they may be sites of active transcription. Others have seen that wild-type and polyglutamine-expanded ataxin-1 protein are dynamic within the nucleus by fluorescence recovery after photobleaching experiments (Chai et al., 2002; Stenoien et al., 2002). Whether ataxin-1 and TAP/NXF1 interact directly, or through association with RNA is unclear at this time. Our observed effect of heat-shock recruitment of TAP/NXF1 to ataxin-1 inclusions indicates that cell stress may recruit TAP/NXF1 to ataxin-1 in messenger ribonucleoprotein particles (mRNPs). Others have shown that TAP/NXF1 dynamics are temperature sensitive and reduce when bound to mRNPs (Calapez et al., 2002).

What might the nuclear function of ataxin-1 be? The requirement of active transcription and RNA for ataxin-1 nuclear inclusions, the nuclear export of wild-type ataxin-1 and the specific co-localization of ataxin-1 inclusions with TAP/NXF1 mRNA export factor suggest that ataxin-1 may have a role in the processing and/or transport of specific mRNAs from sites of transcription. These properties of ataxin-1 might be relevant to SCA1 disease because the ability of ataxin-1 to shuttle from the nucleus was blocked by expansion of its polyglutamine tract. The apparent requirement of RNA for the localization of ataxin-1 to inclusions in the nucleus is a particularly intriguing observation. Previous studies showed that the formation of ataxin-1 nuclear inclusions was linked to functions regulating protein folding (Rimoldi et al., 2001), interaction with molecular chaperones (Cummings et al., 1998) and degradation by the proteasome (Cummings et al., 1999). Our demonstration that inhibition of transcription with actinomycin D or α-amanatin as well as degrading RNA with RNase dramatically decreased inclusion formation implicates other cellular pathways in the formation of ataxin-1 nuclear inclusions. The inability of cyclohexamide, colchicine or MG132 to alter inclusion formation indicates that the effects seen with the other agents is simply not due to the increased or decreased amounts of ataxin-1. Recently, it was discovered that ataxin-1 is phosphorylated at a single serine near its NLS and that nuclear ataxin-1 is also phosphorylated (Emamamian et al., 2003). This suggests that ataxin-1 may respond to signals, possibly to up-regulate important factor protein levels by mediating RNA processing or nuclear export of specific mRNAs to the cytoplasm. Some nuclear proteins, such as β-catenin, are normally regulated by proteasome degradation in the cytoplasm via export by adenomatous polyposis coli (APC) protein (Henderson and Fagotto, 2002). Lack of nuclear export of mutant ataxin-1 could affect RNA export, and/or cause ataxin-1 transcription inhibition by accumulation of toxic mutant protein in the nucleus, while normal ataxin-1 levels would still be regulated by proteolysis in the cytoplasm. We did not observe accumulation of total TAP/NXF1 to ataxin-1 inclusions, suggesting that while TAP/NXF1 co-localization may indicate an activity of normal ataxin-1, it is unlikely that TAP/NXF1 export inhibition would provide any cell specificity of toxicity in SCA1 as TAP/NXF1 inhibition would be a catastrophic event in any cell.

Disruption in RNA processing or export has precedence in neurological disease. In Fragile X syndrome, the product of the *FMR1* gene, FMRP, a mRNA-associated nucleocytoplasmic shuttle protein (Eberhart et al., 1996), is either not expressed because of a CGG repeat expansion in its 5′ untranslated region, or cannot be exported from the nucleus because of loss of an exon encoding its nuclear export signal (Fridell et al., 1996). The net result is lack of nuclear export and down-regulation of important mRNAs for brain development, resulting in mental retardation in Fragile X-affected individuals (Brown et al., 2001; Kaytor and Orr, 2001). In spinal muscular atrophy, the SMN1 protein is expressed at reduced levels

throughout the brain, yet neuronal degeneration is limited to motor neurons in the anterior horn of the spinal cord. Wild-type, but not mutant truncated, SMN1 has been shown to interact with RNA transport factors hnRNP-R and hnRNP-Q (Rossoll et al., 2002). Nova proteins are involved in paraneoplastic opsoclonus myoclonus ataxia (POMA), an autoimmune neurodegenerative disease. Nova proteins regulate neuronal pre-mRNA splicing by directly binding to RNA (Ule et al., 2003). Another putative RNA export factor, NXF5, is absent in some patients of X-linked mental retardation (Frints et al., 2003; Jun et al., 2001).

The data provided here indicate that ataxin-1 nuclear inclusions are not simply protein aggregates. Rather, we suggest they seem to be areas of high ataxin-1 protein concentration correlated with active RNA transcription and the presence of RNA. The lack of nuclear export of polyglutamine-expanded ataxin-1 suggests that a gain of function for ataxin-1 in SCA1 disease may be the retention of specific RNA or proteins specifically associated with RNA within the nucleus. Specific toxicity of ataxin-1 in SCA1 may be conferred at the level of specific mRNA species affected within ataxin-1 inclusions. Identification of any specific species of mRNAs affected by ataxin-1 are the subjects of further study.

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References

Brown, V., Jin, P., Ceman, S., Darnell, J. C., O'Donnell, W. T., Tenenbaum, S. A., Jin, X., Feng, Y., Wilkinson, K. D., Keene, J. D. et al. (2001). Microarray identification of FMRP-associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome. *Cell* **107**, 477-487.

Burd, C. G. and Dreyfuss, G. (1994). RNA binding specificity of hnRNP A1: significance of hnRNP A1 high-affinity binding sites in pre-mRNA splicing. *EMBO J.* **13**, 1197-1204.

Calapez, A., Pereira, H. M., Calado, A., Braga, J., Rino, J., Carvalho, C., Tavanez, J. P., Wahle, E., Rosa, A. C. and Carmo-Fonseca, M. (2002). The intranuclear mobility of messenger RNA binding proteins is ATP dependent and temperature sensitive. *J. Cell Biol.* **159**, 795-805.

Campbell, R. E., Tour, O., Palmer, A. E., Steinbach, P. A., Baird, G. S., Zacharias, D. A. and Tsien, R. Y. (2002). A monomeric red fluorescent protein. *Proc. Natl. Acad. Sci. USA* **99**, 7877-7882.

Chai, Y., Shao, J., Miller, V. M., Williams, A. and Paulson, H. L. (2002). Live-cell imaging reveals divergent intracellular dynamics of polyglutamine disease proteins and supports a sequestration model of pathogenesis. *Proc. Natl. Acad. Sci. USA* **99**, 9310-9315.

Chen, H. K., Fernandez-Funez, P., Acevedo, S. F., Lam, Y. C., Kaytor, M. D., Fernandez, M. H., Aitken, A., Skoulakis, E. M., Orr, H. T., Botas, J. et al. (2003). Interaction of Akt-phosphorylated ataxin-1 with 14-3-3 mediates neurodegeneration in spinocerebellar ataxia type 1. *Cell* **113**, 457-468.

Chen, Y. W., Allen, M. D., Veprintsev, D. B., Lowe, J. and Bycroft, M. (2004). The structure of the AXH domain of spinocerebellar ataxin-1. *J. Biol. Chem.* **279**, 3758-3765.

Cummings, C. J. and Zoghbi, H. Y. (2000). Fourteen and counting: unraveling trinucleotide repeat diseases. *Hum. Mol. Genet.* **9**, 909-916.

Cummings, C. J., Mancini, M. A., Antalffy, B., DeFranco, D. B., Orr, H. T. and Zoghbi, H. Y. (1998). Chaperone suppression of aggregation and altered subcellular proteasome localization imply protein misfolding in SCA1. *Nat. Genet.* **19**, 148-154.

Cummings, C. J., Reinstein, E., Sun, Y., Antalffy, B., Jiang, Y., Ciechanover, A., Orr, H. T., Beaudet, A. L. and Zoghbi, H. Y. (1999). Mutation of the E6-AP ubiquitin ligase reduces nuclear inclusion frequency while accelerating polyglutamine-induced pathology in SCA1 mice. *Neuron* **24**, 879-892.

de Chiara, C., Giannini, C., Adinolfi, S., de Boer, J., Guida, S., Ramos, A., Jodice, C., Kioussis, D. and Pastore, A. (2003). The AXH module: an independently folded domain common to ataxin-1 and HBP1. *FEBS Lett.* **551**, 107-112.

DiFiglia, M., Sapp, E., Chase, K. O., Davies, S. W., Bates, G. P., Vonsattel, J. P. and Aronin, N. (1997). Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. *Science* **277**, 1990-1993.

Eberhart, D. E., Malter, H. E., Feng, Y. and Warren, S. T. (1996). The fragile X mental retardation protein is a ribonucleoprotein containing both nuclear localization and nuclear export signals. *Hum. Mol. Genet.* **5**, 1083-1091.

Emamamian, E. S., Kaytor, M. D., Duvick, L. A., Zu, T., Tousey, S. K., Zoghbi, H. Y. and Orr, H. T. (2003). Serine 776 of Ataxin-1 is critical for polyglutamine-induced disease in SCA1 transgenic mice. *Neuron* **38**, 375-387.

Eskiw, C. H., Dellaire, G., Mymryk, J. S. and Bazett-Jones, D. P. (2003). Size, position and dynamic behavior of PML nuclear bodies following cell stress as a paradigm for supramolecular trafficking and assembly. *J. Cell Sci.* **116**, 4455-4466.

Fischbeck, K. H., Lieberman, A., Bailey, C. K., Abel, A. and Merry, D. E. (1999). Androgen receptor mutation in Kennedy's disease. *Philos. Trans. R. Soc. London Series B* **354**, 1075-1078.

Fridell, R. A., Benson, R. E., Hua, J., Bogerd, H. P. and Cullen, B. R. (1996). A nuclear role for the Fragile X mental retardation protein. *EMBO J.* **15**, 5408-5414.

Frints, S. G., Jun, L., Fryns, J. P., Devriendt, K., Teulingkx, R., van den Berghe, L., de Vos, B., Borghgraef, M., Chelly, J., Des Portes, V. et al. (2003). Inv(X)(p21.1;q22.1) in a man with mental retardation, short stature, general muscle wasting, and facial dysmorphism: clinical study and mutation analysis of the NXF5 gene. *Am. J. Med. Genet.* **119**, 367-374.

Griffis, E. R., Altan, N., Lippincott-Schwartz, J. and Powers, M. A. (2002). Nup98 is a mobile nucleoporin with transcription-dependent dynamics. *Mol. Biol. Cell* **13**, 1282-1297.

Gruter, P., Tabernero, C., von Kobbe, C., Schmitt, C., Saavedra, C., Bachi, A., Wilm, M., Felber, B. K. and Izaurralde, E. (1998). TAP, the human homolog of Mex67p, mediates CTE-dependent RNA export from the nucleus. *Mol. Cell* **1**, 649-659.

Henderson, B. R. and Fagotto, F. (2002). The ins and outs of APC and beta-catenin nuclear transport. *EMBO Rep.* **3**, 834-839.

Herold, A., Suyama, M., Rodrigues, J. P., Braun, I. C., Kutay, U., Carmo-Fonseca, M., Bork, P. and Izaurralde, E. (2000). TAP (NXF1) belongs to a multigene family of putative RNA export factors with a conserved modular architecture. *Mol. Cell. Biol.* **20**, 8996-9008.

Howell, J. L. and Truant, R. (2002). A live cell nucleocytoplasmic protein shuttle assay utilizing laser confocal microscopy and FRAP. *Biotechniques* **32**, 80-87.

Jun, L., Frints, S., Duhamel, H., Herold, A., Abad-Rodrigues, J., Dotti, C., Izaurralde, E., Marynen, P. and Froyen, G. (2001). NXF5, a novel member of the nuclear RNA export factor family, is lost in a male patient with a syndromic form of mental retardation. *Curr. Biol.* **11**, 1381-1391.

Kaytor, M. D. and Orr, H. T. (2001). RNA targets of the fragile X protein. *Cell* **107**, 555-557.

Klement, I. A., Skinner, P. J., Kaytor, M. D., Yi, H., Hersch, S. M., Clark, H. B., Zoghbi, H. Y. and Orr, H. T. (1998). Ataxin-1 nuclear localization and aggregation: role in polyglutamine-induced disease in SCA1 transgenic mice. *Cell* **95**, 41-53.

Misteli, T., Caceres, J. F. and Spector, D. L. (1997). The dynamics of a pre-mRNA splicing factor in living cells. *Nature* **387**, 523-527.

Muratani, M., Gerlich, D., Janicki, S. M., Gebhard, M., Eils, R. and Spector, D. L. (2002). Metabolic-energy-dependent movement of PML bodies within the mammalian cell nucleus. *Nat. Cell Biol.* **4**, 106-110.

Nakamura, K., Jeong, S. Y., Uchihara, T., Anno, M., Nagashima, K., Nagashima, T., Ikeda, S., Tsuji, S. and Kanazawa, I. (2001). SCA17, a novel autosomal dominant cerebellar ataxia caused by an expanded polyglutamine in TATA-binding protein. *Hum. Mol. Genet.* **10**, 1441-1448.

Nucifora, F. C., Jr, Ellerby, L. M., Wellington, C. L., Wood, J. D., Herring, W. J., Sawa, A., Hayden, M. R., Dawson, V. L., Dawson, T. M. and Ross, C. A. (2003). Nuclear localization of a non-caspase truncation product of

atrophin-1, with an expanded polyglutamine repeat, increases cellular toxicity. *J. Biol. Chem.* **278**, 13047-13055.

Orr, H. T. and Zoghbi, H. Y. (2001). SCA1 molecular genetics: a history of a 13 year collaboration against glutamines. *Hum. Mol. Genet.* **10**, 2307-2311.

Orr, H. T., Chung, M. Y., Banfi, S., Kwiatkowski, T. J., Jr, Servadio, A., Beaudet, A. L., McCall, A. E., Duvick, L. A., Ranum, L. P. and Zoghbi, H. Y. (1993). Expansion of an unstable trinucleotide CAG repeat in spinocerebellar ataxia type 1. *Nat. Genet.* **4**, 221-226.

Rimoldi, M., Servadio, A. and Zimarino, V. (2001). Analysis of heat shock transcription factor for suppression of polyglutamine toxicity. *Brain Res. Bull.* **56**, 353-362.

Ross, C. A. (1997). Intranuclear neuronal inclusions: a common pathogenic mechanism for glutamine-repeat neurodegenerative diseases? *Neuron* **19**, 1147-1150.

Rossoll, W., Kroning, A. K., Ohndorf, U. M., Steegborn, C., Jablonka, S. and Sendtner, M. (2002). Specific interaction of Smn, the spinal muscular atrophy determining gene product, with hnRNP-R and gry-rbp/hnRNP-Q: a role for Smn in RNA processing in motor axons? *Hum. Mol. Genet.* **11**, 93-105.

Saporita, A. J., Zhang, Q., Navai, N., Dincer, Z., Hahn, J., Cai, X. and Wang, Z. (2003). Identification and characterization of a ligand-regulated nuclear export signal in androgen receptor. *J. Biol. Chem.* **278**, 41998-42005.

Segref, A., Sharma, K., Doye, V., Hellwig, A., Huber, J., Luhrmann, R. and Hurt, E. (1997). Mex67p, a novel factor for nuclear mRNA export, binds to both poly(A)+ RNA and nuclear pores. *EMBO J.* **16**, 3256-3271.

Siomi, H. and Dreyfuss, G. (1995). A nuclear localization domain in the hnRNP A1 protein. *J. Cell Biol.* **129**, 551-560.

Skinner, P. J., Koshy, B. T., Cummings, C. J., Klement, I. A., Helin, K., Servadio, A., Zoghbi, H. Y. and Orr, H. T. (1997). Ataxin-1 with an expanded glutamine tract alters nuclear matrix-associated structures. *Nature* **389**, 971-974.

Stenoien, D. L., Mielke, M. and Mancini, M. A. (2002). Intranuclear ataxin1 inclusions contain both fast- and slow-exchanging components. *Nat. Cell Biol.* **4**, 806-810.

Tsai, C. C., Kao, H. Y., Mitzutani, A., Banayo, E., Rajan, H., McKeown, M. and Evans, R. M. (2004). Ataxin **1**, a SCA1 neurodegenerative disorder protein, is functionally linked to the silencing mediator of retinoid and thyroid hormone receptors. *Proc. Natl. Acad. Sci. USA* **101**, 4047-4052.

Ule, J., Jensen, K. B., Ruggiu, M., Mele, A., Ule, A. and Darnell, R. B. (2003). CLIP identifies Nova-regulated RNA networks in the brain. *Science* **302**, 1212-1215.

Weis, K. (2002). Nucleocytoplasmic transport: cargo trafficking across the border. *Curr. Opin. Cell Biol.* **14**, 328-335.

Xia, J., Lee, D. H., Taylor, J., Vandelft, M. and Truant, R. (2003). Huntingtin contains a highly conserved nuclear export signal. *Hum. Mol. Genet.* **12**, 1393-1403.

Yue, S., Serra, H. G., Zoghbi, H. Y. and Orr, H. T. (2001). The spinocerebellar ataxia type 1 protein, ataxin-1, has RNA-binding activity that is inversely affected by the length of its polyglutamine tract. *Hum. Mol. Genet.* **10**, 25-30.

Zhuchenko, O., Bailey, J., Bonnen, P., Ashizawa, T., Stockton, D. W., Amos, C., Dobyns, W. B., Subramony, S. H., Zoghbi, H. Y. and Lee, C. C. (1997). Autosomal dominant cerebellar ataxia (SCA6) associated with small polyglutamine expansions in the alpha 1A-voltage-dependent calcium channel. *Nat. Genet.* **15**, 62-69.