Chapter 14
Polyglutamine-Independent Features in Ataxin-3 Aggregation and Pathogenesis of Machado-Joseph Disease

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Abstract The expansion of a trinucleotide (CAG) repeat, translated into a polyglutamine expanded sequence in the protein encoded by the MJD gene, was identified over 20 years ago as the causative mutation in a severe neurodegenerative disorder originally diagnosed in individuals of Portuguese ancestry. This incapacitating disease, called Machado-Joseph disease or spinocebellar ataxia type 3, is integrated into a larger group of neurodegenerative disorders—the polyglutamine expansion disorders—caused by extension of a CAG repeat in the coding sequence of otherwise unrelated genes. These diseases are generally linked with the appearance of intracellular inclusions, which despite having a controversial role in disease appearance and development represent a characteristic common fingerprint in all polyglutamine-related disorders. Although polyglutamine expansion is an obvious trigger for neuronal dysfunction, the role of the different domains of these complex proteins in the function and aggregation properties of the carrier proteins is being uncovered in recent studies. In this review the current knowledge about the structural and functional features of full-length ataxin-3 protein will be discussed. The intrinsic conformational dynamics and interplay between the globular and

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intrinsically disordered regions of ataxin-3 will be highlighted, and a perspective picture of the role of known ataxin-3 post-translational modifications on regulating ataxin-3 aggregation and function will be drawn.

Keywords Amino acid repeats · Amyloid · Conformational plasticity
Post-translational modifications

14.1 Ataxin-3: A Multidomain Protein with Considerable Conformational Plasticity

Ataxin-3 (Atx3) is a modular protein, containing a globular N-terminal domain (Josephin domain, JD) and a flexible C-terminal tail containing ubiquitin interaction motifs (UIM) and a polyglutamine (polyQ)-rich region [1] (Fig. 14.1). Mutations leading to expansion of the repeated polyQ sequence represent the major trigger for

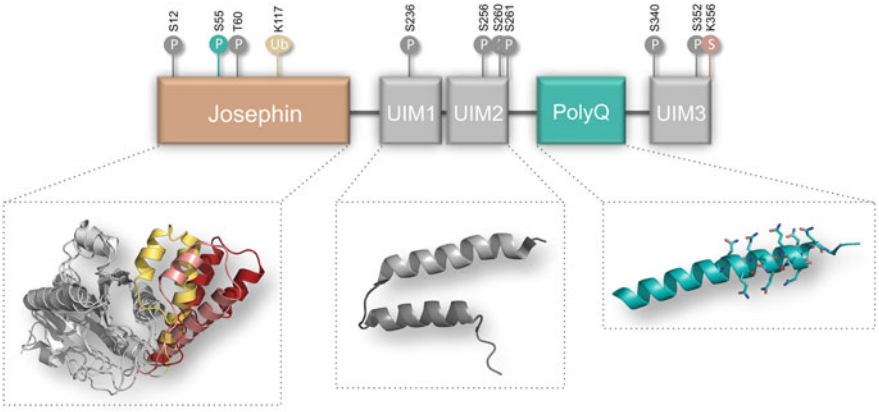

Fig. 14.1 Domain architecture of ataxin-3. Schematic representation of the modular structure of Atx3 composed of a globular domain [the Josephin domain (JD)] and a flexible tail containing two or three ubiquitin interaction motifs (UIMs) and the expandable polyQ stretch. The JD is formed by a catalytic subdomain and a flexible helical hairpin. The cartoon representation of the JD shows two of the structures determined by nuclear magnetic resonance depicting the closed (PDB accession 2aga, catalytic subdomain in grey and helical hairpin in yellow), half-closed (PDB accession 2dos, catalytic subdomain in grey and helical hairpin in pink) and open (PDB accession 1yzb, catalytic subdomain in grey and helical hairpin in red) conformations of the mobile hairpin. UIMs 1 and 2 adopt a predominantly helical conformation that is more compact in the presence of ubiquitin (PDB accession 2klz). The polyQ segment is polymorphic and can adopt a helical conformation (PDB accession 4wth) stabilized by interactions with a neighbor molecule in the crystal lattice. Known phosphorylation sites of Atx3 are represented as grey circles (S12 and T60 at the JD and S236, S256, S260, S261, S340 and S352 at the UIMs) or as a green circle (S55 at the JD, phosphorylation is more pronounced in expanded Atx3). Ubiquitination (K117 at the JD) and SUMOylation sites (K356 at the UIM3) of Atx3 are represented as yellow and pink circles, respectively. Figure prepared with Pymol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC.)

the development of spinocerebellar ataxia type 3 (SCA3) or Machado-Joseph disease (MJD), a neurodegenerative disease for which no pharmacological therapies are available so far [2, 3].

Early bioinformatic studies predicted that Atx3 shared several signatures typically found in ubiquitin-interacting proteins and ubiquitin hydrolases [4, 5]. In agreement, both normal and polyQ-expanded Atx3 were shown to be able to cleave standard ubiquitin hydrolase substrates, an enzymatic activity that was critically dependent on the conserved catalytic residue cysteine 14 [6]. Follow-up studies with the isolated JD corroborated its identity as Atx3 catalytic domain [7], and the link between Atx3 and the ubiquitin-proteasome pathway was further established [8, 9]. Investigation of Atx3 biochemical properties and enzyme specificity showed its preference for K63-linked or heterotypic linkage (mixed K63 and K48 linkages) polyubiquitin chains with four or more ubiquitins [10]. While the JD plays a role in defining the cleavage properties and linkage specificity [11], polyQ expansion does not seem to impose significant differences in enzyme activity or specificity [10]. Accordingly, normal and expanded Atx3 bind to K48-linked tetraubiquitin chains with equivalent affinities [12]. Covalent modification of Atx3 by ubiquitination partly increments its proteolytic activity towards polyubiquitin chains [13], whereas Atx3 phosphorylation decreases its catalytic activity [14] (see below).

The first structural insights of the domain architecture of Atx3 in solution resulted from combined studies using circular dichroism and nuclear magnetic resonance (NMR) spectroscopy [1]. Later, the solution structure of the JD was determined, unveiling a canonical cysteine protease fold [15–18] with a distinctive helical hairpin, whose conformation is variable in the several solution and crystallographic structures determined thus far. This α2/α3 hairpin samples multiple conformations and can be found in a closed (PDB accession 2aga, [15]), half-closed (PDB accession 2dos, [18]) and open conformation (PDB accession 1yzb, [17]), a dynamic behavior that has been linked with ubiquitin substrate recognition and binding [18–21]. The JD encloses two central ubiquitin-binding sites: a proximal site (a.k.a. site 1), located close to the catalytic triad, and a distal site (a.k.a. site 2) including an extended hydrophobic patch, located at the back of this globular domain [18, 19]. The binding region of the ubiquitin-like domain of HHR23B also maps to the distal ubiquitin-binding site [18, 19], a region that modulates Atx3 cellular turnover [22].

The first two UIMs of Atx3 are structurally similar to related motifs in different proteins and form two α-helices connected by a short linker, adopting a more compact structure upon addition of ubiquitin [23]. The crystal structures of a small C-terminal fragment of Atx3 containing the nuclear localization sequence [24] and a polyQ region of 14 glutamines, interrupted by a single lysine residue, were recently solved in fusion with maltose-binding protein (MBP) [25]. The polyQ region was also shown to display conformational plasticity sampling multiple conformations that range from random coil to α-helical structures, the latter stabilized by interactions with neighbouring MBP molecules in the crystal lattice [25]. The essentially polymorphic nature of Atx3 polyQ region is not unusual, and is also common to polyQ segments in other polyQ disease-related proteins [26, 27].

Contrary to what was originally postulated, the expansion of the polyQ region in disease-related Atx3 is not accompanied by global structural changes [28, 29], and has no relevant impact on polyubiquitin binding [12] and hydrolysis [10]. Recent data from ion mobility mass spectrometry revealed that normal and polyQ-expanded Atx3 adopt a wide range of conformational states imparted by the intrinsic plasticity of the C-terminal tail, containing the UIMs and the polyQ stretch, and the α2/α3 hairpin of the globular JD [29–31].

14.2 The Complex Pathway of Ataxin-3 Self-assembly into Amyloid-like Fibrils

Expansion of Atx3 polyQ repeat sequence is directly correlated with the appearance of ubiquitinated intracellular inclusions containing the mutated protein [32]. Early evidence, where expression of an expanded polyQ-containing sequence stimulated recruitment of full-length Atx3 into insoluble inclusions, suggested a central role for the expanded polyQ sequences in protein aggregation and disease pathogenesis. In vitro studies with the full-length protein, with variable polyQ tract sizes, revealed that polyQ expansion promoted protein self-assembly into insoluble SDS-resistant aggregates [28, 33]. These aggregates had fibrillar morphology, were enriched in β-structure, and displayed the capacity to bind the amyloid dye Congo Red, suggesting that they have amyloid-like properties [33]. Interestingly, non-pathological Atx3 is also found in intracellular inclusions under stress-induced conditions [34, 35].

Follow-up studies with recombinant Atx3 demonstrated that the non-expanded protein as well as the isolated JD could also self-assemble into insoluble β-rich structures upon protein destabilization/unfolding [36–39], but also under near-physiological conditions [40]. The modulation of protein aggregation by regions outside the polyQ tract is a common feature in several proteins involved in polyQ expansion diseases [41–44]. The JD has a number of aggregation-prone regions [7, 38, 40, 45] and plays a central role in the early aggregation steps of both normal and polyQ-expanded Atx3 [40, 46, 47]. Small oligomers and protofibrils from non-pathological Atx3 and JD were efficiently detected by a specific antibody, which universally recognizes toxic amyloid oligomeric structures from unrelated amyloid-forming proteins [40, 48]. Those findings suggest that studying the "slower" aggregation pathway of normal Atx3 or the JD will provide clues to identify early intermediates with relevance for neuronal degeneration. Native mass spectrometry data has contributed for the delineation of the early molecular events underlying JD-mediated aggregation: self-association of the JD is initiated through dimerization, followed by a conformational change of the dimer, which can be recognized by a conformation-specific antibody [29, 40]. The more compact dimer further sustains linear oligomer assembly by sequential monomer addition [29].

Current experimental data support a two-step model for Atx3 aggregation as proposed by Ellisdon and coworkers [47]: the first step leads to the formation of SDS-sensitive protofibrils, while the second step generates long-straight and

SDS-resistant mature fibrils (Fig. 14.2). The first aggregation step is mediated by self-association of the JD and occurs in both expanded and non-pathological Atx3, being independent of the polyQ tract. The second step is observed only for Atx3 with a pathological polyQ tract size and leads to the polyQ-dependent formation of SDS-resistant fibrils, as initially reported by Bevivino and Loll [33]. A combination of mass spectrometry and limited proteolysis studies identified the region encompassing helix α4 from the JD as the core of the protofibrils formed in the JD-mediated aggregation step [29, 49]. Additionally, the fibrils formed by polyQ-expanded Atx3 contained two structured regions, the protofibril core and a polyQ-containing core [29]. These results denote that the polyQ stretch is directly involved in the maturation into SDS-resistant fibrils [29], which are stabilized by hydrogen bonds mediated by the glutamine side-chains [50]. However, although both the isolated JD as well as non-pathological and polyQ-expanded protein can self-assemble into amyloid-like fibrils, the in vitro aggregation rates are different for the different constructs [28–30, 33]. This implies that further mechanistic details are required to completely comprehend the complexity of Atx3 aggregation pathway(s).

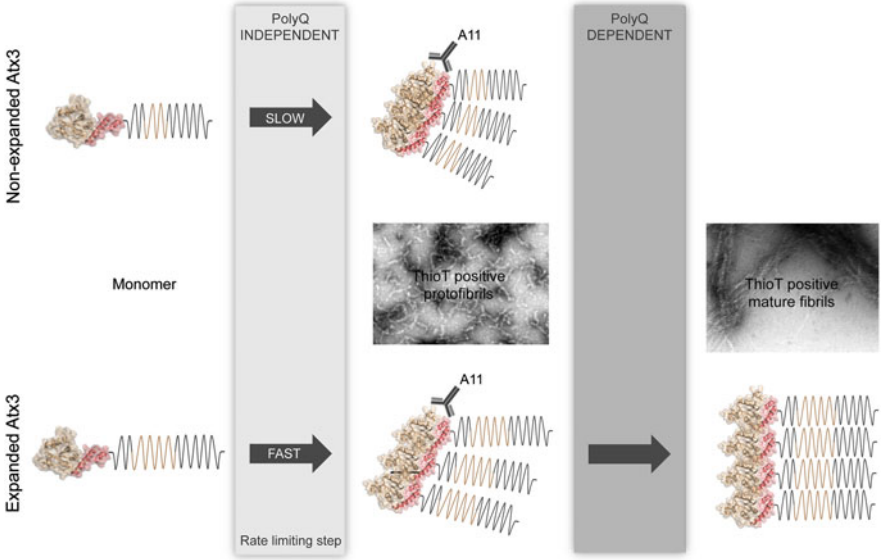

Fig. 14.2 Two-step model of ataxin-3 aggregation. The first step of ataxin-3 self-assembly, common to non-pathological and polyQ-expanded Atx3, is centered on the Josephin domain. The oligomers and small protofibrils formed are sensitive to SDS and can be recognized by the anti-oligomer specific antibody A11 [40, 48]. A second step, exclusively dependent on the polyQ expansion, leads to the generation of mature and SDS-resistant Atx3 fibrils

14.3 Polyglutamine Sequence Expansion and the Conformational Dynamics of the Josephin Domain

Expansion of the polyQ tract has been consistently demonstrated to accelerate Atx3 fibrillation, although multiple studies have demonstrated that the JD and the C-terminal tail are not directly involved in a stable interaction [28, 29, 31, 38] and polyQ expansion does not modify Atx3 overall stability or folding/unfolding [28]. Experimental data from different research groups have demonstrated that polyQ expansion induces local conformational fluctuations in the aggregation-prone regions of the globular JD, exposing them more frequently and thereby increasing the rates of aberrant self-assembly events [29, 49]. PolyQ expansion affects in particular helix α4, identified as part of the protofibrillar core, and helix α1, containing the catalytic residue C14 [49] (Fig. 14.3). This region is adjacent to the mobile α2/α3 hairpin. Interestingly, those aggregation-prone and mobile surfaces of the catalytic JD overlap with the functionally relevant Ub binding sites [51]. In agreement, JD-mediated interactions set off the oligomerization of both normal Atx3 and its expanded counterpart in vitro [31, 40, 45, 47], and interaction with ubiquitin delays JD aggregation [51]. The key role of JD-mediated protein interactions as regulators of Atx3 self-assembly is further reinforced by the findings that a molecular chaperone, transiently interacting with JD catalytic subdomain, significantly reduces aggregation [52]. Those studies expose a role for protein interactions as modulators of the conformational plasticity and self-assembly properties of

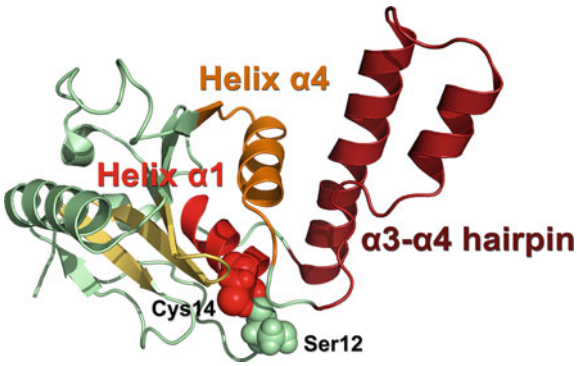

Fig. 14.3 Cartoon representation of the JD structure depicting the aggregation-prone stretch forming the core of the protofibril (orange) and the regions showing structural perturbations characterized by increased solvent exposure upon expansion of the polyQ segment (helix α1, red; helix α4, orange; central β3–β5 strand, yellow). The helical hairpin is shown in dark red. Phosphorylation site serine 12, close to the ubiquitin-binding site of JD and with an impact in Atx3 aggregation, and cysteine 14, the nucleophile of the catalytic triad, are represented as red and green spheres, respectively. Figure prepared with Pymol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC.)

full-length Atx3 (for a recent review on Atx3 interacting proteins see [53]), providing an attractive link between cellular context and Atx3 aggregation and toxicity.

14.4 Ataxin-3 Post-translational Modifications as Modulators of Protein Aggregation and Function

A striking feature of MJD and other polyQ diseases is the selective vulnerability of specific brain regions to the expanded protein, despite its ubiquitous expression [3]. The disease features reflect the loss or dysfunction of specific neuron populations that are affected by expanded Atx3. These observations motivated a series of studies addressing Atx3 regulation by post-translational modifications (which could be cell type-specific) as an attractive mechanism for modulating protein aggregation and toxicity. Important insights have been provided by studying Atx3 regulation by phosphorylation, ubiquitination and SUMOylation (Table 14.1).

14.4.1 Ataxin-3 Phosphorylation

Atx3 is phosphorylated at both the JD and the UIMs (Fig. 14.1), in some cases with consequences on protein aggregation and toxicity. In the JD, Atx3 is phosphorylated at serine 12 [14] (Fig. 14.3), a residue located in proximity to structural elements associated with substrate binding and hydrolysis. Serine 12 is in the loop preceding the α1 helix where the C14 nucleophile of the catalytic triad is located, near Q9, a residue that may contribute to the stabilization of the negatively charged transition state during peptide bond cleavage, and in the vicinity of the loop centered on S27, close to the docking site for the ubiquitin substrate [19]. In fact, mimicking constitutive phosphorylation of Atx3 at residue 12 decreases its deubiquitinase (DUB) activity [14]. Additionally, in cortical neurons phosphorylation of S12 reduces dendritic tract and synapse (excitatory and inhibitory) loss caused by expression of expanded Atx3, and reduces expanded Atx3 aggregation. In a lentiviral rat MJD model, expanded Atx3 phosphorylation at this position is associated with decreased formation of Atx3 aggregates, and decreased neuronal and synapse loss [14], suggesting that modifications targeting S12 may reduce the toxicity of expanded Atx3, and be protective. The effect of S12 phosphorylation on Atx3 aggregation may be related to its proximity to the proximal ubiquitin-binding site in the JD (a.k.a. site 1), which has been described to overlap with regions that take part in JD self-assembly [19, 23, 51]. In addition to this phosphorylation site, S55 and T60 at the JD were also recently described to undergo phosphorylation [54] (Fig. 14.1), but the functional consequences of these phosphorylation events are so far unknown. Interestingly, phosphorylation at S55 is increased in expanded Atx3 compared to the non-expanded protein [54].

Table 14.1 Post-translational modifications in ataxin-3

Modification	Residue in Atx3	Enzyme	Protein domain	Functional effect	References
Phosphorylation	S12	Unknown	JD	Phosphorylation decreases DUB activity; prevents dendritic shrinkage and synapse loss caused by expression of expanded Atx3; decreases expanded Atx3 aggregation	[14]
	S55	Unknown	JD	Unknown	[54]
	T60	Unknown	JD	Unknown	[54]
	S236	Unknown	UIM1	Phosphorylation enhances nuclear localization and transcription repression activity	[56]
	S256	GSK3β	UIM2	Preventing phosphorylation increases expanded Atx3 aggregation	[55]
	S260/261	Unknown	UIM2	Unknown	[56]
	S340	Unknown	UIM3	Phosphorylation enhances nuclear localization and transcription repression activity	[56]
	S352	Unknown	UIM3	Phosphorylation enhances nuclear localization and transcription repression activity	[56]
Ubiquitination	K117	CHIP/E4B	JD	Ubiquitination enhances Atx3 catalytic activity	[13, 58]
SUMOylation	K356	Unknown	UIM3	SUMOylation increases affinity for p97/VCP	[64]

Six other Atx3 phosphorylation sites have been described, localized in the UIMs of the protein (Fig. 14.1). Glycogen synthase kinase 3β (GSK3β) phosphorylates Atx3 in vitro at S256 in the second UIM [55]; preventing Atx3 phosphorylation at S256 by replacing the serine residue by alanine increased Atx3 aggregation, which was prevented by overexpression of the Hsp70 chaperone [55]. Serine 236 in UIM1, serines 260 and 261 in UIM2, as well as serines 340 and 352 in UIM3 are also phosphorylation substrates [56]. Phosphorylation of S236 or of S340 and S352 enhances Atx3 nuclear localization and repression of Atx3-regulated transcription, whereas inhibition of casein kinase 2 (CK2), a kinase that binds to Atx3 [56, 57], leads to cytoplasmatic accumulation of the protein [56].

14.4.2 Ataxin-3 Ubiquitination

Atx3 is ubiquitinated at lysine 117 (Fig. 14.1), near the DUB active site [58]. Ubiquitination enhances the catalytic activity of the protein [13, 58], without affecting its preference for K63-linked ubiquitin chains [13]. A recent study addressed the consequences of Atx3 ubiquitination at K117 at the structural level, and found that K117-covalently linked mono-ubiquitin binds to the proximal ubiquitin-binding site 1, suggesting that the observed increase in catalytic activity could result from locking Atx3 in an active state [59]. Interestingly, regulation of Atx3 DUB activity by ubiquitination is enhanced upon proteasome inhibition or activation of the unfolded protein response [13], suggesting that cellular protein turnover control by Atx3 is regulated by its ubiquitination.

The co-chaperone C-terminal Hsp70-interacting protein (CHIP) as well as the mammalian E4B (UFD2a), a ubiquitin chain assembly factor (E4), interact with Atx3 and promote its ubiquitination and degradation [60–62]. However, cellular degradation of Atx3 does not require its ubiquitination, rather is dependent on binding of Rad23 to its distal ubiquitin-binding site, which rescues it from proteasomal degradation [22]. In a *Drosophila* model of MJD, expression of E4B suppressed the neurodegeneration induced by expanded Atx3 [61], suggesting that enhancing Atx3 ubiquitination is neuroprotective. Accordingly, it was more recently found that ubiquitination of wild-type Atx3 at K117 enhances its protective effect against expanded Atx3-induced degeneration in *Drosophila* [63].

14.4.3 Ataxin-3 SUMOylation

Covalent attachment of small ubiquitin-like modifier (SUMO) protein to lysine residues in target proteins can influence their localization, interactions, activity and stability, and has been shown for several polyQ-containing proteins, including Atx3 [64, 65]. Atx3 is SUMOylated by SUMO1 and SUMO2 at K356 in UIM3 (Fig. 14.1), which decreases Atx3 fibril formation in vitro, and increases Atx3 affinity for the ATPase p97/VCP [64]. P97/VCP is involved in retrotranslocation of misfolded proteins from the endoplasmic reticulum to the cytosol, for degradation by the proteasome, and Atx3 is known to participate in efficient elimination of retrotanslocated misfolded proteins [66, 67]. On the other hand, p97/VCP influences the assembly of Atx3 protein aggregates in vitro, in a concentration-dependent manner, and suppresses Atx3-induced neurodegeneration in *Drosophila* [67]. Atx3 SUMOylation, by impacting Atx3 affinity for p97/VCP, may affect the p97/VCP-mediated ER-associated degradation, as well as the modulation of Atx3 aggregation by VCP.

14.4.4 Concluding Remarks

The modular structure of Atx3, containing a globular domain and an intrinsically disordered flexible tail, outlines its polymorphic character and multifaceted self-assembly process. Multiple studies have delineated the relevance of the regions and domains flanking the polyQ segment in the regulation of Atx3 function and in modulating its aggregation behavior. In particular, the globular JD assumes a central role for Atx3 function and self-assembly, properties that can be partly modulated by post-translational modifications and protein-protein interactions. The identification of a globular region whose conformational dynamics is influenced by polyQ expansion, and is directly involved in the establishment of the protofibrillar core, suggests that JD can constitute a valuable target for rational design of anti-Atx3 aggregation molecules.

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