Stabilization and Degradation Mechanisms of Cytoplasmic Ataxin-1

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ABSTRACT: Aggregation-prone proteins in neurodegenerative disease disrupt cellular protein stabilization and degradation pathways. The neurodegenerative disease spinocerebellar ataxia type 1 (SCA1) is caused by a coding polyglutamine expansion in the Ataxin-1 gene (ATXN1), which gives rise to the aggregation-prone mutant form of ATXN1 protein. Cerebellar Purkinje neurons, preferentially vulnerable in SCA1, produce ATXN1 protein in both cytoplasmic and nuclear compartments. Cytoplasmic stabilization of ATXN1 by phosphorylation and 14-3-3-mediated mechanisms ultimately drive translocation of the protein to the nucleus where aggregation may occur. However, experimental inhibition of phosphorylation and 14-3-3 binding results in rapid degradation of ATXN1, thus preventing nuclear translocation and cellular toxicity. The exact mechanism of cytoplasmic ATXN1 degradation is currently unknown; further investigation of degradation may provide future therapeutic targets. This review examines the present understanding of cytoplasmic ATXN1 stabilization and potential degradation mechanisms during normal and pathogenic states.

KEYWORDS: Ataxin-1, spinocerebellar ataxia type 1, 14-3-3, autophagy, ubiquitin proteasome system, mTOR, nuclear inclusions, aggregation

Introduction
Spinocerebellar ataxia type 1 (SCA1) is a fatal progressive neurodegenerative disease that preferentially affects the Purkinje cells of the cerebellum. SCA1 is caused by an expansion in the polyglutamine tract of the ataxin-1 gene, ATXN1. The known function of ATXN1 protein is as a regulatory binding partner in gene transcription and RNA splicing, indicating a predominantly nuclear role. However in Purkinje neurons, ATXN1 is uniquely cytoplasmic and nuclear. Phosphorylation by a cytoplasmic kinase stabilizes ATXN1 through 14-3-3 binding, a mechanism that ultimately drives the translocation of ATXN1 into the nucleus. Disruption of either phosphorylation or 14-3-3 binding leads to rapid degradation of ATXN1 within the cytoplasm and thus prevents nuclear translocation. Effectively, the polyglutamine expansion shifts ATXN1 from a normal state of degradation and bidirectional translocation to a state favoring stabilization and nuclear localization. Once in the nucleus, expanded ATXN1 remains nuclear, interacts with both native and novel complexes, and may aggregate. Thus, degradation mechanisms are a key factor in understanding the role that aggregation-prone proteins play in disease. This review outlines the potential mechanisms of cytoplasmic ATXN1 degradation that ultimately determine its normal and pathological function.

14-3-3 Stabilization in SCA1
14-3-3 characterizes a family of highly conserved, ubiquitously expressed eukaryotic proteins that constitute 1% of all soluble protein in the brain and are involved in numerous biological processes, including neuronal development, signal transduction, vesicular transport, apoptosis, and cell cycle control. 14-3-3 proteins contain a conserved amphipathic groove that mediates ligand binding at phosphoserine residues, modulating ligand stability and acting as a molecular chaperone.

Importantly, 14-3-3 mediates ATXN1 phosphorylation and localization. ATXN1 homologs across species share the canonical 14-3-3-binding motif RXXXXP, which flanks the critical phosphorylation-dependent serine residue (Ser776 of the nonexpanded human ATXN1 protein). The affinity of 14-3-3 binding for aggregation-prone proteins including expanded ATXN1 promotes phosphorylation, stabilization, and aggregation.
14-3-3ε+/− haploinsufficiency in an SCA1 mouse model (Sca1<sup>154Q-knock-in</sup>) decreases cerebellar ATXN1 levels and leads to a rescue of motor skills and Purkinje cell degeneration. Notably, lethality of haploinsufficient SCA1 mice is unaffected, indicating that ATXN1 expression in noncerebellar regions such as the brainstem may be mediated by differing 14-3-3 isoforms.20,21

### Autophagic Degradation in SCA1

Macroautophagy, a lysosomal degradation pathway in which proteins flagged for degradation are delivered to the lysosome via fusion to a double-membraned vesicle, the autophagosome, has been implicated as dysfunctional in multiple polyglutamine expansion diseases, including SCA1.22-24 Specifically in SCA1, the Purkinje neurons of transgenic mice overexpressing ATXN1 housing 82 polyglutamine residues (ATXN1-82Q) display cytoplasmic vacuoles in concurrence with decreased dendritic spine density prior to the onset of behavioral symptoms.24 The vacuoles stain positive for microtubule–associated protein light chain 3 (LC3), a marker of autophagosomes, and myo-inositol monophosphatase 1 (IMPA1), a mediator of lithium-induced autophagy.24,25 Moreover, cerebellar fractions from the transgenic mice showed significant conversion of the 18 kDa form of LC3 (LC3-I) to the 16 kDa form (LC3-II), indicative of autophagic flux.24 In a cellular model, treatment of COS-7 cells overexpressing ATXN1-97Q with the autophagy activator rapamycin decreased soluble ATXN1 levels, reduced total aggregation, and diminished toxicity, as measured by apoptotic nuclear morphology.22

In the latter study, rapamycin treatment of a Drosophila model overproducing a 108Q free polyglutamine peptide in neuronal cells reduced neuronal toxicity and increased juvenile survival rates in the flies.22 While based on the previous accounts it may appear that autophagic stimulation is a viable strategy for reducing polyglutamine-expanded ATXN1 toxicity, the experimental results remain more complex. Nuclear localization of aggregated ATXN1, in contrast to the cytoplasmic-free peptide, may make autophagic augmentation strategies ineffective.22 A study by Iwata et al (2005) demonstrated colocalization of autophagic markers with polyglutaminic, but not nuclear, ATXN1-85Q inclusions in HeLa cells that had been transfected with either a wild-type ATXN1-85Q construct or one harboring a point mutation in the nuclear localization sequence thus preventing nuclear entry.27 Additionally, knockdown of autophagic genes led to significantly increased aggregation of cytoplasmic, but not nuclear, ATXN1-85Q.27 The finding that nuclear aggregates cannot readily be cleared by autophagy supports the notion of the nucleus as a toxic environment, in which pathogenic proteins may be shielded from degradation.

### Ubiquitin-Proteasome System Degradation in SCA1

The ubiquitin–proteasome system (UPS), a highly regulated degradation pathway, breaks down the majority of short-lived nuclear and cytosolic proteins by first tagging substrates with polyubiquitin recognition chains. The ubiquitination then targets the proteins for degradation by the proteasome.28 Ubiquitinated substrates and proteasomal fragments are detected in the nuclear inclusions of SCA1 patients and SCA1 transgenic mice models.22,23 Additionally, ATXN1 may serve as a UPS substrate; early work demonstrates an interaction between ATXN1 and the protein A1Up, an ataxin-1 interacting protein, which contains a N-terminal ubiquitin-like region, an ubiquitin–associated domain, and a heat shock chaperonin-binding motif. A1Up is highly expressed in Purkinje neurons29 and interacts with the 19S proteasome. Although the interaction between the A1Up and the 19S proteasome is disrupted in the presence of mutant ATXN1, A1Up promotes the polyubiquitination of substrates in the presence of mutant ATXN1 and colocalizes with mutant ATXN1 nuclear inclusions.30 These findings may be evidence of mutant ATXN1 directly driving UPS dysfunction and aberrant ubiquitination, a pathological hallmark of SCA1. In vitro studies reveal that the UPS conjugates ubiquitin to normal and mutant ATXN1 similarly, but mutant ATXN1 is as much as three times more resistant to UPS degradation,31 implicating the polyglutamine expansion as the root of this resistance. As would be predicted, polyglutamine-expanded ATXN1 adopts multiple energetically favorable three-dimensional conformations, making it resistant to unfolding and hydrolysis.31

Inhibition of the UPS by beta lactone–lactacystin in HeLa cells transiently transfected with GFP-ATXN1-82Q promoted aggregation into detergent-insoluble inclusions without altering soluble ATXN1 levels.31 In order to further investigate the effects of UPS inhibition, a single component of the UPS, the ubiquitin-conjugating E3 ligase E6-AP, was characterized for its involvement in mutant ATXN1 degradation. Specifically, mice lacking the E6-AP gene, <i>Ube3a</i>−/− were bred to SCA1 transgenic mice. The progeny displayed delayed inclusion formation, fewer nuclear aggregates, and advanced SCA1 pathology.31 The somewhat paradoxical results obtained by inhibiting the UPS pharmacologically with beta lactone–lactacystin versus deletion of the <i>Ube3a</i> gene suggest that the UPS plays a complex role in the inclusion formation and degradation of polyglutamine-expanded ATXN1. Conceivably, multiple factors including the type of cell, the subcellular localization of the protein, the aggregation state of the protein, and the size of the expansion may affect whether the UPS degrades the substrate or facilitates folding of the substrate into an inclusion. Additionally, the above mentioned results provide evidence that aggregation occurs independently of or perhaps even opposing of toxicity, indicating that facilitation of aggregation by the UPS may be a valid preventive measure for ameliorating cellular toxicity.

Of relevance, of course, is that SCA1 transgenic mice present with both Purkinje cell nuclear inclusions and Purkinje cell toxicity. One of the earliest markers of pathology, occurring by P25, is the presence of cytoplasmic vacuoles32,33 noted in the previous section as containing components of the autophagy system. In addition, cytoplasmic vacuoles in...
Purkinje neurons of SCA1 mice have been noted to stain intensely for ubiquitin and the 20S proteasome by 5 weeks of age.\textsuperscript{14} The consequence of UPS components in cytoplasmic vacuoles remains unclear. Potentially, the UPS remains active, degrading proteins trapped in the vacuoles. Alternatively, the vacuoles serve to sequester UPS components, rendering the UPS system in the cell less effective. A recent review of autophagy in polyglutamine disease, which thoroughly examines multiple polyglutamine expansion proteins beyond the scope of the present ATXN1 review, provides insight into the relationship between the UPS and autophagy.\textsuperscript{26} The authors propose that UPS dysfunction, perhaps through the quarantine of UPS components by cytoplasmic vacuoles, ultimately upregulates autophagy. This increased autophagic activity may continue to degrade cytoplasmic protein effectively but would be unable to degrade nuclear protein, thus having major implications for diseases with nuclear pathogenesis such as SCA1. To further expand on this model, expression, folding, and aggregation of ATXN1 over time in a cell will shut off UPS function gradually. In the meantime, available functional UPS might contribute to the early formation of nuclear inclusions seen in SCA1 mice models. The question of whether the inclusions are protective or pathogenic still persists, and the answer might be that they are both. Early inclusions may be a protective mechanism to inactivate nondegradable, pathogenic aggregation-prone protein. Late-stage inclusions may ultimately overwhelm the cell, particularly in the nucleus where they may obstruct normal transcription and splicing function and further fuel disease pathogenesis.

**Mammalian Target of Rapamycin Pathway: The Missing Link**

A potential molecular link connecting 14-3-3, autophagy, and the UPS is the mammalian target of rapamycin (mTOR) pathway. mTOR is a serine/threonine kinase, which regulates the detection of cellular stress and nutrient levels, as well as autophagic activity.\textsuperscript{35,36} 14-3-3 activates and inhibits autophagy by binding to various mTOR regulatory proteins. For example, under normal growth conditions, 14-3-3 complexes with mTOR to activate downstream factors such as tuberous sclerosis protein 2 and proline-rich Akt substrate 40, ultimately inhibiting autophagy.\textsuperscript{37} During starvation conditions, 14-3-3 dissociates from these complexes, rendering mTOR inactive, thus activating autophagy.\textsuperscript{37} The mTOR inhibitor human ortholog of yeast vacuolar protein sorting 34 (hVps34) is a lipid kinase required for autophagy initiation and is deactivated when bound by the 14-3-3ζ isoform.\textsuperscript{38} The dissociation of 14-3-3ζ from hVps34 results in hVps34 activation and subsequent autophagosome formation.\textsuperscript{38} 14-3-3 also binds to suppressors of mTOR including Unc-51-like kinase 1/2 (ULK1/2) and the mTOR-complex I protein raptor during starvation conditions, demonstrating that 14-3-3 plays a complex role in autophagy activation.\textsuperscript{37}

The UPS also regulates the mTOR pathway. A recently discovered endogenous inhibitor of mTOR, DEPTOR, is a relatively unstable protein that is rapidly degraded in the presence of growth factors.\textsuperscript{39} mTOR self-activates via a signaling cascade that results in the degradation of DEPTOR by the UPS.\textsuperscript{40,41} Inhibition of the UPS blocks autophagy and the resultant DEPTOR accumulation inhibits mTOR, activating autophagy.

Polyglutamine expansion proteins that reside for a considerable time in the cytoplasm such as Huntington, ataxin-3, and ataxin-7 have been reported as mTOR-mediated autophagic substrates, directly interacting with mTOR-mediated autophagic pathway proteins.\textsuperscript{26,42} Furthermore, cytoplasmic inclusions of mutant Huntington protein sequester mTOR and other autophagic inhibitors, activating autophagy and reducing cellular toxicity.\textsuperscript{43} Surprisingly, nuclear inclusions of ATXN1 were also found to sequester mTOR and its target substrate, phosphorylated S6 protein.\textsuperscript{43} The results, obtained via transient transfection into COS7 cells, indicate that early inclusion formation may be a dual protective strategy. The first aim of this strategy may inactivate nuclear pathogenic protein through folding and aggregation, and the second aim may degrade cytoplasmic pathogenic protein via sequestration of mTOR and subsequent activation of autophagy.

**Neuronal Toxicity Without Aggregation in Human Purkinje Neurons**

The link between inclusions and toxicity remains a major paradox, particularly in the environment of Purkinje neurons. The Purkinje neurons of SCA1 mice models display ATXN1-positive nuclear inclusions via two means: 1) early disease stage formation if ATXN1-82Q is overexpressed in the transgenic mouse model\textsuperscript{42} and 2) late disease stage formation if ATXN1-154Q is expressed at endogenous levels in the knock-in model.\textsuperscript{44} However, to date, nuclear inclusions have not been detectable in Purkinje neurons from human SCA1 patient postmortem tissue, despite the enhanced vulnerability of Purkinje neurons (compared to other cerebellar cell types and other regions) in the human disease.\textsuperscript{45} Expression levels and the size of the polyglutamine expansion, which is typically considerably smaller in the human disease than in either mouse model, likely contribute to the above differences. However, nuclear inclusions are found in human cerebellar Golgi neurons,\textsuperscript{45} dentate nuclei neurons,\textsuperscript{45} and neurons of the nucleus pontis centralis,\textsuperscript{13} indicating that intrinsic factors of Purkinje cells must also play a vital role. Importantly, nuclear inclusions have not been found in the Purkinje cells from human SCA2, SCA3, or DRPLA tissue, despite diffuse accumulations of mutant protein in Purkinje nuclei.\textsuperscript{46}

One explanation is that nuclear inclusions perhaps do form, yet the severe degeneration and loss of Purkinje cells\textsuperscript{46} in human autopsy tissue make detection difficult. However, if this is true, it is expected that some nuclear inclusions would be visible. An alternate explanation is that Purkinje cells may be highly vulnerable to minor perturbations and might undergo enhanced toxicity prior to the onset of nuclear inclusion formation.
Perhaps, a soluble form of ATXN1 is primarily responsible for cellular toxicity. Data from the SCA1 knock-in 154Q mouse model show that oligomeric species of mutant ATXN1 protein, conformationally similar to oligomeric species that are detectable in Alzheimer's and Parkinson's disease, propagate in mouse cerebellar Purkinje neurons in a pattern that follows disease progression.\(^47\) Moreover, ATXN1 oligomers mediate toxicity via interactions with the nuclear transcription factor, Capicua.\(^47\) Clearance of the oligomers through passive immunization therapy improves behavior and extends lifespan to a modest degree.\(^48\) Further research is needed to discern the extent of toxicity of ATXN1 oligomers versus nuclear inclusions. Molecular and genetic differences that account for the lack of nuclear inclusion formation in human Purkinje neurons versus human non-Purkinje neurons and mouse Purkinje neurons is also deserving of greater investigation.

A noteworthy point that should be addressed is the mechanism of toxicity and cell death in Purkinje cells. Medium spiny neurons from patients with Huntington's disease show signs of apoptosis,\(^49\) and cell culture studies have determined that transient transfection and expression of mutant Huntington protein in the nucleus of specific cell types induces apoptosis.\(^50\) Interestingly, apoptosis in transfected cells does not correlate with the presence of nuclear inclusions of Huntington protein, another indicator that the nuclear inclusions may initially be protective. Apoptosis, however, does not appear to be the mechanism of Purkinje neuron-mediated toxicity. In a study of a co-cultured rat cerebellar Purkinje and granule neurons, it appears that granule neurons are susceptible to apoptosis but Purkinje neurons are not.\(^51\) Rather Purkinje neuron toxicity, induced by the removal of growth factors from the culture medium, is a result of increased accumulation of autophagic granules.\(^51,52\) Treatment of Purkinje neurons with exogenous insulin-like growth factor reduces cell death and increases the turnover rate of autophagosomes.\(^52\) More research is needed to determine whether the regulation of autophagy in Purkinje cells by growth factors is mediated via the mTOR pathway, as might be predicted\(^53\) and how accumulation of aggregation-prone protein might affect autophagosome turnover.

Model of Potential Autophagy and UPS Degradation Pathways in SCA1

Based on the experimental observations described previously, we have assembled a model that depicts the potential chemical alterations to ATXN1 protein during early-, mid-, and late-stage SCA1 and postulated how those chemical alterations affect autophagy, UPS, mTOR signaling, and ATXN1 nuclear interactions (Fig. 1). For simplicity, only mutant ATXN1 is shown (purple stars). During early disease stage, soluble mutant ATXN1 may fluctuate between two different states. In the disease null state (Fig. 1, upper left panel), ATXN1 is largely cytoplasmic and dephosphorylated. Some ATXN1 does translocate to the nucleus but remains mainly soluble. Autophagic mechanisms are functioning (depicted by the green oval), degrading cytoplasmic ATXN1. UPS mechanisms are functioning (for simplicity, depicted only in the nucleus in green), degrading nuclear ATXN1. In contrast, in the disease prone state (Fig. 1, upper right panel), ATXN1 is phosphorylated (red “P”) at the Ser776 residue and recruits 14-3-3 for stabilization. 14-3-3 may complex with mTOR and inhibit autophagy (depicted by the red oval), allowing for an accumulation of stable ATXN1 in the cytoplasm. In this scenario, nuclear UPS is functional and nuclear ATXN1 remains largely soluble. During early disease stage, wild-type ATXN1 interactions with transcription and spliceosomal binding partners in the nucleus are favored over mutant ATXN1 interactions. The double arrow in the middle of the upper panel illustrates that the disease null and disease prone stages may oscillate in biochemical equilibrium.

During mid-stage disease (Fig. 1, lower left panel), 14-3-3–stabilized, phosphorylated ATXN1 begins to aggregate and enter the nucleus, recruiting mTOR along the way and sequestering it within the nucleus. As a result, autophagy is largely functional and perhaps even pronounced due to the absence of mTOR inhibition. Seeds of aggregated nuclear ATXN1 may eventually reduce or halt nuclear UPS function (depicted in red). Within the nucleus, mutant ATXN1 interactions with binding partners may increase, resulting in a developing gain of function phenotype. During late disease stage (Fig. 1, lower right panel), mTOR is sequestered within ATXN1 nuclear inclusions. Seeds of aggregated ATXN1 in the cytoplasm shut down autophagy function resulting in exacerbated cytotoxicity. ATXN1 nuclear inclusions shut down UPS function, resulting in exacerbated nuclear toxicity. Mutant ATXN1 interactions within the nucleus are favored over wild-type interactions further evolving the gain of function phenotype and initiating a loss of function phenotype due to the diminished role of wild-type ATXN1. Since human Purkinje neurons do not appear to contain nuclear inclusions, they may reach a maximal mid-stage of disease before toxicity yields to neuronal death. This theory would fit with the evidence of increased autophagosomes in Purkinje neurons\(^51,52\) and would account for the lack of nuclear inclusions.\(^45\)

Targeting mTOR-mediated Autophagy as a Form of SCA1 Therapy

At this stage in our understanding of mTOR-mediated autophagy and its potential role in SCA1 pathogenesis, it is unclear whether targeting mTOR can be an effective therapeutic strategy. First, although nuclear interactions of mutant ATXN1 contribute to pathogenesis of SCA1, nuclear interactions of wild-type ATXN1 are important for normal cellular function.\(^4,5,26\) Thus, strategies aimed at autophagic mechanisms need to allow for a chisel rather than a hacksaw approach to augmenting degradation. Second, as stated earlier, therapies aimed at an mTOR-mediated autophagy may be less effective for nuclear proteinopathies including SCA1 compared to cytoplasmic proteinopathies such as Huntington’s disease. Third, in their review on autophagic mechanisms in polyglutamine diseases, Cortes and La Spada (2015) effectively
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explain that successful autophagic-targeting therapy needs to occur in a tissue-specific and stage-specific manner to account for the complexity of autophagic signaling during the progression of normal aging and neurodegenerative disease, as well as for the differential expression of mTOR pathway genes in varying tissues. Additionally, the phenomenon of mosaicism in polyglutamine expansion diseases provides another argument in favor of tissue-specific treatment. Fourth, in treatment of cancerous tumors, rapamycin is believed to block the mTORC1 pathway (complex of mTOR and the proteins mLST8 and raptor), which augments autophagy and increases apoptosis of tumor cells. However, long-term side effects include inhibition of mTORC2 complex assembly (mTOR, mLST8, and rictor), resulting in increased Akt/PKB signaling and enhanced tumor growth. A similarly conflicting study of rapamycin treatment concluded that long-term treatment of rapamycin in mice showed detrimental effects on metabolism early during chronic treatment and beneficial metabolic effects and increased lifespan with prolonged treatment. Certainly, caution is needed before adopting mTOR-mediated autophagic treatment strategies to human neurodegenerative disease.

Conclusions
The determining factors for ATXN1 entry into the nucleus, in both normal and disease states, are stabilization by 14-3-3 and degradation by probable autophagic and/or UPS mechanisms. Given that known normal and pathogenic functions of ATXN1 occur in the nucleus, further study of cytoplasmic stabilization and degradation and their role in facilitation of ATXN1 nuclear transport is vital.

Further experimental analysis is needed to discern the involvement of autophagy and the UPS in the degradation of soluble and aggregated wild-type and mutant ATXN1. Due to the influence of mTOR activity on 14-3-3, autophagy, and the UPS, the contribution of mTOR to ATXN1 stabilization and degradation also deserves investigation, with the caveat that mTOR may play multiple roles in mutant ATXN1 pathogenesis.

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Wrote the first draft of the manuscript: MFK. Contributed to the writing of the manuscript: MFK and SL. Agree with manuscript results and conclusions: MFK and SL.
developed the structure and arguments for the paper: MFK and SL. Made critical revisions and approved final version: SL. Both authors reviewed and approved the final manuscript.

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