Ataxin-7 Can Export from the Nucleus via a Conserved Exportin-dependent Signal*

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Spinocerebellar ataxia type 7 is a progressive neurodegenerative disorder caused by a CAG DNA triplet repeat expansion leading to an expanded polyglutamine tract in the ataxin-7 protein. Ataxin-7 appears to be a transcription factor and a component of the STAGA transcription coactivator complex. Here, using live cell imaging and inverted fluorescence recovery after photobleaching, we demonstrate that ataxin-7 has the ability to export from the nucleus via the CRM-1/exportin pathway and that ataxin-7 contains a classic leucine-type nuclear export signal (NES). We have precisely defined the location of this NES in ataxin-7 and found it to be fully conserved in all vertebrate species. Polyglutamine expansion was seen to reduce the nuclear export rate of mutant ataxin-7 relative to wild-type ataxin-7. Subtle point mutation of the NES in polyglutamine expanded ataxin-7 increased toxicity in primary cerebellar neurons in a polyglutamine length-dependent manner in the context of full-length ataxin-7. Our results add ataxin-7 to a growing list of polyglutamine disease proteins that have the ability to shuttle to and from the nucleus and suggest that the role of ataxin-7 in STAGA/SAGA/crxml complex of trafficking between the nucleus and cytoplasm.

Spinocerebellar ataxia type 7 (SCA7)‡ is a dominantly inherited neurodegenerative disorder characterized by loss of neurons in the cerebellum, brain stem, and retina (1). SCA7 is a member of a family of neurodegenerative diseases in which a CAG DNA triplet repeat expansion results in polyglutamine expansion in the gene product (2). Other members of this polyglutamine expansion disease family include Huntington disease, spinobulbar muscle atrophy, dentatorubral pallidoluysian atrophy, and cone-rod dystrophy (5). The mutant ataxin-7 protein can have nuclear import signals (NLs) defined in both the central (7), and carboxyl-terminal regions of the protein (8). Within the nucleus, ataxin-7 is known to be a subunit of the mammalian GCN5 histone acetyltransferase STAGA transcription coactivator complex (9). Ataxin-7 directly binds GCN5, and mutant ataxin-7 can inhibit the histone acetyltransferase activity of STAGA (10). Although the precise biological function of ataxin-7 is unknown, mutant ataxin-7 is known to interfere with Crx-dependent transcription of retinal photoreceptor-specific genes (11, 12). Ataxin-7 interacts with TFTC/STAGA protein subunits through a central evolutionarily conserved block of residues that have defined an ataxin-7 homology family in species ranging from human to yeast (9). In Saccharomyces cerevisiae, the yeast ataxin-7 homolog, Sgf73, is a member of the SAGA and SLIK histone acetyltransferase complexes (13). Ataxin-7 and the Crx homeodomain transcription factor interact via glutamine regions in each protein (8). In SCA7-affected brains (14, 15) and transgenic mice (11, 16, 17), ataxin-7 subcellular localization varies from cytoplasmic to nuclear with evidence of neuronal intranuclear inclusions (18). Ataxin-7 has been observed to undergo proteolytic cleavage in a mouse model (16) and may be cleaved by caspase-7 at amino acid positions 266 and 344.‡

In this study, we have examined the subcellular localization of ataxin-7 and ataxin-7 fragments in cultured cells by live cell microscopy and aequorin fluorescent protein fusion technology. In fragments of ataxin-7 containing a defined NLS (7) and a leucine-rich evolutionarily conserved region (9), we observed a predominantly cytoplasmic distribution, indicating the presence of a nuclear export signal (NES) in this region of ataxin-7. Treatment of cells expressing these ataxin-7 fragments with the CRM-1/exportin inhibitor compound leptomycin B (20, 21) led to the nuclear accumulation of these fragments, indicating that ataxin-7 could export from the nucleus by the CRM-1/exportin pathway. By a live cell nucleocytoplasmic inverted fluorescence after photobleaching (iFRAP) shutting assay (22), we found that ataxin-7 had the ability to shuttle to and from the nucleus. We precisely defined the position of the NES in ataxin-7 and found it to be typical of leucine-type NESs, highly conserved, and that it could be inactivated by site-directed mutagenesis, preventing ataxin-7 nuclear export. In primary granule cerebellar neurons, mutation of the ataxin-7 NES was seen to significantly increase cell toxicity, but only in the context of full-length ataxin-7 with all of its nuclear localization signals intact. The nuclear shuttling ability of ataxin-7 adds this protein into a growing list of polyglutamine disease proteins that have the ability to shuttle to and from the nucleus and suggests that the role of ataxin-7 in STAGA/SAGA/SLIK may be to traffic between the nucleus and cytoplasm.

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MATERIALS AND METHODS

Expression Plasmids—The plasmids encoding ataxin-7 and ataxin-7 (344–892) were made in the following manner. The plasmids encoding EGFP-ataxin-7 (Q10) (pMV014) and EGFP-ataxin-7 (Q64) (pMV015) were made by digesting pTAG-SCA7 (Q10) and pTAG-SCA7 (Q64) with EcoRI and EcoRV. The resulting 2.7-kb (CAG)10 and 2.8-kb (CAG)64 fragments were ligated into EcoRI/Smal-digested pEGFP-C2 (BD Biosciences). The plasmid encoding EGFP-ataxin-7 (344–892) (pMV020) was made by PCR amplification using pTGA-hr-GFP-SCA7 (Q10) as a template and primers RT0399 and RT0371 to truncate the SCA7 gene and introduce BglII and EcoRI restriction sites at the 5’ and 3’ ends. The PCR product was digested with BglII and EcoRI and ligated into BglII/EcoRI-digested pEGFP-C1 (BD Biosciences). The inserts for the plasmids encoding EGFP-ataxin-7 (1–619) (Q10) (pJT018) and EGFP-ataxin-7 (1–619) (Q64) (pJT019) were made by digesting pTAG-SCA7 (Q10) and pTAG-SCA7 (Q64) with EcoRI and KpnI. The resulting 1.9-kb (CAG)10 and 2.0-kb (CAG)64 fragments were ligated into EcoRI/KpnI-digested pEGFP-C2. The inserts for the plasmids encoding the EGFP-ataxin-7 (1–619) (Q10)/V349S I350T (pJT020) and EGFP-ataxin-7 (1–619) (Q64) V349S I350T (pJT021) NES mutants were made by recombinant PCR. Complimentary mutagenic primers RT0419 and RT420 were used to introduce the point mutations encoding for the V349S I350T amino acid changes in the putative NES of ataxin-7 and a Scal restriction site. The final PCR product was digested at endogenous restriction sites with PstI and BspEII, and the resulting 0.6-kb fragment was ligated into a 6.0-kb fragment resulting from PstI/BspEII digestion of pJT018 and a 6.1-kb fragment resulting from PstI/BspEII digestion of pJT019. The plasmids encoding EGFP-ataxin-7 (228–619) (pJT024) and EGFP-ataxin-7 (228–619) V349S I350T (pJT025) were made by digesting pJT018 and pJT020 with PstI and KpnI and ligating the resulting 1.2-kb fragments into PstI/Kpn1-digested pEGFP-C3. For the plasmid encoding EGFP-ataxin-7 (619–892) (pJT006), pTAG-SCA7 (Q10) cDNA was digested with KpnI and EcoRV, and the resulting 0.8-kb fragment was ligated into KpnI/EcoRV-digested pEGFP-C3 (BD Biosciences). The plasmid encoding EGFP-ataxin-7 (1–230) (Q10) was made by digesting pJT018 and pJT033 plasmids with Nhel and PstI and ligating the resulting 1.4-kb fragment from pJT018 with the 4.0-kb fragment from pJT033. The pJT033 plasmid was made by digesting pJT034 and pJT018 with Nhel and XmnI and ligating the resulting 4.2-kb fragment from pJT034 with the 1.6-kb band from pJT018. The pJT034 plasmid was made by PCR amplification of a region of pTAG-SCA7 (Q10) encoding ataxin-7 (1–343) using primers RT0441 and RT0442 to introduce HindIII and EcoRI restriction sites at the 5’ and 3’ ends. The PCR product was digested with HindIII and EcoRI and ligated into HindIII/EcoRI-digested pEGFP-C1. For the plasmids encoding EGFP-ataxin-7 (1–344) (Q10), the region of cDNA encoding ataxin-7 (201–344) was PCR-amplified using pMV014 as a template to introduce a BamHI restriction site at the 3’ end and a stop codon following amino acid 344. SV40Tag NLS (PKKKRKVR) EGFP-1xKb expression plasmid was purchased from BD Biosciences. All of the constructed plasmids were PCR-sequenced at junctions, mutations, or CAG repeats at the McMaster Sequencing Facility.

Cell Culture and Transfections—The cell lines used were mouse fibroblast NIH3T3 cells (ATCC, CRL-1658) grown and passaged by ATCC specifications. The cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum (Invitrogen) and 10% CO2. For the purposes of live cell microscopy, ~100,000 – 150,000 cells were seeded on 35-mm glass-bottomed dishes 18–24 h before transfection. The culture dishes were prepared using a procedure based on that described elsewhere (22).

 ~100,000 – 150,000 cells were seeded on 35-mm glass-bottomed dishes 18–24 h before transfection. The culture dishes were prepared using a procedure based on that described elsewhere (22). The cells were transfected using polyethylenimine (Exgen 500, MBI Fermentas) according to the manufacturer’s instructions. To minimize expression, transfection conditions were optimized for the limit of EGFP-ataxin-7 protein detection on an imaging system optimized for EGFP detection. One microgram of DNA was transfected per 35-mm culture dish, and imaging was carried out at 14–18 h post-transfection. For CRM-1/exportin inhibition experiments, the cells were treated with 5 ng/ml leptomycin B (Sigma) for 20–23 h in serum-free medium, conditions empirically determined to cause nuclear accumulation of EGFP-1xKb (BD Biosciences). All ataxin-7 EGFP fusion moieties were tested by Western blot analysis and found to be intact with no apparent degradation after 24 h of expression, typically 8 h after the imaging experiments were performed.

Live Cell Microscopy—Epifluorescence microscopy was performed on a Nikon TE2000 inverted fluorescence microscope with a 175 W xenon arc lamp (Sutter Instruments) light source. Nikon 63× plan apochromat (NA 1.3) or 100× plan apochromat (NA 1.4) oil immersion objectives were used. Specific EGFP filter sets were used for EGFP imaging, and Texas Red filter sets were used for mRFP imaging (Chroma Technologies). The images were captured using a Hamamatsu ORCA100 digital camera and SimplePCI 5.2 software (C-imaging). Quantitation of intensity was done using SimplePCI “Mass” (intensity × area) measurement for the nucleus divided by the Mass measurement of the total cell. The cells were imaged at the lower limit of detection after transfection, typically 14–18 h. The results presented are typical of 200 cells observed for each construct accumulated over at least three independent experiments. The statistical significance of results was determined using a Student’s t test. The images were captured “self-blinded” using cotransfection of mRFP expression plasmid, as described elsewhere (23). Briefly, the cells were only visualized by red channel fluorescence (mRFP) at the time of image capture to identify only healthy transfected cells, without observing green fluorescence. The images were then captured in both red and green channels. Green (EGFP) channel data were only measured after cells were blindly observed and imaged.

Live Cell iFRAP Shuttling Assays—The qualitative live cell iFRAP shuttle assays were performed essentially as described elsewhere (22) on a Zeiss LSM510 confocal microscope. iFRAP experiments of NES mutant ataxin-7 in single cells were done 18 h post-transfection in NIH3T3 cells in the presence of cycloheximide. For full-length ataxin-7 export measurements in single cells, the photo-multiplier detector settings were set to maximum to detect cytoplasmic fluorescence. Subsequently, the nucleus was not measured for fluorescence loss, because >80% of pixels in the nucleus were at or above maximum intensity. Only cytoplasmic recovery could be accurately measured. Quantitation of recovery was done on raw data TIFF images exported from LSM 510 5.0 software. Mean pixel intensity levels and area measurements were conducted with NIH Image J software.

Immunocytochemistry Studies with Transiently Transfected Cerebellar Granule Cell Neurons—Neurons were cultured from 7-day-old mice as described previously (24). For purification and coculture of identified cell populations, briefly, the cerebella were dissected from 7-day-old mice and minced in cold Hanks’ buffered saline. Tissue was trypsinized for 25 min at 37°C and then dissociated by titration with a P1000 pipette tip. The cells were counted and plated at a density of 3 × 10^6 cells/ml in basal medium (Invitrogen) with 10% fetal bovine serum, 2 mM glutamine, 25 mM KCl, 9.5 mM glucose, and 100 units/ml penicillin/streptomycin. Twenty-four hours after plating, cytosine arabinoside was added.
This resulted in a culture of 95% granule cell neurons. After 5 days in culture, the neurons were transfected using Lipofectamine 2000 (Invitrogen). The cells were fixed at 24 h post-transfection and stained with a polyclonal antibody for active caspase-3 (1:200; Cell Signaling) and monoclonal mitogen-activated protein 2 antibody (1:500; Chemicon). The nuclei were visualized with Hoechst 33258 dye (4 μg/ml). The images were captured at 40X with an Axiovert 200M microscope (Zeiss). Toxicity was determined by looking at nuclear morphology, caspase-3 activity, and loss of Map2-positive dendrites. All of the plasmids assayed were designated with a coded name only, and the assays were performed blinded.

RESULTS

Subcellular Localization in Live Cells—In mouse model studies and human brains, ataxin-7 is seen in both the nucleus and cytoplasm (15, 16), suggesting that this protein may reside in either compartment or dynamically traffic between the two compartments. In cell culture models, ataxin-7 is predominantly nuclear (7, 25). To examine whether there might be additional nuclear transport signals to those previously described (7, 8), we expressed human wild-type (Q10) and polyglutamine-expanded mutant (Q64) ataxin-7 and fragments as EGFP fusion proteins and observed them in live NIH 3T3 cells (Fig. 1A). As seen in Fig. 1B, both wild-type and mutant ataxin-7 localized primarily to the nucleus (panels a and b), as did a carboxyl-terminal fragment containing two basic stretches of residues, previously shown to be NLSs by fixed cell immunofluorescence (8) (panel g). This indicated that EGFP carboxyl-terminal fusions were not disrupting the normal nuclear localization of ataxin-7. Expansion of polyglutamine from 10 to 64 repeats did not affect the subcellular localization of ataxin-7 but did induce the presence of nuclear inclusions. An amino-terminal truncation of ataxin-7 (1–619), and the central region of ataxin-7 (228–619) (Fig. 1, panels d–f), both displayed weak nuclear localization despite the presence of an NLS in these fragments (7). Thus, ataxin-7 could have either a cytoplasmic retention signal or an NES within a fragment of 228–619 amino acids.

Ataxin-7 Is Capable of Nucleocytoplasmic Trafficking—Many proteins that contain nuclear localization signals also rely on nuclear export signals for their biological function (26). To assess whether ataxin-7 localized statically to both the nucleus and cytoplasm or whether ataxin-7 shuttled dynamically between the two compartments, we used...
a live cell nucleocytoplasmic transport assay by iFRAP analysis (22). In this assay, a nuclear protein is fused to EGFP and expressed in polyethylene glycol-fused cells to generate binucleate cells or bikaryons (Fig. 2, panel a). The cells are treated with cycloheximide to prevent new protein synthesis, and the entire area of the bikaryon except one nucleus (donor) is photobleached by a 488-nm laser to irreversibly destroy the fluorescence of those molecules (Fig. 2, panel c). The images are then successively captured at timed intervals to detect fluorescence recovery into the bleached nucleus (acceptor) (panels d–f). Recovery into the acceptor nucleus indicates active export of ataxin-7 from the donor nucleus and active import into the acceptor nucleus, thus nuclear shuttling. From this assay, we could detect ataxin-7 shuttling as early as 10 min post-bleaching (Fig. 2, panel e), a similar time scale as seen with a known shuttling protein, the mRNA processing factor hnRNPA1 (22).

In these assays, both wild-type (Fig. 2) and polyglutamine-expanded mutant (Q64) ataxin-7 (data not shown) were observed to dynamically shuttle. From this assay, we could detect ataxin-7 shuttling as early as 10 min post-bleaching (Fig. 2, panel e), indicating export of ataxin-7 from the unbleached donor nucleus and import to the acceptor nucleus. The scale bar indicates 10 μm.

FIGURE 2. Ataxin-7 can dynamically traffic in and out of the nucleus. A live cell iFRAP nuclear transport assay in NIH 3T3 cells with wild-type (Q10) EGFP-ataxin-7 expressed in a bikaryon is shown. Panel a, differential interference contrast (DIC); panel b, prebleach fluorescence; panel c, photobleached area of EGFP-ataxin-7 bikaryon. The bleached area is defined by the white dashed line. Panels d–f, bleach recovery post-bleaching at 0 (panel d), 10 (panel e), and 20 min (panel f). Recovery is seen in the acceptor, or bleached nucleus, starting at 10 min (panel e), indicating export of ataxin-7 from the unbleached donor nucleus and import to the acceptor nucleus. The scale bar indicates 10 μm.

The Ataxin-7 NES Is Highly Conserved—We detected ataxin-7 export from the nucleus by both the iFRAP shuttle assay and by leptomycin B treatments. Therefore, we wanted to know whether this nuclear export was being mediated directly by signal sequences in ataxin-7 or potentially by another associated protein. We compared the leucine/methionine-valine-rich region to see whether any sequence existed that could fit into a loose NES consensus (27, 28). As seen in Fig. 3, nuclear accumulation of ataxin-7 fragments, in either the wild-type or polyglutamine-expanded mutant was seen after 20 h of 5 ng/ml leptomycin B treatment (Fig. 3, panels c, e, and g versus panels d, f, and h). The same time frame and leptomycin B concentration was required to see nuclear accumulation of IκBα (panels a and b). Therefore, ataxin-7 uses the CRM-1/exportin pathway to exit the nucleus.

FIGURE 3. Ataxin-7 exports from the nucleus via the CRM-1/exportin pathway. Leptomycin B export inhibition assays of EGFP-ataxin-7 fragments in NIH 3T3 cells are shown. Panels a and b, control GFP-IκB before and after 5 ng/ml leptomycin B treatment. Panels c and d, ataxin-7 (Q10) 1–619 nuclear accumulation before and after leptomycin B treatment. Panels e and f, ataxin-7 (Q64) nuclear accumulation before and after leptomycin B treatment. Panels g and h, ataxin-7 228–619 nuclear accumulation before and after leptomycin B treatment. The images are representative of 200 cells; each construct was observed over three independent experiments. The scale bar indicates 10 μm.

Nuclear Shuttling of a STAGA Complex Member

Ataxin-7 Exports from the Nucleus by the CRM-1/Exportin Pathway—To determine how ataxin-7 was being exported from the nucleus, we visually examined the ataxin-7 amino acid sequence, comparing the sequence to a loose consensus sequence determined for a leucine-type NES (27) and a data base of known NESs (28). We noted the presence of a hydrophobic residue-rich sequence within the defined conserved block II of ataxin-7. Classical leucine-rich nuclear export signals are often seen to export from the nucleus by the CRM-1/exportin pathway (21). The CRM-1/exportin-specific inhibitory compound, leptomycin B (20), was used to treat ataxin-7 fragment expressing cells to see whether these fragments would shift in localization toward the nucleus, because of inhibition of export in the same time frame as a known exportin-dependent NES-containing protein, IκBα (29). As seen in Fig. 3, nuclear accumulation of ataxin-7 fragments, in either the wild-type or polyglutamine-expanded mutant was seen after 20 h of 5 ng/ml leptomycin B treatment (Fig. 3, panels c, e, and g versus panels d, f, and h). The same time frame and leptomycin B concentration was required to see nuclear accumulation of IκBα (panels a and b). Therefore, ataxin-7 uses the CRM-1/exportin pathway to exit the nucleus.
An NES Point Mutant of Ataxin-7 Cannot Export from the Nucleus—
To assess the NES signal in ataxin-7 in live cells, we performed the iFRAP nuclear shuttle assay on the 1–619 fragment of ataxin-7 with or without the NES mutation, with photo-multiplier detector levels set high to be able to measure fluorescence intensity in both the nucleus and cytoplasm. As with Fig. 5, we could see EGFP-ataxin-7 1–619 in both the nucleus and cytoplasm. With either the wild-type fragment (Fig. 6A, panels a–g) or the NES mutant in the same fragment context (panels h–n), we selectively and precisely photo-bleached the entire cytoplasm of the cells (panels a and h) and then measured recovery of fluorescence of the EGFP-ataxin-7 1–619 fragment from the nucleus into the cytoplasm. With ataxin-7 1–619, recovery in the cytoplasm could be seen as soon as 60 s post-bleaching (Fig. 6A, panel c), whereas ataxin-7 1–619 NES mutant was not seen to recover in the cytoplasm (Fig. 6A, panels i–n), even when recovery measurements were extended to 500 s (data not shown). Therefore, the export dynamics of ataxin-7 could be inhibited by point mutation of the critical hydrophobic amino acids of the NES. The presence of EGFP-ataxin-7 NES mutant in the cytoplasm at the start of these assays is likely due to weak-NLS activity in this fragment of the protein, because it lacks two NLSs of the intact full-length protein. A bikaryon shuttle assay with full-length NES mutant ataxin-7 was not possible in our system because of the significant increase of toxicity of this NES mutant protein to several tissue culture cell lines tested. Instead, we turned up the detector gains to maximum and performed iFRAP assays measuring recovery in the cytoplasm only, because nuclear fluorescence levels had exceeded maximum pixel intensity, and therefore loss could not be measured. By these assays, we could see export of full-length wild-type ataxin-7 (Fig. 6D, panels a–f), but not NES mutant full-length ataxin-7 (panels g–l), indicating that the NES we had identified was the only one in ataxin-7. We then compared wild-type full-length ataxin-7 export (panels a–f) to polyglutamine-expanded ataxin-7 (Q64) export (panels m–r) and found that although polyglutamine-expanded ataxin-7 could still export from the nucleus, the amount seen to recover was less than wild-type protein (Fig. 6E). Thus, the polyglutamine expansion in mutant ataxin-7 can partially inhibit ataxin-7 nuclear export.

Mutation of the Ataxin-7 NES Increases Toxicity of Polyglutamine-
expanded Ataxin-7—To determine whether the nuclear export activity we discovered with ataxin-7 could impact ataxin-7 toxicity in neurons, we assayed aggregate frequency and neuronal death in primary cerebellar granule neurons with ataxin-7 and ataxin-7 NES mutants in both wild-type and polyglutamine-expanded contexts (Fig. 7). In the context of 1–619 ataxin-7, mutation of the NES was seen to have a significant effect on the increased formation of polyglutamine aggregates (Fig. 7A). In the full-length polyglutamine-expanded ataxin-7 context, however, NES mutation decreased polyglutamine aggregate frequency. In primary cerebellar neuron cell death assays measured by the presence of activated caspase-3 in the nucleus, no effect of NES mutation was seen upon the (Q64)1–619 fragment context, missing its two NLSs, whereas a significant increase in toxicity was noted for NES mutant, full-length, polyglutamine-expanded (Q64) ataxin-7. No significant effect upon toxicity was noted for the NES mutant, nonexpanded full-length ataxin-7. These results indicate that NES inactivation by point mutation could increase polyglutamine length-dependent ataxin-7 toxicity for full-length protein with all of the NLS signals present. In addition, an inverse correlation between aggregate formation and polyglutamine-expanded ataxin-7 toxicity was noted, as has been seen similarly by others in a SCA7 mouse model (30).
DISCUSSION

SCA7 is unique among the polyglutamine expansion diseases in that SCA7 patients undergo cone-rod dystrophy retinal degeneration that eventually leads to blindness (5, 31). Studies in SCA7 knock-in and SCA7 transgenic mice reveal a comparable retinal degeneration (11, 16), and one study found that this degeneration is due to the Crx homeodomain transcription factor interference resulting in the down-regulation of a set of retinal-specific genes (8, 16). Biochemical data have shown that ataxin-7 is a member of a GCN5 histone acetyltransferase-containing complex known as the STAGA coactivator complex (32), where ataxin-7 directly interacts with GCN5 (9, 10). In yeast S. cerevisiae, the ataxin-7 homolog, Sgf73, is a member of SAGA and SLIK histone acetyltransferase complexes, and mammalian ataxin-7 can complement the loss of Sgf73 in yeast (13). Ataxin-7 has also been detected in a complex with TATA-binding protein-free transcription-activating factors or the TFTC complex (9, 33). Ataxin-7 interaction with these coactivator complexes likely occurs through a central region of ataxin-7 protein defined as conserved block II, found in a family of ataxin-7 homologs and similar proteins (9). The amino terminus of ataxin-7 can sequester the transcription-activating factor II30 TFIIID complex member in nuclear inclusions (34), and the ataxin-7 amino terminus can coimmunoprecipitate Crx via interaction between the glutamine regions in each of the proteins (8). These interactions make an interesting indirect connection between ataxin-7 and the TATA-box binding protein, the polyglutamine disease protein in SCA17 (3). Ataxin-7 mediates the direct interaction of Crx with GCN5/STAGA to form the bridge between an upstream activator protein (Crx) and the RNA polymerase II transcription activation complex (10). Despite the fact that the exact function of ataxin-7 in this complex remains unknown (1), data from SCA7 mouse models and biochemical protein-protein interaction data imply a role for ataxin-7 in transcription control, having interactions with both a transcription activator (Crx) and a coactivator complex, but on different regions of the ataxin-7 protein. Some transcription regulatory factors function by trafficking between the nucleus and the cytoplasm as signal transducers (35), with a well described example being the IκBα regulator of NFκB transcription (29, 36). Although ataxin-7 appears to be a completely nuclear protein in cell culture models, in SCA7 brains and mouse models (15, 16), ataxin-7 is seen in both the nucleus and cytoplasm. The differential nuclear/cytoplasmic localization of ataxin-7 in vivo and association with transcription coactivators and the GCN5 histone acetyltransferase in vitro led us to examine whether ataxin-7 had the potential to export from the nucleus. The amino-terminal 1–230 fragment of ataxin-7, containing the polyglutamine expansion, appeared to be predominantly nuclear, despite a lack of any classical basic-type NLS sequence. We have found that a EGFP-mRFP fusion protein at 52 kDa can still diffuse across the nuclear pore (data not shown).
FIGURE 6. A site-directed NES mutant of ataxin-7 is defective for nuclear export. A, live cell quantitative iFRAP nuclear assay in cells expressing wild-type EGFP-ataxin-7 1–619 and 1–619 NES mutant. The bleached areas are indicated by white dashed lines. B, nuclear loss of wild-type ataxin-7 1–619 (black box) but not NES mutant ataxin-7 1–619 (white box) is seen over 360 s post-bleaching. C, cytoplasmic recovery of wild-type but not NES mutant ataxin-7 1–619. Raw data were measured for pixel intensity, normalized, and plotted over time.
shown). Given the size of this fusion protein at 51 kDa, the nuclear localization of EGFP-1–230 is likely explained by diffusion into the nucleus and affinity of this ataxin-7 fragment for nuclear factors. Our analysis of the ataxin-7 amino acid sequence yielded identification of a typical, leucine-rich, CRM-1/exportin-dependent NES. Such signals have been seen in a wide variety of proteins that modulate transcription activity (26).

In the context of polyglutamine diseases, ataxin-7 now joins a list of polyglutamine disease proteins that have a nuclear export signal and shuttle to and from the nucleus that includes: the spinobulbar muscle atrophy disease protein, the androgen receptor (37); the dentatorubral pallidoluysian atrophy protein, atrophin-1 (38); the SCA1 protein, ataxin-1 (39); the Huntington disease protein, huntingtin (40, 41); likely the ataxin-2 protein (42); and the TATA box-binding protein, because some transcription-activating factors are known to export from the nucleus (43). For huntingtin, androgen receptor, and atrophin-1, proteolysis is predicted to affect nuclear export of the proteins and lead to nuclear accumulation of toxic fragments (38, 40, 44). This is not likely the case for ataxin-7, because fragments used here without all of the nuclear localization signals intact did not increase in toxicity in primary neurons. Ataxin-7 is known to interact with the STAGA complex (9), specifically and directly through GCN5 (10). The GCN5 STAGA complex member can modulate nuclear export of the steroidogenic transcription factor, SF1, by SF1 acetylation (45). A TFTC complex protein is TRRAP (33), and a TRRAP-binding protein, BAF53, is known to shuttle to and from the nucleus (46). In yeast S. cerevisiae, the ataxin-7 homolog, Sgf73, is a SAGA/SLIK complex member and can be functionally interchanged with the human protein (13). Although Sgf73 and human ataxin-7 only have 21% sequence identity overall, in a 50-amino acid region spanning the NES, homology increases to 50% (13), with a

with mean and standard errors across three experiments. The scale bar indicates 10 μm. D, iFRAP assays with wild-type and NES mutant full-length ataxin-7, compared with full-length polyglutamine-expanded (Q64) ataxin-7. E, NES mutant ataxin-7 fails to export, polyglutamine-expanded ataxin-7 can still export, but less recovery is seen relative to wild-type protein. The means and standard errors from five experiments were plotted.

FIGURE 7. Mutational inactivation of the ataxin-7 NES affects aggregation frequency and cell toxicity in primary neurons. The results of 36 h of expression of ataxin-7 moieties as EGFP fusions in primary granule cerebellar neurons. Toxicity was measured by caspase-3 detection in nuclei by immunofluorescence in cells expressing EGFP signal. A, mutational inactivation of the ataxin-7 NES increases aggregate frequency in a polyglutamine-dependent manner in 1–169 (Q64) context, but not in full-length ataxin-7 (Q64) context. B, mutational inactivation of ataxin-7 NES increases toxicity of only full-length, polyglutamine-expanded ataxin-7 (Q64). An inverse correlation between aggregate formation frequency and cell toxicity was observed. *, p value < 0.01. C, primary cerebellar neuron toxicity assay results (negative, panels a–d; positive, panels e–h) with anti-caspase-3 antibody and EGFP moieties. The scale bar indicates 10 μm.
potential NES with HIV-1 Rev-like leucine spacing present in Sgf73. It is possible that ataxin-7 may traffic to and from the nucleus with a STAGA member to regulate STAGA complex function, and this activity may be conserved evolutionarily through to yeast species as a member of SAGA/Slik.

The location of the NES in ataxin-7 at 344–352 is intriguing in that it overlaps a region of sequence similarity with the phosphoprotein-binding domain of β-arrestin proteins (47). Several of these G-protein-coupled receptor-binding proteins have been found to have nuclear shuttling activity (48, 49), suggesting that ataxin-7 may have a cytoplasmic role analogous to a nuclear signaling β-arrestin. Binding of a phosphoprotein to this domain in ataxin-7 may sterically hinder the NES or one NLS, modulating ataxin-7 nuclear levels. Alternatively, the NES in ataxin-7 may normally function to control total levels of ataxin-7- or associated proteins by facilitating degradation of a target protein by the cytosolic proteasome (50), in a manner similar to adenomatous polyposis coli/β-catenin regulation (51). Although no obvious effect on ataxin-7 stability could be seen with the NES mutation, this does not exclude the possibility that the levels of an ataxin-7-exposed protein may be altered by altered nuclear export of mutant ataxin-7.

We observed that nuclear export inhibition of ataxin-7 can modulate ataxin-7 toxicity, but only in a polyglutamine length-dependent manner and only in ataxin-7 constructs containing all of the NLS signals. This is consistent with the concept that NES inactivation in ataxin-7 would be toxic, but only if the rest of the protein had full nuclear import capability. In contrast, an inverse correlation in these assays could be seen between aggregate formation and toxicity. Similar observations were recently noted by others in an ataxin-7 knock-in mouse model that recapitulates several features of SCA7 (30). One explanation for this would be that aggregated protein trapped in nuclear inclusions may not function in the dynamic properties of ataxin-7 and hence cannot confer the toxic gain-of-function related to the normal ataxin-7 activity of nuclear shuttling.

We demonstrated that polyglutamine expansion in ataxin-7 could slow but not stop nuclear export dynamics of ataxin-7, consistent with similar FRAP experiments carried out on other polyglutamine disease proteins (52–54) but different from the block of nuclear export observed for huntingtin, polyglutamine aggregation can be either dynamic properties of ataxin-7 and hence cannot confer the toxic gain-of-function related to the normal ataxin-7 activity of nuclear shuttling. It is likely that the NES inactivation in ataxin-7 would be toxic, but only if the rest of the protein had full nuclear import capability. In contrast, an inverse correlation in these assays could be seen between aggregate formation and toxicity. Similar observations were recently noted by others in an ataxin-7 knock-in mouse model that recapitulates several features of SCA7 (30). One explanation for this would be that aggregated protein trapped in nuclear inclusions may not function in the dynamic properties of ataxin-7 and hence cannot confer the toxic gain-of-function related to the normal ataxin-7 activity of nuclear shuttling.

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Nuclear Shuttling of a STAGA Complex Member

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