Universidade de Aveiro Departamento de Biologia
2011

Sara Matias Carmo Silva

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Professor Doutor Luís Pereira de Almeida do Centro de Neurociências e Biologia Celular de Coimbra e Professor Auxiliar na Faculdade de Farmácia da Universidade de Coimbra da Universidade de Coimbra e de Professora Doutora Margarida da Cruz Fardilha, Professora Auxiliar Convidada da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro.

À minha Mãe

o júri
presidente

Prof. Doutora Odete Abreu Beirão da Cruz e Silva
Professora Auxiliar com Agregação da Secção Autónoma de Ciências da Saúde - Universidade de Aveiro

Prof. Doutora Ana Cristina Carvalho Rego
Professora Auxiliar da Faculdade de Medicina da Universidade de Coimbra; Centro de Neurociências e Biologia Celular de Coimbra

Prof. Doutor Luís Pereira de Almeida
Professor Auxiliar da Faculdade de Farmácia da Universidade de Coimbra; Centro de Neurociências e Biologia Celular de Coimbra

Prof. Doutora Margarida Sâncio da Cruz Fardilha
Professora Auxiliar Convidada da Secção Autónoma de Ciências da Saúde- Universidade de Aveiro

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palavras-chave

DMJ, ataxina-3, ataxina-2, PABP, PAM2, A2BP1,efeitos modulatórios, interação proteica, CAG, SCA3, SCA2, SCA1, ALS.

resumo

A doença de Machado-Joseph também conhecida como ataxia espinhocerebelosa tipo 3 (MJD/SCA3) é uma doença neurodegenerativa hereditária descrita pela primeira vez em emigrantes portugueses da região dos Açores. É a ataxia autossómica dominante mais comum e apresenta manifestações clínicas severas com um desfecho fatal. Esta doença é causada por uma expansão instável do trinucleótido CAG na região codificante do gene *MJD1*, que se traduz numa cadeia com um número excessivo de glutaminas na ataxina-3. A ataxina-3 mutante adquire propriedades tóxicas e desencadeia uma patogénese complexa com manifestações heterogéneas.

Apesar da expansão poliglutamínica na proteína ataxina-3 ser suficiente para induzir a patogénese da doença e do número de repetições se correlacionar inversamente com a idade de início dos sintomas, não permite explicar toda a diversidade na idade de iníco e nas manifestações clínicas. Há evidências de que a interação entre diferentes proteinas pode afectar quer a idade de ínicio dos sintomas quer a progressão da doença.

Na procura de modificadores da neurodegeneração da DMJ estudos recentes sugerem que a ataxina-2, uma proteína envolvida na ataxia espinocerebelosa do tipo 2 (SCA2) que interage com a proteína ligante da cadeia de poliadenina do RNA mensageiro (PABP) e poderá potenciar a neurodegeneração na DMJ. O objectivo principal deste trabalho foi investigar a interacção entre a ataxina-2, PABP e a ataxina-3 na DMJ.

Utilizando material humano (tecido cerebral e fibroblastos), animais trangénicos para a doença, um modelo lentiviral em roedor e ainda um modelo celular da doença, observámos que os níveis de ataxina-2 e PABP sofrem alterações na DMJ. A imunoreactividade para a ataxina-2 encontra-se aumentada no tecido humano dos doentes, o que também ocorreu também no murganos trangénicos. No modelo lentiviral observámos um recrutamento de ambas as proteínas para o núcleo da célula coincidente com a progressão da doença.

O modelo celular revelou um feedback positivo entre os níveis de ataxina-2 e PABP, que poderá estar na base da sua capacidade de reduzir os níveis de ataxina-3 mutante na doença. Provando assim que os níveis das três proteínas parecem ser interdependentes, permanecendo a questão se esta interação ocorre de forma directa ou indirecta.

Estes estudos sugerem que os níveis e consequente toxicidade de uma proteína poliglutamínica mutada pode ser modulada pela actividade normal de outra. Esta relação sinérgica pode ser crítica para a patogénese da doença e desta forma constituir um potencial alvo terapêutico.

keywords

MJD, ataxin-3, ataxin-2, PABP, PAM2, A2BP1,modulatory effect,proteic interactions, CAG, SCA3, SCA2, SCA1, ALS

abstract

Machado-Joseph disease also known as Spinocerebellar ataxia type 3 (MJD/SCA3) is a hereditary neurodegenerative disease first described among Portuguese immigrants from Azores. Is the most common autossomal dominant spinocerebellar ataxia worldwide and it presents severe clinical features with a fatal outcome. This disease is caused by an unstable expansion of the trinucleotide CAG in the coding region of the MJD1 gene, the mutation is translated into an expanded polyglutamine tract in the protein ataxin-3. The mutant ataxin-3 acquires toxic properties and leads to a downstream of pathogenic mechanisms that make MJD a very heterogeneous disease.

Although the polyglutamine expansion in ataxin-3 is sufficient for the pathogenesis of the disease and for the number of repeats is correlated inversely with the age of onset, clinical observations suggest that the polyQ expansion does not account for all the diversity in the age of onset and clinical manifestations. Interactions between different proteins may affect the age of onset as well as the disease progression.

In a screen for modifiers of neurodegeneration in MJD, previous studies found ataxin-2, a protein involved in the spinocerebellar ataxia type 2 (SCA2) which interacts with poly (A)-binding protein (PABP) and may potentiate the neurodegeneration in MJD. The main goal of our research was to investigate the relation between ataxin-2, PABP and ataxin-3 in MJD.

Recurring to human material (brain tissue and fibroblasts), transgenic mice for MJD, a lentiviral-based MJD mouse model and a cellular model of the disease, we observed that levels of ataxin-2 and PABP suffer alterations in MJD. The ataxin-2 immunoreactivity is increased in the human tissue of MJD patients, which also occurred in the transgenic rodents. In the lentiviral model we observed both proteins' recruitment into the nucleus with the progression of the disease.

The cellular model revealed a positive feedback between ataxin-2 and PABP that may to be responsible for their capacity in reducing mutant ataxin-3 levels in the disease. Attesting that the levels of the three proteins seem to be interdependent, remaining only the question whether this interaction occurs direct or indirectly.

These studies indicate that the levels and the consequent toxicity of a polyglutamine disease protein can be modulated by the normal activity of another. This synergicall relation may be critical for the pathogenesis of the disease and therefore be a potential therapeutic target.

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Chapter I

Introduction

1.1. Diseases of unstable triplet repeat expansions

An important development in the understanding of the pathogenesis of a group of neurodegenerative diseases occurred more than 20 years ago, with the identification of a mutational mechanism that is behind their pathogenesis, the expansion of unstable nucleotide repeats (*Koshy and Zoghbi, 1997; Gatchel and Zoghbi 2005*). In the case of triplet repeat diseases, they are caused by an unstable expansion of a trinucleotide repeat (*Gatchel and Zoghbi 2005*). In normal conditions, there is a stable transmission of the number of repeats from the parents, but when there is a mutation involving excessive repetition c triplet over a certain threshold it becomes unstable in the parental transmission, leading to variations of the number of repeats from one generation to the next. These intergenerational instability leads to an earlier onset and a more severe phenotype in successive generations of an affected family *(Timchenco and Caskey, 1996; Tsuji, 1997; Koshy and Zoghbi, 1997*).

These diseases are classified depending on the genetic context of the unstable repeat; it may occur an expansion of coding repeats (exons) or an expansion of non-coding repeats (5'UTR, 3'UTR or introns) (Fig.1). The expansion of non-coding repeats, leads to the alteration of gene expression, which reflects in either a loss of protein function or altered RNA function (*Tsuji, 1997; Gatchel and Zoghbi, 2005*). The other subclass of these disorders is caused by the expansion of an unstable Cytosine-Adenine-Guanine (CAG) trinucleotide repeat within the protein-encoding region, which translates into an abnormally long stretch of glutamine residues, conferring a toxic function to the protein unrelated to its normal function. These diseases are called polyglutamine diseases and are characterized by mutant proteins with expanded polyglutamine (polyQ) tracts (*Tsuji, 1997; Ross and Poirer, 2004; Gatchel and Zoghbi, 2005; Riley and Orr, 2006*).

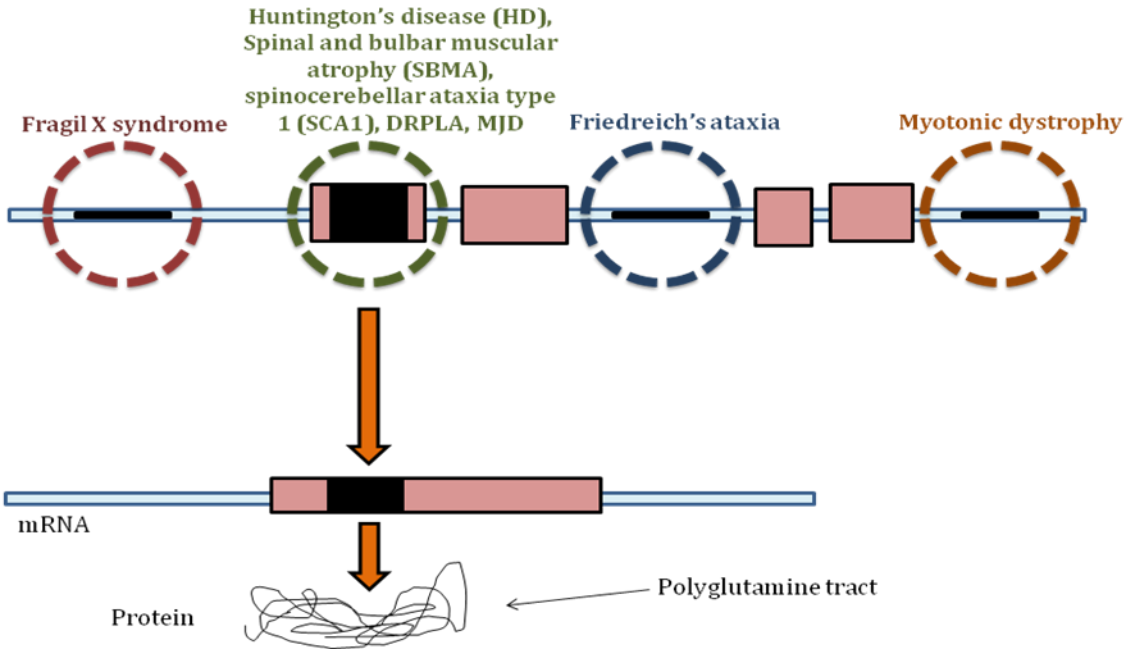

Figure 1: **Representation of the possible localization of triplet repeats for various diseases.** (Adapted from *Tsuji, 1997*).

1.2. Polyglutamine diseases

The polyglutamine disorders are autosomal and are dominantly inherited (except SBMA (spinal bulbar muscular atrophy), an X-linked recessive disorder), representing the most common form of inherited neurodegenerative disease. The first polyglutamine disease to be discovered was SBMA in 1991 (*La Spada and Keneth Fischbeck, 1991*). They are caused by a polyglutamine (polyQ) expansion within their active coding unrelated proteins, including Huntington's disease (HD), dentatorubral-pallidoluysian atrophy (DRPLA), spinal bulbar muscular atrophy (SBMA), and the spinocerebellar ataxias (SCA1, 2, 3, 6, 7 and 17) (Table1) (*Orr and Zoghbi, 2000*).

Table 1. Polyglutamine diseases

Disease	Disease Protein	Normal subcellular localization	Affected brain regions*
Huntington's disease (HD)	*Huntingtin (htt)*	*Cytoplasmic*	*Striatum and Cortex*
Spinal and Bulbar Muscular Atrophy (SBMA)	*Androgen receptor (AR)*	*Nuclear and Cytoplasmic*	*Motor Neurons*
Dentatorubal- Pallidoluysian Atrophy (DRPLA)	*Atrophin-1*	*Nuclear and Cytoplasmic*	*Central Cortex*
Spinocerebellar ataxia 1 (SCA1)	*Ataxin 1*	*Nuclear and Cytoplasmic*	*Cerebellum*
Spinocerebellar ataxia 2 (SCA2)	*Ataxin-2*	*Cytoplasmic*	*Cerebellar Purkinje Cells*
Spinocerebellar ataxia 3/ Machado-Joseph disease (SCA3/MJD)	*Ataxin3*	*Nuclear and Cytoplasmic*	*Cerebellum, Brain Stem, Basal Ganglia, Ventral pons and Substantia nigra*
Spinocerebellar ataxia 6 (SCA6)	*Ataxin6*	*Nuclear and Cytoplasmic*	*Cerebellar Purkinje Cells*
Spinocerebellar ataxia 7 (SCA7)	*Ataxin7*	*Nuclear and Cytoplasmic*	*Cerebellar Purkinje Cells, Brain Stem and Spinal Cord*
Spinocerebellar ataxia 17 (SCA17)	*TBP*	*Nuclear*	*Cerebellar Purkinje Cells*

**Main regions affected.*

The polyglutamine expansion diseases have onset in midlife, leading to the death of the patient 10-30 years after the appearance of the first symptoms (*Cummings and Zoghbi, 2000; Orr and Zoghby, 2007; Macedo-Ribeiro et al., 2009*). In general the age of onset inversely correlates with the size of expanded CAG repeats, and there are evidences that point to the interaction of the normal repeat with the expanded CAG repeat influencing the age of onset (*Maciel et al., 1995; Maruyama et al., 1995*; *Gusella and MacDonald, 2000, Djousse et al., 2003*).

The genetic similarities among these disorders strongly support the hypothesis that these diseases share a common mechanism of pathogenesis, mainly based in the toxic properties of the polyglutamine tract. The expanded proteins of polyQ disorders have an enhanced ability to form intranuclear aggregates/inclusions (*Ross and Poirer 2004*), and in some cases also cytoplasmic inclusions in the disease affected regions (*Ciechanover and Brundin, 2003; Bennet et al., 2005*). Neuronal nuclear inclusions (NII) are considered to be a histopathological hallmark of the polyQ diseases. Although the role of nuclear inclusions in pathology is not completely understood, it is clear that these inclusions result from the nuclear accumulation of polyQ expanded proteins, which are normally in the cytoplasm. Mutant polyQ proteins in the nucleus can abnormally interact with nuclear proteins, such as transcription factors, leading to transcriptional dysregulation (*Havel et al., 2009*). The presence of these NI of mutant protein focused attention on the nucleus as the subcellular site determinant for the pathogenesis of these diseases, with some suggesting that inclusions were pathogenic (*Arrasate et al. 2004*) and others questioning this pathogenicity of aggregates/inclusions (*Orr and Zoghbi, 2007*).

It is becoming more apparent that the polyQ tract by itself; even though toxic and sufficient to cause polyQ pathology, does not account for all the symptoms of the polyQ disorders nor for all the similarities observed between them. It is possible that each polyglutamine disorder may be the result of the expanded polyglutamine tract action in the context of the "host" protein. This means that the normal function and the interactions of each polyglutamine protein is determinant to define the pathogenic pathway of each disorder, which explains the similarities found between disorders as well as their differences (*Orr 2001; Gatchel & Zoghbi 2005; Orr and Zoghbi, 2007*).

1.3. Spinocerebellar ataxias

Autossomal dominant Spinocerebellar Ataxias (SCAs) are a group of neurodegenerative diseases, with some of them being also polyglutamine disorders (SCA1, 2, 3, 6, 7 and SCA17) (Fig. 2). These disorders are clinically and genetically very divergent and are characterized by progressive cerebellar ataxia, which consists in the loss of motor coordination, showing unsteady movements and staggering gait (*Zoghbi, 2000*). Ataxia results from variable degeneration of neurons in the cerebellar cortex, brain stem, spinocerebellar tracts and their afferent/efferent connections. Although ataxia is the prominent symptom in SCAs, few mutations cause an almost pure cerebellar syndrome isolating neurodegeneration to the cerebellar cortex. On the contrary, most SCAs are multisystemic disorders presenting clinical variability. The ataxia may be accompanied by numerous other symptoms that vary between different SCAs. One common ground between all of these diseases is the progressive neurodegeneration and its fatal outcome (*Maruyama et al., 2002; Dueñas et al., 2006*).

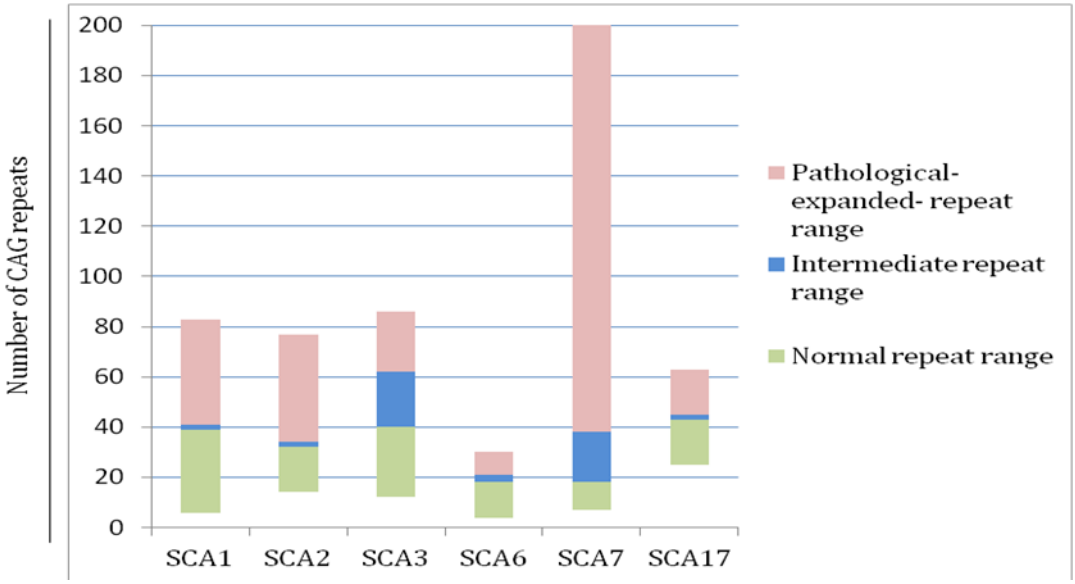

Figure 2. **Different CAG repeat expansions in Polyglutamine disorders.** (Adapted from *Schöls et al., 2004*).

The most striking characteristics in SCAs as unstable triplet expansion diseases are its unstable and heterogeneous expression, observed even in the same family (*Moseley, 1998; Schöls, 2004*); and the age of onset of the disease that is inversely correlated with the repeat length. The age of onset of the clinical symptoms is between 30 and 50 years of age, but there are cases of childhood onset caused by extreme expansions between generations. (*Schöls et al., 2000; Zoghbi, 2000*).

1.4. Machado Joseph Disease/Spinocerebellar ataxia type 3

Machado-Joseph disease (MJD) or spinocerebellar ataxia type 3 (SCA3) is the most frequent autossomal dominantly inherited cerebellar ataxia worldwide, comprising between 15% and 45% of all SCAs (*Schöls et al., 1995; Riess et al., 2008*). This neurodegenerative disease was first described in 1972 among immigrants native of the Portuguese Azorean Island, São Miguel. Later on, other cases were described in descents of Azorean immigrants from the Flores Island (*Nakano et al., 1972; Rosenberg et al., 1976; Rosenberg et al., 1978*); and the disorder was subsequently also identified in Brazil, Japan, China, and Australia (*Sequeiros and Coutinho, 1993; Sudarsky and Coutinho, 1995*).

MJD is caused by an unstable expansion of a CAG repeat in the coding region of the *MJD1* gene mapped on chromosome 14q32.1 (*Kawaguchi et al., 1994*). The *MJD1* gene encodes ataxin-3, a 42 kDa protein that resides in both nucleus and cytoplasm (*Paulson et al., 1997a*). Ataxin-3 is a polyubiquitin-binding protein whose physiological function is related to ubiquitin-mediated proteolysis (*Burnett et al., 2003; Chai et al., 2004*). The mutation results in a long polyglutamine chain at the C-terminus of ataxin-3; the CAG repeats range from 10 to 44 in normal population and from 45 to 87 in MJD patients (*Cummings and Zoghbi, 2000; Maciel et al., 2001; Padiath et al., 2005*) In MJD, like for other polyQ disorders, an inverse correlation between the number of CAG repeat and the age of onset of the disease is verified (*Maruyama et al., 1995*)

1.4.1. Genetic features

The common underlying genetic basis of MJD is the expansion of a CAG repeat region beyond a certain threshold. There is an overlap of normal repeat sizes (up to 47 repeats) in the CAG tract of the *MJD1* gene. As shown in other polyQ diseases such as Huntington's disease, individuals with a repeat length in the overlapping region do not always manifest the disease in what is designated as incomplete penetrance of the disease (*Padiath et al., 2005*). This repeat tract is not exclusively a CAG stretch, as variant CAA and AGG triplets were also found in normal alleles (*Kawaguchi et al., 1994*).

It was described somatic mosaicism of the repeat size in CAG repeat disorders, where different cells of the same individual carry different repeat sizes. This somatic instability has

also been described for MJD in different brain areas, there were found smaller repeat sizes in cerebellar cortex that in other brain regions (*Lopes-Cendes et al., 1996*; *Hashida et al., 1997*).

Even though the correlation between CAG repeats and the age at onset in MJD is well documented and proved to be a genetic feature of MJD, the length of CAG repeats does not account for all the variation observed in the patients. Also, the variance of the age at onset in MJD patients with similar number of repeats, suggest that additional factors influence the age at onset. It is predicted that a healthy life style leads to a later disease manifestation; it has also been shown that females manifest the disorder later in live as compared to their brothers even with the same expanded repeat size or longer normal CAG repeat sizes (*DeStefano et al., 1995*). CAG repeats are thought to be responsible for 45-60% of variability in age of onset, nevertheless other factors remain to be identified, and there is evidence that similarly to other polyQ diseases, proteic interactions may influence the variability observed in clinical phenotypes and age of onset (*Maciel et al., 1995; Van de Warrenburg et al., 2002*).

1.4.2. Clinical features

The clinical spectrum of MJD is highly pleomorphic. Based on these clinical manifestations four subphenotypes have been suggested (*Coutinho and Andrade, 1978; Schöls et al., 1996; Riess et al., 2008*), which during the progression of the disease may evolve from one type to other type (*Fowler, 1984*). Recently it an additional MJD type (V) has been proposed based in a homozygous 33-years old patient of Portuguese/Brazilian descent (*Lysenko et al. 2010*). Therefore the proposed five clinical subtypes of MJD are:

- Type I: Extrapyramidal (dystonia and bradykinesia)+ Pyramidal deficits + early onset (10-30 years);
- Type II: Pyramidal + cerebellar deficits + intermediate onset (20-50 years);
- Type III: Cerebellar deficits + peripheral neuropathy + late onset (40-75 years);
- Type IV: Neuropathy + Parkinsonism with variable onset;
- Type V: Pronounced Extrapyramidal signs + Pyramidal deficits + spastic paraplegia + variable age of onset (4-43 years).

Other clinical features of MJD include ophtalmoplegia, double vision, faciolingual fasciculation, dysphagia, weight loss without loss of appetite, incontinence, restless legs syndrome, eyelid retraction, postural instability, difficulty with swallowing. Cognitive disturbances are mild and rarely develop to relevant dementia (*Sequeiros and Coutinho, 1993; Sudarsky and Coutinho, 1995; Bürk et al., 2003*). Clinically these symptoms may fluctuate in different subtypes according to individual characteristics, the number of CAGs and the age of

onset. For example, individuals with early onset of MJD may suffer more from dystonia and bradykinesia than from ataxia (type I). In addition, the latest onset patients, beginning after age 50, may experience as much difficulties resulting from neuropathy as from ataxia (*Paulson et al., 2007; Riess et al., 2008*).

1.4.3 Neuropathological features

The neuropathological alterations of MJD in the brain consist in neuronal loss in multiple systems. The degeneration is not confined to a single specific region of the brain, but on the contrary is quite widespread which contributes to the multiple clinical impairments observed in the disease. The disorder involves a combination of neurodegeneration in the spinocerebellar tract with other areas of the central nervous system (Fig. 3). The neuropathology involves cerebellar systems (dentate nucleus and pontine nuclei), cranial nerve motor nuclei, *substantia nigra*, and striatum. A relative preservation of the cerebellar cortex, particularly Purkinje cells at initial stages and inferior olive is reported (*Rosenberg, 1992; Coutinho and Andrade, 1995; Durr et al., 1996; Alves et al., 2008a*). A marked degeneration of Clarkes's column nuclei and vestibular and pontine nuclei is observed (*Durr et al., 1996*). Marked neuronal loss is also observed in the anterior horn of the spinal cord, and motor nuclei of the brainstem (*Rub et al., 2008*).

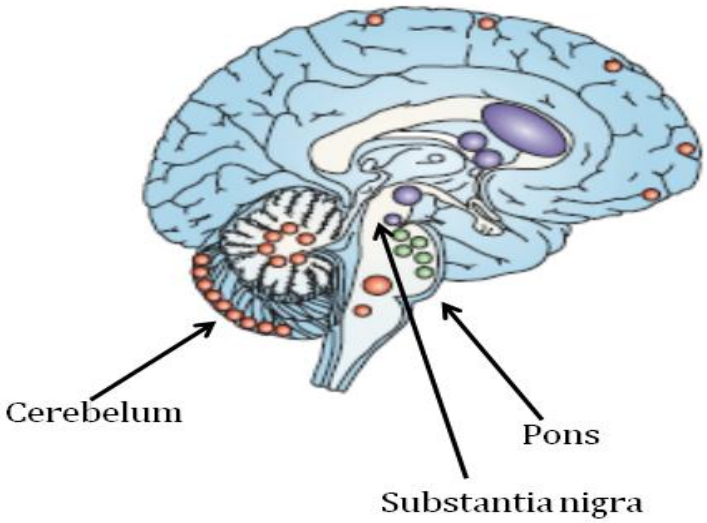

Figure 3. The main areas exhibiting neuronal loss in MJD. Indicated in the figure three of the most affected regions, the cerebellum, the ventral pons and *substantia nigra*. Large dots indicate severe neuronal loss. Blue dots indicate involvement of extrapyramidal nuclei. Green dots indicate cranial nerve involvement. (Adapted from *Taroni and DiDonato, 2004*)

Studies regarding the localization of mutant ataxin-3 in the brain and its preferential accumulation allowed to describe with much more accuracy the regions affected by the disease itself. Upon macroscopic examination MJD patients brains (disease duration over 15 years) display depigmentation of the *substantia nigra*, atrophy of the cerebellum as well as atrophy of cranial nerves III to XII. The depigmentation of the *substantia nigra* is the cause of parkinsonian features, however, not all the patients with degenerated *substantia nigra* show parkinsonism (*Rub et al., 2003; Rub et al., 2006*). The majority of these MJD patient's brains has lower weight than brains from individuals without medical histories of neurological or psychiatric diseases (*Iwabuchi et al., 1999*). The study of human brains of normal individuals and MJD patients suggests that the cerebellar dentate nucleus and the *substantia nigra* are among early targets of the degenerative process of MJD, while the thalamus and the cranial nerves (dorsal motor vagal and ambiguous nuclei) are affected only late during the course of the disease (*Riess et al., 2008*). The involvement of the striatum in the affected brain areas of MJD was recently described (*Alves et al., 2008a*) and may provide an explanation for symptoms found in some MJD patients such as dystonia and chorea (*Lee et al., 2003*). Chorea and dystonia have been consistently associated with lesions in the striatum. Chorea involves dysfunction of the indirect pathway from the caudate and putamen to the internal *globus pallidus*, whereas dystonia is generated by dysfunction of the direct pathway (*Janavs et al., 1998*).

One important hallmark of neurodegeneration in the brains of MJD patients is the neuronal intranuclear inclusions (NIIs), which have been observed in patients, transgenic animal models and cellular models (*Ikeda et al., 1996; Schmidt et al., 1998; Evert et al., 1999*). The NIIs role in the pathogenesis is controversial. Initially associated to the pathogenic mechanism, they may actually contribute to delay the pathogenesis by concealing the toxic mutant ataxin-3 within inert structures reducing its toxicity.

1.4.4. *MJD1* gene

The genetic locus for Machado-Joseph disease, *MJD1*, was identified in 1994 and mapped to chromosome 14q32.1 (*Kawaguchi et al., 1994*). The *MJD1* gene spans about 48Kb with one long open reading frame (17776-base pairs) and is composed of 11 exons. It was identified as a novel gene containing CAG repeats that are located in exon 10 (*Ichikawa et al., 2001*). The normal *MJD1* gene encodes ataxin-3 (*ATXN3*), a polyubiquitin-binding protein that is mutated in MJD. The mutation results in an expanded polyglutamine tract at the C-terminus of ataxin-3 (*Durr et al., 1996*). The CAG repeat contains two variant sequences, CAA and AAG, at three

positions that are also translated into a polyglutamine tract at C-terminal region of the protein ataxin-3. The normal allele of *MJD1* has usually from 10 to 44 CAG repeats, there is an intermediate state where between 45 and 51 CAG repeats some individuals may manifest the disease and MJD patients alleles range from 52 up to 87 repeats (*Cummings and Zoghbi, 2000; Maciel et al., 2001;Padiath et al., 2005*). MJD's high threshold is a particularity of this disorder since in other polyglutamine disorders repeats over 36 to 40 become pathogenic (*Kawaguchi et al., 1994; Ichikawa et al., 2001; do Carmo Costa et al., 2004; Rodrigues et al., 2007*).

1.4.5. Ataxin-3

Human ataxin-3 is the *MJD1* gene product, a ubiquitously expressed protein found in the genome of several species, ranging from nematodes to humans, and plants (*Albrecht et al., 2003*). In mice and humans, despite the localized neuronal degeneration observed in MJD patients, ataxin-3 displays a ubiquitous expression among different body tissues and cell types. *MJD1* was found to be widely expressed throughout the brain, though different regions present varying expression levels (*Trottier et al., 1998*). Although the precise biological function of ataxin-3 still remains poorly understood, evidence supports its participation in several pathways related to protein homeostasis maintenance (clearance of misfolded and damaged proteins) (*Buchberger, 2002; Doss-Pepe et al., 2003; Burnett et al., 2003; Albrecht et al., 2004; Chai et al., 2004; Mao et al., 2005; Berke et al., 2005*), transcriptional regulation (*Evert et al., 2003; Rodrigues et al., 2007*), cytoskeleton regulation (*Rodrigues et al., 2010*) and myogenesis (*do Carmo Costa et al., 2010*), cellular activities whose deregulation can compromise cell functioning and survival.

1.4.5.1. Structure

The wild type ataxin-3 has an approximated globular weight of 42 KDa in normal individuals, possesses 376 amino acids (including 22 glutamines of the polyQ tract) and is widely expressed in the brain and in the body existing in both nucleus and cytoplasm of various cell types (*Paulson et al., 1997a; Trottier et al., 1998; Ichikawa et al., 2001*). Ataxin-3 consists of a globular deubiquitinating N-terminal Josephin domain (20 KDa), able to resist proteolysis, a flexible protease accessible C-terminal containing two ubiquitin interacting motifs (UIM's) and a polyQ region of variable length, whose expansion beyond a certain threshold is associated with MJD (Fig.4) (*Burnett et al., 2003; Masino et al., 2003; Scheel et al., 2003*). The most

common isoform found in the human brain has an extra UIM localized in the C-terminal region, downstream the polyQ sequence (*Harris et al., 2010*). The two conserved UIMs located N-terminally of the polyQ region are α-helical structures separated by a short flexible linker region and act cooperatively when binding ubiquitin (Ub); the affinity of the two tandem motifs is greater than that of each individual UIM (*Song et al., 2010*).

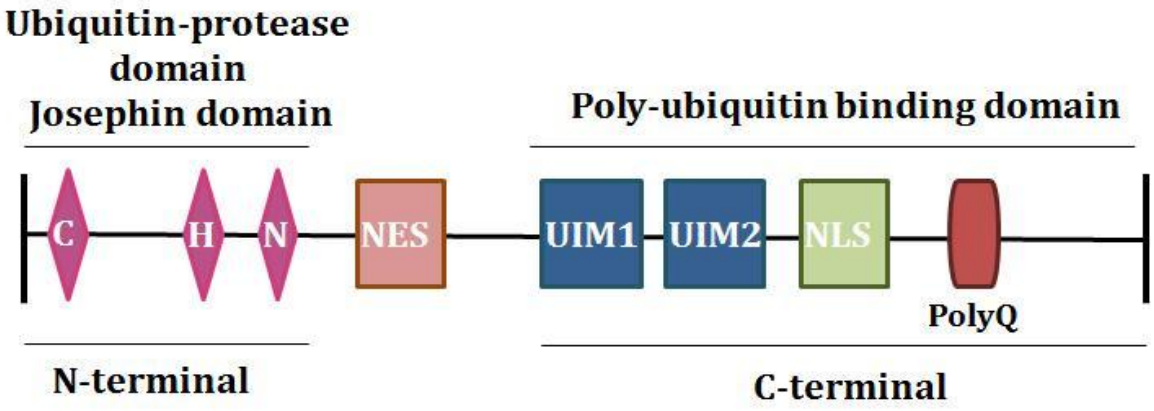

Figure 4. Structure of the human Ataxin-3. Ataxin-3 is mainly composed of a conserved Josephin domain in the N-terminal side, encoding the ubiquitin protease with the catalytic triad (Cys, His and Asn) and a Nuclear Export Signal (NES). In the flexible C-terminal there are 2 or 3 ubiquitin-interacting motifs (UIM), a Nuclear Localization signal (NLS) and the polyQ tract.

A highly conserved, putative nuclear localization signal (NLS) is found upstream of the polyQ tract (*Tait et al., 1998; Albrecht et al., 2004*), this signal may determine the rate of ataxin-3 transported into the nucleus but is shown to have a weak nuclear import activity (*Antony et al., 2009*). Furthermore, two nuclear nuclear export signals (NES) with significant activity were identified in ataxin-3, following the Josephin domain. (*Antony et al., 2009; la Cour et al., 2003*). Even though the NLS has already been identified, the non-expanded protein is present both in the nucleus and in the cytoplasm; indicating that ataxin-3 possesses nucleocytoplasmic shuttling activity (*Tait et al., 1998; Trottier et al., 1998; Antony et al., 2009*). Evidences support the hypothesis that when in a non-aggregated state, the ataxin-3 polyQ is in a random coil conformation (*Chen et al., 2001*). Polyglutamine abnormal expansion causes ataxin-3 monomeric peptides to aggregate into β-rich amyloid-like fibrillar structures. The identity of pathogenic species remains unknown – whether the protein alone, the putative intermediates of the fibrillization pathway, or the soluble mature fibrils. Nevertheless, taking in account the similarities with other misfolding amiloidophaties it is possible that protofibrillar intermediates may be far more toxic than large aggregates (*Gales et al., 2005*).

Wild-type ataxin-3 is evenly distributed throughout the cell, particularly around the nuclear envelope, whereas mutant ataxin-3 accumulates into intranuclear aggregates and is depleted from the cytoplasm. Wild-type ataxin-3 co-localizes to ubiquitin-rich aggregates found in normal aged brains, known as Marinesco bodies (*Fujigasaki et al., 2000*), is also found in ubiquitinated inclusions of mutant polyQ proteins (*Chai et al., 2001*) and directly combined with the proteasome (*Doss-Pepe et al., 2003*).

1.4.5.2. Ataxin-3 function

Ataxin-3 is a de-ubiquitinating (DUB) enzyme present in the nucleus and in the cytoplasm of the cell that appears to function in cellular pathways regulating cellular vigilance, homeostasis and transcriptional processes. DUBs are a group of enzymes responsible in the cell for removing ubiquitin (Ub) or polyUb chains from a target protein, processing Ub pre-proteins and remodeling or disassembling bound or unbound polyUb chains (*Reyes-Turcu et al., 2009; Reyes-Turcu and Wilkinson, 2009*). Ataxin-3 is an ubiquitin-binding protein capable of binding and cleaving specific types of large ubiquitin chains, removing ubiquitin from substrates to allow proteosomal degradation. Ataxin-3 binds to ubiquitin from polyubiquitinated substrates by the UIM's, allowing cleavage by ataxin-3 N-terminal Josephin domain. Ubiquitination fulfills many cellular functions in the cytoplasmic trafficking, guiding specific proteins through the endocytic pathways and targeting proteins for the proteasome (*Buchberger, 2002; Doss-Pepe et al., 2003; Burnett et al., 2003; Chai et al., 2004; Mao et al., 2005; Berke et al., 2005*). In the case of ataxin-3, inhibition of the catalytic activity results in the increase of polyubiquitinated proteins (localized primarily in the nucleus), to a degree similar to what is observed when the proteasome is inhibited (*Berke et al., 2005*). This supports the idea of ataxin-3 involvement with the polyubiquitinated proteins targeted for proteasomal degradation, therefore modulating ubiquitin-dependent mechanisms.

These ataxin-3 characteristics suggested a possible role in the Ubiquitin-Proteasome System (UPS); the UPS is involved in the processing of mutant or damaged proteins (UPP), DNA repair, chromatin remodeling, cell cycle progression, subcellular localization and pathway signaling (*Reyes-Turcu et al., 2009; Reyes-Turcu and Wilkinson, 2009; Weissman, 2001*). Its impairment is related to several human diseases, such as polyQ diseases and other neurodegenerative pathologies. When the enzymatic activity of ataxin-3 is suppressed, it no longer can suppress abnormal protein's toxicity and becomes somewhat toxic by itself leading to the impairment of the UPS, resulting in cell death (*Burnett and Pittman, 2005*). Considering its

function it is no surprise that several genes involved in the UPS vigilance pathway were altered in the absence of wild-type ataxin-3 or in the presence of mutant ataxin-3 (*Rodrigues et al.,* 2007). This evidence may be supported by the presence of ubiquitin in ataxin-3 aggregates; that may be a consequence of ataxin-3 being itself a substrate for ubiquitination, and thus being degraded by the UPS (*Matsumoto et al., 2004; Berke et al., 2005*).

Ataxin-3 function may also have relevance in the Endoplasmic Reticulum Associated Protein Degradation (ERAD). There is an ongoing controversia regarding whether ataxin-3 promotes or decreases degradation by this pathway (*Wang et al., 2006; Zhong and Pittman, 2006*); however, unexpectedly recent evidences suggest that ataxin-3 inhibits the ERAD, which can be explained by proteosomal degradation of ataxin-3 (*Riess et al., 2008*). Ataxin-3 has been found to associate with the proteasome itself through its N-terminal region (*Doss-Pepe et al., 2003*), but this interaction may not be very strong or even direct (*Todi et al., 2007*). Functioning with these interactors, ataxin-3 may act in a number of different ways, (a) trimming polyUb chains of a substrate, thus facilitating the subsequent disassembly of the chain by proteasome-associated DUBs, (b) editing polyUb chains in order to guarantee that the substrate is correctly targeted for degradation, or (c) functioning as a transiently associated subunit of the proteasome and recognizing some of its substrates (*Boeddrich et al., 2006; Wang et al., 2008*).

Another instance where ataxin-3 has been associated with cellular quality control mechanisms, is its proposed role in aggresome formation. Aggresomes are misfolded protein aggregates that form near the microtubule-organizing center (MTOC) when the proteasome is not able to deal with misfolded proteins. These structures seem to be of physiologic importance, since those defective proteins are then degraded by lysosomes, contributing to the maintenance of cellular homeostasis (*Markossian and Kurganov, 2004*). Endogenous ataxin-3 seems to play a role in the regulation of aggresome formation, having been shown to co-localize with aggresome and preaggresome particles and to be important in the formation of the aggresomes themselves (*Burnett and Pittman, 2005*). Ataxin-3 also associates with dynein and histone deacetylase 6 (HDAC6), both constituents of the complex responsible for the transport of misfolded proteins to the MTOC (*Burnett and Pittman, 2005*), and was recently shown to interact with tubulin and microtubule-associated protein 2 (MAP2), two other constituents of the cytoskeleton (*Mazzucchelli et al., 2009; Rodrigues et al., 2010*). Along with its effect in increasing ataxin-3 catalytic activity, ubiquitination of this protein also potentiates its ability to promote aggresome formation (*Todi et al., 2010*).

Considering the structure of ataxin-3, as well as its localization within the cell and its interactions with other proteins, it is possible that ataxin-3 might be involved in transcriptional processes. Due to its characteristics, ataxin-3 has several ways of interacting with the transcription. First and indirectly, through its DUB activity, ataxin-3 can modulate the degradation of transcription factors and repressors by removing the polyubiquitin degradation signal (*Li et al., 2002*). In addition, ataxin-3 may have a more direct effect over transcription regulation since it has a NLS that allows its transportation to the nucleus, where it can regulate transcription. Recent studies demonstrated that ataxin-3 is actively transported across the nuclear envelope, being actively shuttled from the cytoplasm to the nucleus and vice versa (*Chai et al., 2002; Antony et al., 2009; Macedo-Ribeiro et al., 2009*). The nuclear accumulation of ataxin-3 following stress supports the hypothesis of a nuclear function such as transcription regulation (*Reina et al., 2010*). Accordingly, it has also been suggested that ataxin-3 is able to bind histones through its Josephin domain and interact with histone acetyltransferases, which work as transcriptional co-activators, thus repressing histone acetylation and transcription. Altered protein acetylation has already been implicated in polyQ disease processes (*Li et al., 2002; Bodai et al., 2003; Zhang, 2003*). The possible role of ataxin-3 in degradation pathways and in transcriptional regulation may be cell-specific, potentially explaining why only a subset of neurons is affected in the human disease despite the widespread expression of normal and mutant ataxin-3 (*Rodrigues et al., 2007*).

The importance of ataxin-3 interaction with components of the cytoskeleton such as tubulin, MAP2 and dynein may not be limited to its possible role in aggresome formation (*Burnett and Pittman, 2005; Mazzucchelli et al., 2009*). Recent findings indicate that ataxin-3 may play a role in the organization of the cytoskeleton itself, since its absence leads to morphologic alterations in cell lines, which are accompanied by the disorganization of the several cytoskeleton constituents (microtubules, microfilaments and intermediate filaments) and a loss of cell adhesions (*Rodrigues et al., 2010*).

Adding to these functions, in vitro silencing of ataxin-3 in differentiating mouse myoblasts, not only leads to the generation of an immature cytoskeleton, but also compromises myoblasts transition into muscle fibers, thus suggesting a potential role for ataxin-3 in myogenesis (*do Carmo Costa et al., 2010*). This process comprises several events where remodeling of the cytoskeleton as well as of cell–cell and cell–extracellular matrix interactions is of crucial importance, with a tight control of protein expression and turnover (frequently by the UPP) being essential (*Baylies and Michelson, 2001; Bryson-Richardson and Currie, 2008*).

1.4.6. Nuclear inclusions and pathogenesis mechanisms

Since the association between polyglutamine expansions and neurodegeneration was made (*La Spada et al., 1994*), a number of mechanisms had been suggested to participate in the pathology of these diseases such as altered gene expression (*Okazawa, 2003*), impairment of cell's quality control machinery: proteasome and chaperones (*Ferrigno and Silver, 2000*) and alterations in intracellular trafficking and apoptotic cell death (*Rego and de Almeida, 2005*).

1.4.6.1. Nuclear inclusions

A common feature of polyglutamine diseases is the deposition of insoluble intracellular inclusions containing the misfolded disease protein (*Paulson, 1999*). Although their correlation with pathology is controversial (*Bates, 2003; Michalik and Van Broeckhoven, 2003*), nuclear and sometimes cytoplasmic inclusions are considered the identifying hallmark of polyglutamine diseases (*Ross and Poirier, 2004*). The type of aggregated proteins and the regional and cellular distribution of the protein deposits vary from disease to disease, indicating that the individual proteins carrying the polyglutamine expansion modulate the associated pathogenesis (*Paulson, 2003*).

Mutant ataxin-3 in MJD, like other pathogenic proteins with expanded polyQ tracts, appears to undergo a conformational alteration and aggregate in cells forming ubiquitinated intranuclear neuronal inclusions (NIIs) with many different neurons containing more than one inclusion body, both in and outside areas affected by the selective neurodegeneration (*Paulson et al., 1997b; Schimdt et al., 1998; Rub et al., 2006*). Ataxin-3 aggregates were shown to contain β-rich fibrillar structures of amyloid nature (*Bevivino and Loll, 2001; Chen et al., 2002*). As for most amyloid-forming proteins, several pathways may drive the conversion of the soluble protein to amyloid aggregates, through the formation of different conformationally altered monomeric or self-assembled multimeric species (*Uversky, 2010*). Polyglutamine monomers of ataxin-3 acquire β-strand conformations that have been shown to be cytotoxic in cultured cells (*Nagai et al., 2007*), assembling into oligomers (*Bevivino and Loll, 2001; Takahashi et al., 2008)* able to simultaneously dissociate into monomers (*Schaffar et al., 2004*). Thus, it seems that β-stranded polyglutamine monomers are important for pathogenesis in MJD and other polyglutamine diseases, however is still controversial if their toxicity is sufficient to be neurotoxic. Polyglutamine oligomers, have been shown to induce greater toxicity than polyglutamine monomers or inclusion bodies in neuronaly

differentiated cells (*Takahashi et al., 2008*). This and other findings support the hypothesis that polyglutamine oligomers may have a crucial role in cytotoxicity (*Poirier et al., 2002; Sanchez et al., 2003; Kayed et al., 2003; Ross and Poirier, 2004; Behrends et al., 2006*), as well as β-stranded monomers, however the role of amyloid fibrillar aggregates is still controversial as well as the NIIs in the pathogenesis of the disease (*Popiel et al., 2011*)(Fig. 5).

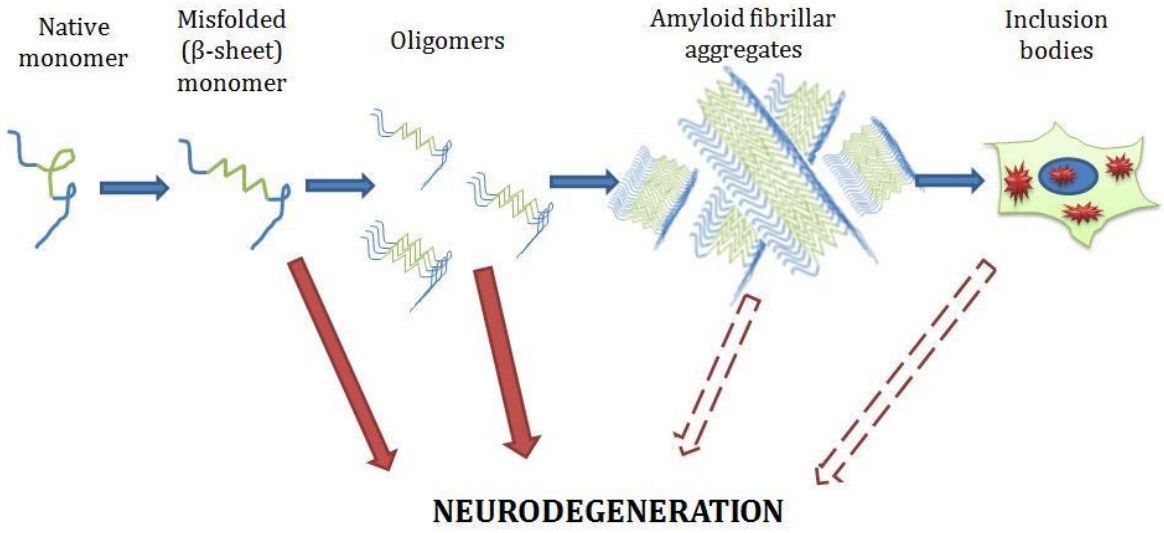

Figure 5. Inclusion bodies formation. Proteins with an expanded polyQ stretch are prone to misfold into a β-sheet dominant structure, leading to their assembly into oligomers and amyloid fibrillar aggregates, followed by their accumulation as inclusion bodies within neurons, eventually resulting in neurodegeneration. Dashed arrows indicate structures for which cytotoxicity remains controversial. (Adapted from *Popiel et al., 2011*).

NIIs are eosinophilic structures, which vary in size from 0.7 to 3.7 μm; are membrane-bound and contain a mix of granular and filamentous structures. These aggregates were also found in the cytoplasm of neurons in several affected areas (*Hayashi et al., 2003*), and in axons in fiber tracts known to be targets of neurodegeneration in MJD (*Seidel et al., 2010*). Nuclear inclusions of mutant polyglutamine proteins are characteristic of these diseases. Even in those diseases where the aggregates are found in cytoplasm like in SCA2 and SCA6, there is evidence for the presence of polyQ inclusions in the nuclei of neuronal cells (*Ishikawa et al., 2001; Pang et al., 2002; Schols et al., 2004*). The nuclear membrane serves as barrier segregating activities related to gene transcription from other activities of the cytoplasmic compartment. This function does not exclude its part in the cellular mechanisms of these polyQ diseases, in fact, it has been more accepted that the functional and structural changes in the nucleus are central for the pathogenesis of several neurodegenerative diseases. The

presence of NII is the proof that the neuronal nucleus suffers changes that allows the nuclear aggregation of certain proteins that in their normal conformation are localized in the cytoplasm (*Woulfe, 2007*).

In NIIs, normal and mutant ataxin-3 colocalize with cellular components such as ubiquitin, the proteasome, heath shock proteins (HSPs) and transcription factors (*Cummings et al., 1998; McCampbell et al., 2000; Schmidt et al., 2002*). Other proteins have also been found in the NIIs of pontine neurons of MJD patients, such as ataxin-2 (the causative protein of SCA2) and the TATA box binding protein (TBP) (*Uchihara et al., 2001*).

The NIIs in MJD are distributed in many neurons of the central and peripheral nervous regions, including the cerebral cortex, thalamus and autonomic ganglia (*Schilling et al., 1999*). However, the presence of NIIs in regions not affected in MJD raises the possibility that NIIs are not directly pathogenic (*Rub et al., 2006*). It has been suggested that NIIs are in fact just the result of protective cellular mechanisms to cope with the toxicity of the expanded proteins. Studies concerning MJD demonstrate that there is no perfect overlap between the brain regions that degenerate and the regions presenting NIs (*Klement et al., 1998; Saudou et al., 1998; Cummings et al., 1999; Evert et al., 2006; Paulson et al., 1997b; Ross and Poirier, 2004; Trottier et al., 1998; Yamada et al., 2001*). Interestingly, upon blockage of the ubiquitin machinery fewer inclusions are observed but neurodegeneration proceeds. Therefore the mutant proteins may be far more toxic if they are not sequestered into inclusions, which supports the hypothesis that NIIs formation can be a protective mechanism against the toxicity of polyQ expanded proteins (*Schols et al., 2004*).

1.4.6.2. Pathogenesis mechanisms

Although in different levels, SCAs show as a common characteristic the progressive neurodegeneration and mutant proteic aggregate formation, which accumulate in the nucleus or in the cytoplasm. Despite the fact that the causative genes are widely expressed, the central nervous system is commonly and selectively attained, with neurodegeneration patterns that are unique to each disease. The inherited ataxias are caused by either gain of function or loss of function mutations in seemingly unrelated genes. Because of the overlap in the phenotypes and the prominence of the Purkinje cells pathology, it is hypothesized that the gene products involved in this class of neurodegenerative diseases might play a role in common molecular pathways that are essential for cerebellar function and survival (*Gatchel and Zoghbi, 2005*)(Fig. 6).

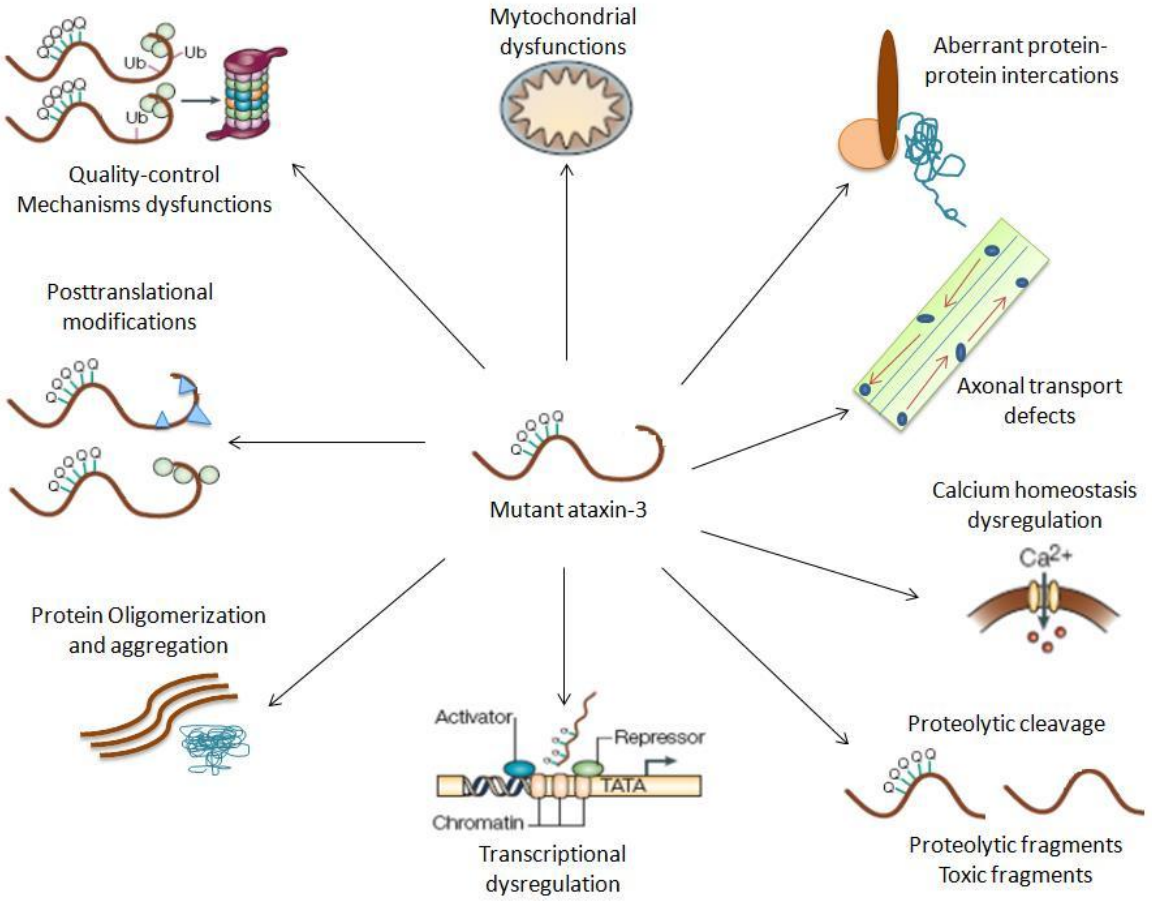

Figure 6. **Mechanisms of pathogenesis in Machado-Joseph disease.** Expanded polyglutamine proteins might mediate pathogenesis trough a range of common mechanisms; MJD is no exception. The MJD1 gene with expanded CAG repeat is transcribed and translated into ataxin-3 with expanded polyglutamine tract. The expansion results in an aberrant structure of ataxin-3, which forms possible new proteic interactions that wouldn't happen in normal situation and triggers several events that lead to neurodegeneration in selective areas of the brain. Accumulation of mutant ataxin-3 to form insoluble NIIs recruits components of the UPS and other protein quality-control pathways, furthermore, the normal function of ataxin-3 in the cell can contribute to the impairment of UPS, thus leading to a dysfunction in these quality-control mechanisms. The full-length polyglutamine protein can also be cleaved by proteases to form fragments, which are toxic and might also mediate pathogenic effects. The oligomerization and aggregation and the posttranslational modifications may also contribute to the increase of the neuronal cytotoxicity and dysfunction. The mutant protein affects many other cellular processes, including transcription and RNA metabolism. Other cellular processes that can be affected include mitochondrial function, calcium homeostasis, and axonal transport, ultimately leading to neuronal dysfunction and death. Q, glutamine; Ub, ubiquitin. (Adapted from *Gatchel and Zoghbi, 2005*)

1.5. Ataxin-3 interactions

Several studies reported the importance of protein-protein interactions in understanding the normal function of the disease-causing proteins (*Yoshida et al., 2002; Chen et al., 2003; Goehler et al., 2004; Ravikumar et al., 2004; Kaytor et al., 2005; Tsuda et al. 2005*); thus suggesting that interactions between ataxin-3 and other proteins could be important in the development of MJD pathogenesis.

One of the most intriguing facts that points to a possible protein interaction of Ataxin-3 with other proteins is the age of onset. As previously referred an inverse correlation between the number of CAG repeats and the age of onset is present in MJD. The CAG repeats are responsible for most of the variability in the age of onset (about 45-60%) (*Maciel et al., 1995; Van de Warrenburg et al., 2002*). Nevertheless, there are cases where this correlation it is not verified suggesting that other factors can contribute to the age of onset.

Another evidence that not only the CAG repeats account for the phenotype is the variability in the symptoms and overall phenotype of heterozygous individuals with similar number of CAG repeats. As previously discussed, CAG repeats are thought to be responsible for some part of the variability but there is a lack of evidence about other factors that can influence the phenotype and the disease progression. In other polyQ diseases correlations between specific proteic interactions and the variability of the phenotypes and diseases progression have been reported. It has been suggested that also in MJD proteic interactions can determine certain aspects of the disease and be the cause of the divergence observed (*DiStefano et al., 1995; Maciel et al., 1995; Van de Warrenburg et al, 2002*).

Studies revealed that silencing of the mutant Ataxin-3 is not enough to stop the pathology, leading to a diminished inclusion formation but unable to stop completely the pathogenesis of the disease (*Hübener and Riess, 2010*). This phenomenon could imply that pathogenesis is related to other factors besides the action of the mutant ataxin-3, becoming more clear the possible role of other proteins interacting with ataxin-3 in MJD. Moreover, the toxicity might be related with the expanded polyglutamine tract, but this does not explain the selective vulnerability of specific neurons affected in MJD. Thus, the normal function of ataxin-3 and protein-protein interaction mechanisms might also be crucial for pathogenesis (*Takahashi et al., 2010*).

1.5.1. Ataxin-3 interaction with other polyglutamine proteins

Among the SCAs, there is a remarkable overlap of symptoms, including progressive loss of gait and limb muscle coordination, with neurodegeneration in the cerebellum and often brainstem nuclei (*Paulson, 2007*). In addition to the shared mechanism of mutation and several phenotypes, there are intriguing hints of interactions between the polyglutamine genes that cause different SCAs.

For example, relatively long CAG repeats lengths (within the normal range) in the causative gene for SCA6 are correlated with an early age of onset of SCA2 (*Pulst et al., 2005*). Moreover, between SCA2 and SCA3 there are also some observations that suggest links between the causing genes. A phenotypic characteristic in SCA3 patients is correlated with the length of CAG repeat in the normal alleles of SCA2 causing gene (*Simón-Sanchez et al., 2005*). Both SCA2 and SCA3 are unusual among the dominantly inherited ataxias in that they can manifest with parkinsonism (*Jardim et al., 2003*). The normal ataxin-2 (Atx2) protein can be detected in the pathogenic inclusions of SCA3 patients, and, likewise, normal ataxin-3 protein localizes to the inclusions formed in SCA2 patients (*Uchihara et al., 2001*). In a search for modifiers of degeneration induced by the human pathogenic protein causing SCA3, the ataxin-2 gene was identified (*Bilen and Bonini, 2007*). The normal activity of Ataxin-2 was shown to be critical to SCA3 neurodegeneration, suggesting that toxicity of one polyglutamine disease protein can be modulated by the normal activity of another (*Lessing and Bonini, 2008*). The causing gene for SCA2 (ataxin-2) had already been associated to SCA1, in a search for genetic modifiers for this disease (*Fernández-Funez et al., 2000*). This genetic interaction between these two causing polyQ diseases was shown to be due to physical direct interaction (*Lim et al., 2006*). However, it has been suggested that ataxin-2 and ataxin-3 do not interact with each other directly (*Lim et al. 2006*) and may be dependent of an additional transcript target. The ataxin-2 protein is thought to be related with regulation of translation of specific transcripts (*Satterfield and Pallanck, 2006*), taking into account that ataxin-2 physically interacts with poly-A-binding protein (PABP) in polyribosomes (*Ralser et al. 2005*) and with putative RNA-binding protein: ataxin-2-binding-protein (A2BP) (*Shibata et al. 2000*). However, it seems that ataxin-3 mRNA cannot be directly the target of ataxin-2-PABP interaction (*Lessing and Bonini, 2008*). Nevertheless, other proteins could be implicated in this interaction. In fact, network studies of ataxia-causing proteins revealed hub proteins that interconnect several proteins, including several ataxia-causing proteins (*Baravabi and Albert,*

1999; Lim et al. 2006). Three of these proteins seem to be very relevant and important: RBM9, A2BP1, and RBPMS. The last two proteins were found to be genetic modifiers of SCA1 (*Fernández-Funez et al., 2000*). Also, these three proteins are involved in RNA binding or splicing (*Jin et al., 2003; Nakahata and Kawamoto, 2005; Underwood et al., 2005*). Finally, these three proteins interact together in an ataxia-causing protein network, linking ataxin-1, ataxin-2 and ataxin-3 (*Lim et al., 2006*).

Several studies have revealed the importance of protein-protein interactions in understanding the normal function of the disease-causing protein (*Stefan et al., 2001; Yoshida et al., 2002; Chen et al., 2003; Goehler et al., 2004; Ravikumar et al., 2004; Kaytor et al., 2005; Tsuda et al. 2005*). Unraveling the molecular mechanisms underlying these interactions could lead to identification of new targets and pathways that could be used as therapeutic approaches.

1.6. Ataxin-2

Human Ataxin-2 (ATX2) is the gene product of the spinocerebellar ataxia type 2 (*SCA2*) gene. This gene was localized to chromosome 12q24.1 in 1993 but it was not until 1996 that Ataxin-2 was identified as its product (*Gispert et al., 1993; Pulst et al., 1996; Sanpei et al., 1996*). The *SCA2* gene contains 25 exons, encompassing approximately 130kb of genomic DNA (*Sahba et al., 1998*) and it has an unstable triplet-repeat expansion as the pathogenic mutation, coding for a polyglutamine domain in the N-terminal region of the protein Ataxin-2. This triplet-repeat reads $(CAG)_8$-CAA-$(CAG)_4$-CAA-$(CAG)_8$ in DNA and mRNA of most individuals, but can vary in length in the human population. The presence of more than 31 triplets in the SCA2 gene may cause clinically manifest neurodegeneration (*Pulst et al., 1996; Sanpei et al., 1996; Pulst et al., 2005*).

1.6.1 Ataxin-2 Structure

Ataxin-2 is composed of 1312 amino acid residues (including the 22 glutamines of the polyQ stretch), with an approximated molecular mass of 140kDa. Except for the polyglutamine domain in its N-terminal, ATX2 has no similarity with other proteins involved in polyglutamine diseases (*Albrecht et al., 2004*). Ataxin-2 is a highly basic protein with an isoelectric point of 9.6 due to the elevated content in glutamine residues, the 22 glutamines are flanked by a region of proline-rich and serine-rich domains. The protein is not exclusively basic, containing one acidic region of 46 acidic amino acids (amino acid 254-475). This region covers roughly exons 2-7 and is predicted to consist of two globular domains named Lsm (Like Sm, amino acid 254-345) and LsmAD (Lsm- associated domain, amino acid 353-475) (Fig. 7) (*Neuwald et al., 1998*).

The Lsm domain contains Sm1 and Sm2 motifs, which are found in proteins involved in RNA splicing, suggesting that wild-type ataxin-2 might function as a component of the RNA splicing complex. The LsmAD with mainly α-helices, contains both a clathrin-mediated trans-Golgi signal and a endoplasmic reticulum exit signal (*Neuwald et al., 1998; Huynh et al., 2003; Albrecht et al., 2004*). Ataxin-2 also has a highly conserved domain in its C-terminal, which mediates association with the polyadenylate (poly(A))-binding protein, PABP (Fig. 6). This domain is called PAM2, which stands for PABP interacting motif, due to its function. This domain is contained in a number of proteins including an ataxin-2 ortholog, Ataxin-2 related protein (A2RP) (*Kozlov et al., 2001*).

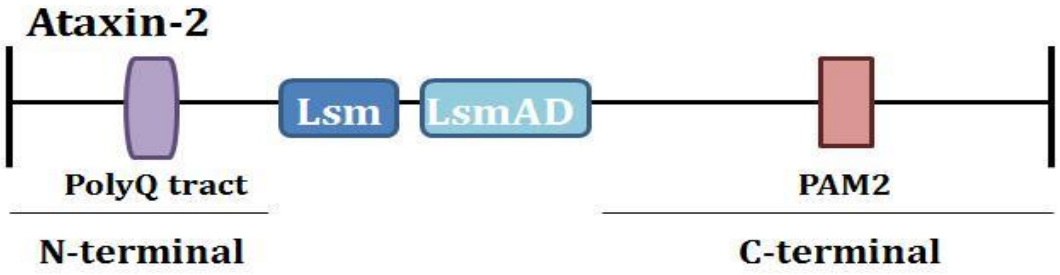

Figure 7. Protein structure of human ataxin-2. The PolyQ tract in the N-terminal region, the two globular domaisn, Lsm and LsmAD domains, and the PAM2 motif in the C-terminal region.

1.6.2. Ataxin-2 function

Many of the proteins affected by polyQ expansions normally function as transcription factors and the transcriptional dysregulation appears to be a central feature of the neurodegenerative mechanism in the disease (*Hughes et al., 2001*). Ataxin-2 is one of these polyQ proteins, implied directly in SCA2, and in a dominantly inherited form of Parkinsonism (*Shan et al., 2001; Lu et al., 2004*). More recently it has been implicated in the neurodegenerative diseases ALS (*Elden et al., 2010*) and SCA1 (*Al-Rahami et al., 2007*). This makes ataxin-2 an important and interesting protein by the suggestion that in its wild type form it can be a modulatory protein for other diseases. Therefore ataxin-2 may be an important key to understand the disease mechanisms and possibly allow the discovery of new and promising therapeutic targets. However, despite the several studies conducted to understand wild type ataxin-2 function, it is still currently not fully understood. Previous work on ataxin-2 and its related homologues, have implicated this protein in diverse biological processes including secretion, cell specification, actin filament formation and apoptotic and receptor-mediated signaling. Several observations suggest that the influence of the ataxin-2 in all these processes results from a conserved biochemical function in cytoplasmic RNA metabolism (*He et al., 2000; Satterfield and Pallanck, 2006*).

A role of ataxin-2 in RNA metabolism is credible in view of the Lsm and LsmAD domains contained in its N-terminal. The Lsm domain is known to function as an RNA-binding domain to Lsm proteins that are generally involved in important processes of RNA metabolism, including RNA modification, splicing and degradation. Ataxin-2 may fulfill similar functions in RNA metabolism. Ataxin-2 interacts with the translational machinery by assembling with polyribosomes through its Lsm/LsmAD domain and independently through its PAM2 motif (*He et al., 2000; Satterfield and Pallanck, 2006*).

Ataxin-2 functions may be accessed by its interactions with other proteins; to achieve that, several studies were conducted using ataxin-2 homologous. From these studies it became clear the role of this protein in RNA metabolism, observed specially by its interaction with PABP and polyribossomal machinery.

1.6.2.1 Stress granules (Ataxin-2 and PABP co-localization site)

It has been demonstrated that ataxin-2 is a component of mammalian stress granules (*Ralser et al., 2005*). Stress granules (SG) are cytoplasmic sites where untranslated mRNAs are stalled during environmental stress, and both RNA stabilizing and destabilizing proteins such as PABP are recruited to stress granules. PABP is found in distinct cytoplasmic granules (stress granules) arising in the cytoplasm of mammalian cells in response to environmental stresses (*Kedersha and Anderson, 2002; Villace et al., 2004*). Preliminary genetic analyses of Ataxin-2 and its homologs indicate that the role of this gene family in RNA metabolism may involve translational regulation. Ataxin-2 was co-localized with PABP in stress granules, which demonstrated that ataxin-2 is a part of these in heat stressed cells. It is possible that ataxin-2 may be recruited to the SG so that PABP can perform its normal function of RNA modification. Since it was discovered that ataxin-2 is a component of these sites of mRNA triage, one might speculate that ataxin-2 plays a role in regulating cellular mRNA turnover (*Ralser et al., 2005*).

Another protein localized to the stress granules was TDP-43 that interestingly interacts with ataxin 2 in ALS (*Liu-Yesucevitz et al., 2010*), as we discuss ahead. The regulatory role of ataxin-2 in the SGs, and the interaction with TDP-43 and SGs, is likely to reflect a mixed mechanism involving both protein-protein interactions, as well as protein-RNA interactions, since TDP-43 is a well known protein interactor with ataxin-2 (*Hua and Zhou, 2004; Nonhoff et al., 2007*). The type of interaction might depend on the particular species of TDP-43 and on the conditions at the binding site. A recent study demonstrated that ataxin-2 binds TDP-43 and expanded polyglutamine domains in ataxin-2 promote formation of TDP-43 inclusions. The same group also identified cases of ALS that were TDP-43 positive and associated with expanded polyglutamine stretches in ataxin-2. SG proteins might enhance inclusion formation related to these proteins as a result of their mutual associations (*Elden et al., 2010*).

1.6.2.2. A2BP1

Further strengthening the concept of ataxin-2 function in RNA metabolism; it has been shown that the C-terminus of ataxin-2 binds to A2BP1 (*Shibata et al., 2000*), a novel protein containing a RNP motif which is highly conserved among RNA-binding proteins (*Ralser et al., 2005*) and whose RNA-binding *C. elegans* homologue, fox-1, regulates tissue-specific alternative splicing (*Jin et al., 2003*). Disruption of the human A2BP1 gene may cause epilepsy or mental retardation (*Bhalla et al., 2004*). A bulk of evidence indicates that ataxin-2 homologues might be involved in RNA splicing, mRNA stabilization or degradation and other essential RNA-processing pathways. Further studies are required to define its function in RNA metabolism (*Ralser et al., 2005*).

1.6.3. Ataxin-2 localization

Ataxin-2 has a widespread expression in adult and embryonic tissues, predominantly in the brain, but also in the heart, muscle, intestine, liver and lung (*Pulst et al., 1996*). Ataxin-2 is strongly expressed in neuronal cells of adult mouse brain such as large pyramidal neurons and subpopulations of hippocampus, thalamus and hypothalamus. Its major localization is seen in the cerebellar Purkinje cells, the primary site of SCA2 neurodegeneration (*Nechiporuk et al., 1998*).

In its normal distribution was found in apical cytoplasm of pyramidal neurons of some layers of the frontal cortex, but not in white matter (*Huynh et al., 1999*). In the hippocampus, ataxin-2 is more expressed in pyramidal neurons than in the granule neurons of the dentate gyrus. In the basal glia, in the neurons of globus pallidus and amygdaloid nuclei, a moderate ataxin-2 expression was found (*Huynh et al., 1999*). In the large neurons of *substantia nigra* a strong expression was found, as well as in the cerebellum. In Purkinje cells a strong expression of ataxin-2 was observed whereas in the dentate nucleus this expression was moderate (*Huynh et al., 1999; Lastres-Becker et al., 2008*). Recurring to fluorescent labeling, it was observed that in Purkinje cells, the expression of ataxin-2 besides intense was distributed throughout the cells including the dendrites with an intense labeling around the nucleus, with the particularity of being concentrated in only one pole of the cytoplasm (*Huynh et al., 1999; Becker et al., 2008*).

The levels of Ataxin-2 expression increased with age in neurologically normal brains and were greatly increased in brains from SCA2 patients (*Huynh et al., 1999*).

1.7. Ataxin-2 proteic interactions

Despite the genetic heterogeneity, many ataxias show striking similarities. These similarities suggest that SCAs, and perhaps other ataxias, may also share common mechanisms of pathogenesis. Interaction proteic networks can explain the phenotypic similarities in inherited cerebellar ataxias (*Lim et al., 2006; Soong and Paulson, 2007*). Several ataxia proteins interact together and there are common processes and pathways involved in this class of neurodegenerative diseases. A surprising and interesting outcome of its study was that the majority (18/23) of the ataxia-causing proteins interact either directly or indirectly with each other (*Lim et al., 2006*).

1.7.1 Ataxin-2 mediates Ataxin-1 induced neurodegeneration

Spinocerebellar ataxia type 1 (SCA1) is caused by the expansion of a CAG repeat encoding the polyQ tract within the protein ataxin-1, which induces a toxic gain of function (*Orr and Zoghbi, 2001*). Although the polyglutamine expansion triggers the toxicity of ataxin-1, experiments in *Drosophila* and mouse SCA1 models have shown that protein context plays a determinant role in expanded ataxin-1 induced neurodegeneration (*Gatchel and Zoghbi, 2005*). In addition, certain interacting partners of unexpanded ataxin- 1 are critical to the expanded ataxin-1 toxicity (*Tsai et al., 2004; Tsuda et al., 2005; Al-Rahami et al., 2007*).

Expanded ataxin-1 by itself causes an abnormal phenotype in the *Drosophila* eye and overexpression of ataxin-2 also leads to an abnormal eye phenotype. Upon co-expression of the two ataxins, an increase in the severity of the phenotype was observed. Decreased levels of endogenous ataxin-2 also modified expanded ataxin-1 toxicity. However nor the SCA1 mRNA transcripts nor the levels of ataxin-1 protein were affected by altering the levels of ataxin-2. Flies with expanded ataxin-1 in the nervous system displayed a progressive impairment of motor function, which was suppressed by the decrease of ataxin-2 levels. SCA1 flies heterozygous for an ataxin-2 mutation showed a significantly improved motor performance when compared to flies with normal ataxin-2 (*Al-Rahami et al., 2007*). It was also observed that ataxin-1 is able to precipitate endogenous ataxin-2 from human cells, which suggests that these proteins may be functional interactors *in vivo* (*Al-Rahami et al., 2007*).

Regarding subcellular localization, ataxin-1 is normally found in the nucleus while ataxin-2 is clearly a cytoplasmic protein. In neurons, endogenous ataxin-2 is not normally found in the nucleus but when co-expressed with expanded ataxin-1, it was possible to observe ataxin-2 in the nucleus. These results indicate that expanded ataxin-1 causes ataxin-2 to localize to the nucleus and suggest that both proteins co-aggregate in NIIs. This accumulation in the nucleus occurs when the expanded ataxin-1 is present and is not promoted in the presence of unexpanded ataxin-1. . Ataxin-2 accumulation is paticularly harmful in the nucleus where it contributes to the exacerbated toxicity of expanded ataxin-1. This nucleoshuttling of ataxin-2 into the nucleus is an important mechanism of SCA1 pathogenesis (*Al-Rahami et al., 2007*).

1.7.2 Ataxin-2 intermediate-length polyglutamine expansions are a risk factor for ALS

Amyotrophic lateral sclerosis (ALS) also known as Lou Gehrig's disease, is a progressive, fatal neurodegenerative disease caused by degeneration of motor neurons (*Cleveland et al., 2001*). To date, various genes have been identified as associated with familial cases, among these, mutations in superoxide dismutase 1 (SOD1), which account for 15–20% of cases of familial ALS (*Valentine et al., 2003*). Transactivate reponse DNA-binding protein (TARDBP, or TDP-43) is a major disease protein of the ubiquitin-positive cytoplasmic inclusions in ALS without SOD1 mutations (*Neumann et al., 2006; Winton et al., 2008*). Ataxin-2 was found to be a possible modifier of TDP-43 toxicity in ALS (*Elden et al., 2010*), since the motor neurons features of SCA2 are sufficiently prominent to mimic an ALS presentation, indicating the potential for a clinicopathological overlap (*Infante et al., 2004; Nanetti et al., 2009*).

Pbp1, the ortholog of human Ataxin-2 gene, was identified as a gene that enhanced TDP-43 toxicity. Upregulation of Pbp1 enhanced TDP-43 toxicity in yeast, whereas Pbp1 loss-of-function suppressed toxicity. It was also observed that mutant TDP-43 aggregates in the cytoplasm were able to recruit Ataxin-2 (*Elden et al., 2010*).

Recent observations in patients with ALS indicate that intermediate-size expansions (polyQ27-33) of ataxin-2 act as a risk factor modulating the manifestation and age at onset of motor neuron degeneration (*Chen et al., 2011; Daoud et al., 2011; Elden et al., 2010; Lee et al., 2011; Ross et al., 2011; Soraru et al., 2011; Van Damme et al., 2011*). Regarding the effects of ataxin-2 in the pathogenesis of ALS, it was observed that ataxin-2 expansions just above the threshold (Q32–37) may lead to ALS instead of SCA2 (*Daoud et al., 2011; Ross et al., 2011*).

1.8. Ataxin-2 connection to PABP determines its function?

Pbp1 (PABP binding protein 1) is the yeast homologue of human ataxin-2, a non-essential protein in contrast to *Caenorhabditis elegans* and *Drosophila melanogaster* homologues, which were proven to be essential proteins. Ataxin-2 shows significant homology to the yeast protein Pbp1, which also contains the Lsm and LsmAD domains. The regions outside of these two globular domains are predicted to be mainly unstructured in Pbp1 as in ataxin-2 (Fig. 8) (*Albretcht et al., 200*4). Although the C-terminal tail of Pbp1 does not contain a PAM2 motif, this yeast protein regulates polyadenylation after pre-mRNA splicing and interacts with the C-terminal part of the yeast homologue Pab1 of the human cytoplasmic poly(A)-binding protein 1 (PABP) (*Mangus et al., 1998*). Ataxin 2-binding protein 1 (A2BP1) and PABP are also evolutionarily related and possess RNA recognition motifs (*Shibata et al., 2000*). These observations strongly suggest that ataxin-2 is involved in similar mRNA processing tasks (*Albrecht et al., 2004*).

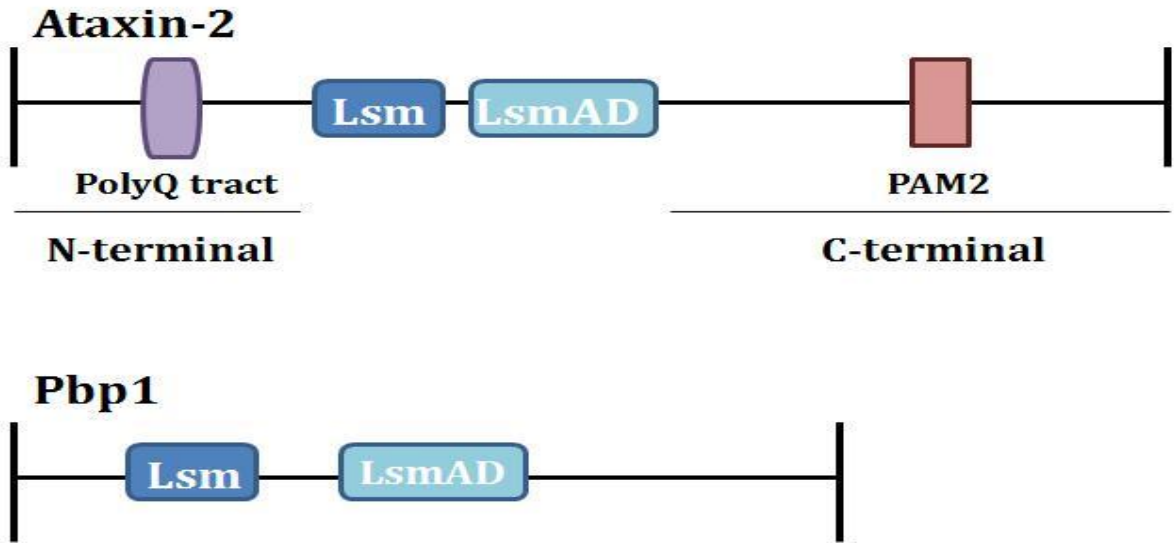

Figure 8. Protein structures of human Ataxin-2 and its yeast homologue Pbp1. Both proteins have a putative RNA binding Lsm domain followed by an as yet uncharacterized Lsm-associated domain LsmAD. ATX2 also contains a PABC-interacting motif named PAM2, which Pbp1 does not contain. (Adapted from *Ralser et al., 2005*).

Pbp1 regulates polyadenylation and binds the C-terminal part of the poly(A)-binding protein Pab1, the yeast homolog of human cytoplasmic poly(A)-binding protein 1 (PABP). In the

absence of Pbp1, the 3'- ends of the pre-mRNAs are cleaved properly, but lack a complete poly(A) tail (*Mangus et al., 1998; Mangus et al., 2003; Mangus et al., 2004*). Pbp1 is linked to the nuclear export machinery of mRNA (*Nakielny and Dreyfuss, 1997*), therefore taking in account its structural and functional similarity with ataxin-2, it is possible to extrapolate that ataxin-2 is also linked to this function.

C. elegans ataxin-2 homolog, ATX2, is widely expressed in the cytoplasm in form of a complex together with a poly(A)- binding protein (PAB1) (*Ciosk et al., 2004*). The complex formed suggests a possible role for ATX2 in PAB1 mediated processes. However, not all PAB1 functions were affected by the silencing of ATX2, raising the hypothesis that ATX2 does not play a mandatory role in all of PAB1 functions and may instead have specialized functions. ATX2 seems to be responsible for the ability of PAB1 to interact with components of the translational machinery, therefore playing an important role in the translation regulation as suggested in studies with other homologues. It was also shown that reduced ataxin-2 function was able to alter the abundance of particular proteins, without altering the abundance of their corresponding transcript, suggesting that these transcripts may be translationally regulated by ataxin-2 (*Ciosk et al., 2004*). Several studies achieved similar evidences that loss of ataxin-2 increases the abundance of some proteins while reducing the abundance of others, suggesting that the possible regulatory role in translation can be both positive and negative (*Ciosk et al., 2004; Satterfield and Pallanck, 2006*).

A direct interaction between ATX2 and PAB1 was not shown in *C. elegans*. Nevertheless, this possible interaction was found upon transfection of mammalian cell lines (COS-1) with ATX2 since endogenous PABP and ATX2 are present in the cytoplasm of COS-1 cells, and co-localize in certain areas of the cytoplasm (*Ralser et al., 2005*). Noticeably, it seems that ATX2 (*C. elegans* ataxin-2 homologous) and Pbp1 (yeast ataxin-2 homologous) use different mechanisms to interact with the C-terminal part of PABP and Pab1, respectively. While ATX2 binds the PABC domain itself, the major interaction site of Pbp1 with Pab1 appears to be a region preceding the PABC domain of Pab1 (*Mangus et al., 2004*).

Despite the evidence that the ATXN2 family is involved in translational regulation, there was no direct evidence that ataxin-2 would engage the translational apparatus and itself function as a translational regulator. Later it was, demonstrated that human ataxin-2 and its *Drosophila* homolog (ATX2) interact with the translational machinery by assembling with polyribosomes (*Satterfield and Pallanck, 2006*), suggesting that the ataxin-2 protein directly influences the translation of particular mRNAs. ATX2 bears at least two polyribosome binding regions, one envolving the N-terminal Lsm/LsmAD domain and the other the C-terminal

region (PAM2 motif), thus its assembly is mediated independently by the Lsm/LsmAD domains and the PAM2 motif. The Lsm portion of the Lsm/LsmAD domain is a known RNA-binding motif and could thus allow ataxin-2 to directly bind to polyribossomal mRNA; whereas ataxin-2 assembles with PABP in a PAM2 motif dependent manner and its interaction appears to link ataxin-2 to polyribosomes by allowing it to interact with the polyribosome-bound dPABP (*Satterfield and Pallanck, 2006*).

Given the evidence supporting a role for Ataxin-2 in translational regulation, the question arises to the mechanism by which ataxin-2 imposes this regulation. The cytoplasmic localization of ataxin-2 indicates that this protein most likely does not influence directly transcription, but its interaction with PABP as well as its assembly with polyribosomes, seem to be solid indicators of an ataxin-2 role in the mediation of translational regulatory activities.

1.8.1. PABP

The poly(A)-binding protein (PABP) is a highly conserved, abundant and essential protein which can shuttle between the nucleus and the cytoplasm of all eukaryotes exhibiting different functions depending on its location in the cell (*Afonina et al., 1998; Brune et al., 2005*). It is a ubiquitous RNA-binding protein, which preferentially associates with the 3'-poly(A) tail of eukaryotic mRNA (*Amrani et al., 1997*). This multifunctional protein is involved in an extensive range of cellular functions, including translation, mRNA metabolism, and mRNA export (*Afonina et al., 1998; Brune et al., 2005*).

Structurally, PABPs consist of four N-terminal RNA-recognition motifs (RRMs) and a C-terminal that consists in a proline-rich region and a globular domain termed PABC (Fig. 9) that has been shown to be important for functional regulation by interacting with many cytosolic and nuclear proteins (*Kozlov et al., 2001*). In humans, three types of PABP proteins are observed: cytoplasmic PABPs (PABPC1, PABPC3, and iPABP); nuclear PABP (PABPN1); and X-linked PABP (PABPC5). PABC structure plays an important and determinant role in the cellular localization of PABP (*Kleene et al., 1998; Mangus et al., 2003*).

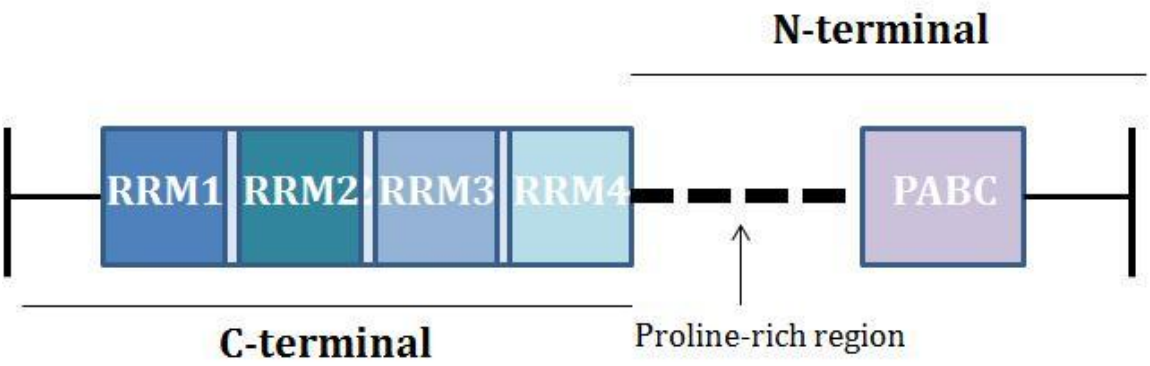

Figure 9. PABP1 structure diagram. The N-terminal consists in the four RRMs, different and not functionally equivalent. The C-terminal consists in a proline rich region and the PABC. (Adapted from *Smith and Gray, 2010*).

The RRMs associate with the poly(A) tail of mRNA and can also act as a scaffold for binding other proteins, which appear to differ in their RNA- and protein-binding specificities (*Mangus et al., 2003; Gorgoni et al., 2004*). The association of PABPs with poly(A) requires a minimal binding site of 12 adenosines, and multiple PABP molecules can bind to the same poly(A) tract, forming a repeating unit covering approximately 27 nucleotides (*Baer et al., 1983; Sachs et al., 1987*). PABPs interact with poly(A) via RNA-recognition motifs (RRMs). Because of the essential function of the RRM domains, they have received more attention than the PABC domain. The C-terminal of PABP does not bind RNA, but is required for oligomerization. Both C-terminal regions bind proteins, but the PABC domain, in particular, mediates interactions with proteins that contain a PAM2 motif to regulate translation and mRNA stability (*Roy et al., 2002; Albrecht and Lengaver, 2004; Bravo et al., 2005*). There are many proteins that have a PAM-2 motif, ataxin-2 for example. Each modulates translation efficiency via interaction with PABP, other translation factors, or *cis*-elements within the mRNA transcript. The structures of PABC domain adopt a comparable fold consisting of four or five α-helices and act in an analogous manner by recognizing a similar PAM-2 sequence. The human PABC-PAM2 complex structure displays the PAM-2 motif binding to specific residues within the PABC domain (*Kozlov et al., 2004*).

PABPs have crucial roles in the pathways of gene expression. They bind the poly(A) tails of newly synthesized or mature mRNAs and appear to act as *cis*-acting effectors of specific steps in the polyadenylation, export, translation, and turnover of the transcripts to which they are bound (*Mangus et al., 2003*). As an initiator factor, PABP plays a global role in the translation of adenylated mRNAs. However, the translation of some mRNAs is more sensitive

to PABP than others (*Smith and Gray, 2010*). In addition to its global activity, PABP also has specific roles in translation. PABP binding to internal poly(A) stretches in 3'-UTRs can promote translation (*Skabkina et al., 2003*). PABP can also be recruited to the 3'-UTR to activate translation by direct protein-protein interactions (*Mangus et al., 2003*).

Moreover, PABP participates in mRNA turnover and stability. Removal of the poly(A) tail is the initial step in different mRNA turnover pathways an PABP-mediated end-to-end initiation complexes generally protect the poly(A) tail from deadenylases and block deccaping. Lacking any evident of catalytic activity, PABPs provide a scaffold for the binding of factors that mediate these steps and also apparently act as antagonists to the binding of factors that enable the terminal steps of mRNA degradation (*Mangus et al., 2003*).

Therefore, PABP principal function is related to global gene regulation both by stimulating translational initiation and by enhancing RNA stability (*Sachs and Davis, 1989; Wang and Kiledjian, 2000*). Its connection to ataxin-2 through the PAM2 motif places PABP in an interesting position, since it is the only known protein to bind ataxin-2 through that motif. The observed role of ataxin-2 in translational regulation may therefore be mediated by its connection to PABP.

1.9. Ataxin-2 may mediate Ataxin-3 induced neuro-degeneration in MJD/SCA3

Mutant polyQ proteins in the corresponding diseases are found to be aggregated with similar chaperones besides the proteasome subunits; which may imply the sharing of important pathogenic features (*Bilen and Bonini, 2007*). This established common ground in the pathogenesis of these neurodegenerative diseases opened the door for the study of proteic interactions as modifiers of the toxicity mechanisms involved in several diseases. A number of modifiers of polyQ disease proteins have been already identified using animal models; including chaperones, transcriptional co-regulators and microRNAs. Several studies have been conducted trying to unravel potential modulators of ataxin-3 (*Kazemi-Kazemi-Esfarjani and Benzer, 2000; Fernandez-Funez et al., 2000; Nollen et al., 2004; Bilen et al., 2006*). Ataxin-3 is a unique protein due to its implication in ubiquitin pathways and degradation pathways. These characteristics not only make ataxin-3 an interesting protein regarding possible proteic interactions but also convert these studies searching for modifiers in a determinant tool to understand MJD and provide potential therapeutic targets to this disease that is the most frequent SCA.

Heat shock proteins (Hsp) were already involved in ataxin-3 toxicity in studies with *Drosophila*. Hsp70 substantially ameliorated Ataxin-3 retinal toxicity in a model of SCA, whereas the expression of a dominant negative form of *Drosophila* Hsp40, enhanced retinal toxicity (*Warrick et al., 1999*). In a screening study to search modifiers of ataxin-3 toxicity, the majority of modifiers fell into chaperone and ubiquitin pathways, consistent with ataxin-3 function in the UPS (*Bilen and Bonini, 2007*).

As previously discussed, ataxin-2 participates as a modulator in some disorders including PolyQ diseases modulating the neurodegeneration induced by expanded ataxin-1 in SCA1 and, in its intermediate length form, increasing the risk for ALS. Another study revealed that ataxin-2 intermediate expansions also modulated the risk of another neurodegenerative disorder within the spectrum of Parkinson-plus syndromes, progressive supranuclear palsy (*Ross et al., 2011*). Several lines of evidence led researchers to consider ataxin-2 as holder of a strong potential as a common mediator in neurodegenerative diseases (*Ghosh and Feany, 2004*).

In SCA1 and MJD some overlap has been observed in the symptoms due to the same affected areas (*Ghosh and Feany, 2004*). Different genes that modify SCA1 toxicity have been

shown to encode proteins that interact directly with pathogenic ataxin 1, and further, to form an interaction network that includes ataxin-3 (*Lim et al., 2006*). Is it possible that similarly to what happens with ataxin 1, ataxin-2 may also influence the pathogenesis of MJD by interacting with ataxin-3?

Several evidences support the hypothesis that ataxin-2 may act downstream of ataxin-3 in shared pathways, a concept strengthened by the parallels between the following neurodegeneration syndromes. Similar to the polyQ-expansions of sizes ≥34 in the mutant ataxin-2 causing SCA2, polyQ expansions of sizes ≥52 in the ataxin-3 (ATXN3) cause MJD. In both SCAs the pattern of affected neuron populations extends beyond the spinocerebellar circuits to involve the peripheral and central motor neurons as well as the dopaminergic neurons of *substantia nigra* (*Munoz et al., 2002; Lastres-Becker et al., 2008*). In consequence, SCA2 and MJD usually manifest with ataxia and dysarthria in their clinical pictures. As a result of the affection of dopaminergic nigra neurons, a clinical presentation of levodopa- responsive Parkinsonism can be present, caused both by ataxin-2 and ataxin-3 expansions in SCA2 and MJD, respectively (*Gwinn-Hardy et al., 2000, 2001; Shan et al., 2001; Payami et al., 2003; Simon-Sanchez et al., 2005; Lu et al., 2006; Socal et al., 2009*). Due to the motor neurons degeneration, the manifestation of prominent areflexia may be present in combination with pyramidal signs mimicking ALS, which may be caused by polyQ expansions in ataxin-2 and in ataxin-3 (*Landau et al., 2000; Infante et al., 2004; Rosa et al., 2006; Pinto de Carvalho, 2008; Nanetti et al., 2009*). Interestingly, ALS-typical inclusion bodies containing TDP-43 were observed in the cytoplasm of affected motor neurons both in SCA2 and in MJD (*Tan et al., 2009; Elden et al., 2010; Seidel et al., 2010*), suggesting the possibility of common pathogenic mechanisms for these disorders. In addition to all of these evidences, ataxin-2 can be detected in the NII in neurons of MJD patients as well as ataxin-3 can be reported in SCA2 inclusions, suggesting that these two proteins share some type of connection, otherwise this would not be observed (*Uchihara et al., 2001; Lessing and Bonini, 2008*). It was also found a correlation between the length of the CAG repeat length at the *SCA2* gene and fasciculations in MJD patients (*Jardim et al., 2003*).

In the *Drosophila* eye the presence of mutant expanded ataxin-3 by itself induced degeneration, but when coexpressed with ataxin-2 a dramatical enhancement in the degeneration was verified. In contrast, when the levels of ataxin-2 where reduced a significant mitigation of degeneration was observed. Similar results were observed in *Drosophila* neurons (*Lessing and Bonini, 2008)*. Taken together, these studies suggest that normal endogenous ataxin-2 activity facilitates ataxin-3 dependent toxicity, with up-regulation of ataxin-2 enhancing and

loss of ataxin-2 functions dramatically slowing the progression of neurodegeneration caused by pathogenic ataxin-3. The formation of NIIs as a hallmark of polyglutamine disease was also correlated with these previous reports. Up-regulation of ataxin-2 which enhanced ataxin-3 toxicity, also accelerated inclusion formation when compared to the inclusions in animals expressing solely mutant Ataxin-3 (*Lessing and Bonini, 2008*).

To further clarify the mechanism by which ataxin-2 modulated ataxin-3 in Drosophila, it was tested whether the PAM2 motif of ataxin-2 was required for the enhancement of MJD toxicity. The PAM2 motif is required for ataxin-2 to associate with PABP, and together with the RNA-binding Lsm domain, it mediates colocalization of ataxin-2 with PABP in polyribosomes. Using an ataxin-2 lacking its PAM2 motif it was observed that this motif was determinant for the accelerated inclusion formation, since the coexpression of this ataxin-2 and mutant ataxin-3 did not induce the results observed earlier on the study with normal and complete ataxin-2 (*Lessing and Bonini, 2008*).

Because PABP is the only protein known to date to interact directly with ataxin-2 through its PAM2 motif, its involvement in MJD neurodegeneration became a possibility. Using flies expressing mutant ataxin-3 and with deletion of the PABP gene, an enhancement in degeneration of the *Drosophila* eye was observed. On the contrary, overexpression of PABP significantly suppressed neurodegeneration. These results suggested that PABP has an opposite activity to ataxin-2 with respect to ataxin-3 dependent neurodegeneration, playing a protective role. Further tests were conducted revealing that the toxicity of ataxin-2 is mitigated by PABP, therefore consistent with PABP also playing a crucial role in modulating ataxin-3 toxicity. The importance of the PAM2 motif for ataxin-2's toxicity and for the enhancement of ataxin-3 neurodegeneration suggests a mechanism of interaction between these two proteins, even though indirect. PABP is possibly the connection between these two proteins since ataxin-2 does not interact directly with another known protein through its PAM2 motif. It may happen that in the absence of the connection of PABP to the PAM2 motif of ataxin-2, there would be an induction of MJD neurodegeneration by ataxin-2 action. When this interaction occurs, the PAM2 motif is blocked and it may have a suppressor effect on ataxin-2 action in MJD neurodegeneration (*Lessing and Bonini, 2008*).

These findings point to an interaction between the gene that causes SCA2 and the gene responsible for MJD, leaving the suggestion of a determinant role of PABP as a mediator of this interaction. This interactive and regulative proteic network may be a key to underline new therapeutical strategies and unravel further therapeutic targets. Nevertheless, further studies are necessary to confirm these observations in mammalian models.

Objectives

The general objective of this work is the evaluation of the modulator effect of ataxin-2 and PABP in Machado-Joseph disease, concerning a possible interaction between these proteins and Ataxin-3.

The specific objectives are as follows:

- To study Ataxin-2 and PABP levels in Machado-Joseph disease patient tissue.
- To analyze Ataxin-2 and PABP levels in a transgenic mouse model of Machado-Joseph disease.
- To study how overexpression of Ataxin-3 modulates Ataxin-2 and PABP expression in cellular models of Machado-Joseph disease.
- To investigate the effects of Ataxin-2 and PABP overexpression in cellular models of Machado-Joseph disease, regarding the expression levels of Ataxin-3, Ataxin-2 and PABP.
- To investigate if silencing PABP in a cellular model of Machado-Joseph disease aggravates the pathogenesis of the disease.

Chapter II

Methods and Material

2.1. Tissue preparation

2.1.1. Human

Postmortem cortex and striatal tissue from three MJD patients with morphologically and genetically confirmed MJD (1: 62 years, 26/70 CAG repeats; 2: 53 years, 23/69 CAG repeats; 3: 62 years, 22/74 CAG repeats) were obtained from the "Tissue donation program of the National Ataxia Foundation, Minneapolis, MN, USA" (VA Medical Center, Albany, N.Y., USA). Two controls with no evidence of neurological disease (1: 55 years; 2: 56 years) were obtained from Neurology and Pathology Services of University Hospital of Coimbra (H.U.C). The tissue was fresh when dissected and then placed in 10% neutral buffered formalin and kept at 4ºC.

The tissue was cryopreserved in 25% sucrose- 0.1M PBS solution for 48h. The tissue was then dry-ice frozen and cut at a cryostat-microtome (Leica CM3050S, Leica Microsystems Nussloch Gmbh, Nussloch, Germany) in 25-30 μm coronal sections. Slices were collected and stored in 48-well trays (Corning Inc., NY, USA), free-floating in 0.1M PBS supplemented with 0.12 μmol/L sodium azide. The plates were stored at 4°C until the immunohistochemical processing.

2.1.2. Transgenic and Wild-type Mice

The Machado Joseph's disease transgenic mouse model expressing the N-terminal-truncated ataxin-3 with 69 glutamine repeats and an N-terminal hemagglutinin (HA) epitope driven by Purkinje-cell-specific L7 promoter (Tg-MUT) was previously described (*Torashima et al., 2008; Oue et al., 2009*). A colony of these transgenic mice was established in the Center for Neurosciences and Cell Biology of the University of Coimbra and the line was maintained by backcrossing heterozygous males with C57BL/6 females. Genotyping was performed by PCR. The present study used 6 heterozygous animals and 6 wild-type.

Transgenic mice (Q69) and wild type C57BL/6 mice, with 14 weeks old, were killed by sodium pentobarbital overdose, transcardially perfused with 0.1M PBS and a 4% paraformaldehyde fixate solution (PFA 4%, Fluka, Sigma, St. Louis, USA) followed by brain removal. After retrieving cerebellar punches, the tissue was cryoprotected in 25% sucrose-0.1M PBS solution for 48h, dry ice- frozen and cut at a cryostat-microtome (Leica CM3050S, Leica Microsystems Nussloch Gmbh, Nussloch, Germany) in 20 μm coronal sections. Slices of

each animal were collected in *superfrost* plus microscope slides (Thermo Fisher Scientific, U.S.A) and stored at -20°C before immunohistochemical processing.

Brains of wild type C57BL/6 mice and transgenic mice (Q69) (*Torashima et al., 2008*), with 14 weeks old were dissected fresh; and small punches of striatum, cortex, hipocamppus and cerebellum were collected; the punches were harvested with a Harris Uni-Core pen, with 2.0 mm of diameter (Ted Pella, Inc, CA, USA) and kept at -80°C.

2.1.3. Lentiviral-based MJD rat model

In the lentiviral-based MJD rat model, LV encoding human full length wild-type (Atx3-27Q) (*Alves et al., 2008b*) and human full length mutant ataxin-3 (Atx3-72Q) (*Alves et al., 2008b*), were stereotaxically injected into striatum of anesthetized animals.

Two (n=3), four (n=3) and eight (n=3) weeks after injection in the striatum (4 weeks old at the time of the injection), the animals were killed by sodium pentobarbital overdose, transcardially perfused with 0.1M PBS and a 4% paraformaldehyde fixate solution (PFA 4%, Fluka, Sigma, St. Louis, USA) followed by brain removal. The tissue was cryoprotected in 25% sucrose- 0.1M PBS solution for 48h, dry ice- frozen and cut at a cryostat-microtome (Leica CM3050S, Leica Microsystems Nussloch Gmbh, Nussloch, Germany) in 20µm coronal sections. Three slices of each animal were collected and stored in 48-well trays (Corning Inc., NY, USA), free-floating in 0.1M PBS supplemented with 0.12 µmol/L sodium azide. The plates were stored at 4°C until immunohistochemical processing.

2.2. Cell cultures

2.2.1. Neuroblastoma cell culture

Mouse neuroblastoma cell line (Neuro-2a cells) obtained from the ATCC cell biology bank (CCL-131) were incubated in DMEM medium supplemented with 10% FBS, 100U/ml penicillin and 100µg/ml streptomycin (Gibco, Paisley, Scotland, UK) (complete medium) at 37°C in 5% CO_2/air atmosphere.

2.2.2. Human fibroblasts culture

Human fibroblast cells were obtained from Coriel cell repositories and skin biopsy (Control: 38 years, not affected; MJD1: 38 years, 74 CAG repeats; MJD2: 28 years, 82 CAG repeats). Cells were kept in culture in DMEM supplemented with 10% bovine serum, 1% non essential amino-acids, 2mM L-glutamine 100U/ml penicillin and 100µg/ml streptomycin (Gibco, Paisley, Scotland, UK) (complete medium) at 37°C in 5% CO_2/air atmosphere. Cells were then plated in 12-wells plaque and 12-weels plates with slides, at 48h post-plating cells were lysed for western blotting and the plaque with slides was fixated with PFA 4% for immunocitochemistry.

2.3. Infection/Transfection

2.3.1. Lentiviral vectors

Viral vectors encoding for the human Ataxin-2, PABP, human full length wild type ataxin-2 with 27 glutamines (ATX3 WT) (*Alves et al., 2008b*) and human full length mutant ataxin-3 with 72 glutamines (ATX3 MUT) (*Alves et al., 2008b*) were produced in human embryonic kidney cells (HEK) 293T cells using a four-plasmid system described previously (*de Almeida et al., 2002*). The lentiviral particles were produced and resuspended in phosphate-buffered saline with 0.5% bovine serum albumin (PBS-BSA) and samples were matched for particle concentration by measuring HIV-1 p24 antigen content (RETROtek, Gentaur, France). Viral stocks were stored at -80°C until use.

2.3.2. N2A infection

Mouse neuroblastoma cell line (Neuro-2a cells) obtained from the ATCC cell biology bank (CCL-131) were incubated in DMEM medium supplemented with 10% FBS, 100U/ml penicillin and 100µg/ml streptomycin (Gibco, Paisley, Scotland, UK) (complete medium) at 37°C in 5% CO_2/air atmosphere. We plated the cells in 12 well plates; 2 x 10^5 cells per well in 1000µl of medium and 24h later were infected with lentiviral vectors.

For the infection, we replaced the medium with 400µl of new medium and 1.6µl of hexadimethrin bromid (Aldrich) (8µg/ml) and added the corresponding lentivirus according to the condition (human full length wild type ataxin 3, human full length mutant ataxin-3, human ataxin-2 and human PABP), the plates were then incubated at 37°C in 5% CO_2/air atmosphere, and after 6h of infection, we added 600µl of new fresh medium to the wells. At two weeks post-infection cells were lysed for western bloting analysis.

2.3.3. Lipofectamine transfection

Mouse neuroblastoma cell line (Neuro-2a cells) were incubated in DMEM medium supplemented with 10% FBS, 100U/ml penicillin and 100µg/ml streptomycin (Gibco, Paisley, Scotland, UK) (complete medium) at 37°C in 5% CO_2/air atmosphere. Cells were plated in a 24 well plaque and 24 hours later were lipofectamine transfected. For the transfection we plate 2 x 10^5 cells in 500µl of growth medium without antibiotics per well. 0.8µg of DNA (shPABP and shLuc accordingly with the situation) were diluted in 50µl of Opti-MEM® I Reduced Serum Medium without serum. Also diluted 2.0µl of Lipofectamine ™ 2000 (Invitrogen, CA, USA) in 50µl of Opti-MEM and incubated both mixtures for 5min at room temperature. After the 5 min incubation, we combined the two mixtures and incubated for more 20min at room temperature, allowing the DNA-Lipofectamine ™ 2000 (Invitrogen, CA, USA) complexes to form. Next the 100µl of the mixture were added to each well and the plaques were incubated at 37°C in 5% CO_2/air atmosphere, after 4h the medium was changed back to DMEM with FBS. At 48h post-transfection cells were lysed for western blot analysis.

2.4. Immunochemical procedures

2.4.1. Immunohistochemistry for human brain tissue

The immunohistochemical procedure for human tissue was initiated by mounting the sections in *superfrost* plus microscope slides (Thermo Fisher Scientific, U.S.A) followed by the drying of those slides at 37°C approximately 45min. Once dried the sections were incubated 30min at 100°C with a Tris-buffered saline (TBS) pH 9 antigen retrieval solution and then blocked for 1h at room temperature in a 0.3% Triton in PBS solution supplemented with 10% NGS. After the blocking, sections were incubated with monoclonal mouse anti-ataxin-2 antibody (BD Biosciences, San Jose, California, USA) diluted in the blocking solution (1:1000, overnight 4°C). After the overnight incubation, the sections were washed three times with PBS and incubated with the corresponding secondary antibodies anti-mouse conjugated to alexa 488 or 594 fluorophores (Alexa Fluor, Invitrogen, Paisley, UK) diluted in 0.1% Triton in PBS supplemented with 10% NGS for 2h at room temperature. The sections were washed three times with PBS after the incubation, counterstained with DAPI and mounted with Fluorsave (Calbiochem, Germany). Fluorescent images were acquired with a Zeiss Axiovert 200 imaging microscope or LSM Zeiss microscope for double staining. All immunohistochemical analyses were performed in triplicate and for an n=3 for MJD patients and n=2 for controls.

2.4.2. Immunohistochemistry for Transgenic and Wild-type mouse sections

The immunohistochemical procedure was initiated by drying the slides with the sections at 37°C for 1 h., followed by hydratation with PBS and 1h at room temperature blocking in 0.3% Triton in PBS supplemented with 10% of NGS (normal serum goat, Gibco). After the blocking, sections were incubated with the appropriate antibodies diluted in the blocking solution: mouse monoclonal anti-ataxin-2 antibody (BD Biosciences, San Jose, California, USA.; 1:1000, overnight 4°C) and mouse monoclonal anti-PABP antibody (Millipore, Temecula, California, USA;1:1000, overnight 4°C). After the overnight incubation, the sections were washed three times with PBS and incubated with the corresponding secondary antibodies goat-mouse conjugated to alexa 488 or 594 fluorophores (Alexa Fluor, Invitrogen, Paisley, UK) diluted in 0.1% Triton in PBS supplemented with 10% NGS for 2h at

room temperature. The sections were washed three times with PBS after the incubation, counterstained with DAPI and mounted with Fluorsave (Calbiochem, Germany). Fluorescent images were acquired with a Zeiss Axiovert 200 imaging microscope. All immunohistochemical analyses were performed in triplicate; for the sections incubated with ataxin-2 there was an n=6 and for those incubated with PABP there was an n=5.

2.4.3. Free-floating Immunohistochemistry for Lentiviral-based MJD rat model sections

Free-floating immunochemistry was initiated incubating brain sections on phenylhidrazine diluted in PBS (1:1000; 15min, 37°C) for peroxidase inhibition, followed by three washes with PBS (5'; 5'; 10'). Once washed the sections were blocked 1h at room temperature in PBS 0.1% Triton solution with 10% NGS and then incubated overnight with the primary antibodies: rabbit polyclonal anti-ataxin-3 antibody (Immunostep, Salamanca, Spain; 1:2000, overnight 4°C); mouse monoclonal anti-ataxin-2 antibody (BD Biosciences, San Jose, California, USA; 1:1000, overnight 4°C) and mouse monoclonal anti-PABP antibody (Millipore, Temecula, California, USA; 1:1000, overnight 4°C). After the overnight incubation, the sections were washed three times with PBS and incubated with the corresponding secondary antibody goat-mouse and rabbit conjugated to alexa 488 or 594 fluorophores (Alexa Fluor, Invitrogen, Paisley, UK) diluted in blocking solution (1:200) for 2h at room temperature. The sections were washed three times with PBS after the incubation, counterstained with DAPI, premounted in *superfrost* plus microscope slides (Thermo Fisher Scientific, U.S.A) followed by a drying period of approximately 45min at 37°C. Once dried the sections were mounted with Fluorsave (Calbiochem, Germany). Light images were acquired with a Ziess Axiovert 200 imaging Microscope. All immunohistochemical analyses were performed in triplicate and using three sections of each animal with at least two or three snaps per section, three animals per each period of time (2W, 4W and 8W).

2.4.4. Immunohistochemistry quantitative analysis

The human brain tissue analysis for the ataxin-2 positive cells was carried out counting for each staining, eight fields (x200 magnification) of three different sections. Cytoplasmic puncta-like immunoreactivity positive for ataxin-2 were counted by means of image analysis software (ImageJ, National Institute of Health, USA). Measurements were performed in triplicate and results are represented as the mean value of ataxin-2 positive punctuates per section ±SEM.

The lentiviral-based model rat sections analysis for the ataxin-2, PABP and ataxin-3 positive cells were carried out counting for each staining, eight fields (x100 magnification) of three different sections per each period of time evaluated.

The transgenic and wild-type mouse sections analysis for ataxin-2 and PABP positive cells were carried out counting for each staining, eight fields (x400 magnification). Measurements were performed in triplicate and results are represented as the mean value of ataxin-2 and PABP immunoreactivity per section ±SEM.

2.4.5. Immunocitochemistry of human fibroblasts

Human fibroblasts were plated in 24-wells plates with slides; at 48h post-plating cells were fixated with PFA 4% for immunocitochemistry.

To initiate the immunocitochemistry, cells were permeabilized with PBS 1% Triton solution (5min; 500μL per well) and then washed with PBS. Once washed it was performed the blocking in a solution of PBS with 3% BSA for 1h at room temperature. Primary antibodies mouse monoclonal anti-ataxin-2 (BD Biosciences, San Jose, California, USA) and mouse monoclonal anti-PABP (Millipore, Temecula, California, USA) conjugated with rabbit polyclonal anti-ataxin-3 (Immunostep, Salamanca, Spain) accordingly to the situation, were diluted in the blocking solution (1:100) and incubated overnight at 4°C. After the overnight incubation, we incubated with the corresponding secondary antibodies anti-mouse conjugated to alexa 488 or 594 fluorophores (Alexa Fluor, Invitrogen, Paisley, UK) diluted (1:200) in PBS 1% BSA for 2h at room temperature. The sections were washed three times with PBS after the incubation, counterstained with DAPI and mounted with Fluorsave (Calbiochem, Germany). Light images were acquired with a Ziess Axiovert 200 imaging Microscope. All immunocitochemical analyses were performed in triplicate; with two slides per situation and at least 12 snaps per slide, at the same exposure in the same day.

2.4.6. Immunocitochemistry quantitative analysis

The fibroblast slides analysis for the ataxin-2, PABP and ataxin-3 positive cells were carried out counting for each staining, several fields (x400 magnification) of two slides for the different conditions. Ataxin-2 and PABP immunoreactivity was measured per cell and per nucleus. Measurements were performed in triplicate and results are represented as the mean value of ataxin-2 and PABP immunoreactivity per slide ±SEM.

2.5. Protein extraction and Western Blotting

For the protein extraction protocol, brain tissue and cells were lysed in RIPA-buffer solution (50mM Tris HCl pH 8, 150nM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing proteases inhibitors (Roche diagnostics GmbH, Mannheim, Germany) followed by a 4s ultra-sound chase (1 pulse/s). Protein concentration was determined with the Bradford protein assay (BioRad, Munich, Germany). Depending on the analysis, twenty (Neuro-2a), forty (mouse brain punches) and sixty (human fibroblasts) micrograms of protein extract were resolved in SDS-polyacrylamide gels (8% or 12% accordingly with the analysis). The proteins were transferred onto PVDF membrane (GE Healthcare, Amersham, UK) according to standard protocols (*Alves et al., 2008b*). The membranes were blocked by incubation in 5% nonfat milk powder in 0.1% Tween 20 in Trisbuffered saline (TBS-T) for 1 h at room temperature, and were then incubated overnight with the following primary antibodies diluted in blocking buffer: anti-ataxin 3 (1H9; 1:2000; Chemicon, Temecula, CA, USA), anti-ataxin-2 (1:1000; BD Biosciences, San Jose, California, USA), anti-PABP (1:2000,Millipore, Temecula, California, USA) and anti-tubulin (1:2000; Sigma, Saint Louis, MO, USA). Blots were washed three times in TBS-T, for 15min each, and incubated with the secondary antibody goat anti-mouse (1:10 000; Vector Laboratories, Burlingame, CA) for 2h at room temperature. After washing, bands were visualized with Enhanced Chemifluorescent substract (ECF) (GE Healthcare, Amersham, UK) and chemifluorescence imaging (Versadoc Imaging System Model 3000, Bio-Rad, Munich, Germany. Semi quantitative analysis was carried out based on the optical density (OD) of scanned films (Quantity One 1-D image analysis software version 4.4; Biorad, Hercules, CA, USA). Specific ODs were normalized with respect to those for tubulin for experiments on total homogenates. The specific OD was then normalized with respect to the amount of tubulin loaded in the corresponding lane of the same gel. A partition ratio was calculated and expressed as a percentage. The western blotting analyses were performed in triplicate for each experimental set when possible.

Chapter III

Results

3.1. Ataxin-2 immunoreactivity is increased in Machado-Joseph disease patient brain tissue

An abnormal connection between ataxin-2 and ataxin-3 was described in a Drosophila model, in which ataxin-2 was shown to have a determinant role in MJD pathogenesis (*Lessing and Bonini, 2008*). Moreover, it has also been shown that normal ataxin-2 is present in nuclear inclusions of mutant ataxin-3 in MJD patients (*Uchihara et al., 2001*). Therefore we investigated if the levels of ataxin-2 were changed in post-mortem brain tissue of Machado-Joseph disease patients.

The immunoreactivity of ataxin-2 in two brain regions affected – the striatum and cortex of three autopsy-confirmed Machado-Joseph disease patients was compared to two age matched controls. Increased ataxin-2 immunoreactivity was observed in MJD patients compared to the controls in striatum (Figure 10B left panel; $P<0,001$), and cortex (Figure 10B right panel; $P<0,01$). This increase in ataxin-2 levels was more significant in striatum, which is an area more affected in MJD as compared with the cortex that is relatively spared (*Sudarsky and Coutinho, 1995; Coutinho and Andrade, 1995*; *Alves et al., 2008*).

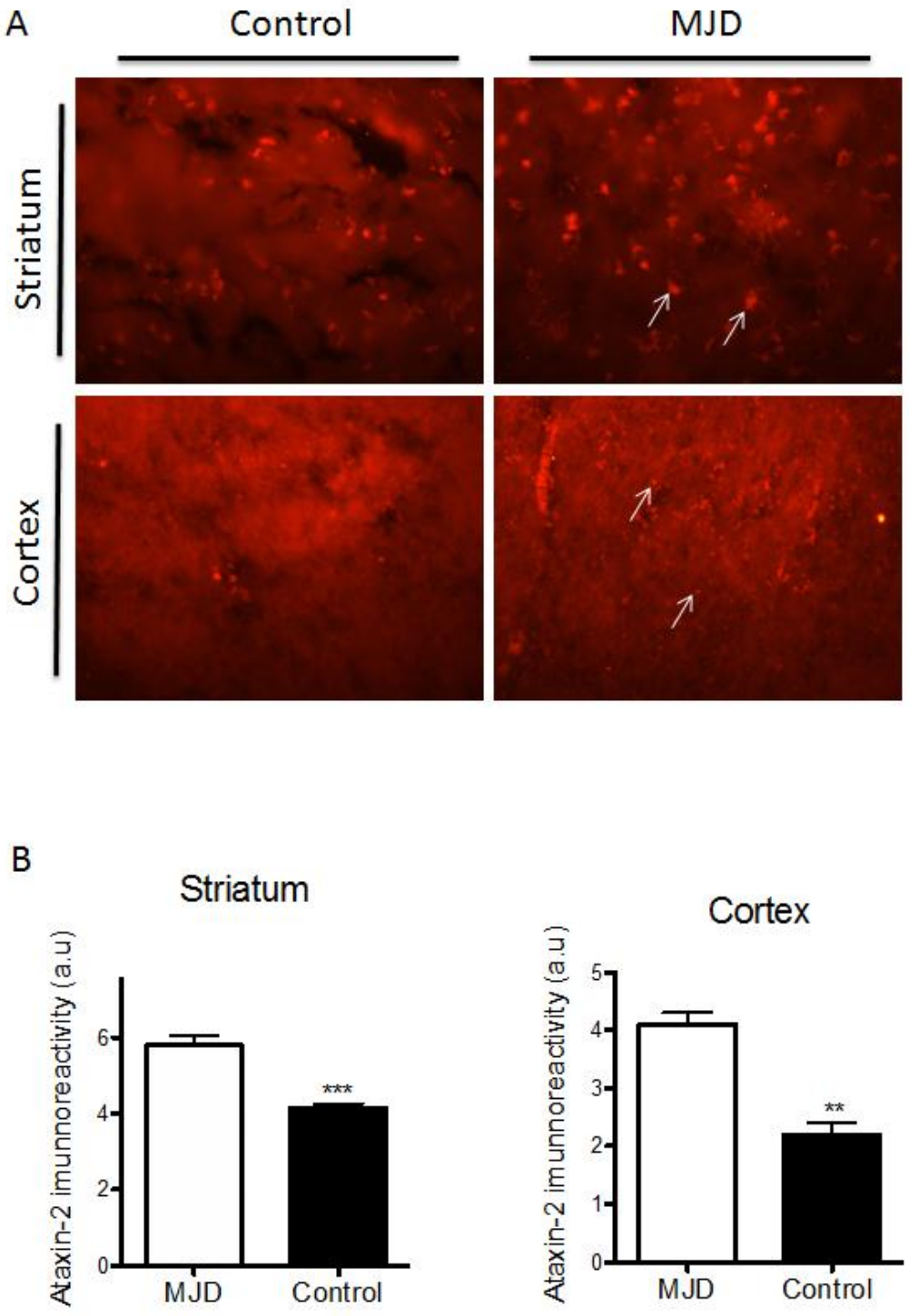

Figure 10. Ataxin-2 immunoreactivity is increased in the brain tissue of Machado-Joseph disease patients. (A,B) Ataxin-2 immunohistochemistry in striatum and cortex of MJD patient's brains (MJD, n=3), and age-matched controls (Control, n=2). (A) Representative brain sections stained for ataxin-2 to highlight ataxin-2 localization in the striatum and cortex of MJD patients and control (fluorescence microscopy). (B) Quantitative analysis of ataxin-2 immunoreactivity in the striatum and

cortex. The immunohistochemistry was performed in triplicate, analyzed in the same day at same exposure. Values are expressed in mean ± SEM. ***P*<0.01; ****P*<0.001 (unpaired Student's t-test).

3.2. Ataxin-2 and PABP levels are increased in Machado-Joseph disease patient fibroblasts

To further investigate the interaction between ataxin-2 and ataxin-3 we also studied the role of poly-A-binding protein (PABP) (*Ralser et al. 2005*), as it has been suggested that ataxin-2 and ataxin-3 proteins do not interact directly, but rather through PABP (*Lim et al. 2006*). Therefore the next step for our investigation was evaluating ataxin-2 and PABP levels in the disease (MJD fibroblasts). In order to obtain these results, we performed an immunocitochemistry of fibroblasts derived from MJD patients (n=2) and controls (n=1), stained for ataxin-2 (red), ataxin-3 (green) (Figure 11A) and PABP (red) (Figure 12A). The immunoreactivity was analyzed in several slides and quantified for ataxin-2 (Figure 11B) and PABP (Figure 12B).

In MJD patient's fibroblasts it was possible to observe the presence of ataxin-2 in the nucleus of the cells similarly to ataxin-3, a subcellular pattern that was not present in control fibroblasts where both ataxin-2 and ataxin-3 were confined to the cytoplasm (Figure 11A). Furthermore, we observed that the immunoreactivity for ataxin-2 was increased in the MJD fibroblasts at the cellular level when compared with the control (Figure 11B left panel; *P*<0.001), and in the nucleus where this difference was even more significant (Figure 11B right panel; *P*<0.0001).

In addition, western blotting analysis of fibroblasts from MJD patients and controls fibroblast (Figure 11C) revealed that ataxin-2 levels relative to tubulin were significantly increased in MJD patient fibroblasts (Figure 11D) when compared with the controls (Figure 11D; *P* <0,01).

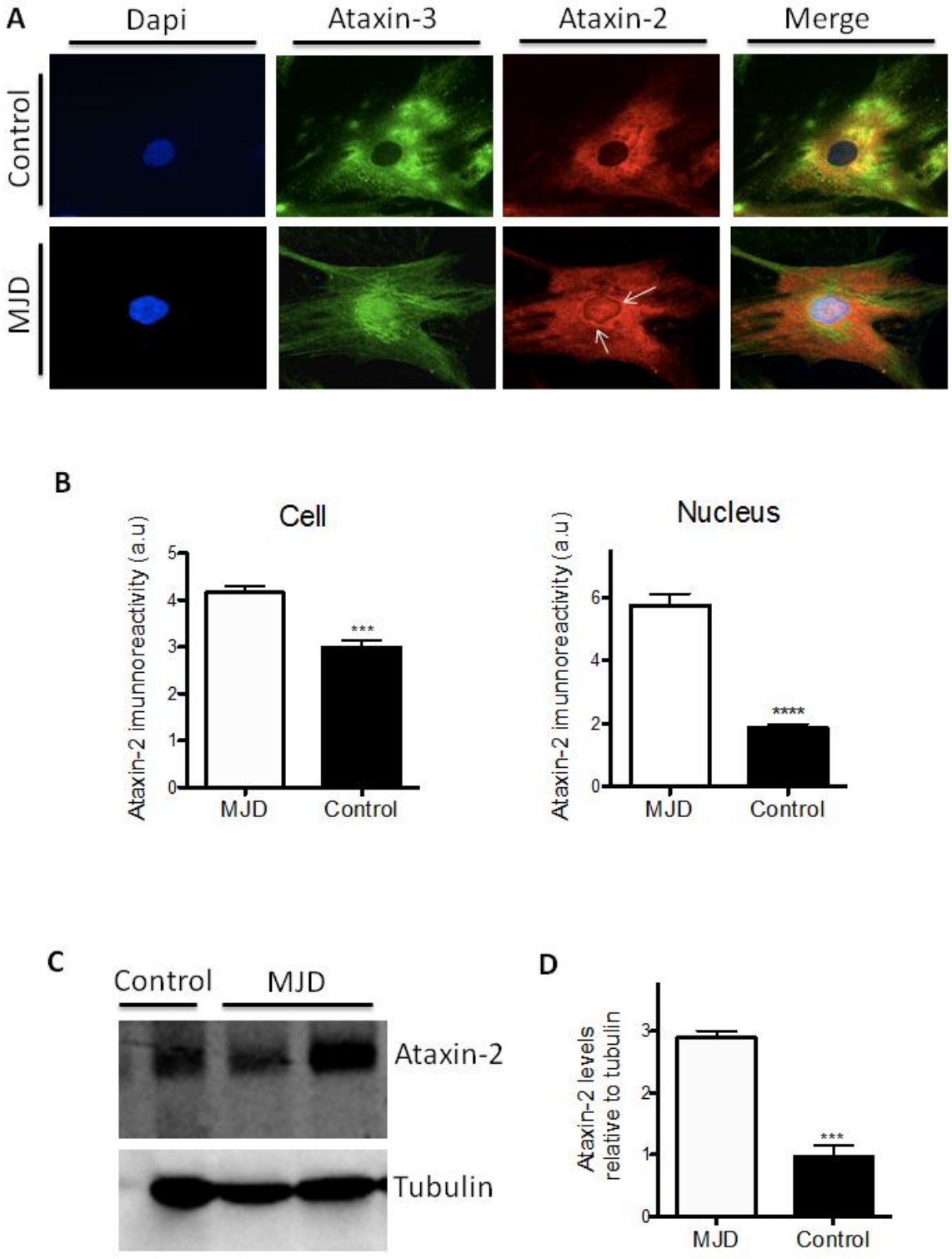

Figure 11. Ataxin-2 is recruited to the nucleus and upregulated in MJD patients fibroblasts (A,B) Immunocytochemical analysis of fibroblasts of MJD patients (MJD, n=2) and fibroblasts of controls (Control, n=1) by staining nuclei (DAPI, blue), ataxin-3 (Ataxin-3, green) and ataxin-2 (Ataxin-2, red). (A) In MJD, ataxin-2 was recruited to the nucleus, a phenomenon that was not observed in the controls,

despite the absence of intranuclear inclusions in MJD patient's fibroblasts. (B) Immunoreactivity of ataxin-2 in the whole cell was increased in MJD, as well as in the nucleus, comparative to controls. Immunocitochemistry was performed in triplicate; with two slides per group and at least 12 snaps per slide, at the same exposure at the same time. Values are expressed in mean ± SEM. ***P<0.001; ****P<0.0001 (unpaired Student's t-test). (C, D) Western blotting analysis of lysates of human fibroblasts derived from MJD patients and from a control individual. (C) Representative western-blot probed for endogenous human ataxin-2 (MW: ≅ 148kDa) and tubulin (MW: 55kDa) proteins. (D) Optical densitometry analysis reveals an increase in the ataxin-2 levels as compared to the control. Each ataxin-2 lane was normalized according to the tubulin band. Results were expressed as ratio Ataxin-2/ Tubulin (n=3 for each experimental set). Values are expressed as mean ± SEM. ***P<0.001 (Student's t-test). Three replicates of the blots were performed to ensure the reliability of data.

We then analyzed the levels of PABP in MJD patient's fibroblasts and controls (Figure 12). Even though PABP was not found extensively recruited into the nucleus of MJD patient fibroblasts (Figure 12A) it is possible to observe differences relatively to the control fibroblasts. An increased immunoreactivity for PABP was observed in MJD patient fibroblasts as compared to control fibroblasts. Moreover, we detected a significant increased immunoreactivity of PABP in the MJD fibroblasts when compared with the controls (Figure 12B; P<0.001). In addition, western blotting analysis of fibroblasts from MJD patients (Figure 12C) and control fibroblasts (Figure 12C) confirmed that PABP levels normalized to tubulin were increased in MJD patients (Figure 12D) when compared with the controls (Figure 12D; P <0,05), as previously observed for ataxin-2 levels.

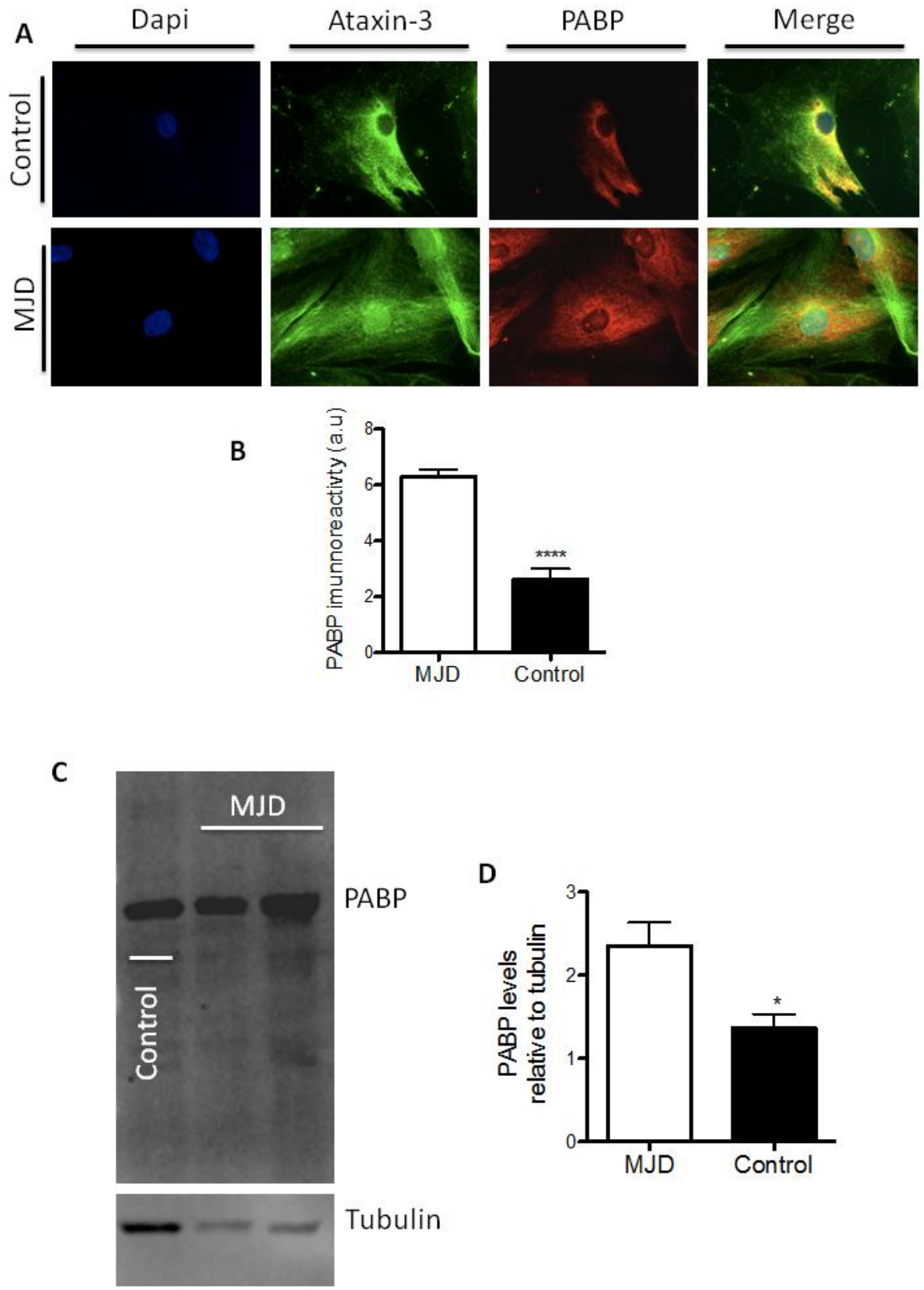

Figure 12. PABP levels are increased in MJD patient's fibroblasts. (A,B) Immunocytochemical analysis of fibroblasts from MJD patients (MJD, n=2) and fibroblasts from control individuals (Control,

n=1) with staining for nuclei (DAPI, blue), ataxin-3 (Ataxin-3, green) and PABP (PABP, red). (A) PABP staining is slightly increased in the nucleus of fibroblasts from MJD patients as compared to controls. (B) Immunoreactivity of PABP was significantly higher in MJD, comparative to controls. Immunocitochemistry was performed in triplicate; in two slides per group and at least 12 snaps per slide, at the same exposure in the same day. Values are expressed in mean ± SEM. ****P<0.0001 (unpaired Student's t-test). (C,D) Western blotting analysis of lysates of fibroblasts derived from MJD patients (MJD) and from a control individual. (C) Representative western-blot probed for endogenous human PABP (MW: 71kDa) and tubulin (MW: 55kDa) proteins. (D) Optical densitometry analysis. An increase in the PABP levels as compared with the control is observed. Each PABP lane was normalized according to the tubulin band. Results were expressed as ratio PABP/ Tubulin (n=3 for each experimental set). Values are expressed as mean ± SEM. *P<0.05 (Student's t-test). Three replicates of the experiment were performed.

3.3. Ataxin-2 and PABP levels decrease with the progression of the disease in a lentiviral-based MJD model

After accessing data regarding human tissue, we next studied if we would obtain the same results in a lentiviral MJD model (*Alves et al., 2008*). We tried to unravel if it was observed altered expressions of ataxin-2 and PABP in mice with MJD, similarly to our results in human tissue. For this purpose mice were injected with lentiviral vectors encoding mutant ataxin-3 (72Q) in one hemisphere and wild type ataxin-3 (27Q) in the contralateral hemisphere, thus acting as an internal control. The immunohistochemistry and quantitative analysis for ataxin-2 revealed a significant reduction in the levels of ataxin-2 from 2 weeks (n=3, Figure 13A upper lane, Figure 13B) to 4 weeks (n=3, Figure 13A middle lane, Figure 13B, P<0.01). This reduction of ataxin-2 levels was also confirmed at 8 weeks (n=3, Figure 13A lower lane, Figure 13B, P<0.05).

Upon evaluation of the PABP levels in the same model (Figure 14A, B), the results were similar as the ones obtained with ataxin-2. Immunohistochemistry and quantitative analysis for PABP revealed a significant reduction in its levels from 2 weeks (n=3, Figure 14A upper lane, Figure 14B) to 4 weeks (n=3, Figure 14A middle lane, Figure 14B, P<0.01) and then to 8 weeks (n=3, Figure 14A lower lane, Figure 14B, P<0.05).

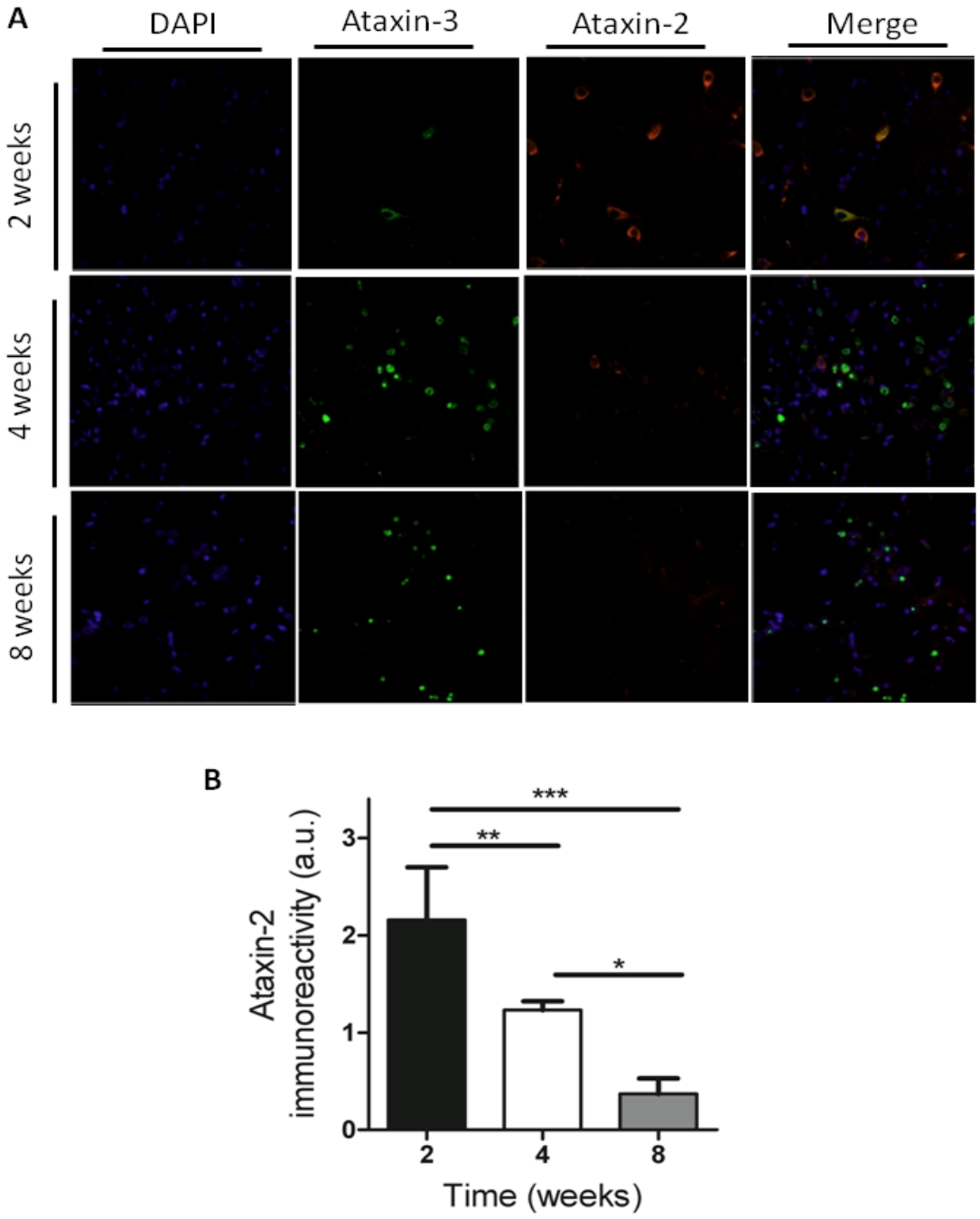

Figure 13. Ataxin-2 levels decrease with the progression of MJD in a lentiviral-based model. (A,B) Immunohistochemical analysis of mouse sections expressing human wild type and mutant ataxin-3 upon lentiviral injection. The analyses were performed at 2 weeks (n=3, upper lane), 4 weeks (n=3, middle lane) and 8 weeks (n=3, lower lane) post-injection by staining for the nuclei (DAPI, blue), for ataxin-3 (ataxin-3, green) and for ataxin-2 (ataxin-2, red). (A) Representative brain sections stained for ataxin-2 (confocal microscopy), it was notable the decrease of ataxin-2 levels with the progression of time. (B) The immunoreactivity decreased over time, being the decrease more evident in the interval

of 2 to 4 weeks. The immunohistochemical analysis was performed in triplicate, with at least three sections per animal, at the same color of staining, analyzed in the same day at same exposure. Values expressed in mean ± SEM. **P*<0.05, ***P*<0.01; ****P*<0.001 (One-way Anova).

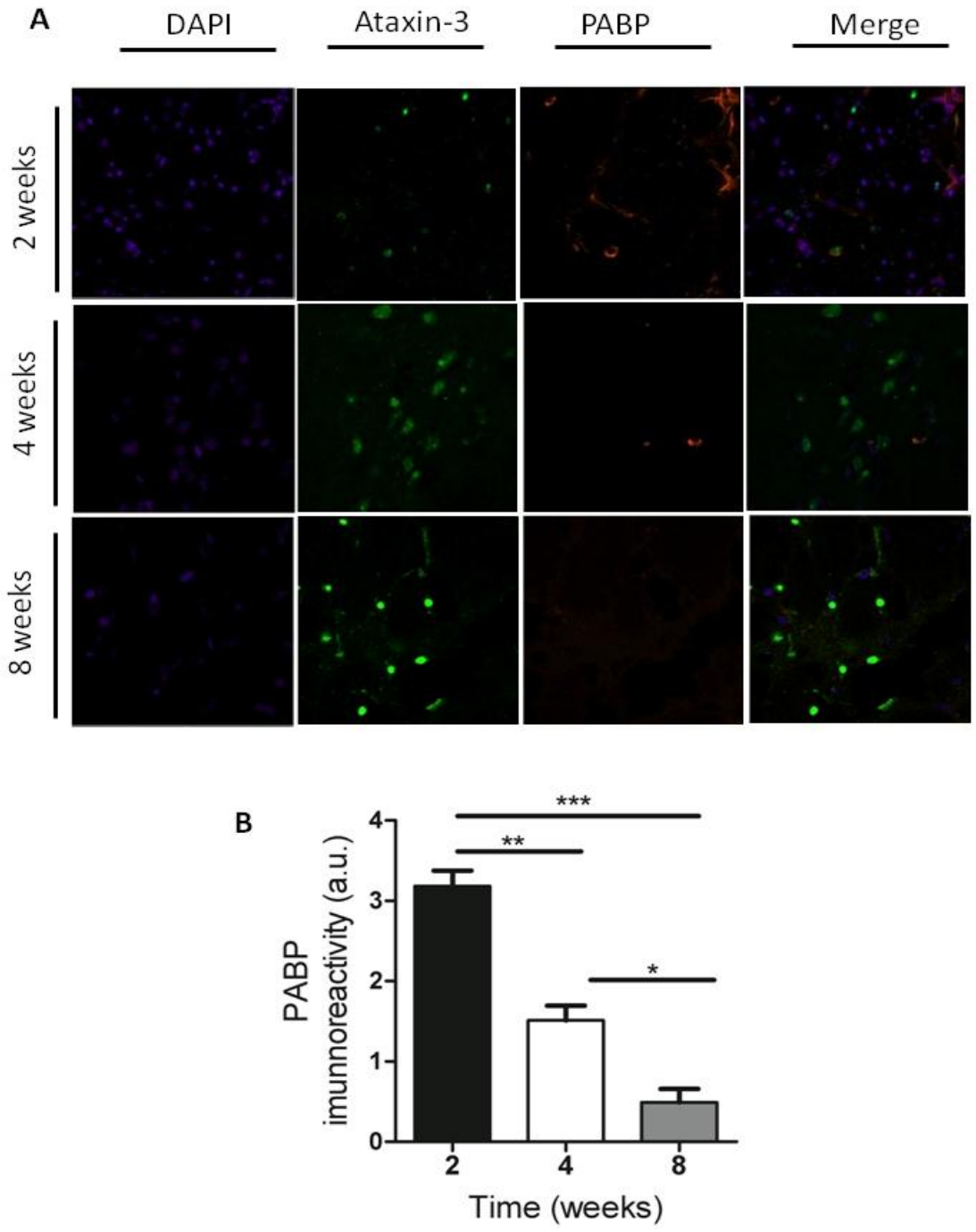

Figure 14. PABP levels decrease with the progression of MJD in a lentiviral model. (A,B) Immunohistochemical analysis of rat sections expressing human wild type and mutant ataxin-3 upon lentiviral injection. The analyses were performed at 2 weeks (n=3, upper lane), 4 weeks (n=3, middle

lane) and 8 weeks (n=3, lower lane) post-injection by staining for the nuclei (DAPI, blue), for ataxin-3 (ataxin-3, green) and for PABP (PABP, red). (A) Representative brain sections stained for PABP and ataxin-3 to highlight PABP levels in the brain sections, it was notable the diminishing of PABP levels with the progression in time (confocal microscopy). (B) The immunoreactivity analysis leads to the conclusion that PABP levels are significantly decreasing over time, being the decrease more accentuated in the interval of 2 to 4 weeks. The immunohistochemmical was performed in triplicate, with at least three sections per animal, at the same color of staining, analyzed in the same day at same exposure. Values expressed in mean ± SEM. **P*<0.05, ***P*<0.01; ****P*<0.001 (One-way Anova).

3.4. Ataxin-2 and PABP levels are altered in a MJD transgenic mouse model

To further investigate how the levels of ataxin-2 and PABP are modified by MJD pathogenesis we analyzed a MJD transgenic mouse model (*Torashima et al., 2008*). For this purpose we performed immunohistochemical staining for ataxin-2 and PABP in brain sections, and western blotting analysis of cerebellar punches and other brain regions of transgenic MJD Q69 mice, using wild-type C57BL6 mice as control.

Immunohistochemistry of ataxin-2 revealed a reduction in the immunoreactivity for ataxin-2 at the level of the Purkinje cell layer in the MJD Q69 compared to WT mice, which was confirmed by quantification of fluorescence (n=6, Figure 15A, B, *P*<0.05). Nevertheless, in western-blot of punches of cerebellum, the opposite was observed: there was a significant increase in ataxin-2 levels in the MJD-Q69 punches (n=3, Figure 15C, Figure 15D left panel, *P*<0.001), relatively to the WT punches (n=3, Figure 15C, Figure 15D).

Additionally, western blot of punches from different brain regions, revealed the ataxin-2 expression in different brain areas. It was clear that decreased levels of ataxin-2 were found in the striatum (Figure 15E) as compared to other brain regions of this mouse model using tubulin as reference.

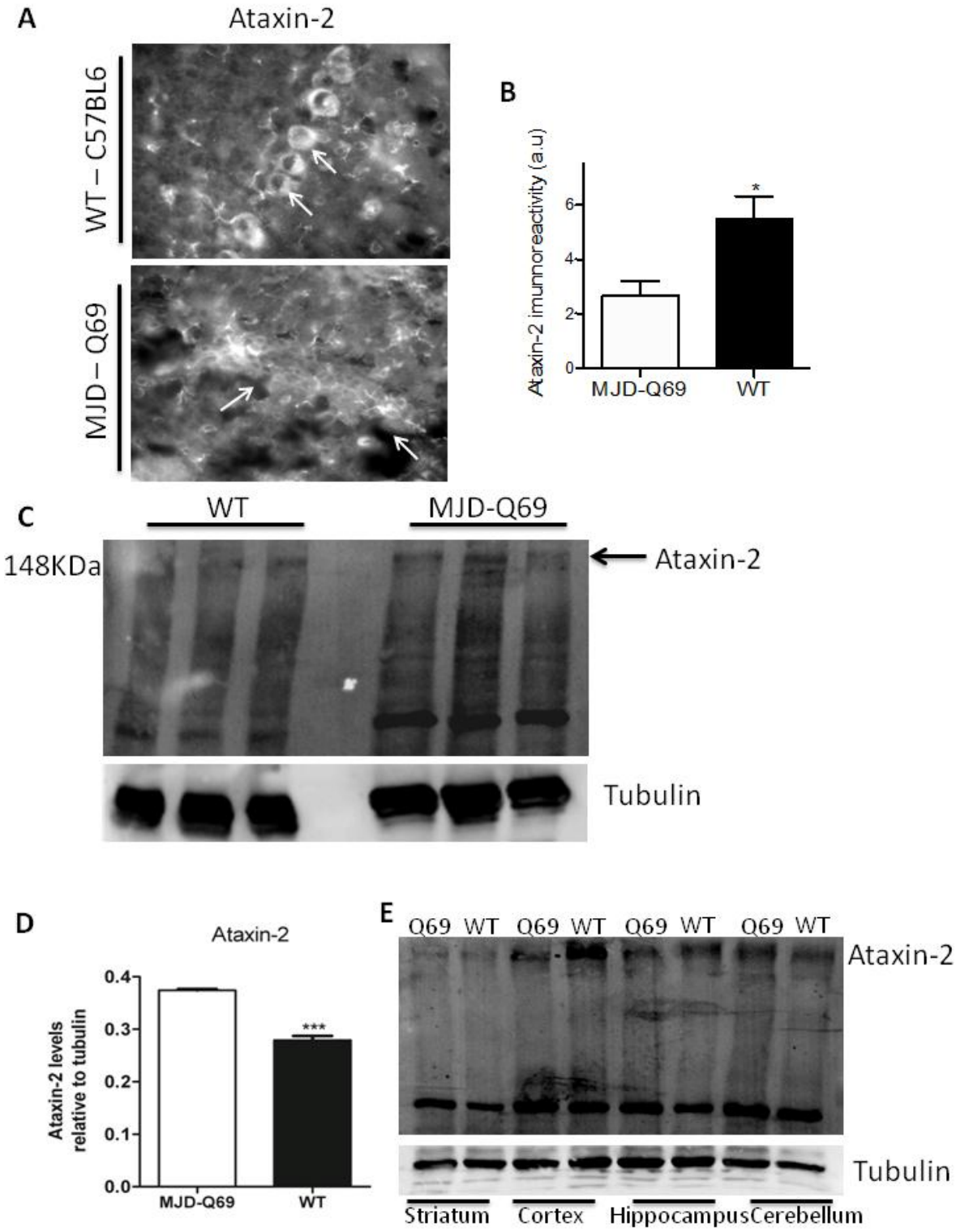

Figure 15. Ataxin-2 levels are altered in a MJD transgenic mouse model. (A,B) Immunohistochemical analysis of brain sections of MJD-Q69 mice (n=6) and WT C57BL6 (n=6) for ataxin-2. (A) Ataxin-2 immunoreactivity was observed in sections of WT mice, mainly in the Purkinje cells, and was reduced in the MJD-Q69 mice at this level. (B) Immunoreactivity of ataxin-2 is

significantly lower in the MJD Q69 mice, comparative the controls. Immunohistochemical fluorescence analysis was performed in triplicate; with at least 3 snaps per section, at the same exposure in the same day. Values expressed in mean ± SEM. **P*<0.05 (unpaired Student's t-test). (C, D) Western blotting analysis of cerebellar punches of WT C57BL6 (n=3) and MJD Q69 (n=3) mice. (C) Representative western-blot probed for endogenous human ataxin-2 (MW: ≅ 148kDa) and tubulin (MW: 55kDa) proteins. (D) Optical densitometric analysis reveals an increase in the ataxin-2 levels in MDJ-Q69 punches. Each ataxin-2 lane was normalized according to the tubulin band, used as a loading control. Results are expressed as ratio ataxin-2/ Tubulin (n=3 for each experimental set). Values are expressed as mean ± SEM. ***P*<0.01; *** *P*<0.001 (t-test). Three replicates of the protocol were performed. (E) Representative western blot of punches of striatum, cortex, hippocampus and cerebellum of WT C57BL6 (n=3) and MJD Q69 (n=3) mice, probed for endogenous human ataxin-2 (MW: ≅ 148kDa) and tubulin (MW: 55kDa) proteins. Decreased levels of ataxin-2 were found in the striatum.

The immunocitochemistry (Figure 16A) and quantitative analysis (Figure16B) for PABP revealed that its levels were significantly reduced in the MJD-Q69 brain sections (Figure 16A) compared to WT mice at the level of the Purkinje cell layer (n=5, Figure 16B, *P*<0.001). In western-blot of cerebellar punches, it was also observed a decrease in PABP levels of the MJD Q69 (n=3, Figure 16C, Figure 16D), relatively to the WT (n=3, Figure 16C, Figure 16D), even though this decrease did not prove to be significant. Additionally, western blot of different brain regions punches, revealed that levels of PABP are different in those areas, with higher density of PABP lane in the cerebellum (Figure 16E) and lower density of the striatal lane, as compared to the other regions (Figure 16E).

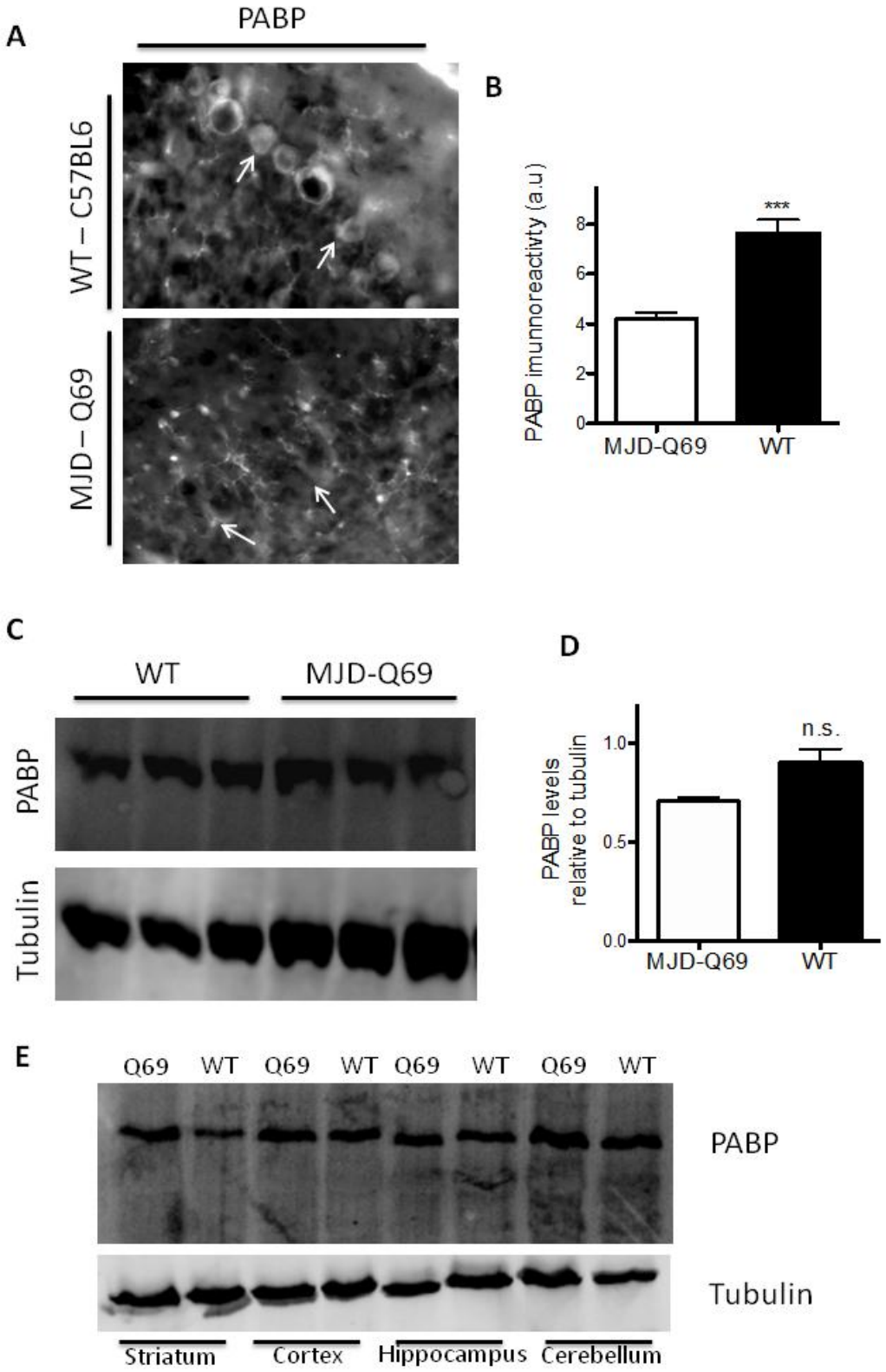

Figure 16. PABP levels are altered in a MJD transgenic mouse model. (A,B) Immunohistochemical analysis of brain sections of MJD-Q69 mice (n=5) and WT C57BL6 (n=5) mice for PABP. (A) PABP

immunohistochemical staining was observed in the sections of WT mice, particularly in the Purkinje cell layer but was significantly decreased in the MJD Q69 brain sections. (B) Immunoreactivity of PABP is significantly lower in the MJD-Q69 mice, compared to the controls. Immunohistochemistry was performed in triplicate; with at least 3 snaps per section, at the same exposure in the same day. Values are expressed in mean ± SEM. ***P<0.001 (unpaired Student's t-test). (C, D) Western blotting analysis of cerebellar punches of WT C57BL6 (n=3) and MJD Q69 (n=3) mice. (C) Representative western-blot probed for endogenous PABP (MW: 71kDa) and tubulin (MW: 55kDa) proteins. (D) Optical densitometric analysis. A non-significant decrease in the PABP levels was observed in MDJ-Q69 punches. Each PABP lane was normalized according to the tubulin band used as a loading control. Results were expressed as ratio PABP/ Tubulin (n=3 for each experimental set). Values are expressed as mean ± SEM. ns.P>0.05 (t-test). Three replicates of the protocol were performed. (E) Representative western blot of punches of striatum, cortex, hippocampus and cerebellum of WT C57BL6 (n=3) and MJD Q69 (n=3) mice, probed for endogenous PABP (MW: 71kDa) and tubulin (MW: 55kDa) proteins.

3.5. Ataxin-2 and PABP levels are altered in a cellular model of Machado-Joseph disease

After accessing data from the human tissue, lentiviral and transgenic MJD models, we developed a cellular MJD model in order to further investigate the changes in ataxin-2 and PABP expression levels associated to the disorder. Neuro-2a cells (obtained from the ATCC cell biology bank (CCL-131)) were infected with lentiviral vectors encoding human normal length ataxin-3 (27Q) and human mutant expanded ataxin-3 (72Q).

Western blot analysis suggests that the levels of ataxin-2 were decreased in cells expressing mutant ataxin-3 (n=3, ATX3 MUT, Figure 17A) relatively to controls. Densitometric quantification using tubulin as loading control confirmed that the levels of ataxin-2 were significantly diminished in cells expressing mutant ataxin-3 (Figure 17B), relatively to the non-infected cells (Figure 17B, P<0.01), and also significantly reduced when compared with the cells expressing wild type ataxin-3 (Figure 17B, P<0.001). These data suggest that in the cellular model of MJD, levels of ataxin-2 are reduced similarly to the results found in the lentiviral MJD mouse model in which ataxin-2 tends to decrease with the progression of the disease.

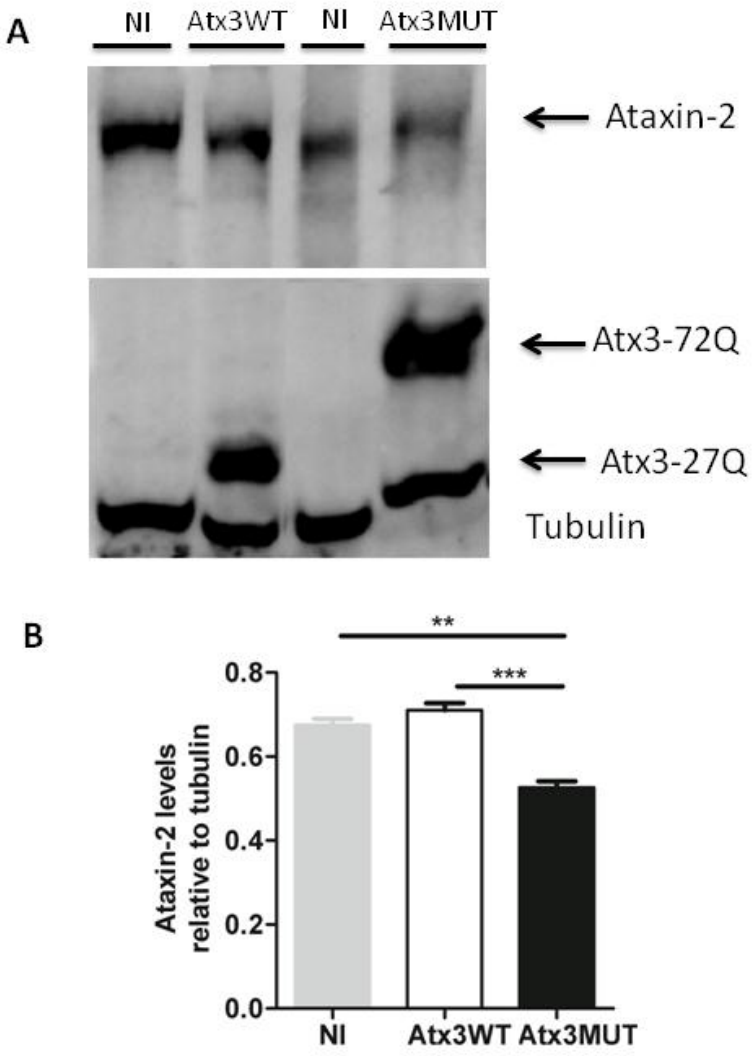

Figure 17. Ataxin-2 levels are decreased in a MJD cellular model. (A,B) Western blotting analysis of non-infected (NI, n=3), expressing human wild-type ataxin-3 (Atx3WT, n=3) and human expanded ataxin-3 (Atx3MUT, n=3) cell lysates. (A) Representative western-blot probed for ataxin-2, ataxin-3 and tubulin. Note the presence of endogenous ataxin-2 (MW: ≅ 148kDa), human Atx3 WT/ATx3-27Q (MW: 50kDa), Atx3Mut/Atx3-72Q (MW: 67kDa) and tubulin (MW: 55kDa). (B) Optical densitometry analysis. Significant decrease in the levels of ataxin-2 was observed in the cells expressing Atx3MUT relatively to the NI cells and the Atx3WT expressing cells. Each ataxin-2 lane was normalized according to the tubulin band. Tubulin was used as a loading control. Results were expressed as ratio Ataxin-2/ tubulin (n=3 for each experimental set). Values are expressed as mean ± SEM. ***P*<0.01; ***P<0.001 (One-Way Anova test). Three replicates of the protocol were performed.

Regarding the levels of PABP in this MJD cellular model, an increase in PABP levels was observed in cells expressing wild-type ataxin-3 (n=3, Atx3WT, Figure 18A, Figure 18B) when compared with non-infected cells (n=3, NI, Figure 18A, Figure 18B, *P*<0.01). The same

increase was observed in cells expressing mutant ataxin-3 (n=3, Atx3MUT, Figure 18A, Figure 18B, *P*<0.05) compared with non-infected cells. No significant differences were detected between Atx3WT and Atx3MUT cells.

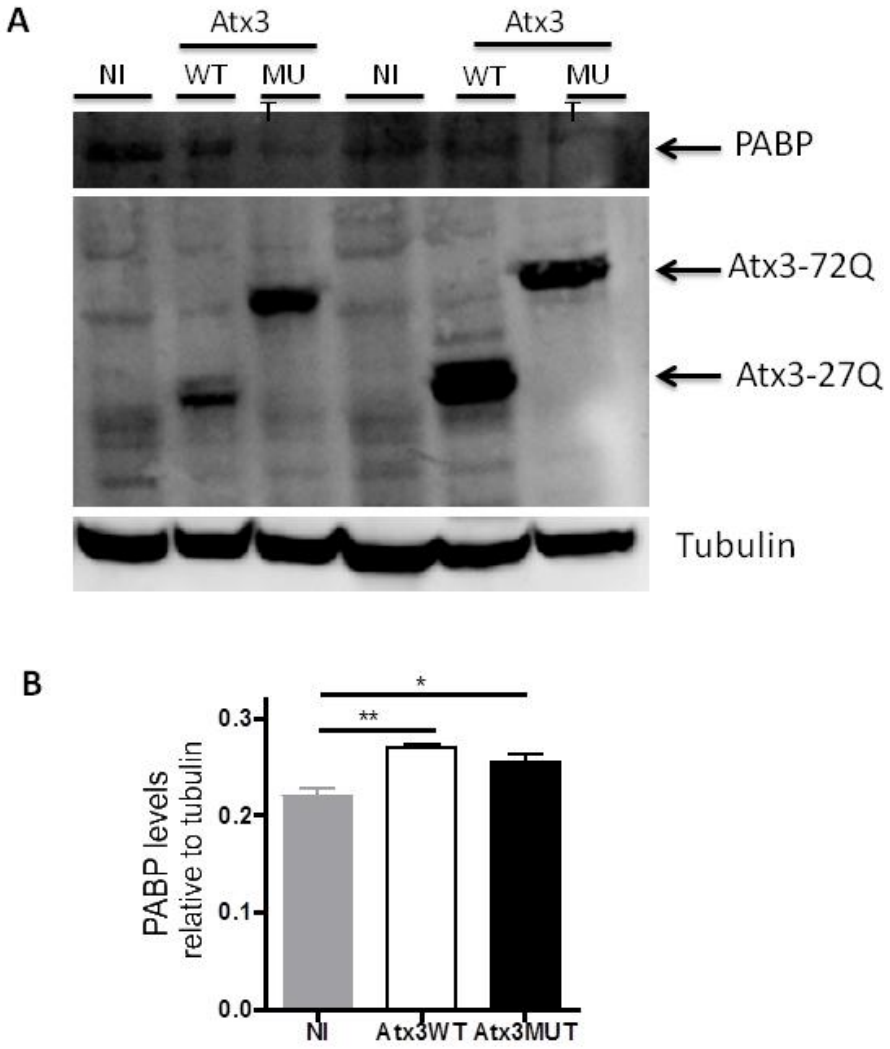

Figure 18. PABP levels are increased in a MJD cellular model. (A, B) Western blotting analysis of neuro2A cells: non-infected (NI, n=3), expressing human wild-type ataxin-3 (Atx3WT, n=3) and expressing human expanded ataxin-3 (Atx3MUT, n=3) cell lysates. (A) Representative western-blot probed for PABP, ataxin-3 and tubulin. Note the presence of endogenous PABP (MW: 71kDa), human Atx3 WT/ATx3-27Q (MW: 50kDa), Atx3Mut/Atx3-72Q (MW: 67kDa) and tubulin (MW: 55kDa). (B) Optical densitometry analysis. A significant increase in the levels of PABP was observed in cells expressing Atx3WT and in those expressing Atx3MUT relatively to the NI cells. Each PABP lane was normalized according to the tubulin band. Tubulin was used as a loading control. Results were

expressed as ratio PABP/ Tubulin (n=3 for each experimental set). Values are expressed as mean ± SEM. **P*<0.05; ***P*<0.01 (One-Way Anova test). It was performed three replicates of the protocol.

3.6. Overexpression of ataxin-2 and PABP reduce ataxin-3 levels in a neuro2a MJD model

We further analyzed ataxin-3, ataxin-2, and PABP network connection, by co-infecting N2a cells with lentiviral vectors encoding Atx3WT or Atx3MUT and Ataxin-2 or PABP, to access the effect of these co-infections not only in the levels of ataxin-3, but also in ataxin-2 and PABP levels.

Upon infection of cells expressing Atx3WT and Atx3MUT with ataxin-2-encoding vectors we performed western-blotting of cell lysates (Figure 19A). The membranes were probed for ataxin-2, PABP, ataxin-3 and tubulin in order to access the levels of the different proteins upon co-infection (Figure 19A). We found that the levels of ataxin-3 (relative to tubulin) in Atx3WT cells infected with ataxin-2 (Atx2) (Atx3Wt+Atx2, n=3) were significantly decreased (Figure 19B upper panel, *P*<0.01) relatively to the Atx3WT cells non-infected with Atx2 (Atx3WT, n=3). A similar effect was observed in Atx3MUT cells co-infected with vectors encoding ATX2 (Atx3MUT+Atx2, n=3), with an even more significant decrease (Figure 19B upper panel, *P*<0.001) compared to the Atx3-MUT cells non-infected with Atx2 (Atx3MUT, n=3). Densitometric analysis confirmed a significant decrease of wild-type (Figure 19B lower panel, *P*<0.001) and mutant ataxin-3 (Atx3MUT cells; Figure 19B lower panel, *P*<0.001) levels upon ataxin-2 co-expression.

Analysis of PABP levels (relative to tubulin) also yielded significant results. An increase (Figure 19C, *P*<0.05) of PABP levels in the Atx3WT+Atx2 cells when compared to the non-infected, Atx3WT cells was detected. This increase was also observed in the Atx3MUT+Atx2 cells, even though much more accentuated (Figure 19C, *P*<0.001).

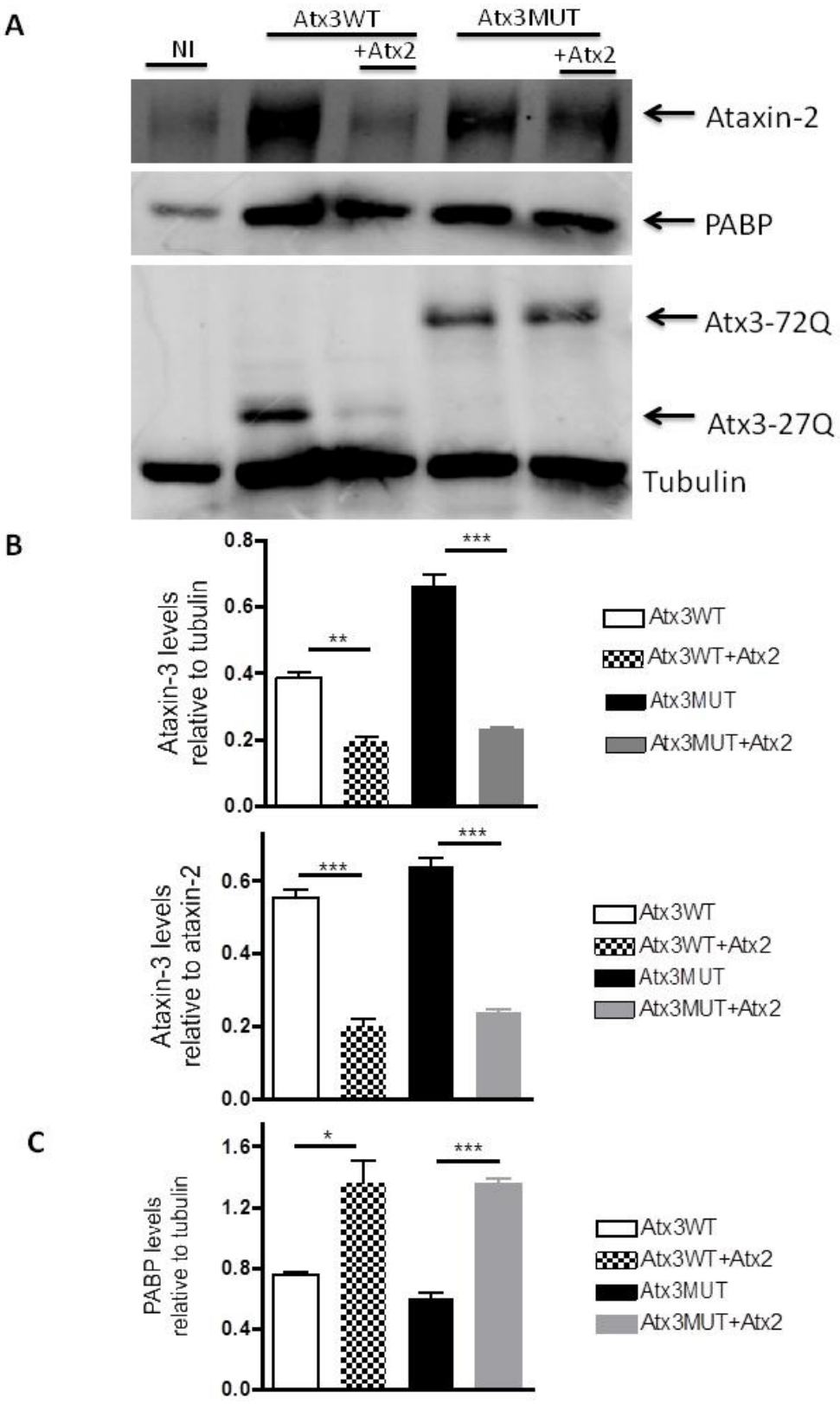

Figure 19. Ataxin-2 overexpression mediates decrease of ataxin-3 and increase of PABP levels. (A-C) Western blotting analysis of: non-infected (NI, n=3), expressing human wild-type ataxin-3

(Atx3WT, n=3), expressing human wild-type ataxin 3 infected with ataxin 2 (Atx3WT+Atx2, n=3), expressing human expanded ataxin-3 (Atx3MUT, n=3), expressing human expanded ataxin-3 infected with ataxin-2 (Atx3MUT+Atx2, n=3) cells. (A) Representative western-blot probed for Ataxin-2, PABP, ataxin-3 and tubulin. Note the presence of endogenous Ataxin-2 (MW: ≅ 148kDa), PABP (MW: 71kDa), human Atx3 WT/ATx3-27Q (MW: 50kDa), Atx3Mut/Atx3-72Q (MW: 67kDa) and tubulin (MW: 55kDa). (B) Optical densitometry analysis. Significant decrease in the ataxin-3 levels was observed (relative to tubulin and to ataxin-2) in Atx3WT+Atx2 cells relatively to the Atx3WT cells. A similar occurrence was observed in the levels of ataxin-3 with Atx3MUT+Atx2 relatively to non-infected Atx3MUT cells. The ataxin-3 levels were normalized according to the amount loaded (tubulin band). Results are expressed as ratio ataxin-3/tubulin and ataxin-3/ataxin-2 (n=3 for each experimental set). Values are expressed as mean ± SEM. ***P*<0.01; ****P*<0.001 (Student's t-test). Three replicates of the protocol were performed. (C) Optical densitometry analysis. Significant increase in PABP levels was detected (relative to tubulin) in Atx3WT+Atx2 cells relatively to the Atx2WT cells. A similar occurrence was observed in the levels of PABP in Atx3MUT+Atx2 relatively to Atx3MUt cells. Each PABP lane was normalized according to the tubulin band. Tubulin was used as a loading control. Results were expressed as ratio PABP/ Tubulin (n=3 for each experimental set). Values are expressed as mean ± SEM. **P*<0.05; ****P*<0.001 (Student's t-test).

A similar experience was conducted by infecting cells expressing Atx3WT and Atx3MUT with lentiviral vectors encoding for PABP and performing western-blotting of the cell lysates (Figure 20A). The membranes were probed for ataxin-2, PABP, ataxin-3 and tubulin in order to access different expressions due to the co-infection (Figure 19A). The levels of ataxin-3 (relative to tubulin) in Atx3WT cells infected with PABP (Atx3Wt+PABP, n=3) were significantly decreased (Figure 20B upper panel, *P*<0.001) relatively to the Atx3WT cell non-infected (Atx3WT, n=3). The same occurred in the levels of ataxin-3 in Atx3MUT cells infected with PABP (Atx3MUT+PABP, n=3), with a significant decrease (Figure 20B upper panel, *P*<0.01) compared to the Atx3-MUT cells non-infected with PABP (Atx3MUT, n=3). Analyzing the results of ataxin-3 levels relative to PABP levels, the results were very similar; a significant decrease of ataxin-3 levels was observed in Atx3WT+PABP cells compared to Atx3WT cells (Figure 20B lower panel, *P*<0.01). A similar decrease was observed in Atx3MUT+PABP relatively to Atx3MUT cells (Figure 20B lower panel, *P*<0.001).

Analysis of ataxin-2 levels (relative to tubulin) did not show significant differences in the ataxin-2 levels between Atx3WT+PABP and Atx3WT (Figure 20C, *P*>0.05). However, in Atx3MUT+PABP cells a significant increase of ataxin-2 levels was observed relatively to Atx3MUT cells (Figure 20C, *P*<0.001).

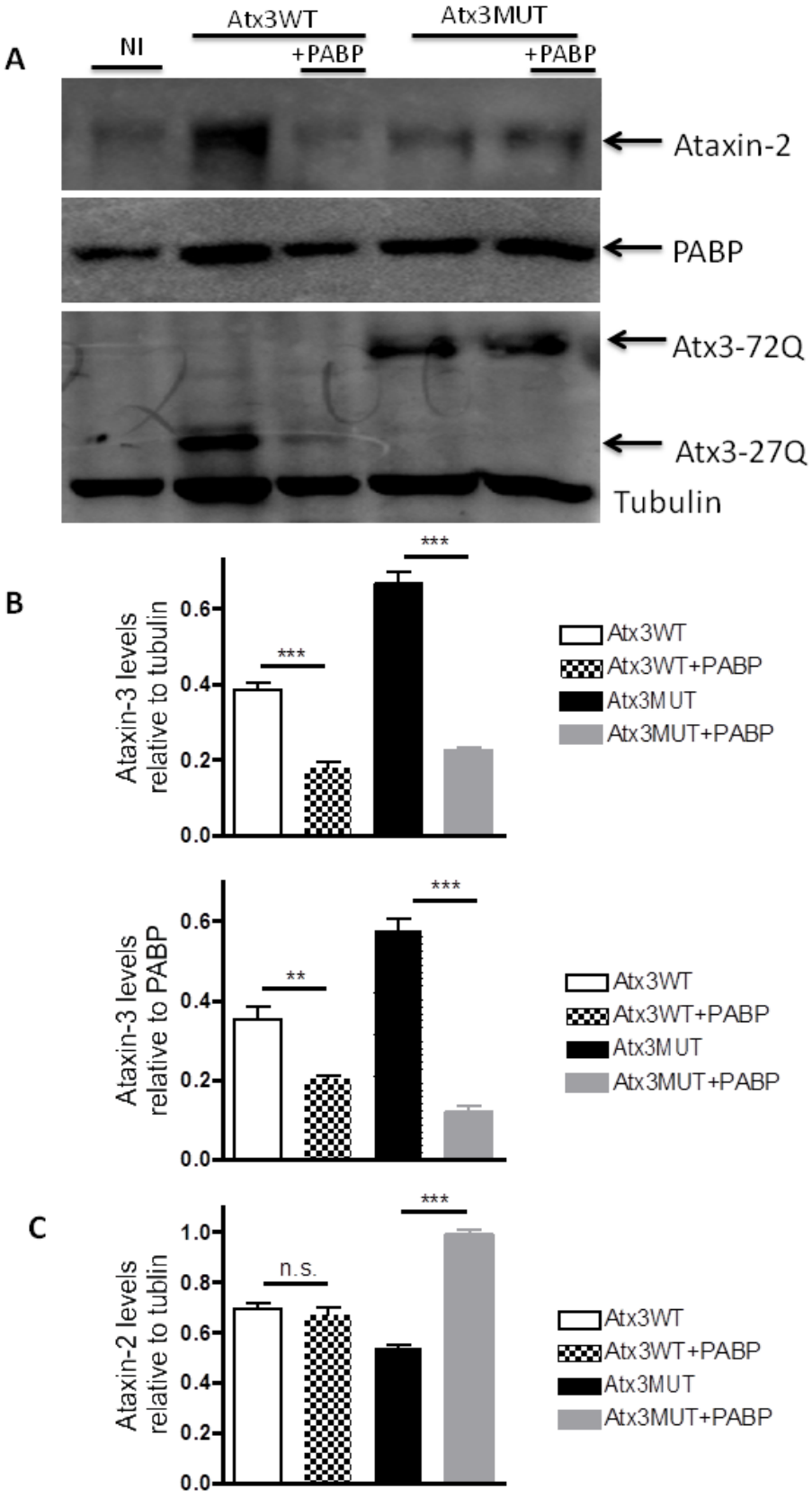

Figure 20. PABP overexpression decreases the levels of ataxin-3 expression and increases the levels of ataxin-2 expression. (A-C) Western blotting analysis of non-infected cells (NI, n=3), or cells

infected with lentiviral vectors encoding, respectively: human wild-type ataxin-3 (Atx3WT, n=3), human wild-type ataxin 3 and PABP (Atx3WT+PABP, n=3), human expanded ataxin-3 (Atx3MUT, n=3), human expanded ataxin-3 and PABP (Atx3MUT+PABP, n=3) cells. (A) Representative western-blot probed for ataxin-2, PABP, ataxin-3 and tubulin. Note the presence of endogenous ataxin-2 (MW: ≅ 148kDa), PABP (MW: 71kDa), human Atx3 WT/ATx3-27Q (MW: 50kDa), Atx3Mut/Atx3-72Q (MW: 67kDa) and tubulin (MW: 55kDa). (B) Optical densitometry analysis. Significant decrease in ataxin-3 levels (relative to tubulin and to PABP) was observed in Atx3WT+PABP cells relatively to the Atx3WT cells. Similarly, the levels of ataxin-3 decreased with Atx3MUT+PABP relatively to non-infected Atx3MUt cells. Ataxin-3 levels were normalized according to the amount loaded (tubulin band). Results are expressed as ratio ataxin-3/tubulin and ataxin-3/PABP (n=3 for each experimental set). Values are expressed as mean ± SEM. $^{**}P<0.01$; $^{***}P<0.001$ (Student's t-test). Three replicates of the protocol were performed. (C) Optical densitometry analysis. Note that there were no significant differences in Atx3WT+PABP and Atx3WT cells. Significant increase in ataxin-2 levels (relative to tubulin) in Atx3MUT+Atx2 relatively Atx3MUT cells. Each Ataxin-2 lane was normalized according to the tubulin band. Tubulin was used as a loading control. Results were expressed as ratio PABP/ Tubulin (n=3 for each experimental set). Values are expressed as mean ± SEM. ns; $P>0.05$; $^{***}P<0.001$ (Student's t-test).

3.7. Silencing PABP does not alter ataxin-3 neither ataxin-2 levels but increases mutant ataxin-3 aggregates

To further investigate the dynamics of the ataxin-3, ataxin-2 and PABP network we performed silencing of PABP. Evidence from the previous section suggested that overexpression of PABP could decrease ataxin-3 levels and increase ataxin-2 levels therefore we investigated if knocking-down PABP would mediate symmetric effects.

For our experiments we used a short-hairpin control (shLuc) and a mix of four short hairpin RNA silencing sequences targeting PABP (shPABP), which was used to transfect mutant Neuro-2a cells already expressing human mutant ataxin-3. The cells lysates were collected 48h after the transfection for western blotting analysis (Figure 21A). The membranes were probed for ataxin-2, PABP, ataxin-3 and tubulin in order to access their expression levels (Figure 21A). A decrease in the levels of PABP (relative to tubulin) was observed in the cells transfected with shPABP (n=3, $P<0.01$; Figure 21B) relatively the control cells (shLuc, n=3; Figure 21B). Nevertheless, there were no significant differences in ataxin-2 (Figure 21C) and ataxin-3 levels (Figure 21D) (related to tubulin) between the control cells and the silenced cells. This may result from the low knocking-down that was obtained with the shRNAs. However, a small but significant increase in the aggregate levels of mutant

ataxin-3 (found in upper portion of the blot corresponding to the stacking gel) was observed in the PABP silenced cells when compared to the control cells (Figure 21E, $P<0.01$). Further experiments with more efficient silencing sequences are necessary to confirm these results.

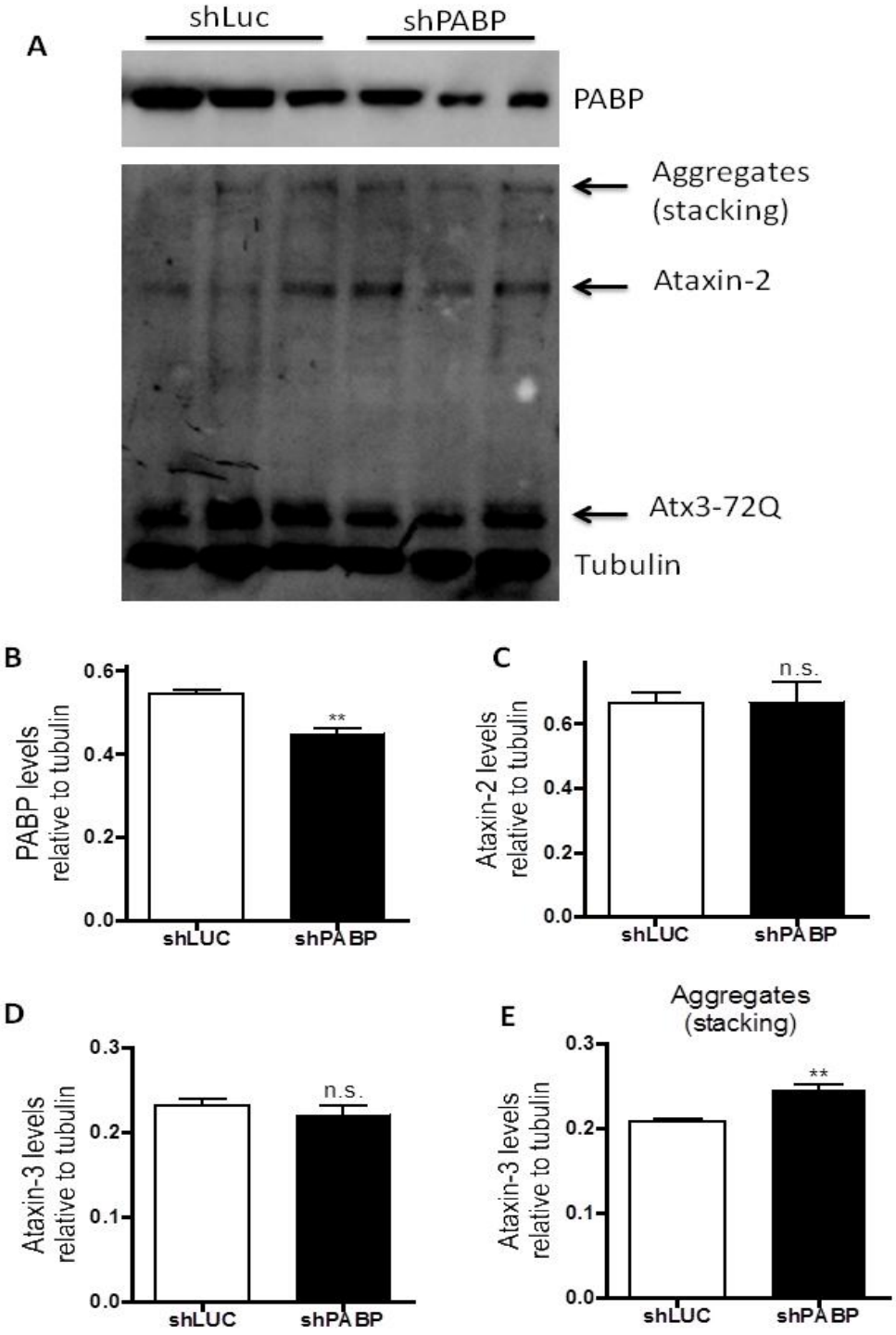

Figure 20. PABP silencing increases aggregate formation. (A-E) Cell lysates western blotting analysis of Atx3MUT cells transfected with shLuc (controls) (n=3) and transfected with shPABP (n=3).

(A) Representative western-blot probed for Ataxin-2, PABP, ataxin-3 and tubulin. Note the presence of endogenous ataxin-2 (MW: ≅ 148kDa), PABP (MW: 71kDa), ataxin-3 aggregates (MW> 250kDa), Atx3Mut/Atx3-72Q (MW: 67kDa) and tubulin (MW: 55kDa). (B) Optical densitometry analysis. It was possible to observe a decrease in the levels of PABP (relative to tubulin) in the cells transfected with shPABP relatively the control cells (shLuc), as expected. The PABP levels were normalized according to the amount loaded (tubulin band). Results were expressed as ratio PABP/tubulin (n=3 for each experimental set). Values are expressed as mean ± SEM. ***P*<0.01; ***P*<0.01 (Student's t-test). (C) Optical densitometry analysis. There were no significant differences in ataxin-2 between the control cells and the silenced cells. The ataxin-2 levels were normalized according to the amount loaded (tubulin band). Results were expressed as ratio ataxin-2/tubulin (n=3 for each experimental set). Values are expressed as mean ± SEM. ns.*P*>0.05 (Student's t-test). (D) Optical densitometry analysis. There were no significant differences in ataxin-3 between the control cells and the silenced cells. The ataxin-3 levels were normalized according to the amount loaded (tubulin band). Results were expressed as ratio ataxin-3/tubulin (n=3 for each experimental set). Values are expressed as mean ± SEM. ns.*P*>0.05 (Student's t-test). (E) Optical densitometry analysis. It was verified an increase in the aggregates of mutant ataxin-3 (found in the stacking) in the silenced cells when compared to the control cells. The aggregates levels were normalized according to the amount loaded (tubulin band). Results were expressed as ratio aggregates/tubulin (n=3 for each experimental set). Values are expressed as mean ± SEM. ***P*<0.01 (Student's t-test).

Chapter IV

Discussion

4. Discussion

In this study we provide evidence of an established interacting network between the causative protein of MJD, ataxin-3, its known interactor, ataxin-2 (*Lim et al., 2006; Lessing and Bonini, 2008*), and the only PABC containing protein that interacts with ataxin-2, PABP (*Kozlov et al., 2001*). This interactive network was already proven to exist in previous research (*Lessing and Bonini, 2008*), were it was found a toxic effect of ataxin-2 and a protective effect of PABP in the MJD phenotype. However, we obtained new evidence concerning these three proteins interactions and how affecting expression of one of them reflects in the others.

In the first part of this study, it was shown that ataxin-2 and PABP levels are altered in Machado-Joseph disease patients. In post-mortem human brain tissue of MJD patients, it was found higher levels of ataxin-2 in striatum and cortex, compared to the normal controls. This increase in the expression of ataxin-2 was higher in the striatum, a more affected region in MJD when compared to the cortex, which is relatively spared (*Sudarsky and Coutinho, 1995; Coutinho and Andrade, 1995*; *Alves et al., 2008*). These results are concordant to a possible neurodegenerative role of ataxin-2, already proven in other neurodegenerative diseases such as SCA1 (*Fernández-Funez et al., 2000; Al Rahami et al., 2007*) and ALS (*Elden et al., 2010; Chen et al., 2011*). In MJD it was previously found an association between the CAG repeat length at the SCA2 gene and the severity of fasciculations in MJD phenotype (*Jardim et al., 2003*). More data (*Uchichara et al., 2001*) fortified this possible role with the finding of non-expanded ataxin-2 in MJD intranuclear inclusions. Ataxin-2 incorporation in NIIs, even with pathological expansion of the polyQ tract in SCA2, was considered limited (*Huynh et al., 1999; Koyano et al., 1999; Koyano et al., 2000*), therefore its incorporation in MJD may suggest a real and active role in the disease pathogenesis for ataxin-2. Moreover, in fibroblasts from MJD patients, we also found an increase in ataxin-2 and PABP levels; also consonant with data from brain. Levels of ataxin-2 in the nucleus were found to be much higher than cytoplasmic levels in the patients. It was verified a recruitment of ataxin-2 to the nucleus, the possibility of an ataxin-2 shuttling may explain these observations. If contrarily to what is currently though (*Huynh et al., 1999; Uchichara et al., 2001*), ataxin-2 is able to acquire some type of shuttling capacity, it may explain why protein levels are also elevated in the cytoplasm, even while being recruited to the nucleus. Alternatively, ataxin-2 in the cytoplasm, its normal localization, may be required for the development of certain cellular responses, which may involve ataxin-2's metabolization or processes which impair the recognition of its epitopes,

leading to an inaccurate reading. Several studies were conducted regarding the subcellular localization of ataxin-2, however is still not clarified; it is currently a controversial matter whether if it is a cytoplasmic or nuclear protein (*Huynh et al., 1999; Lastres-Becker et al., 2008*), but evidence point it to be an almost exclusively cytoplasmic protein. It is known however, that in SCA2, mutant ataxin-2 accumulates both in the cytoplasm and in the nucleus, even though differently. The presence of pathogenic expansions does not lead to intranuclear inclusions contrarily to other polyglutamine diseases, but rather produces stranger and more diffuse cytoplasmic signals; in addition its nuclear localization it's not required to induce toxicity (*Huynh et al., 1999*). However the recruitment of ataxin-2 has already been reported in SCA1 (*Al Rahami et al., 2007*), where ataxin-2 located in the nucleus was verified much more harmful than in the cytoplasm once it interferes with the transcriptional machinery and becomes a potent pathogenesis mechanism (*Al Rahami et al., 2007*). It seems that mutant ataxin-2 with expanded polyQ tract does not increases the toxicity in SCA2 if translocated into the nucleus, however wild type ataxin-2 gains a destructive function once recruited into the nucleus in other polyQ diseases (such as ataxin-1). PABP levels were also increased in the MJD patients, although the evidences point to partial localization in the nucleus, its recruitment is not as obvious as the observed for ataxin-2. PABP is a RNA-binding protein which associates with the 3'-poly(A) tail of eukaryotic mRNA (*Amrani et al., 1997*), it is a multifunctional protein involved in many different cellular functions, including translation, mRNA metabolism, and mRNA export (*Afonina et al., 1998; Brune et al., 2005*). It is known its nucleoshuttling capacity; it can be present in the nucleus or in the cytoplasm depending on its functions (*Afonina et al., 1998; Brune et al., 2005*). In our results, we found some PABP in the nucleus; however this may not result from direct recruitment due to the pathology. Data found leaves us the doubt whether PABP is located in the nucleus or not in MJD. If in the control fibroblast it is not verified PABP in the nucleus, but in MJD patient fibroblast there are evidence of a certain nuclear localization. If indeed the recruitment of PABP into the nucleus in MJD is occurring, may concern PABP's functions requiring its nuclear localization. On the other hand, PABP in the nucleus may be compromising transcriptional processes since its localization in the cytoplasm is required for those functions, which can be damaging for the cell and lead to the worsening of the pathology.

A previous work (*Lessing and Bonini, 2008*) related ataxin-2's toxicity to its PAM2 motif. The PAM2 motif binds specifically to the PABC domain, and PABP is currently the only known protein containing a PABC domain able to interact with ataxin-2 (*Kozlov et al., 2001*). Using an ataxin-2 lacking the PAM2 motif, it was verified an accelerated inclusion formation

(*Lessing and Bonini, 2008*); since the PAM2 motif is required for the connection of PABP it is safe to say that the impossibility of PABP connection to ataxin-2 leads to an exacerbation of the pathology. Therefore, these results could point to a regulatory activity towards ataxin-2, what could account for the elevation of PABP levels to annul some of the toxic activity of ataxin-2 in MJD but also a possible protective role of PABP in MJD by connecting to ataxin-2 and preventing its toxic effect.

In the attempt to better understand the correlation between the levels of ataxin-3, ataxin-2 and PABP, we continued our study by accessing levels of ataxin-2 and PABP in MJD models.

In the lentiviral MJD model (*Alves et al., 2008*) we further obtained data and evidence of ataxin-2 and PABP in the progression of the disease. With disease progression, and the increase in the number of intranuclear inclusions of mutant ataxin-3, the levels of ataxin-2 and PABP were drastically reduced when compared to an earlier state. These results were opposite to the increase of their levels observed in MJD patients. In this model like in patients there is an ataxin-2 recruitment in to the nucleus, which can lead to its depletion in the cytoplasm in a more advanced stage of the disease. This cytoplasmic depletion and ataxin-2's nuclear accumulation are consistent with the neuropathology aggravation with the progression of the disease. However, in this lentiviral MJD model, the ataxin-3 provided is human, due to the inter-species discrepancy it is possible that it doesn't exist compensatory mechanisms to restore ataxin-2 levels in the cytoplasm, therefore its depletion over time. PABP, as referred, is a protein with shuttling capacity accordingly to its functions (*Afonina et al., 1998; Brune et al., 2005*); it is possible that the advance of the pathology and the decline of cellular functions create the necessity of elevated PABP levels in the nucleus, which can also lead to its depletion in the cytoplasm. The possible regulatory effect in ataxin-2's levels may also account for its nuclear shuttling, if ataxin-2 is being recruited to the nucleus, then PABP can't regulate its levels in the cytoplasm, therefore shuttling in to the nucleus. However, this shuttling may have drastic consequences to the cell integrity; since PABP is essential in transcriptional processes (*Amrani et al., 1997; Afonina et al., 1998; Mangus et al., 2003; Brune et al., 2005*) and for those purposes its cytoplasmic localization is required, the cytoplasmic depletion of PABP over time may cause irreparable damages to the cell, leading to the enhancement of neurodegeneration.

In a MJD transgenic mice model (*Torashima et al. 2008*) we observed a decrease of ataxin-2 and PABP levels in the cerebellum (by immunoreactivity) compared with normal wild-type mice. In MJD patients, the cerebellum is one of the most attained regions

(*Rosenberg, 1992; Sudarsky and Coutinho, 1995; Durr et al., 1996; Rub et al., 2006., Alves et al., 2008*), and this model has a predominantly expression of mutant Ataxin-3 in Purkinje cells. Ataxin-2 has a widespread expression in the brain but its major localization is seen in the Purkinje cells (*Nechiporuk et al., 1998; Huynh et al., 1999*) Thus, a depletion and degeneration of these cells, results in a reduction of the ataxin-2 and PABP levels. However, total protein levels of ataxin-2 are increased in the MJD Q69 cerebellum comparatively to the WT mice. Regarding the total PABP protein levels was verified a decrease in MJD Q69 mice when compared to the WT mice, although this decrease can't be considered significant. This decrease may be a result from PABP metabolization or its epitopes may be hidden or harder to access if PABP is localized in the nucleus performing its normal function or enhancing the pathology as referred. These results are similar to the human tissue analysis, where we found an increase in ataxin-2 levels in MJD patients; in human tissue analysis we also found an increase in PABP levels, but as explained, the non-significant decrease observed in the MJD 69 mice may not correspond to the real scenario.

In order to better understand how the ataxin-3-ataxin-2-PABP could interact and to gather further evidence of ataxin-2 and PABP role in MJD, we looked for the effect of expression of one protein in the levels of expressions of the others, in a cellular model. The infection of N2a cells with mutant ataxin-3 diminished ataxin-2 levels and increased PABP levels. However, ataxin-2's decrease may be due to its recruitment to the nucleus where once incorporated in the aggregates may be difficult to detect. Also, wild type ataxin-3 has an important neuroprotective role, depending on a fully functioning proteasome (*Warrick et al., 2005*), it's possible that the endogenous wild type ataxin-3 may be fulfilling its normal function and activating clearance mechanisms, leading to the destruction of the aggregates (induced by the infection of human expanded ataxin-3) that contain ataxin-2 (*Uchihara et al., 2001*), therefore causing a decrease in endogenous ataxin-2 levels. This outcome is probable since infection with wild type ataxin-3 didn't altered ataxin-2's levels when compared to the non-infected cells; highlighting that in normal and non-pathological conditions, ataxin-3 does not alter ataxin-2 levels, indicating that this phenomenon may be a protective cellular mechanism verified in the disease. Levels of PABP were found to be increased upon infection of both ataxin-3 wild type and mutant. These results could point to an interaction between PABP and ataxin-3 (normal and mutant), or at least to sub-effect in PABP levels due to the expression of ataxin-3. However, contrarily to ataxin-2, this interaction doesn't seem to be verified only in pathological conditions.

Once established the effects of ataxin-3 infection in ataxin-2 and PABP's levels, we tried to access the possible effect of ataxin-2 and PABP overexpression in ataxin-3 levels. To date, there was only the evidence that ataxin-2 overexpression exacerbated neurodegeneration in a MJD model in Drosophila eye. On the contrary, PABP overexpression suppressed neurodegenerative phenomenons (*Lessing and Bonini, 2008*). When co-expressing human ataxin-2 with human mutant ataxin-3 in N2a cells, the levels of ataxin-3 were drastically diminished, while levels of PABP increased, which could support PABP possible regulatory effect facing the overexpression of ataxin-2. Since ataxin-2's levels are increased there is an increase in PABP levels in order to annul the ataxin-2 possible toxic effect. Moreover the co-expression of wild-type ataxin-3 and ataxin-2 also decreased ataxin-3 levels, which further support a possible interaction between ataxin-2 and ataxin-3. It's known that ataxin-2 has a role in RNA metabolism, regulating translational processes, and that modifications in ataxin-2 levels are able to alter the abundance of some proteins (*Ciosk et al., 2004; Satterfield and Pallanck, 2006*). In some way still not understood it appears that by translational regulation ataxin-2 is able to decrease ataxin-3 levels in a pathological situation as MJD.

The overexpression of PABP also led to a decrease in the overall ataxin-3 levels. Co-expression of human PABP and human mutant ataxin-3 led to a significant decrease in ataxin-3 levels, and also in ataxin-2 levels. The same decrease in ataxin-3 levels was observed in the co-infection of PABP and wild-type ataxin-3, however, levels of ataxin-2 remained the same, did not suffer any alteration when compared to the cells infected only with wild-type ataxin-3. These results may reflect this correlation between these three proteins, in normal conditions (co-infection of wild-type ataxin-3 and PABP), ataxin-2 levels are not altered by PABP, but in pathological conditions (co-infection of mutant ataxin-3 and PABP), overexpression of PABP leads to an increase in ataxin-2 levels. The possible regulatory role of PABP regarding ataxin-2 may therefore be reciprocal and it seems to occur in pathological conditions; high levels of ataxin-2 are accompanied by the elevation in PABP levels and vice-versa, implying some kind of feedback and balance between these two proteins. The results point to this interaction as a protective cellular mechanism.

All together these results points to a synergicall relation between ataxin-2 and PABP, which can actively decrease ataxin-3 levels, both in normal and pathological conditions.

We also tried silencing PABP in order to decipher better its role in the disease, and our results corroborate all of our previous findings. Silencing PABP did not altered levels of ataxin-3 neither of ataxin-2, which in light to our previous findings shouldn't have happened;

unless the possible feedback mechanism existing between ataxin-2 and PABP also involves ataxin-3. If this were the case, silencing PABP would trigger the feedback and ataxin-2 and ataxin-3 may not respond by altering significantly their levels in the attempt to cope with the decrease in PABP levels. What we found was a significant increase in aggregate formation; reinforcing the possibility of a protective role of PABP, that once silenced would not connect to the PAM2 motif of ataxin-2, leaving room for ataxin-2's toxicity. Although these results might be explained, the efficiency of the silencing was not ideal, since levels of PABP didn't decreased how it was expected. Levels of PABP should have lowered around 50%, which did not occurred and so doesn't allow us to take more appropriated and accurate insights about the effect of PABP silencing in ataxin-2 and ataxin-3 levels. The silencing was performed by lipofectamine transfection of shPABP, in order to obtain a more efficient silencing we should have used lentiviral vectors encoding shPABP.

Although the PABP silencing didn't work entirely it was still possible to observe significant differences in the aggregate formation. It is described that PABP connection to ataxin-2's PAM2 motif can suppress its toxicity (*Lessing and Bonini, 2008*), in the absence of PABP it wasn't verified a decrease in ataxin-3 as observed in infection with ataxin-2. These results may indicate that the decrease in ataxin-3 levels observed with mutant ataxin-3 and ataxin-2 co-infection, might derivate from a PABP action, since ataxin-2's overexpression clearly induces PABP expression and PABP is able to decrease ataxin-3 levels. This synergy between the levels of ataxin-2 and PABP results in a protective condition.

In our work we collected evidence for a strong and significant correlation between ataxin-3, ataxin-2 and PABP. Ataxin-2 might play a role in amplifying toxicity in MJD similarly to other neurodegenerative diseases; however, PABP might play the opposite role, protecting cells from ataxin-2 induced toxicity and alleviating neurodegeneration. Once all the pending questions that remain are answered, then perhaps dampening ataxin-2 toxicity by stimulating PABP activity could lead to a MJD treatment.

Chapter V

Conclusions and perspectives

5. Conclusion

In our work, we tried to unravel more about the proteic interaction that seem to determine some of MJD's characteristics. As referred earlier, the CAG repeats don't account for all the variability observed in the disease; therefore understanding the factors that can interfere with the age of onset and some phenotypic aspects of MJD may be a stepping stone for a new and unexplored field in therapeutics. It's possible that some these situations may be due to proteic network interactions that may somehow lead to an earlier age of onset or even certain polymorphisms still not discovered.

We followed evidences regarding the intriguing proteic interaction between MJD's causative protein Ataxin-3, Ataxin-2 and PABP. We found that in fact there is a network interaction which can be determinant for the progression of the disease. It is clear that ataxin-2 and PABP expressions are co-dependent, probably by feedback mechanisms, and that ataxin-3 becomes a part of this interaction in pathological conditions, such as in MJD. Ataxin-2 levels seem to be regulated by PABP, in its turn; PABP plays a neuroprotective role protecting the cell from ataxin-2 induced toxicity. Ataxin-2 and PABP appear to create a balance in which they are able to diminish Ataxin-3 levels. High levels of ataxin-2 are present in the disease and its recruitment to the nucleus involves this protein in MJD's pathogenesis mechanisms; it's still not understood how this interactive connection functions and what triggers PABP's regulatory functions over ataxin-2. If we could be able to understand why this protective effect doesn't always work and why in certain stage of MJD it becomes impaired leading to the exacerbation of neurodegeneration maybe with ataxin-2's influence; it would be possible to find new strategic therapeutics to suppress or delay the MJD's fatal outcome. For this matter it is relevant and possibly determinant to further invest in the study of this proteic interactive network.

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