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Toward understanding Machado-Joseph Disease

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Abstract

Machado-Joseph disease (MJD), also known as Spinocerebellar ataxia type 3 (SCA3), is the most common inherited spinocerebellar ataxia and one of many polyglutamine neurodegenerative diseases. In MJD, a CAG repeat expansion encodes an abnormally long polyglutamine (polyQ) tract in the disease protein, ATXN3. Here we review MJD, focusing primarily on the function and dysfunction of ATXN3 and on advances toward potential therapies. ATXN3 is a deubiquitinating enzyme (DUB) whose highly specialized properties suggest that it participates in ubiquitin-dependent proteostasis. By virtue of its interactions with VCP, various ubiquitin ligases and other ubiquitin-linked proteins, ATXN3 may help regulate the stability or activity of many proteins in diverse cellular pathways implicated in proteotoxic stress response, aging, and cell differentiation. Expansion of the polyQ tract in ATXN3 is thought to promote an altered conformation in the protein, leading to changes in interactions with native partners and to the formation of insoluble aggregates. The development of a wide range of cellular and animal models of MJD has been crucial to the emerging understanding of ATXN3 dysfunction upon polyQ expansion. Despite many advances, however, the principal molecular mechanisms by which mutant ATXN3 elicits neurotoxicity remain elusive. In a chronic degenerative disease like MJD, it is conceivable that mutant ATXN3 triggers multiple, interconnected pathogenic cascades that precipitate cellular dysfunction and eventual cell death. A better understanding of these complex molecular mechanisms will be important as scientists and clinicians begin to focus on developing effective therapies for this incurable, fatal disorder.

Keywords

polyglutamine disease; deubiquitinating enzyme; protein quality control; spinocerebellar ataxia; neurodegeneration; ataxin-3

1. Introduction

Many hereditary neurodegenerative diseases manifest later in life and are characterized by the progressive and selective loss of neuronal cell bodies, axons, dendrites and/ or synapses. For decades scientists have sought to clinically define specific neurodegenerative diseases and their genetic causes in order to achieve a molecular diagnosis, offer presymptomatic and prenatal testing to affected families, generate cellular and animal models toward

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understanding pathogenic mechanisms and facilitate the development of potential therapies. Studies over the past 20 years have established that an unusual type of mutation, dynamic repeat expansions, cause many inherited neurodegenerative diseases.

Among the dynamic repeat expansion diseases, the polyglutamine (polyQ) disorders caused by CAG repeat expansions represent the most common class, although each polyglutamine disease is relatively rare. In all polyQ diseases the CAG repeat expansion is translated into an abnormally long stretch of glutamine residues in the corresponding disease protein. Spinal Bulbar Muscular Atrophy (SBMA) was the first discovered polyQ disease, identified 20 years ago (La Spada *et al.*, 1991). Since then nine additional polyQ diseases have been identified: the Spinocerebellar Ataxias (SCA) types 1, 2, 3 (also known as Machado-Joseph disease), 6, 7 and 17, Dentatorubral-Pallidoluysian Atrophy (DRPLA), Huntington disease (HD), and, most recently, Huntington Disease-like 2 (HDL2). All polyQ diseases are dominantly inherited disorders except SBMA, which is X-linked. The current review focuses on MJD/SCA3 and its disease protein, ataxin-3 (ATXN3).

Development of rational, targeted therapies for these diseases will be facilitated by knowing the pathogenic mechanism of the disease-causing mutation. As a class, polyQ diseases share certain features that suggest a general toxic mechanism triggered by expanded polyQ, which might be targetable in class-wide therapeutics. All ten polyglutamine diseases are characterized by selective neurodegeneration in the central nervous system (CNS) despite widespread expression of the disease proteins. Indeed there is little correlation between the expression pattern of polyQ proteins and the sites of CNS pathology. The disease proteins are widely expressed throughout the CNS with two notable exceptions: the $CACNA1_A$ calcium channel subunit in SCA6, which is mainly expressed in affected cerebellar Purkinje cells, and the androgen receptor in SBMA, which is primarily expressed in vulnerable motor neurons. Another shared feature of polyQ disease proteins is their propensity to misfold, oligomerize, and form intracellular aggregates and inclusions that constitute a pathological disease hallmark. The misfolding and aggregation of polyQ disease proteins have been targets of some proposed therapeutic strategies (Bauer and Nukina, 2009; Di Prospero and Fischbeck, 2005; Matos *et al.*, 2011; Williams and Paulson, 2008).

Despite these shared features, however, each polyQ disease is a distinctive disorder with characteristic symptomatology and pathology occurring in specific brain regions. PolyQ disease proteins differ in size, cellular localization and biological function, suggesting that the toxic effect of a given polyQ expansion depends on the specific protein context and that the particular details of pathogenesis may be unique to each disease.

Here we review Machado-Joseph disease (MJD), also known as Spinocerebellar Ataxia Type 3 (SCA3), focusing primarily on the molecular properties of the disease protein, ATXN3, both in normal and pathogenic contexts, and on recent progress toward therapeutic development for this fatal disorder.

2. MJD

2.1. Clinical features

The discovery of MJD (OMIM#109150) illustrates the difficulty of defining a disease as a single entity when variable symptoms themselves represent a hallmark of the disease. MJD was first described in Northern American families of Azorean ancestry. Between 1972 and 1977 the disease was identified in four families, reported as four distinct entities named “Machado disease” (Nakano *et al.*, 1972), “nigrospino-dentatal degeneration” (Woods and Schaumburg, 1972), “Joseph disease” (Rosenberg *et al.*, 1976), and “Azorean disease of the nervous system” (Romanul *et al.*, 1977). In 1975, Coutinho and Andrade studied 15 families

from the Azorean Islands and proposed that the above mentioned diseases were simply variations of the same clinical disorder (Coutinho and Andrade, 1978). They defined it as "Machado-Joseph disease," a single disorder characterized by an unusually high degree of clinical variability.

Most frequently in affected individuals, a slowly progressive "ataxia-plus" syndrome appears, typically beginning between the ages of 20 and 50 years (Coutinho and Andrade, 1978; Paulson, 1998 Oct 10 [updated 2011 March 17]). Cerebellar ataxia, progressive external ophthalmoplegia, dysarthria, dysphagia, pyramidal signs, dystonia, rigidity, and distal muscle atrophies are common features of MJD. The highly variable clinical presentation led to a description of four distinct clinical subtypes of MJD (Coutinho and Andrade, 1978; Lima and Coutinho, 1980; Paulson, 2007; Riess *et al.*, 2008; Rosenberg, 1992). Type 1 begins early in life, often before age 20, may progress more quickly and is characterized by prominent pyramidal signs (rigidity and spasticity) and extrapyramidal features (bradykinesia and dystonia) as well as ataxia. Type 2, the most common type, has an intermediate age-at-onset (20–50 years) with cerebellar ataxia, progressive external ophthalmoplegia and pyramidal signs. Type 3 has a later onset (40–75 years) and is characterized by peripheral signs such as motor neuronopathy and muscle atrophy together with ataxia. Type 4, the rarest presentation, is characterized by parkinsonism associated with other core clinical features. More recently, a type 5 MJD was proposed for rare cases presenting pure spastic paraplegia (Landau *et al.*, 2000; Sakai and Kawakami, 1996; Wang *et al.*, 2009). Other common features not confined to a specific subtype are weight loss and restless legs syndrome (Paulson, 2007; Riess *et al.*, 2008). Less commonly, mild cognitive and behavioral problems can be observed (Burk *et al.*, 2003; Kawai *et al.*, 2004).

Since the original clinical description of MJD, many affected families have been identified worldwide, both of Portuguese and nonPortuguese ancestry (Eto *et al.*, 1990; Healton *et al.*, 1980; Lima and Coutinho, 1980; Livingstone and Sequeiros, 1984; Sakai *et al.*, 1983; Sequeiros and Suite, 1986; Takiyama *et al.*, 1993; Taniguchi and Konigsmark, 1971). MJD is currently thought to be the most common dominantly inherited ataxia in the world, comprising 15–45% of dominantly inherited ataxia in different countries and ethnic populations (Margolis, 2002; Paulson, 2007; Schols *et al.*, 2004).

2.2. The disease brain

Despite the fact that MJD is classified as a form of spincocerebellar ataxia, brain imaging and neuropathological studies indicate that the range of CNS involvement extends well beyond the brainstem and cerebellum.

Enlargement of the fourth ventricle is the most consistent feature observed by magnetic resonance imaging (MRI) in MJD. Neuroimaging studies have revealed atrophy of the pons, cerebellar vermis and hemispheres, basal ganglia (globus pallidus, caudate and putamen), midbrain and medulla oblongata (Etchebehere *et al.*, 2001; Klockgether *et al.*, 1998; Murata *et al.*, 1998; Taniwaki *et al.*, 1997; Yoshizawa *et al.*, 2003). The atrophy in the cerebellum and brainstem is progressive and dependent on the length of the CAG repeat and the age of the patients (Abe *et al.*, 1998; Eichler *et al.*, 2011; Onodera *et al.*, 1998). However, different brain regions present different rates of atrophy progression. While atrophy of the cerebellum and pontine base seem to correlate with patient's age, atrophy of the midbrain and pontine tegmentum show no significant progression (Horimoto *et al.*, 2008).

More recent quantitative imaging studies using large cohorts of patients show that MJD is a more widespread disorder throughout the CNS involving the cerebellar hemispheres and vermis, the thalamus, and the frontal, parietal, temporal, occipital and limbic lobes (D'Abreu *et al.*, 2011; D'Abreu *et al.*, 2010; De Oliveira *et al.*, 2010; Etchebehere *et al.*, 2001).

Furthermore, magnetic resonance spectroscopy analysis of deep white matter has shown metabolic abnormalities suggestive of axonal dysfunction in MJD patients (D'Abreu *et al.*, 2009). Glucose utilization deficits in cerebellum, brainstem and cerebral cortex can be observed in MJD carriers even before clinical signs of disease (Soong and Liu, 1998). Likewise, decreased binding for dopamine transporter in these regions and in the striatum is observed in symptomatic MJD patients (Taniwaki *et al.*, 1997; Wullner *et al.*, 2005). Brain abnormalities detected by advanced and quantitative neuroimaging techniques may offer effective biomarkers to monitor interventional trials in MJD patients.

Brains of MJD patients with advanced disease weigh significantly less than brains from individuals without neurological or psychiatric disease (Iwabuchi *et al.*, 1999). Macroscopically, MJD brains show depigmentation of the substantia nigra and atrophy of the cerebellum, pons, medulla oblongata, as well as multiple cranial nerve nuclei (III to XII) (Rub *et al.*, 2008).

Neurodegeneration in MJD was initially described in the olivopontocerebellar regions (Coutinho and Sequeiros, 1981; Ross, 1995). Recent pathoanatomical studies, however, revealed more extensive damage affecting areas of the cerebellothalamocortical motor loop, the basal ganglia-thalamocortical motor loop, and several other systems: visual, auditory, somatosensory, vestibular, oculomotor, ingestionrelated brainstem, precerebellar brainstem, cholinergic and dopaminergic midbrain, and pontine noradrenergic systems (Rub *et al.*, 2008). Retained integrity of the cortical and subcortical regions of the limbic system and mild degeneration of the white matter of cerebellum, brainstem and spinal cord are also characteristic of MJD (Riess *et al.*, 2008; Rub *et al.*, 2008).

The disease protein, ATXN3, was initially reported to accumulate in neuronal nuclear inclusions (NNIs) in vulnerable regions of the MJD brain (Paulson *et al.*, 1997b; Schmidt *et al.*, 1998). The NNIs stain positively for ubiquitin (Ub) and contain other proteins such as Ub-like proteins, heat-shock proteins (HSPs), proteasome subunits, transcription factors, and other polyQ proteins (Chai *et al.*, 1999a; Chai *et al.*, 1999b; Chai *et al.*, 2001; Mori *et al.*, 2005; Paulson *et al.*, 1997b; Schmidt *et al.*, 1998; Takahashi *et al.*, 2001). Recent studies using other pathoanatomical techniques have established the occurrence of NNIs in both affected and unaffected brain regions (Rub *et al.*, 2008; Rüb *et al.*, 2006; Rub *et al.*, 2007; Yamada *et al.*, 2004). In fact, no clear-cut correlation exists between the distribution of NNIs and the pattern of neurodegeneration, suggesting that NNIs do not play a direct role in determining the survival or death of an affected neuron (Rüb *et al.*, 2006).

In addition to NNIs, neuronal cytoplasmic inclusions (NCIs) immunopositive for expanded polyQ ATXN3 have been described in MJD brains, displaying a similar distribution pattern as NNIs (Hayashi *et al.*, 2003; Yamada *et al.*, 2008; Yamada *et al.*, 2004). NCIs are mainly Ub-negative, consist of fine granules about 1.5 μm in diameter, and are proposed to correspond to electron-dense minute structures scattered in pale, small primitive lysosomes (Yamada *et al.*, 2002). ATXN3 aggregates are also observed in axons; these widespread axonal inclusions contain Ub and p62 and are found in fiber tracts known to degenerate in MJD (Seidel *et al.*, 2010).

In summary, in the MJD brain different types of ATXN3 aggregates accumulate in specific cellular compartments. Defining the molecular composition of each type of aggregate structure may lead to a better understanding both of their possible role in disease pathogenesis and of their correlation to neurodegeneration and other aspects of disease.

3. The disease gene, *ATXN3*

3.1. Genetics of MJD

From its initial description in 1972, MJD was recognized to be a dominantly inherited genetic disorder. Eleven years later, the MJD disease gene was mapped to chromosome 14q32.1 (Takiyama *et al.*, 1993). That same year, the presence of clinical features of ataxia apparently distinct from MJD in some French families that did not map to the SCA1 or SCA2 loci led researchers to propose the existence of a novel, dominantly inherited ataxia which they named Spinocerebellar Ataxia Type 3 (SCA3) (Gispert *et al.*, 1993). Once the gene defect in MJD was discovered, however, it became clear that SCA3 and MJD are in fact the same disease.

The *MJD1* gene was cloned in 1994 (Kawaguchi *et al.*, 1994). Now designated *ATXN3*, the disease gene was found to have a polyglutamine-encoding CAG repeat that was expanded in affected individuals (Kawaguchi *et al.*, 1994). MJD thus joined SBMA, SCA1 and HD as diseases caused by CAG/polyQ expansions.

The molecular diagnosis of MJD based on the *ATXN3* CAG repeat expansion rapidly led to confirmation of the disease in families of many different ethnic origins (Gaspar *et al.*, 2001; Higgins *et al.*, 1996; Lindblad *et al.*, 1996; Maciel *et al.*, 1995). SCA3 was discovered to be the same disease as MJD (Durr *et al.*, 1996; Haberhausen *et al.*, 1995; Matilla *et al.*, 1995), explaining why the disease is designated both as MJD and SCA3. Presymptomatic and prenatal testing soon became available to MJD families through genetic counseling programs (Lima *et al.*, 2001; Maciel *et al.*, 2001; Rolim *et al.*, 2006; Sequeiros *et al.*, 1998).

The expanded repeat in *ATXN3* is nearly a pure CAG tract, interrupted by a single lysine codon near the beginning of the repeat $(CAG)_2CAAAAG(CAG)_n$ (Kawaguchi *et al.*, 1994). This trinucleotide repeat ranges from 12 to 44 triplets in healthy individuals and from ~60 to 87 in MJD patients (Lima *et al.*, 2005; Maciel *et al.*, 2001). Rare alleles of intermediate repeat length fall between the clearly normal and mutant ranges and are not associated with classical clinical features of disease (Gu *et al.*, 2004; Maciel *et al.*, 2001; Padiath *et al.*, 2005; Paulson, 2007; Takiyama *et al.*, 1997; van Alfen *et al.*, 2001; van Schaik *et al.*, 1997). Cases of homozygosity are extremely rare in MJD; the few described homozygous patients appear to show a more severe form of disease suggesting a gene dosage effect (Carvalho *et al.*, 2008; Fukutake *et al.*, 2002; Lang *et al.*, 1994; Lerer *et al.*, 1996; Sobue *et al.*, 1996; Takiyama *et al.*, 1995).

As in other polyQ diseases, the CAG repeat size in MJD inversely correlates with age of disease onset and directly correlates with disease severity (Durr *et al.*, 1996; Jardim *et al.*, 2001; Maciel *et al.*, 1995; Schols *et al.*, 1996). Because intergenerational instability of the CAG repeat occurs in MJD families, the repeat may be of different lengths in progenitors and offspring. Paternal mutant alleles are slightly more unstable than maternal ones, and thus are more prone to expand or contract when transmitted to the next generation (Igarashi *et al.*, 1996; Maciel *et al.*, 1995; Maruyama *et al.*, 1995). This dynamic feature of the disease mutation explains the phenomenon of anticipation observed in some MJD families, in which affected offspring tend to manifest disease earlier than an affected parent (Coutinho and Sequeiros, 1981; Sequeiros and Coutinho, 1993). CAG repeat instability can also occur in different cells from the same tissue, a phenomenon known as somatic mosaicism. In MJD, somatic mosaicism may occur in the brain, but larger repeats are not preferentially associated with affected brain regions (Lopes-Cendes *et al.*, 1996; Maciel *et al.*, 1997).

The existence of several single nucleotide polymorphisms (SNPs) and single tandem repeats (STRs) neighboring the CAG tract have allowed for a better understanding of both the

mechanisms of repeat instability and the origin of the MJD mutation. Specific SNPs neighboring the repeat were shown to have both *cis* and *trans* effects on CAG repeat instability (Igarashi *et al.*, 1996; Maciel *et al.*, 1999; Martins *et al.*, 2008). Evolution of the CAG repeat in the *ATXN3* gene appears to have been driven by a multistep mutational mechanism (Martins *et al.*, 2006). Interestingly, two mutational events may explain the fact that MJD is spread worldwide: the mutation probably originated in Asia, later spreading throughout Europe, with a founder effect explaining the high prevalence in Portugal and the second mutational event perhaps explained by Portuguese emigration (Gaspar *et al.*, 2001; Martins *et al.*, 2007).

Although CAG repeat length is strongly correlated with several clinical aspects of disease, other genetic and/or environmental factors likely contribute to disease presentation. For example, DNA methylation in the promoter region of the *ATXN3* gene was recently proposed to have a small, positive effect on the age at onset of MJD patients, indicating that epigenetic factors might contribute to clinical variability in MJD (Emmel *et al.*, 2011).

3.2. Genomic structure and transcripts

The *ATXN3* gene spans ~48 Kb and comprises 11 exons with the CAG repeat residing in exon 10 (Ichikawa *et al.*, 2001). Four different transcripts of approximately 1.4, 1.8, 4.5, and 7.5 Kb are ubiquitously expressed in human brain and in non-nervous tissues (Ichikawa *et al.*, 2001; Schmitt *et al.*, 1997). These multiple transcripts may result from alternative splicing in exons 2, 10, and 11 in combination with different polyadenylation signals (Goto *et al.*, 1997; Ichikawa *et al.*, 2001; Kawaguchi *et al.*, 1994). A recent study proposed the existence of two novel exons 6a and 9a, located downstream of the corresponding exons, and 50 potential new alternative splice variants of the *ATXN3* gene (Bettencourt *et al.*, 2010). The biological relevance of these numerous variants, however, remains unclear.

The regulation of *ATXN3* gene expression is still poorly understood. The 5'-flanking region is a TATA-less promoter, comprising GC-rich regions, a CCAAT box, multiple putative SP1 binding sites, and a core promoter region within ~300 bp of the start codon (Schmitt *et al.*, 2003). The *ATXN3* 3' untranslated region (UTR) remains unstudied but the existence of transcripts carrying different 3'UTRs suggests additional gene regulation at this level (Ichikawa *et al.*, 2001). The field would benefit from greater clarification of the mechanisms regulating *ATXN3* gene expression as they could represent potential therapeutic targets.

4. The *ATXN3* product, ATXN3

An evolutionarily conserved protein, ATXN3 has a long list of orthologs in a wide range of species (Costa *et al.*, 2004; Linhartova *et al.*, 1999; Rodrigues *et al.*, 2007; Schmitt *et al.*, 1997). Normal (i.e. nonexpanded) human ATXN3 has a molecular weight of approximately 42 KDa, varying slightly in size depending on the length of the polymorphic glutamine repeat. Defining the function, localization, stability and physiological role of wild-type ATXN3 is critically important if scientists want to understand how polyQ expansion in this protein causes its dysfunction and triggers a toxic mechanism.

4.1. Structure and function as a deubiquitinating enzyme

ATXN3 is a deubiquitinating enzyme (DUB) that binds Ub and polyUb chains, and is itself regulated by ubiquitination (Burnett *et al.*, 2003; Chai *et al.*, 2004; Donaldson *et al.*, 2003; Nicastro *et al.*, 2009; Scheel *et al.*, 2003; Todi *et al.*, 2009). Current data support the view that ATXN3 functions, at least in part, to edit polyUb chains added by Ub ligases to target proteins (Kuhlbrodt *et al.*, 2011; Scaglione *et al.*, 2011). Ubiquitination of proteins occurs through sequential reactions involving the Ub activating enzyme (E1), Ub conjugating enzymes (E2), and Ub ligases (E3). Several types of polyUb chains can be formed by a Ub

ligase depending on the specific lysine linkages made between Ub molecules. Different linkage types confer divergent functions on polyUb chains. For example, K48-linked polyUb chains typically target proteins for proteasomal degradation whereas K63-linked chains play diverse roles in subcellular localization, vesicular trafficking, DNA repair, and translation (Glickman and Ciechanover, 2002; Woelk *et al.*, 2007).

ATXN3 contains a structured globular N-terminus of 198 amino acids – the catalytic Josephin domain (JD) - followed by an unstructured, flexible C-terminus containing the polyQ stretch and two or three ubiquitin interacting motifs (UIM), depending on the protein isoform (Goto *et al.*, 1997; Masino *et al.*, 2003) (Figure 1).

While several potential ATXN3 isoforms may be translated, only two have been studied in detail. Both are full length proteins that contain the polyQ tract and UIMs1 and 2 but differ in their C-termini, either lacking or containing UIM3 (designated 2UIM ATXN3 and 3UIM ATXN3, respectively). Studies using antibodies that recognize either both isoforms or only 3UIM ATXN3 indicate that 3UIM ATXN3 is the predominant isoform expressed in brain (Harris *et al.*, 2010; Schmidt *et al.*, 1998; Trottier *et al.*, 1998).

The JD adopts a semi-elongated L-structure composed of a globular catalytic subdomain and a helical hairpin (Mao *et al.*, 2005; Nicastro *et al.*, 2006; Nicastro *et al.*, 2005). The ubiquitin protease activity resides in the catalytic subdomain, which comprises the cleavage pocket (Q9, C14, H119, N134) typical of papain-like cysteine proteases and two binding sites for Ub (Mao *et al.*, 2005; Nicastro *et al.*, 2006; Nicastro *et al.*, 2009). ATXN3 Ub-protease activity requires the active site cysteine 14 (Berke and Paulson, 2003; Burnett *et al.*, 2003; Chai *et al.*, 2004). PolyUb chains with at least four Ub units, but not shorter chains, are cleaved *in vitro* by ATXN3. ATXN3 also shows preference for K63-linked and K48/K63-mixed linkage polyUb chains over K48-linked chains *in vitro* (Burnett *et al.*, 2003; Chai *et al.*, 2004; Winborn *et al.*, 2008). Although favoring the cleavage of long polyUb chains, ATXN3 can also deubiquitinate specific monoubiquitinated substrates once in functional protein complexes (Scaglione *et al.*, 2011).

Ub binding to the JD occurs through an induced-fit mechanism mediated by the helical hairpin (Nicastro *et al.*, 2009). Lying close to the active cleft, Ub-binding site 1 is essential for cleavage of all Ub chains whereas site 2, residing on the opposite side and overlapping with the interaction surface of the HHR23B Ub-like (Ubl) domain, may confer polyUb-linkage preference since mutating this site reduces cleavage of K48-linked and K63/K48-mixed, but not K63-linked, polyUb chains (Nicastro *et al.*, 2010). Intriguingly, only a K48-linkage di-Ub molecule appears capable of simultaneously occupying both Ub sites on the isolated JD (Nicastro *et al.*, 2010) (Figure 1).

Specific recognition and positioning of Ub chains for proteolytic cleavage by ATXN3 requires cooperation between its Ub-binding sites in the JD and the UIMs in the C-terminus. The UIMs are essential for higher affinity polyUb chain binding but dispensable for cleavage (Winborn *et al.*, 2008). Though capable of binding K48 or K63-linkage polyUb chains, the UIMs likely position chains in a way that promotes K63-linked chain cleavage and inhibits K48-linked chain cleavage *in vitro*.

A fraction of the cellular pool of ATXN3 is itself ubiquitinated. Although ATXN3 can be mono- and oligo-ubiquitinated, the major ATXN3-Ub species is monoubiquitinated (Berke *et al.*, 2005; Todi *et al.*, 2009). This posttranslational modification enhances ATXN3 DUB activity toward ubiquitinated substrates and free polyUb chains (Todi *et al.*, 2010; Todi *et al.*, 2009). Among the several lysine residues that can be ubiquitinated on ATXN3, residue K117 near the catalytic pocket is predominantly modified (Todi *et al.*, 2010) (Figure 1). Ubiquitination at K117 increases ATXN3 activity independent of other cofactors/interactors,

other types of posttranslational modification, or the known Ub binding domains (Todi *et al.*, 2010).

How does ATXN3 function as a DUB? Most likely, C-terminal UIMs and JD Ub binding site 1 cooperate to position the polyUb chains for Ub isopeptide-bond cleavage at the catalytic site. Although ATXN3 shows preference for cleaving long polyUb chains containing at least four units, it can also deubiquitinate select monoubiquitinated substrates. The properties described for ATXN3 *in vitro* may represent an overly simplistic view of how ATXN3 actually functions in cells when interacting with its multiple known partners and potential substrates.

4.2. Localization

ATXN3 is widely expressed throughout peripheral and neuronal tissues in many different cell types (Paulson *et al.*, 1997a; Schmidt *et al.*, 1998; Trottier *et al.*, 1998; Wang *et al.*, 1997). A similar expression pattern is observed for ATXN3 orthologs in other species (Costa *et al.*, 2004; Rodrigues *et al.*, 2007; Schmitt *et al.*, 1997). Intracellular localization of ATXN3 is regulated at different levels, and its primary subcellular site of action remains uncertain. ATXN3 has been reported in the cytoplasm, nucleus and even in mitochondria (Paulson *et al.*, 1997a; Pozzi *et al.*, 2008; Tait *et al.*, 1998; Trottier *et al.*, 1998).

ATXN3 is highly mobile in the cytoplasm and nucleus with its diffusion limited by the rate of transport across the nuclear membrane (Chai *et al.*, 2002). Nucleocytoplasmic shuttling of ATXN3 is mediated, in part, by a weak nuclear localization signal (NLS), 282RKRR285, and two nuclear export signals (NES), NES77 and NES141 (CRM1/exportin-dependent) (Figure 1) (Antony *et al.*, 2009; Macedo-Ribeiro *et al.*, 2009; Reina *et al.*, 2010; Tait *et al.*, 1998). Interestingly, NES77 overlaps with Ub-binding site 1, but whether Ub binding actually modulates transport into or out of the nucleus remains unknown.

Under basal conditions nuclear import of ATXN3 appears to be mainly controlled by casein kinase 2 (CK2)-mediated phosphorylation of residues S236 in UIM1 and S340/S352 in UIM3 (Mueller *et al.*, 2009). Posttranslational modification by ubiquitination does not affect subcellular localization of ATXN3 in cell lines, but enzymatically active ATXN3 has been reported to localize to the nucleus more often than catalytically inactive ATXN3 (Todi *et al.*, 2007; Todi *et al.*, 2010).

Intracellular localization of ATXN3 is also regulated by specific proteotoxic stressors. Heat shock or oxidative stress leads ATXN3 to accumulate in the nucleus (Reina *et al.*, 2010). Whether CK2-mediated phosphorylation contributes to ATXN3 nuclear translocation under these stress conditions is currently uncertain (Mueller *et al.*, 2009; Reina *et al.*, 2010). Phosphorylation of S111 in the JD seems to be required for nuclear localization of ATXN3 upon heat-shock (Reina *et al.*, 2010).

In summary, the subcellular localization of ATXN3 is highly regulated, depending on interactions between internal localization signals, posttranslational modifications, protein-protein interactions and specific cellular conditions (Antony *et al.*, 2009; Macedo-Ribeiro *et al.*, 2009; Mueller *et al.*, 2009; Reina *et al.*, 2010; Trottier *et al.*, 1998). Understanding the events that modulate intracellular trafficking of ATXN3 may help to elucidate disease pathogenesis, as mutant ATXN3 tends to accumulate in the nucleus and the nucleus is a preferential site for polyQ-induced toxicity.

4.3. Stability

Knowledge of specific cellular events that modulate ATXN3 stability and function is important to understand the cellular dysfunction caused by polyQ expansion. The stability of

ATXN3 can be affected by its solubility or propensity to aggregate, by its susceptibility to proteolytic cleavage, and by signals that alter its rate of degradation.

4.3.1. Aggregation—ATXN3 has an intrinsic propensity to aggregate *in vitro* under native conditions. As is true for other polyQ disease proteins, the fibrillization of ATXN3 is modulated by flanking domains in the protein – in the case of ATXN3, the N-terminal JD influences aggregation. Nonpathogenic (i.e. nonexpanded) ATXN3 is able to undergo a single step aggregation event via JD self-association into dimers. These dimers then aggregate into spheroidal oligomers that in turn assemble into elongated "beads-on-strings" fibrils that are SDS-soluble and Thioflavin T (ThT)-positive (Ellisdon *et al.*, 2007; Ellisdon *et al.*, 2006; Gales *et al.*, 2005; Masino *et al.*, 2011b).

In vitro fibrils formed by nonpathogenic ATXN3 are structurally similar to the ones formed by other amyloidogenic proteins (Masino *et al.*, 2011b). Importantly, ATXN3 enzymatic activity is lost in the fibrils, probably because the native α-helical structure of the JD is converted into a β-sheet-enriched conformation as aggregation proceeds (Masino *et al.*, 2011b). To slow down ATXN3 aggregation, strategies have been pursued to increase JD thermodynamic stability and inhibit self-interaction (Masino *et al.*, 2011a; Robertson *et al.*, 2010; Saunders *et al.*, 2011). Interaction of Ub or alpha B-crystallin (αβ-c) with JD potently inhibits JD self-association *in vitro* (Masino *et al.*, 2011a; Robertson *et al.*, 2010). Interestingly, in cells the 2UIM ATXN3 isoform carrying a hydrophobic C-terminal is more prone to aggregate than the 3UIM isoform (Harris *et al.*, 2010).

These results suggest that protein-protein interactions involving the JD and C-terminus of ATXN3 may help prevent ATXN3 aggregation *in vivo* and thereby preserve its enzymatic function. Such interactions might explain why ATXN3 aggregates are not seen in normal individuals despite the propensity of nonpathogenic ATXN3 to aggregate *in vitro*. Because aggregation of *mutant* ATXN3 is a pathological hallmark of MJD, it will be even more important to understand the fibrillization pathway of mutant ATXN3 and identify potential altered intermolecular interactions that modulate the formation of insoluble aggregates.

4.3.2. Proteolysis—Caspases and calpains have been reported to cleave ATXN3 at specific sites, both in cell lines and in animal models (Berke *et al.*, 2004; Colomer Gould *et al.*, 2007; Goti *et al.*, 2004; Haacke *et al.*, 2006; Haacke *et al.*, 2007; Jung *et al.*, 2009; Mauri *et al.*, 2006; Wellington *et al.*, 1998). Caspase-1 and caspase-3 cleave ATXN3 *in vitro* (Wellington *et al.*, 1998) but in cell lines undergoing apoptosis ATXN3 is cleaved mainly by caspase-1, resulting in the release of a polyQ-containing fragment (Berke *et al.*, 2004). Apoptotic cleavage of ATXN3 is abolished by mutating all nine potential caspase recognition sites in ATXN3 and is markedly reduced by mutating a cluster of aspartate residues within UIM2 (D241/244/248) (Berke *et al.*, 2004). Evidence in *Drosophila* shows that proteolytic processing of ATXN3 is conserved across species and may be caspase-dependent (Jung *et al.*, 2009).

The role of calpains in ATXN3 proteolysis is unsettled, given the opposing results in the literature (Berke *et al.*, 2004; Haacke *et al.*, 2007; Jung *et al.*, 2009; Wellington *et al.*, 1998). Some studies suggest that ATXN3 proteolysis is not affected by calpain inhibition (Berke *et al.*, 2004; Jung *et al.*, 2009; Wellington *et al.*, 1998), however another study *in vitro* and in cells showed that calcium-dependent calpains cleave ATXN3 in regions around amino acids 60, 200, 260 and 318 (Haacke *et al.*, 2007). Divergent results regarding calpain-mediated proteolysis of ATXN3 are probably explained by the use of different systems in which protein-protein interactions essential for this process may be affected by cellular environment and overexpression of ATXN3, among other factors.

Though details of ATXN3 cleavage *in vivo* remain uncertain, perhaps multiple proteolytic events can occur as in other polyQ disease proteins, carried out by different proteases acting sequentially or concomitantly. The specific cellular conditions under which normal and mutant ATXN3 are cleaved, and the functional properties of the protein fragments generated, remain to be determined. Further defining the proteolytic processing of ATXN3 is important because a leading hypothesis regarding polyQ-mediated toxicity is the generation of aggregation-prone, "toxic" protein fragments. Thus, implicated proteases could prove to be druggable targets.

4.3.3. Degradation—A relatively long-lived protein, ATXN3 is degraded at least partly by the proteasome (Berke *et al.*, 2005; Jana *et al.*, 2005; Matsumoto *et al.*, 2004). In transfected cells, 3UIM ATXN3, which might also be degraded by macroautophagy, is more stable than 2UIM ATXN3 (Harris *et al.*, 2010) perhaps because additional phosphorylation within UIM3 stabilizes the protein (Mueller *et al.*, 2009). Several E3 ligases and proteasome shuttle proteins are reportedly involved in ATXN3 polyubiquitination and shuttling to the proteasome for degradation, including the E3/shuttle complexes E4B/VCP, CHIP/Hsp70, and E6-AP/Hsp70 (Jana *et al.*, 2005; Matsumoto *et al.*, 2004; Mishra *et al.*, 2008). Gp78, an ER-associated E3, also promotes ATXN3 polyubiquitination and proteasomal degradation (Ying *et al.*, 2009). Interestingly, ATXN3 turnover may also be controlled by its own catalytic activity, though this has not been confirmed in cells expressing physiological levels of the protein (Todi *et al.*, 2007). Once ATXN3 reaches the proteasome its degradation seems to be enhanced by direct binding to the 19S proteasome (Wang *et al.*, 2007). Because ATXN3 is a DUB and participates in protein quality control pathways, its interactions with E3/shuttle complexes might even regulate their function while also affecting ATXN3 turnover.

Wild-type ATXN3 can be degraded by the proteasome, but whether other protein quality control pathways are involved in its turnover remains unknown. Further clarification of ATXN3 degradation pathways might suggest therapeutic strategies to facilitate clearance of the mutant protein in the disease state.

4.4. Normal cellular and physiological role of ATXN3

Identification of ATXN3-interacting proteins and studies in various model systems have shed light on the biological roles of ATXN3. Because knockout models of *ATXN3* orthologs in mouse and *C. elegans* do not display an obvious phenotype, ATXN3 is a nonessential protein (Rodrigues *et al.*, 2007; Schmitt *et al.*, 2007). Conceivably, three other JD-like containing proteins may exert similar functions to ATXN3 and compensate for its absence in knockout models (Todi and Paulson, 2011). Given the diversity of ATXN3's identified protein partners (at least 46) (Table 1) and putative interactors (at least 93) (Araujo *et al.*, 2011; Sowa *et al.*, 2009), ATXN3 likely participates in many cellular pathways.

Multiple lines of evidence implicate ATXN3 in cellular protein quality control, particularly the ubiquitin proteasome system (UPS) responsible for degradation of short-lived and misfolded proteins. ATXN3 may regulate the ubiquitination status of many proteins since total ubiquitinated protein levels are increased in *Atxn3* knockout mouse brain (Scaglione *et al.*, 2011; Schmitt *et al.*, 2007). Overexpression of ATXN3 in polyQ-neurodegeneration models in *Drosophila* suppresses toxicity and cell death, implying that ATXN3 is a neuroprotective protein; its neuroprotective action, moreover, depends both on its DUB activity and proper functioning of the proteasome (Warrick JM *et al.*, 2005). ATXN3 also appears to be involved in the cellular response to heat stress (Reina *et al.*, 2010; Rodrigues *et al.*, 2011).

ATXN3 associates with several E3 ligases, cleaves and edits long polyUb, and participates in substrate delivery to the proteasome by interacting with shuttle proteins (Durcan *et al.*, 2010; Kuhlbrodt *et al.*, 2011; Scaglione *et al.*, 2011; Wang *et al.*, 2006; Zhong and Pittman, 2006). Table 1 lists the E3 ligases and proteasome shuttle factors that interact with ATXN3. ATXN3 interacts via an arginine/lysine motif (aa 277–291) with the AAA ATPase Valosin-Containing Protein or ATPase p97 (VCP/p97) (Boeddrich *et al.*, 2006; Doss-Pepe *et al.*, 2003), which functions coordinately with ubiquitinating complexes to target proteins for proteasomal degradation. The VCP/ATXN3 complex might serve to transfer polyubiquitinated substrates, after editing by ATXN3, directly to the proteasome or to other proteasomal shuttling factors like Ubiquilin/PLIC1 and the Rad23 homologues HHRB23A/B (Doss-Pepe *et al.*, 2003; Heir *et al.*, 2006; Kuhlbrodt *et al.*, 2011; Wang *et al.*, 2000).

The VCP/ATXN3 functional complex also regulates endoplasmic reticulum-associated degradation (ERAD) (Wang *et al.*, 2006; Zhong and Pittman, 2006). Through interaction with ER membrane components including ER-specific E3 ligases, VCP and ATXN3 control the dislocation and degradation of misfolded proteins from the ER (Wang *et al.*, 2006; Zhong and Pittman, 2006). As a scaffold protein, VCP interacts with many adaptors that modulate its activity. SARKS, an ubiquitin regulatory X (UBX) domain-containing protein and VCP adaptor, is able to inhibit VCP/ATXN3-mediated ERAD (LaLonde and Bretscher, 2011). The synergistic cooperation of VCP and ATXN3 in proteostasis is also important in aging as double knockouts in *C. elegans* have a longer lifespan than wild-type worms (Kuhlbrodt *et al.*, 2011). In coordination with VCP and possibly the E3 ligase E4B, ATXN3 also seems to regulate the ubiquitination status and subsequent degradation of components of the insulin/ insulin-like growth factor 1 (IGF1) signaling pathway implicated in lifespan regulation (Kuhlbrodt *et al.*, 2011).

C-terminus of Hsc70 interacting protein (CHIP) is an ATXN3-interacting E3 (Jana *et al.*, 2005) that has been linked to many neurodegenerative diseases. Important insights into the functional interaction between ATXN3 and CHIP have recently been elucidated (Scaglione *et al.*, 2011). ATXN3 is recruited to the ubiquitination complex by monoubiquitinated CHIP, where it then limits the length of polyUb chains formed on substrates and terminates the ubiquitination cycle by removing monoubiquitin from CHIP (Scaglione *et al.*, 2011). Based on these findings, we speculate that ATXN3 could regulate the activity of E3s in a manner similar to the way deneddylases modulate E3 activity in SCF complexes (Scaglione *et al.*, 2011). In fact, ATXN3 also displays deneddylase activity *in vitro*, cleaving isopeptide bonds between a substrate and the Ubl protein neural precursor cell expressed developmentally downregulated 8, or NEDD8 (Ferro *et al.*, 2007). ATXN3-mediated cleavage of monoubiquitinated or neddylated E3s may prove to regulate several ubiquitinating E3 complexes.

Parkin, an E3 implicated in Parkinson disease, also interacts functionally with ATXN3. Parkin undergoes autoubiquitination in vitro, forming mainly K27 and K29-linked polyUb chains which are linked to lysosomal and autophagic degradation. Normal ATXN3 interacts with and deubiquitinates parkin but does not affect its stability (Durcan *et al.*, 2010). It is possible that ATXN3 controls the abundance and edits the architecture of the Ub chains linked to parkin, thereby targeting this protein for specific cellular pathways (Durcan *et al.*, 2010).

When the ubiquitin-proteasome system is compromised or overwhelmed, accumulating proteins become concentrated in perinuclear inclusions called aggresomes. ATXN3 helps regulate the formation of aggresomes in a manner that requires an active DUB site and the UIMS (Burnett and Pittman, 2005). Additional interactions of ATXN3 with other components implicated in aggresome organization, such as dynein, histone deacetylase 6

(HDAC6), protein linking IAP to the cytoskeleton (PLIC1) and microtubules, support the importance of ATXN3 to this cellular process (Burnett and Pittman, 2005; Heir *et al.*, 2006; Mazzucchelli *et al.*, 2009; Rodrigues *et al.*, 2010).

Indeed, ATXN3 is important to cytoskeletal organization and to the formation of focal adhesions (Rodrigues *et al.*, 2010). In the specific case of myoblast differentiation into muscle fibers (myogenesis), ATXN3 is critical to the initial differentiation steps to organize the cytoskeleton and to regulate the levels of integrin subunits and other proteins involved in integrin-mediated signaling (do Carmo Costa *et al.*, 2010). Because ATXN3 interacts with and stabilizes α5 integrin in a DUB activity-dependent manner, it most likely regulates the degradation of this protein through its role in ubiquitin-dependent proteostasis (do Carmo Costa *et al.*, 2010). This basal function of ATXN3 may be common to many cell types.

ATXN3's ability to bind DNA and interact with transcription regulators points toward a role for ATXN3 in transcriptional regulation, most likely as a transcriptional corepressor (Li *et al.*, 2002). Through interaction with cAMP-response element binding (CREB)-binding protein (CBP), p300, and p300/CREBBP associated factor (PCAF), ATXN3 inhibits CREB-mediated transcription (Chai *et al.*, 2001; Li *et al.*, 2002). ATXN3 also regulates histone acetylation, inhibiting p300-mediated histone acetylation and promoting histone deacetylation by interaction with histone deacetylase 3 (HDAC3) and nuclear receptor co-repressor 1 (NCOR1) (Evert *et al.*, 2006; Li *et al.*, 2002). The specific biochemical role of ATXN3 in these reactions, however, is unknown.

Although ATXN3 has been shown to bind a consensus site in DNA (GAGGAA) through a putative basic leucine zipper motif (bZIP) located in its C-terminus (223–270 aa) (Evert *et al.*, 2006), it is still unclear whether ATXN3 functions as a classical repressor. The UPS modulates transcription by regulating chromatin and controlling levels of various transcriptional machinery components, and thus the involvement of ATXN3 in transcriptional regulation might be coupled to its DUB activity. A potential mechanism of action of ATXN3 in transcriptional regulation is to target chromatin by directly binding DNA or histones, which might then favor the ability of ATXN3 to inhibit histone acetylation, recruit corepressors like HDAC3 or NCOR1, and, through its DUB activity, stabilize repressor complexes that enhance histone deacetylation (Evert *et al.*, 2006).

Recently, a specific activity of ATXN3 in transcriptional modulation was suggested by its potential physiological role in response to oxidative stress. ATXN3 interacts with and stabilizes the forkhead box O (FOXO) transcription factor FOXO4, and upon oxidative stress they both translocate to the nucleus and activate manganese superoxide dismutase (SOD2) transcription which in turn protects cells from oxidative damage (Araujo *et al.*, 2011).

In summary, the unique properties of ATXN3 as a DUB suggest that it helps regulate the stability or activity of many proteins in diverse cellular pathways implicated in a wide range of physiological events. It is conceivable that the diverse physiological roles of ATXN3 result not only from its DUB activity, but also from its ubiquitin binding capacity and potential protease activity against other Ub-like proteins such as NEDD8 and SUMO.

5. Mutant ATXN3 and disease pathogenesis

Expansion of the polyQ track likely induces a conformational change in ATXN3 that affects many properties of the protein: stability and degradation, subcellular localization, molecular interactions with other proteins, and propensity to aggregate. These altered properties result in loss and/or gain of function, leading to cellular dysfunction and selective neuronal cell death.

5.1. Dysfunction of mutant ATXN3

Mutant (expanded) ATXN3 still binds K48- and K63-linked polyUb chains, gets activated by ubiquitination, and retains DUB catalytic activity *in vitro* against K48 and K63 chains similarly to normal ATXN3 (Burnett *et al.*, 2003; Todi *et al.*, 2007; Todi *et al.*, 2009; Winborn *et al.*, 2008), but expanded ATXN3 does appear to have an enhanced capacity to deubiquitinate K27- and K29-linked Ub chains (Durcan *et al.*, 2010). Ubiquitination of expanded ATXN3 in cells still occurs at many of the same sites as in normal ATXN3 (Todi *et al.*, 2010). The neuroprotective features of ATXN3 are also at least partly preserved in expanded ATXN3 (Warrick *et al.*, 2005).

Much like normal ATXN3, soluble expanded ATXN3 diffuses rapidly in cytoplasm and nucleoplasm, but unlike normal ATXN3 is highly prone to aggregate, primarily in the nucleus (Chai *et al.*, 2002). The nuclear compartment appears to be the primary site of cellular toxicity in polyQ diseases (Perez *et al.*, 1998). Translocation of both normal and pathogenic ATXN3 from the cytoplasm to the nucleus is mediated by CK2 phosphorylation under basal conditions, and increases in heat-shock and oxidative stress (Mueller *et al.*, 2009; Reina *et al.*, 2010).

Aggregates formed by mutant ATXN3 differ from aggregates generated by normal ATXN3. In contrast to normal ATXN3, mutant ATXN3 undergoes a two-stage aggregation process *in vitro* (Ellisdon *et al.*, 2006). The first stage (formation of SDS-soluble fibrils) is similar to the one occurring for normal ATXN3 but occurs at a faster rate, and the second stage (generation of SDS-insoluble aggregates) occurs through the polyQ segment (Ellisdon *et al.*, 2006). Glutamine side-chain hydrogen bonding in the polyQ track, possibly adopting a β-helical turn or hairpin conformation, contributes to the irreversible aggregation of expanded ATXN3 (Natalello *et al.*, 2011; Perutz *et al.*, 2002; Sikorski and Atkins, 2005). Functional interactions of wild-type ATXN3 with other molecules have been shown to reduce its aggregation propensity and increase solubility (see section 4.3.1). In the case of mutant ATXN3 it is possible that these modifier interactions are lessened, leading to a faster rate of aggregation.

As in other polyQ diseases, the “toxic fragment hypothesis” may apply to MJD. Aggregation of mutant ATXN3 is thought to be enhanced by proteolysis that generates C-terminal fragments containing expanded polyQ tract, which then can act as seeds for aggregation (Goti *et al.*, 2004; Ikeda *et al.*, 1996; Paulson *et al.*, 1997b; Teixeira-Castro *et al.*, 2011). With the exception of one study reporting less proteolysis of expanded ATXN3 than the normal protein (Pozzi *et al.*, 2008), pathogenic and normal ATXN3 are thought to undergo the same types of caspase- or calcium-dependent calpain cleavage (Berke *et al.*, 2004; Haacke *et al.*, 2007; Jung *et al.*, 2009). The stability of generated fragments containing the polyQ track, however, probably differs for normal and expanded ATXN3. For example, ATXN3 C-terminal fragments are not found in brains from healthy humans but are present in brain homogenates from MJD patients and MJD transgenic mice (Goti *et al.*, 2004). These fragments, however, have not been detected in human MJD brains in all studies (e.g., Berke *et al.*, 2004), possibly reflecting the use of different antibodies. C-terminal ATXN3 fragments cleaved proximal to amino acid 190 and containing the UIMs, expanded polyQ and NLS, are more abundant in the nuclear fraction from affected brain regions of MJD patients (Colomer Gould *et al.*, 2007; Goti *et al.*, 2004). Nuclear accumulation of these fragments may be due less to the presence of a weak NLS than to the absence of NES signals and, consequently, escape from chaperone-mediated clearance by the UPS in the cytoplasm (Antony *et al.*, 2009; Breuer *et al.*, 2010). The generation of C-terminal polyQ-containing fragments not only favors aggregation but also eliminates the putative protective action of the JD which is cleaved away by proteolysis (Warrick *et al.*, 2005).

At least two E3s, parkin and the mitochondrial ubiquitin ligase MITOL, are reportedly involved in the proteasomal degradation of mutant ATXN3 C-terminal fragments (Sugiura *et al.*, 2010; Tsai *et al.*, 2003). Though, the mode of action of MITOL with full-length ATXN3 is unknown, parkin seems not to be involved in degradation of full-length wild-type or mutant ATXN3 (Durcan *et al.*, 2010). Like normal ATXN3, expanded ATXN3 is polyubiquitinated and degraded by the UPS (Jana *et al.*, 2005; Matsumoto *et al.*, 2004; Mishra *et al.*, 2008; Wang *et al.*, 2009; Ying *et al.*, 2009) but may have a longer half-life (Matsumoto *et al.*, 2004). While it is still unknown if wild-type ATXN3 is appreciably degraded by macroautophagy, some evidence suggests that mutant ATXN3 can be degraded by this protein quality control pathway (Berger *et al.*, 2006). Furthermore, pathogenic ATXN3 induces autophagy in a *Drosophila* model (Bilen and Bonini, 2007).

Increased stability of expanded ATXN3 might be explained by enhanced binding to VCP, thereby delaying its release from the VCP/E4B complex for subsequent proteasomal degradation (Boeddrich *et al.*, 2006; Matsumoto *et al.*, 2004). The stronger interaction between expanded ATXN3 and VCP may also impair ERAD and other cellular processes that depend on this functional interaction (Zhong and Pittman, 2006).

Expanded ATXN3 binds with higher affinity to CHIP and may target CHIP for degradation (Scaglione *et al.*, 2011). Though normal and expanded ATXN3 bind similarly to polyubiquitinated parkin, pathogenic ATXN3 shows increased DUB activity towards polyUb-parkin, promoting its degradation via autophagy (Durcan *et al.*, 2010). Consistent with these findings, CHIP and parkin levels are decreased in brains of transgenic mice expressing expanded ATXN3 (Durcan *et al.*, 2010; Scaglione *et al.*, 2011).

Mutant ATXN3 displays altered DNA binding which reduces its ability to form deacetylase complexes and repress transcription of target genes (Evert *et al.*, 2006; Li *et al.*, 2002). Another example of ATXN3-mediated transcriptional regulation in which expanded ATXN3 is impaired is its ability to promote FOXO4-mediated *SOD2* transcription during oxidative stress (Araujo *et al.*, 2011).

In summary, mutant ATXN3 appears to be dysfunctional at several levels: (i) it forms insoluble aggregates; (ii) it is susceptible to proteolysis resulting in the generation of expanded polyQ-containing ATXN3 fragments that favor aggregation, primarily in the nucleus; (iii) and it interacts abnormally with at least some of its native partners, impeding its own degradation and leading in some cases to a gain-offunction and in other cases to partial loss of normal ATXN3 function. Examples of gain-of-function of mutant ATXN3 are insoluble aggregate formation and induction of parkin degradation. Conversely, ATXN3's reduced ability to form deacetylating repressor complexes at target genes and to function as an activator of FOXO4-mediated *SOD2* transcription exemplify partial loss of function of expanded ATXN3. As has been well established for SCA1 (Lim *et al.*, 2008; Zoghbi and Orr, 2009), it will be interesting to determine whether ATXN3 dysfunction induced by polyQ expansion includes a partial loss of wild-type ATXN3 function.

5.2. Animal models of ATXN3 overexpression

Many animal models overexpressing specific forms of ATXN3 are available to study the molecular and phenotypic aspects of MJD (Table 2). Models recapitulating aspects of disease exist in mice, rats, flies and worms.

Most *in vivo* MJD models are stable transgenic lines that show highly reproducible features over time. Known for their ease of maintenance and genetic manipulation, several invertebrate models of ATXN3 overexpression in *Drosophila* and *C. elegans* have provided important insights regarding the pathogenic mechanisms involving aggregation, proteolysis

and toxicity of expanded ATXN3, as well as the apparent neuroprotective role of wild-type ATXN3 (Jung *et al.*, 2009; Khan *et al.*, 2006; Teixeira-Castro *et al.*, 2011; Warrick *et al.*, 2005; Warrick *et al.*, 1998). These models have also proved to be important tools for screening potential therapeutic molecules and genetic modifiers of disease.

Albeit more laborious to generate and less genetically manipulable than invertebrate models, rodent models share important molecular, anatomical and physiological similarities with humans. Numerous transgenic mouse models of MJD have been generated with the aim of mimicking general clinical and molecular aspects of the human disease or of studying a specific hypothesis regarding disease pathogenesis (Table 2).

With the exception of a mouse model generated by genomic integration of the entire human *ATXN3* gene housed in a yeast artificial chromosome (YAC) (Cemal *et al.*, 2002), the transgene employed in most mouse models has been a complementary DNA (cDNA) encoding a particular isoform of ATXN3 driven behind a foreign promoter (Table 2). Resulting from DNA random integration, these models differ from each other partly with respect to the copy number of integrated transgenes. Severity of MJD-like symptoms and pathology in the mouse model is generally proportional to the expression levels of mutant ATXN3 in MJD transgenic mice, with homozygous mice displaying a more pronounced phenotype than heterozygous mice, similar to the case in MJD patients. Table 2 describes the major pathological and phenotypic presentations for each mouse model.

Different mouse models expressing full-length ATXN3 isoforms are currently available. Selective expression of mutant ATXN3 in Purkinje cells did not have an apparent deleterious effect in the first generated MJD mouse model, which might have been expected as these cells are not primarily affected in MJD patients (Ikeda *et al.*, 1996). In contrast, three models expressing a pathogenic ATXN3 isoform in the CNS under the direction of the prion protein (Prnp) promoter present relatively early onset motor dysfunction with NNI, although there is little neurodegeneration (Bichelmeier *et al.*, 2007; Chou *et al.*, 2008; Goti D *et al.*, 2004; Goti *et al.*, 2004). Interestingly, the use of the rat huntingtin (Htt) promoter to direct expression of mutant ATXN3 in brain more closely recapitulates the human disease, presenting late onset symptoms, intranuclear inclusions and significant neurodegeneration (Boy *et al.*, 2010). Aiming to reproduce the ubiquitous pattern of ATXN3 expression, an additional model was generated in which the cytomegalovirus (CMV) promoter directs expression of an expanded 3UIM ATXN3 isoform (Silva-Fernandes *et al.*, 2010). These transgenic mice show relatively early onset, non-progressive, mild motor incoordination together with ATXN3-positive cytoplasmic puncta and neurodegeneration in several brain regions affected in MJD patients (Silva-Fernandes *et al.*, 2010).

Other models expressing an ATXN3 fragment or modified versions of ATXN3 have been generated to study specific aspects of disease pathogenesis. Models generated to study the potential toxicity of an ATXN3 C-terminal fragment showed that its expression in Purkinje cells resulted in the formation of intranuclear inclusions and a severe neurodegenerative phenotype in mice (Ikeda H *et al.*, 1996; Torashima *et al.*, 2008). Transgenic mice expressing ATXN3 lacking the protein segment between amino acid residues 190 and 220 revealed that the potential major proteolytic cleavage site on ATXN3 is N-terminal of amino acid residue 190 (Colomer Gould *et al.*, 2007). And mice expressing ATXN3 with additional NLS or NES signals established that mutant ATXN3 is more toxic in the nucleus (Bichelmeier *et al.*, 2007).

While some of the above models recapitulate aspects of the human disease, they all overexpress a single isoform of ATXN3 under the control of an exogenous promoter. In this respect, the YAC MJD transgenic model more closely mirrors what happens in human MJD

because all elements of the *ATXN3* gene are present, including the 5' and 3' regulatory regions (Cemal *et al.*, 2002). As a result, all potential ATXN3 protein isoforms can be expressed. The YAC MJD84.2 transgenic mice also reproduce certain MJD-like features includingearly onset motor incoordination, cytoplasmic and nuclear ATXN3 aggregates, and neurodegeneration in later stages (Cemal *et al.*, 2002; Chen *et al.*, 2008; Shakkottai *et al.*, 2011).

Some properties associated with the expanded CAG repeat in MJD are also replicated in the YAC MJD mice and two cDNA models, HDpromMJD148 and CMVMJD94 (Boy *et al.*, 2010; Cemal *et al.*, 2002; Silva-Fernandes *et al.*, 2010). Similar to MJD patients, in one or more of these models the CAG repeat results in: (i) intergenerational repeat instability, tending to expand upon paternal transmission and contract upon maternal transmission (Boy *et al.*, 2010; Cemal *et al.*, 2002; Silva-Fernandes *et al.*, 2010); (ii) somatic mosaicism, although expansions in repeat length are not preferentially associated with affected brain regions (Silva-Fernandes *et al.*, 2010); and (iii) a direct correlation between repeat length and disease severity (Silva-Fernandes *et al.*, 2010).

A conditional mouse model using the Tet-off system was generated to test whether disease symptoms, once manifest, can be reversed by switching off expression of mutant ATXN3 (Boy *et al.*, 2009). Though expanded ATXN3 is mainly expressed in glial cells in this model, the mice do present phenotypic and pathological MJD-like features that are indeed rescued once the transgene is turned off (Boy *et al.*, 2009). While this result indicates that reducing levels of pathogenic ATXN3 could be a promising strategy to treat SCA3, it will be important to confirm the reversibility of disease features in an equivalent inducible model expressing ATXN3 in neurons.

Finally, the injection of lentivirus expressing human ATXN3 into rat brains reproduces several aspects of MJD neuropathology and provides a very useful tool to study disease pathogenesis in specific brain regions (Alves *et al.*, 2008a; Alves *et al.*, 2008b), although there are some inherent limitations to acute overexpression models.

The importance of having many different animal models of MJD cannot be overestated, as collectively they advance our understanding of many different aspects of MJD and disease pathogenesis. Currently, some models are useful to study the mechanisms of disease progression, others display early MJD-like signs and thus allow for testing therapeutic approaches, and still others serve to test specific hypotheses about pathomechanisms. While the higher severity typically observed in homozygous models probably reflects increased levels of mutant ATXN3, we need to keep in mind the possibility that silencing an endogenous gene at the site of transgene integration could confound analysis. Despite their unquestioned value, the models described above are all overexpression models. Hence, the observed physiological signs could result, in part, from cellular overload of an exogenous protein that is unrelated to the intrinsic pathogenicity of mutant ATXN3. The generation of a knock-in mouse model expressing murine ATXN3 with a polyQ expansion under the control of its endogenous regulatory regions would be a welcome addition to the collection of MJD models, as it should represent a genetically and physiologically accurate model of the human disease.

5.3. Pathogenic mechanisms

While the precise pathogenic mechanism triggered by CAG repeat expansion in the *ATXN3* gene in MJD patients remains unknown, numerous *in vitro* and *in vivo* studies have begun to shed light on the problem. Although a hyperexpanded CAG repeat RNA transcript is toxic and causes degeneration in a *Drosophila* model of MJD (Li *et al.*, 2008), most evidence suggests that the key toxic species is instead the mutant ATXN3 protein with its polyQ

expansion. Expanded ATXN3 is thought to undergo conformational changes and acquire toxic properties, either as a monomer or as part of oligomeric/ aggregate species, resulting in altered molecular interactions. The fact that the majority of suppressors of MJD toxicity affect protein misfolding and protein quality control pathways (Table 3) supports the view that altered conformation and protein misfolding are central to the disease process.

How does mutant ATXN3 cause cellular dysfunction and cell death? As discussed below, several hypotheses have been put forth as the potential toxic mechanism triggered by misfolded mutant ATXN3 and its altered protein interactions: (i) formation of aggregates; (ii) failure of cellular protein homeostasis; (iii) impairment of axonal transport; (iv) transcriptional dysregulation; (v) mitochondrial dysfunction and oxidative stress; and (vi) abnormal neuronal signaling. These hypotheses are not mutually exclusive.

Neuronal inclusions formed by mutant ATXN3 – both NNIs and NCIs (described in section 2.2) - represent pathological hallmarks of MJD. Whether intraneuronal inclusions formed by polyQ disease proteins are directly toxic, however, has been hotly debated. When first discovered, inclusions in human MJD brain were speculated to mediate neurodegeneration as their abundance correlated with CAG repeat size and disease severity. Moreover, the inclusions also contain several key proteins including ubiquitin, proteasomal components, chaperones, transcription factors, and wild-type ATXN3 suggesting that various cellular pathways might be depleted of crucial components (Chai *et al.*, 1999a; Chai *et al.*, 1999b; Mori *et al.*, 2005; Paulson *et al.*, 1997b; Schmidt *et al.*, 1998; Takahashi *et al.*, 2001). Neuronal inclusions, however, do not correlate directly with degeneration and are currently viewed instead as biomarkers of cellular failure to clear mutant ATXN3.

As direct toxicity of large inclusions appears doubtful, what is the toxic species in MJD? Because misfolded β-rich polyQ protein monomers and oligomers are toxic to cells (Nagai *et al.*, 2007) and a conformational change from α to β structure is observed in ATXN3 during its aggregation, β-rich ATXN3 monomers and oligomers may exist and be toxic to cells in MJD (Masino *et al.*, 2011b). Efforts to detect and identify such species in brains of MJD patients and animal models of disease should help elucidate the aggregation process of ATXN3 and might support a strategy of targeting ATXN3 misfolding and early oligomerization as potential therapy for MJD.

Cells have different protein quality control systems to clear misfolded proteins and maintain cellular homeostasis. Indeed, molecular chaperones, the UPS and autophagy have all been implicated in the refolding and clearance of mutant ATXN3 (Berger *et al.*, 2006; Chai *et al.*, 1999a; Chai *et al.*, 1999b). A study in a mouse model revealed a failure of some proteostasis systems in later stages of disease but before appreciable cell death (Chou *et al.*, 2008). At earlier stages of disease, pathogenic ATXN3 induces cellular stress pathways, resulting in increased expression of certain molecular chaperones that are known to suppress expanded ATXN3 toxicity by facilitating its folding and decreasing aggregates (Bilen and Bonini, 2007; Chai *et al.*, 1999a; Chou *et al.*, 2008; Huen and Chan, 2005; Warrick *et al.*, 1999). Consistent with this, heat-shock factor 1 (HSF1) was recently shown to have an early protective role by decreasing protein aggregation in *C. elegans* models of MJD (Teixeira-Castro *et al.*, 2011). In later stages of disease in MJD transgenic mice, however, there is downregulation of HSP70 and HSP40 which would impair the ability of neurons to handle the cellular stress caused by mutant ATXN3 (Chou *et al.*, 2008; Huen and Chan, 2005). This depletion of chaperones was recently extended to the small heat-shock protein HSP27 in cellular models of MJD (Chang *et al.*, 2009). Pathogenic ATXN3 may have an equivalent effect on autophagy as disease progresses, because this clearance pathway is induced in MJD *Drosophila* larvae but is depleted in brains of MJD patients and transgenic mouse models (Bilen and Bonini, 2007; Nascimento-Ferreira *et al.*, 2011). It will be important to

determine whether this decrease in authophagic function happens before neuronal loss. Interestingly, proteasomal protein degradation does not seem to be compromised in cellular and *Drosophila* models of MJD (Berke *et al.*, 2005; Bilen and Bonini, 2007; Chai *et al.*, 1999b). Thus, the decrease of some cellular protein clearance pathways correlates with the accumulation of mutant ATXN3 with aging, implying an impairment of cellular protein homeostasis. This impairment of proteostasis (at least of the chaperone machinery) supports the view that neuronal dysfunction precedes cell loss in MJD.

Possibly as a byproduct of a failure in cellular homeostasis, ATXN3 aggregates are found not only in the soma but also in fiber tracts known to undergo neurodegeneration in MJD patients (Seidel *et al.*, 2010). In *C.elegans* and *Drosophila* models of MJD, mutant ATXN3 aggregates induce swelling and aberrant branching of neuronal processes, which impairs synaptic transmission (Gunawardena and Goldstein, 2005; Khan *et al.*, 2006). ATXN3 interacts with cytoskeletal components and is important for cytoskeletal organization, thus it might normally play a role in cytoskeletal transport that becomes dysregulated in mutant ATXN3 (Burnett and Pittman, 2005; do Carmo Costa *et al.*, 2010; Rodrigues *et al.*, 2010). Insights from these studies suggest that axonal dysfunction may play a role in MJD pathogenesis and may explain why MJD patients often have motor neuropathy or neuronopathy.

Abnormal interactions of mutant ATXN3 with its native protein partners, or novel interactions with new partners, may be a recurring theme underlying many of the potential pathogenic mechanisms in MJD. Numerous interactors of wild-type ATXN3 have been idenitified, but little is known about their relative interaction with mutant ATXN3. For example, expanded ATXN3 interacts differently with two native partners, CHIP and parkin, which may contribute tothe depletion of these neuroprotective proteins in models of MJD (Durcan *et al.*, 2010; Scaglione *et al.*, 2011). Because ATXN3 is implicated in ubiquitin and protein quality control pathways, aberrant interactions of mutant ATXN3 could, in principle, lead to altered stability of a numerous substrate proteins including critically important, cell-specific factors. This could help explain selective neurodegeneration in MJD. Comparative analysis of the behavior of wild-type versus mutant ATXN3 in specific protein complexes will likely be crucial to a full understanding of cellular dysfunction in MJD.

Aberrant interactions of mutant ATXN3 also could influence its activity as a putative transcriptional regulator. Upon polyQ expansion, the action of ATXN3 as a transcriptional corepressor or activator seems to be compromised (Araujo *et al.*, 2011; Evert *et al.*, 2006). Altered interactions with transcription factors and co-activators and the recruitment of certain transcription factors to polyQ protein aggregates are common themes in the polyQ diseases, suggesting that transcriptional dysregulation contributes to disease pathogenesis in MJD and other polyQ disorders. Transcriptome analyses in a neuronal cell model of MJD and cerebella from transgenic MJD mice revealed altered transcription of many genes (Chou *et al.*, 2008; Evert *et al.*, 2001; Evert *et al.*, 2003). Mutant ATXN3 leads to downregulation of genes involved in glutamatergic neurotransmission, intracellular calcium signaling/mobilization or MAP kinase pathways, $GABA_{A/B}$ receptor subunits, HSPs, and transcription factors regulating neuronal survival and differentiation (Chou *et al.*, 2008). Conversely, upregulated genes include ones that are involved in neuronal cell death and inflammation (Chou *et al.*, 2008; Evert *et al.*, 2001; Evert *et al.*, 2003). As posttranslational modifications including ubiquitination are known to regulate transcription, an alteration in the DUB activity of mutant ATXN3 could contribute to cell-specific dysregulation of target genes.

One recently described example is the reduced ability of expanded ATXN3 to activate FOXO4-mediated *SOD2* transcription thereby leading to increased cellular susceptibility to oxidative stress (Araujo *et al.*, 2011). Interestingly, levels of HSP27, which also has anti-

oxidant features, are decreased in MJD suggesting that, as in other polyQ diseases, the cellular defenses to reactive oxygen species (ROS) may be depleted in this disease (Chang *et al.*, 2009). In fact, some cellular models of MJD show decreased antioxidant enzyme activity and increased mitochondrial-mediated cell death via apoptosis (Tsai *et al.*, 2004; Yu *et al.*, 2009). However, question of apoptosis-mediated cell death in MJD is controversial as it isnot activated in several other cellular and mouse MJD models (Evert *et al.*, 1999; Silva-Fernandes *et al.*, 2010). Nonetheless, the view that mitochondrial damage and increased oxidative stress contribute to disease has gained strength recently as a potential pathomechanism in MJD.

The upregulation of inflammatory genes in cells expressing expanded ATXN3 was confirmed by detection of neuroinflammatory markers in the pons of MJD patients, suggesting that glia contribute to MJD pathogenesis (Evert *et al.*, 2001; Evert *et al.*, 2003). Neuroinflammation, however, may not be activated until later stages of disease as it was not detected in early stages of disease in a transgenic mouse model of MJD (Silva-Fernandes *et al.*, 2010). Although there is evidence that glial cells expressing expanded ATXN3 can alter Purkinje cell function in another MJD transgenic mouse model (Boy *et al.*, 2009), the precise role of glia in the pathogenic mechanism of MJD is largely unexplored. Systematic temporal and functional analysis of the several types of glial cells in brains from MJD patients and MJD mouse models will help to understand their specific role during the development of this disease.

Consistent with the downregulation of genes involved in intracellular calcium signaling/mobilization and MAP kinase pathways (Chou *et al.*, 2008), neurons expressing mutant ATXN3 display a decrease in intracellular Ca^{2+} through an abnormal association with the type 1 inositol 1,4,5-triphosphate receptor (InsP3R1) (Chen *et al.*, 2008). Indeed, as in other polyQ diseases, the electrophysiological properties of neurons are altered in MJD. In addition to impaired calcium signaling (Chen *et al.*, 2008), potassium channel dysfunction and depolarized resting membrane potential were observed in neuronal cells expressing mutant ATXN3 (Jeub *et al.*, 2006). Consistent with this finding, in a mouse model of MJD we recently identified robust early changes in cerebellar Purkinje neuron firing that are associated with altered kinetics of voltage-activated potassium (Kv) channels (Shakkottai *et al.*, 2011). Importantly, the silencing of repetitively firing Purkinje cells in MJD transgenic mice occurs at an early stage of disease when mice display motor incoordination but have not yet manifested any neuronal degeneration (Shakkottai *et al.*, 2011). In parallel studies in transfected cells, mutant ATXN3 affected slightly the kinetics of Kv3 and Kv1 channels, suggesting that early Purkinje cell dysfunction in MJD mice may reflect a direct effect of ATXN3 on channel activity (Shakkottai *et al.*, 2011). Clearly, further studies are needed to explore potential electrophysiological changes in other affected neuronal populations in MJD, but early evidence suggests that altered neuronal physiology contributes to MJD pathogenesis and likely underlies some motor symptoms before neuronal cell death has begun to occur (Shakkottai *et al.*, 2011). Understanding the role of altered neuronal physiology in MJD and other polyQ diseases may reveal new, potentially shared therapeutic targets for these incurable neurodegenerative diseases.

In summary, mutant ATXN3 misfolding and its altered molecular interactions with numerous proteins may well trigger a series of interrelated pathogenic cascades in MJD. In a slowly progressive chronic disease extending over decades and involving various types of cells within the brain, it may be unrealistic to expect a single mechanism to emerge as *the* cause. Even though much has already been revealed about MJD pathogenesis, much more needs to be known before we will clearly understand the molecular events driving this process.

6. Development of MJD therapeutics

MJD and the other polyQ diseases are currently untreatable. Despite the absence of preventive treatment, many symptoms of disease can be treated using pharmacological and nonpharmacological measures. Details about symptomatic therapy and clinical trials in MJD can be found in several recent reviews (Bettencourt and Lima, 2011; Paulson, 1998 Oct 10 [updated 2011 March 17]).

Seeking a preventive therapy, MJD researchers have explored pharmacological and genetic approaches, both of which have shown some promise. As described above, efforts are also being made to generate animal models of disease with robust phenotypes that will facilitate therapeutic testing and further advance our understanding of the pathogenic mechanisms in MJD (see section 5). Silencing strategies that target the causative RNA or disease protein have the advantage of hitting proximally in pathogenic cascade and thus not requiring a detailed understanding of downstream mechanisms, which remain unclear in MJD. On the other hand, approaches targeting crucial events in disease pathogenesis may be more potent but depend on accurate knowledge of the central pathogenic pathways and epiphenomena elicited by a specific mutation. Both of these strategies have been explored in the development of potential therapies for MJD.

The fact that phenotypic and pathological features of disease could be reversed In a conditional mouse model of MJD by switching off the transgene indicates that strategies targeting RNA or protein clearance might be useful as future preventive therapies for patients even after symptoms and signs of disease have begun (Boy *et al.*, 2009). Current nucleotide-based gene suppression strategies (e.g. RNAi and antisense molecules) can target specific genes with high specificity, mediating specific suppression of alleles differing by as little as one or a few base pairs. Hence, a gene silencing strategy in MJD is within the realm of possibility.

Silencing *ATXN3* transcripts by RNA interference (RNAi) has been shown to mitigate degeneration in a rat model of MJD (Alves *et al.*, 2008a; Alves *et al.*, 2010). This rescue occurred in a nonallele-specific manner as intracerebral viral-injections of short hairpin RNAs (shRNAs) specific or nonspecific for the mutant *ATXN3* transcript showed similar effects (Alves *et al.*, 2008a; Alves *et al.*, 2010). Thus, silencing of wild type *ATXN3* along with mutant *ATXN3* appears not to be deleterious, at least in this model, which concurs with the lack of neurodegeneration in *Atxn3* knockout mice (Schmitt *et al.*, 2007). Other types of RNA-targeting molecules such as peptide nucleic acid (PNA) and locked nucleic acid (LNA) antisense oligomers also have been shown to be effective in reducing ATXN3 levels in fibroblasts from MJD patients and constitute promising molecules to test *in vivo* (Hu *et al.*, 2011; Hu *et al.*, 2009).

Targeting mutant ATXN3 clearance is also a promising approach toward therapy. Pharmacological induction of autophagy using a rapamycin ester (temsirolimus) increases mutant ATXN3 degradation, reduces the number of aggregates, and improves the motor phenotype in a mouse model of MJD (Menzies *et al.*, 2010). As temsirolimus has been designed for long-term use in patients, it represents a potential therapy for MJD. Additional evidence supporting autophagy induction as a viable therapeutic target is the fact that lentiviral-mediated overexpression of the autophagy protein beclin-1 increases clearance of mutant ATXN3 and decreases aggregates in a MJD rat model (Nascimento-Ferreira *et al.*, 2011). Thus, protein quality control systems involved in mutant ATXN3 degradation represent promising therapeutic targets in MJD.

Though much remains to be learned about the pathogenic mechanisms of MJD, several key molecular events in pathogenesis including aggregate formation, transcriptional

dysregulation and abnormal neuronal signaling, have been targeted in the development of potential therapies. Additionally, several genetic modifiers of mutant ATXN3 toxicity have been identified which themselves represent potential targets (Table 3).

Mutant ATXN3 aggregation has been successfully decreased by treatment of *Drosophila* and *C. elegans* models of MJD with 17-(allylamino)-17-demethoxygeldanamycin (17-AAG), an HSP90 inhibitor (Fujikake *et al.*, 2008; Teixeira-Castro *et al.*, 2011). 17-AAG also improved locomotor activity in MJD transgenic *C.elegans* (Teixeira-Castro *et al.*, 2011). Y-27632, an inhibitor of Rho kinases, also decreased soluble and insoluble expanded ATXN3 in cells (Bauer *et al.*, 2009).

Mutant ATXN3-mediated transcriptional downregulation and H3/H4 histone hypoacetylation has been significantly reversed by treating MJD transgenic mice with sodium butyrate, a HDAC inhibitor (Chou *et al.*, 2010) with a corresponding improvement in motor performance (Chou *et al.*, 2010). Recently, treatment of MJD transgenic *C.elegans* with valproic acid, another HDAC inhibitor, also led to improved locomotor activity accompanied by a decrease in aggregates (Teixeira-Castro *et al.*, 2011). Therefore, compounds that promote histone acetylation over deacetylation may hold promise as preventive therapy in MJD.

Another therapeutic strategy is to use pharmacological approaches to correct abnormal neuronal signaling. The use of dantrolene to stabilize intracellular Ca2+ signaling in MJD transgenic mice resulted in improved motor phenotype and reduced neuronal loss (Chen *et al.*, 2008). And finally, short term administration of an activator of calcium-activated potassium channels, SKA-31, partially corrected Purkinje neuronal firing and improved motor function in MJD transgenic mice (Shakkottai *et al.*, 2011).

In conclusion, there have been several promising developments in the search for therapies for MJD. Agents targeting different molecular events thought to contribute to disease pathogenesis have shown some success in animal models of MJD, though none has yet been advanced to the point of clinical trials in MJD patients. It may be necessary to develop safer, and more efficacious, second generation molecules with reduced toxicity or adverse effects and enhanced delivery to the CNS. Pre-clinical trials of promising molecules using gene delivery or pharmacological approaches in mammalian models of MJD will be crucial for the eventual, successful generation of an effective treatment for MJD.

7. Final remarks

This year marks the 20th anniversary of an historical moment in human genetics: the discovery that heritable human diseases can be caused by dynamic repeat mutations. In 1991, expanded CCG and CAG repeat sequences were reported as the molecular basis of Fragile X syndrome and SBMA, respectively (Kremer *et al.*1991; LaSpada *et al.* 1991). Soon afterward, expansion of polyQ-encoding CAG repeats was revealed to be the cause of the largest class of dynamic repeat disorders, the polyQ neurodegenerative diseases, of which MJD is one of at least ten. In the 17 years since the discovery of the MJD disease gene, many advances have taken place both with respect to the disease itself and to the underlying pathomechanism. ATXN3, the MJD protein, is now known to be a highly specialized DUB that participates in several ubiquitin-related cellular pathways. This knowledge of ATXN3 function, together with the development of a wide range of cellular and animal models of MJD, has greatly advanced our understanding of disease pathogenesis in MJD. This progress notwithstanding, much more needs to be learned before we have a firm grasp of the molecular mechanisms driving neuronal dysfunction and neuronal cell death in MJD. As is true for all polyQ diseases, clarifying the normal functions of ATXN3 and defining the ways it is dysfunctional in the disease state are critical steps toward a full

understanding of disease mechanisms. Several potential pathogenic pathways triggered by expanded polyQ proteins are shared among the polyQ diseases. Thus, there is the potential for class-wide therapeutic strategies to disease prevention; indeed, several therapeutic agents along these lines have shown some success in animal models of MJD. At the same time, the search is on for disease-specific strategies that target proximal steps in the pathogenic cascade (e.g. drugs or nucleotide-based approaches to reduce levels of the *ATXN3* transcript or disease protein) or target pathways implicated specifically in MJD further downstream in the cascade (e.g. pharmacological agents that alter ion channel dysfunction, as recently described in MJD). To date, no potential preventive strategies have been tested in MJD patients. But it is our hope that the remarkable, recent advances in MJD research will open the door to such clinical trials soon.

Article Highlights

- MJD is a neurodegenerative disease caused by polyQ expansion in ATXN3
- ATXN3 is a deubiquitinating enzyme participating in ubiquitin-mediated proteostasis
- PolyQ expansion in ATXN3 triggers cellular dysfunction and selective neuronal death
- Improved understanding of disease mechanisms is suggesting routes to therapy

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Abbreviations

ATXN3	ataxin-3
bZIP	basic leucine zipper motif
CBP	CREB binding protein
cDNA	complementary DNA
CHIP	C-terminus of Hsc70 interacting protein
CK2	casein kinase 2
CMV	cytomegalovirus
CNS	central nervous system
CREB	cAMP-response element binding
DRPLA	Dentatorubral pallidoluysian atrophy
DUB	deubiquitinating enzyme
ERAD	endoplasmic reticulum-associated degradation
FOXO	forkhead box O
HD	Huntington disease
HDAC	histone deacetylase

HDL2	Huntington Disease-like 2
HSF1	heat-shock factor 1
HSP	heat-shock protein
Htt	huntingtin
IGF1	insulin/ insulin-like growth factor 1
JD	Josephin domain
Kv	voltage-activated potassium
LNA	locked nucleic acid
MITOL	mitochondrial ubiquitin ligase
MJD	Machado-Joseph disease
MRI	magnetic resonance imaging
NCIs	neuronal cytoplasmic inclusions
NCOR1	nuclear receptor co-repressor 1
NEDD8	neural precursor cell expressed developmentally downregulated 8
NES	nuclear export signal
NLS	nuclear localization signal
NNIs	neuronal nuclear inclusions
p97	ATPase p97
PCAF	p300/CREBBP associated factor
PLIC1	protein linking IAP to the cytoskeleton
PNA	peptide nucleic acid
polyQ	polyglutamine
Prnp	prion protein
RNAi	RNA interference
ROS	reactive oxygen species
SCA	Spinocerebellar ataxia
SBMA	Spinal bulbar muscular atrophy
shRNAs	short hairpin RNAs
SNPs	single nucleotide polymorphisms
SOD	superoxide dismutase
STRs	single tandem repeats
SUMO	small ubiquitin-like modifier
Ub	ubiquitin
Ubl	ubiquitin-like
UIM	ubiquitin interacting motif
UPS	ubiquitin proteasome system

UTR	untranslated region
VCP	valosin containing protein
YAC	yeast artificial chromosome
17-AAG	17-allylamino-17-demethoxygeldanamycin

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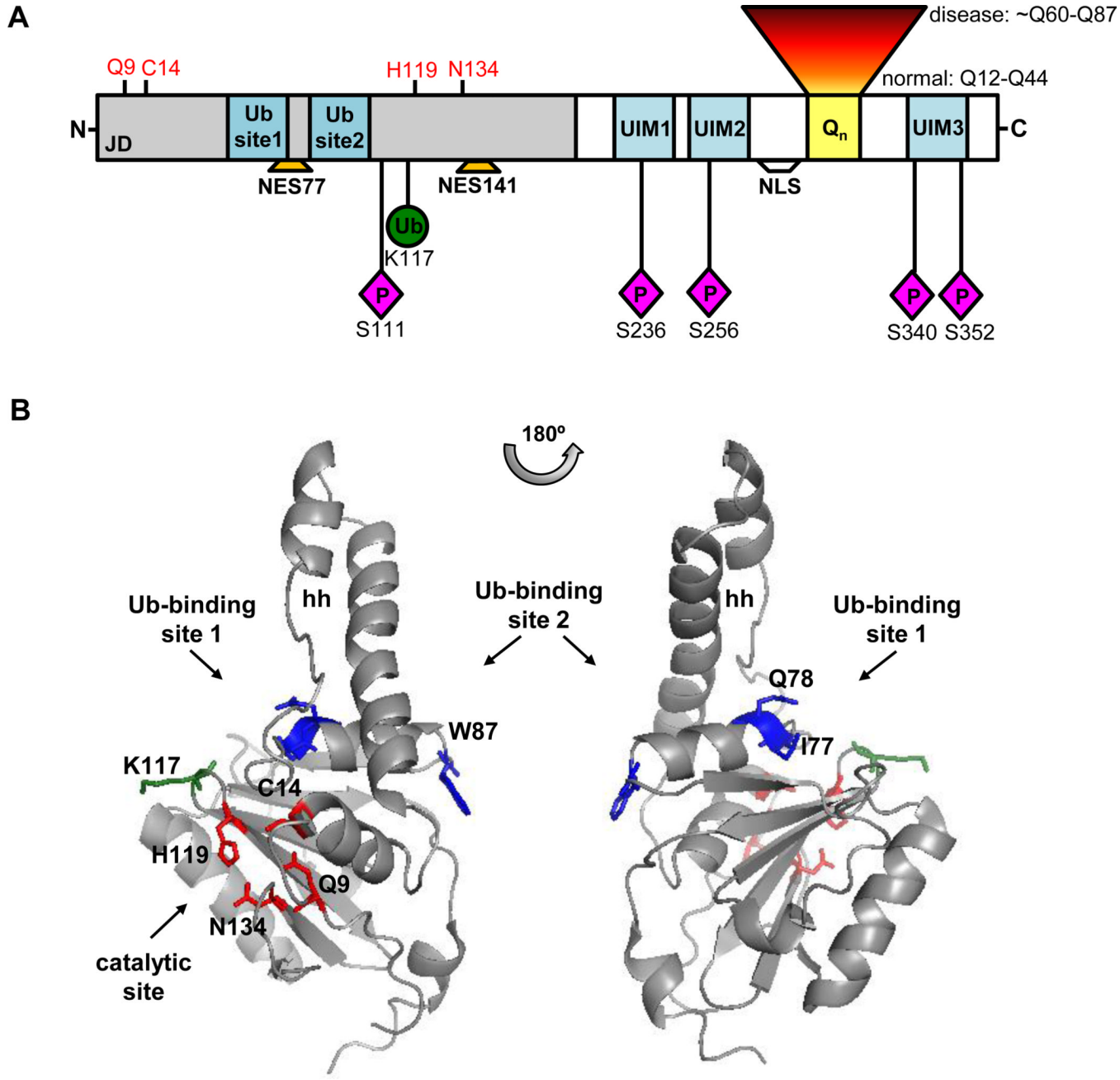

Figure 1.

Functional domains and posttranslational modifications of ATXN3. A) ATXN3 contains a N-terminal DUB catalytic domain (Josephin domain, JD) centered on residues Q9, C14, H119, and N134 (red), two Ub-binding sites, and two nuclear export signals (NES77 and NES141). The C-terminal region of ATXN3 contains 2 or 3 UIMs (depending on the isoform), the polyQ tract (Qn), and the NLS. ATXN3 is mono-ubiquitinated primarily at residue K117 and phosphorylated at residues S111, S236, S256, S340, and S352. B) Solution structure of JD (PDB: 1YZB) highlighting the catalytic residues (red) and critical residues of Ub-binding sites (blue). Ub-binding site 1 (I77Q78) resides near the catalytic pocket while Ub-binding site 2 (W87) resides on the opposite side, separated from site 1 by

a helical hairpin (hh). The main ATXN3 mono-ubiquitination site, K117 (green), also localizes near the catalytic cleft.

Table 1

Known protein interactors of ATXN3.

Protein name	Description	Reference
ACCN2	amiloride-sensitive cation channel 2, neuronal	Shen *et al.*, 2006
ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha	Shen *et al.*, 2006
ARHGAP19	Rho GTPase activating protein 19	Lim *et al.*, 2006
ATXN3	ataxin-3	Todi *et al.*, 2007
CBP	CREB binding protein	Chai *et al.*, 2002
CHIP	STIP1 homology and U-box containing protein 1	Jana *et al.*, 2005
CK2 beta	casein kinase II subunit beta	Tao *et al.*, 2008
Derlin-1	Der1-like domain family, member 1	Wang *et al.*, 2006
Dynein	dynein, cytoplasmic 2, heavy chain 1	Burnett *et al.*, 2005
E6-AP	ubiquitin ligase E3A	Mishra *et al.*, 2010
EWSR1	Ewing sarcoma breakpoint region 1	Lim *et al.*, 2006
FOXO4	forkhead box other 4	Araujo *et al.*, 2011
Gp78	autocrine motility factor receptor (AMFR)	Ying *et al.*, 2009
GSK 3β	glycogen synthase kinase 3β	Fei *et al.*, 2007
H3	histone H3	Li *et al.*, 2002
H4	histone H4	Li *et al.*, 2002
HAP1	huntingtin-associated protein 1	Takeshita *et al.*, 2011
HDAC3	histone deacetylase 3	Evert *et al.*, 2006
HDAC6	histone deacetylase 6	Burnett *et al.*, 2005
HHR23A	RAD23 homolog A (*S. cerevisiae*)	Wang *et al.*, 2000
HHR23B	RAD23 homolog B (*S. cerevisiae*)	Wang *et al.*, 2000
Itga5	α5 integrin subunit (*Mus musculus*)	do Carmo Costa *et al.*, 2010
KCTD10	BTB/POZ-domain containing protein KCTD10	Sowa *et al.*, 2010
Lin-10	lin-10	Lim *et al.*, 2006
NCOR1	nuclear receptor co-repressor 1	Evert *et al.*, 2006
NEDD8	neural precursor cell expressed, developmentally down-regulated 8	Ferro *et al.*, 2007
OTUB2	ubiquitin thioesterase OTUB2	Sowa *et al.*, 2009
PARK2	parkin	Durcan *et al.*, 2010
PCAF	p300/CREBBP-associated factor	Li *et al.*, 2002
PLIC-1	protein linking IAP to the cytoskeleton	Heir *et al.*, 2006
PML	promyelocytic leukemia antigen	Chai *et al.*, 2001
PRKCABP	protein kinase C-alpha-binding protein	Lim *et al.*, 2006
p300	E1A binding protein p300	Li *et al.*, 2002
p45	proteasome (prosome, macropain) 26S subunit, ATPase, 5	Wang *et al.*, 2007
SELS	selenoprotein S	Wang *et al.*, 2006
SUMO1	SMT3 suppressor of mif two 3 homolog 1 (*S. cerevisiae*)	Shen *et al.*, 2006
SYVN1	synovial apoptosis inhibitor 1, synoviolin	Wang *et al.*, 2006

Protein name	Description	Reference
TBP	TATA box binding protein	Araujo *et al.*, 2011
TEX11	testis expressed 11	Lim *et al.*, 2006
TUBB	tubulin, beta	Zhong *et al.*, 2006
UBB	ubiquitin B	Ferro *et al.*, 2007
UBE4B	ubiquitination factor E4B (UFD2 homolog, yeast)	Matsumoto *et al.*, 2004
UBXN-5	UBX-containing protein in Nematode family member (ubxn-5, *C. elegans*)	Rodrigues *et al.*, 2009
USP13	ubiquitin carboxyl-terminal hydrolase 13	Sowa *et al.*, 2009
VCP	valosin-containing protein	Kobayashi *et al.*, 2002
WT1	Wilms tumor 1	Araujo *et al.*, 2011

Table 2

Animal models of ATXN3 overexpression.

Species	Gene promoter	Expressed protein (isoform)	PolyQ size	Line	Motor dysfunction	Neuropathology		Reference
						Aggregates	Cell death	
M. musculus	*L7*	HA-ATXN3(2UIM)	Q79	MJDQ79	−	−	−	Ikeda *et al.*, 1996
		HA-[aa290-Ct]ATXN3(2UIM)	Q35	Q35C	−	−	−	
			Q79	Q79C	+++	ND	++	
	ATXN3	all ATXN3 isoforms	Q15	MJD15.4	−	−	−	Cemal *et al.*, 2002
			Q84	MJD84.2	+++	+++	++	
	Prnp	ATXN3(2UIM)	Q20	Q20-A	−	−	−	Goti *et al.*, 2004
			Q71	Q71-B	+++	+++	−	
				Q71-C	+++	++	+	
	Prnp	ATXN3delta[aa190–220](2UIM)	Q20	deltaQ20	−	−	−	Colomer Gould *et al.*, 2007
			Q71	deltaQ71	+++	ND	ND	
	Prnp	ATXN3(3UIM)	Q15	15.1	−	−	−	Bichelmeier *et al.*, 2007
			Q70	70.61	+++	++	+	
			Q148	148.19	+++	++	ND	
		ATXN3-3NLS(3UIM)	Q148	148.NLS	+++	+++	+	
		ATXN3-NES(3UIM)	Q148	148.NES	+	+	−	
	Prnp	HA-ATXN3(2UIM)	Q22	Ataxin-3-Q22	−	−	−	Chou *et al.*, 2008
			Q79	Ataxin-3-Q79	++	+	+	
	L7	HA-[aa287-Ct]ATXN3(2UIM)	Q69	polyQ	+++	+++	ND	Torashima *et al.*, 2008
	TetOff-*Prnp*	ATXN3(3UIM)	Q77	Prp/MJD77	++	++	+	Boy *et al.*, 2009
	Htt	ATXN3(3UIM)	Q148	HDpromMJD148	+	+	+	Boy *et al.*, 2009
	CMV	ATXN3(3UIM)	Q83	CMVMJD83	−	−	−	Silva-Fernandes *et al.*, 2010
			Q94	CMVMJD94	++	+	++	
R. norvegicus	*PGK* (lentivirus)	Myc-ATXN3(2UIM)	Q27	−	ND	−	−	Alves *et al.*, 2008
			Q72	−	+	++	++	
D. melanogaster	*elav-GAL4* or *gmr-GAL4*	HA-[aa281-Ct]ATXN3(2UIM)	Q27	UAS-MJDtr-Q27	ND	−	−	Warrick *et al.*, 1998

Species	Gene promoter	Expressed protein (isoform)	PolyQ size	Line	Motor dysfunction	Neuropathology		Reference
						Aggregates	Cell death	
C. elegans	*elav-GAL4* or *gmr-GAL4*	Myc-ATXN3(2UIM)	Q78	UAS-MJDtr-Q78	+++	++	+++	Warrick *et al.*, 1998
			Q27	UAS-SCA3-Q27	–	–	–	
			Q84	UAS-SCA3-Q84	+++	++	++	
		HA-ATXN3(2UIM)	Q78	UAS-MJDtr-Q78	+++	++	++	
		Myc-ATXN3UIM*(2UIM)	Q27	UAS-SCA3-Q27-UIM*	ND	ND	ND	
			Q80	UAS-SCA3-Q80-UIM*	ND	ND	++	
		Myc-ATXN3C14A(2UIM)	Q27	UAS-SCA3-Q27-C14A	ND	ND	ND	
			Q80	UAS-SCA3-Q88-C14A	ND	ND	+++	
		Myc-[Nt-aa287]ATXN3(2UIM)	–	UAS-SCA3-delta	ND	ND	ND	
		HA-[aa281-Ct]ATXN3-CAA/G(2UIM)	Q78	UAS-SCA3tr-Q78	+	++	+	Li *et al.*, 2008
	elav-GAL4	Myc-ATXN3-FLAG(2UIM)	Q84	UAS-Myc-Atx3Q84-FLAG(WT)	ND	++	+++	Jung *et al.*, 2009
		Myc-ATXN3(6M)-FLAG(2UIM)	Q84	UAS-Myc-Atx3Q84-FLAG(6M)	ND	++	++	
	unc-119	[Nt-aa344]ATXN3-GFP(2UIM)	Q17	MJD1-17Q-GFP	ND	–	ND	Khan *et al.*, 2006
			Q91	MJD1-91Q-GFP	ND	–	ND	
			Q130	MJD1-130Q-GFP	+	+	ND	
		[aa283–344]ATXN3-GFP(2UIM)	Q19	19Q-GFP	–	–	ND	
			Q33	33Q-GFP	ND	–	ND	
			Q63	63Q-GFP	ND	+	ND	
			Q127	127Q-GFP	++	++	ND	
	F25B3.3	ATXN3-YFP(3UIM)	Q14	AT3q14	–	–	ND	Teixeira-Castro *et al.*, 2011
			Q75	AT3q75	–	–	ND	
			Q130	AT3q130	++	+	ND	
		[aa257-Ct]ATXN3-YFP(3UIM)	Q14	257cAT3q14	–	–	ND	
			Q75	257cAT3q75	+	+	ND	
			Q80	257cAT3q80	++	++	ND	
			Q128	257cAT3q128	+++	+++	ND	

–absent;

+mild;

++ intermediate;

+++ severe;

ND, Not Determined; Ct, C-terminus; Nt, N-terminus; UIM*. S236/256A;(WT), wild type; (6M), D171/208/217/223/225/228N

Table 3

Genetic modifiers of mutant ATXN3 toxicity.

Protein name	Function	Effect on toxicity	MJD model	Reference
ATXN3	DUB	suppressor	*D. melanogaster*	Warrick *et al.*, 2005
		none	*R. norvegicus*	Alves *et al.*, 2010
		none	*M. musculus*	Hubener *et al.*, 2010
USP47	DUB	suppressor	*D. melanogaster*	Bilen *et al.*, 2007
CHIP	Ub E3 ligase	suppressor	*M. musculus*	Williams *et al.*, 2009
E4B	Ub E3 ligase	suppressor	HEK293T cells	Matsumoto *et al.*, 2004
Ubiquitin	post-translational tag	suppressor	*D. melanogaster*	Bilen *et al.*, 2007
VCP	AAA ATPase	suppressor	*D. melanogaster*	Boeddrich *et al.*, 2006
HSP70	chaperone	suppressor	*D. melanogaster*	Warrick *et al.*, 1999
			D. melanogaster	Bilen *et al.*, 2007
HSP40	chaperone	suppressor	*D. melanogaster*	Bilen *et al.*, 2007
αB-Crystallin	chaperone	suppressor	*D. melanogaster*	Bilen *et al.*, 2007
HSPB8	chaperone	suppressor	HEK293T cells	Carra *et al.*, 2010
			D. melanogaster	
TPR2	chaperone	suppressor	*D. melanogaster*	Bilen *et al.*, 2007
CRAG	Guanosine triphosphatase	suppressor	*M. musculus*	Torashima *et al.*, 2008
Beclin-1	protein binding	suppressor	neuronal cells	Nascimento-Ferreira *et al.*, 2011
			R. norvegicus	
Bantam	microRNA	suppressor	*D. melanogaster*	Bilen *et al.*, 2006
MBNL1	nucleic acid binding	suppressor	*D. melanogaster*	Li *et al.*, 2008
IGF2BP2	mRNA binding	suppressor	*D. melanogaster*	Bilen *et al.*, 2007
PABP	poly(A) RNA binding	suppressor	*D. melanogaster*	Lessing *et al.*, 2008
UBXD2	UBX protein	suppressor	*D. melanogaster*	Bilen *et al.*, 2007
UBXD8	UBX protein	suppressor	*D. melanogaster*	Bilen *et al.*, 2007
Exportin1	protein transporter	suppressor	*D. melanogaster*	Bilen *et al.*, 2007
HSF1	transcription factor	suppressor	*C. elegans*	Teixeira-Castro *et al.*, 2011
DAF16	transcription factor	suppressor	*C. elegans*	Teixeira-Castro *et al.*, 2011
NFAT5	transcription factor	suppressor	*D. melanogaster*	Bilen *et al.*, 2007
ATF2	transcription factor	suppressor	*D. melanogaster*	Bilen *et al.*, 2007
SIN3A	transcription regulator	suppressor	*D. melanogaster*	Bilen *et al.*, 2007
TRIM71	transcription regulator	suppressor	*D. melanogaster*	Bilen *et al.*, 2007
Dbr	zinc ion binding	suppressor	*D. melanogaster*	Bilen *et al.*, 2007
ACOX1	palmitoyl-CoA oxidase	suppressor	*D. melanogaster*	Bilen *et al.*, 2007
Sup35	yeast prion domain	suppressor	*D. melanogaster*	Li *et al.*, 2007
UL97	HCMV kinase	supressor	HeLa cells	Tower *et al.*, 2011
FBXL11	transcription regulator	enhancer	*D. melanogaster*	Bilen *et al.*, 2007
ATXN2	RNA and protein binding	enhancer	*D. melanogaster*	Lessing *et al.*, 2008