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Post-Translational Modifications of Ataxin-3, the Protein Involved in Machado-Joseph Disease:

Evidences for Phosphorylation and Sumoylation

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Abbreviations

6His	hexahistidine
AR	androgen receptor
ATM	ataxia telangiectasia mutated kinase
atx3	ataxin-3
BCA	bicinchoninic acid
CACNA1A	calcium channel, voltage dependent, P/Q type, α1A subunit
CaMK2	calmodullin-dependent kinase 2
CBP	CREB-binding protein
Cdc2	cell division cycle 2 kinase
Cdk5	cyclin-dependent kinase 5
CHIP	C-terminus of Hsp70-interacting protein
CK1/2	casein kinase 1 or 2
CLAP	chymostatin, pepstatin, antipain and leupeptin
CREB	cAMP-response element-binding protein
CRM1	chromosome region maintenance 1
D-MEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA PK	DNA-dependent protein kinase
DRPLA	dentatorubral-pallidoluysian atrophy
DUB	deubiquitinating enzyme
ECF	Enhanced Chemifluorecense substrate
EGFR	epidermal growth factor receptor kinase
ERAD	endoplasmic reticulum-associated degradation
Erk1	extracellular signal-regulated kinase 1
FBS	fetal bovine serum
Fgr	Gardner-Rasheed feline sarcoma viral oncogene homolog kinase

GFP	green fluorescent protein
GSK3β	glycogen synthase kinase 3 β
HD	Huntington's disease
HDAC3/6	histone deacetylase 3 or 6
HEK	human embryonic kidney
HHR23A/B	human homolog of Rad23 A or B
HS	heat shock (figures only)
Hsp70	70 KDa heat shock protein
IgG	immunoglobulin G
IMAC	immobilized metal ion affinity chromatography
IP	immunoprecipitation
IPB	IP buffer
iPP	inorganic pyrophosphate
IR	insulin receptor kinase
JD	Josephin domain
JNK	c-Jun NH_2-terminal kinase
KO	knockout
LC	liquid chromatography
LC-ES-MS	LC followed by ion spray MS
MAP2	microtubule-associated protein 2
MAPK	mitogen-activated protein kinase
MEM	Minimum Essential Medium
MJD	Machado-Joseph disease
MS	mass spectrometry
MTOC	microtubule-organizing center
Na_3VO_4	sodium orthovanadate
NCBI	National Center for Biotechnology Information
NCor	nuclear receptor co-repressor

NEAA	Non-Essential Amino Acids
NEDD8	neural precursor cell expressed, developmentally downregulated gene 8
NES	nuclear export signal
NI	nuclear inclusion
NLS	nuclear localization signal
NT	non-transfected cells (figures only)
OA	okadaic acid
PCAF	p300/CBP-associated factor
PDB	Protein Data Bank
PDGFR	platelet-derived growth factor receptor kinase
PKC	protein kinase C
PMA	phorbol 12-myrisate 13 acetate
PMSF	phenylmethanesulphonyl fluoride
polyQ	polyglutamine
PTM	post-translational modification
PVDF	polyvinylidene difluoride
RanGAP1	Ras-related nuclear protein GTPase activating protein 1
SAE1/2	SUMO1-activating enzyme subunit 1 or 2
SBMA	spinal and bulbar muscular atrophy
SCA1/2/3/6/7/17	spinocerebellar ataxia type 1, 2, 3, 6, 7 or 17
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrilamide gel electrophoresis
SGK	serum/glucocorticoid regulated kinase
SNP-1/-2	SUMO-specific proteases 1 or 2
SRC	steroid hormone receptor coactivator
SUMO	small ubiquitin-like modifier
TBP	TATA box binding protein

TBS-T	Tris-buffered saline-Tween 20
Ub	ubiquitin
Ubc9	Ub-conjugating enzyme 9
UCH	Ub C-terminal hydrolases
UCH-L3	Ub C-terminal esterase L3
UIM	Ub-interacting motif
UPP	Ub-proteasome pathway
USP	Ub-specific processing proteases
Usp4/25	Ub-specific protease 4 or 25
VCP	valosin-containing protein
WB	Western blot (figures only)
YUH1	yeast Ub C-terminal hidrolase 1

Abstract

Machado-Joseph disease (MJD), the most common form of autosomal dominantly-inherited ataxia, belongs to a group of neurodegenerative disorders sharing the attribute of being caused by unstable expansions of CAG codon repeats in the respective genes. These disorders are collectively named as polyglutamine (polyQ) expansion diseases, since that expansion is translated as a lengthy glutamine sequence in the otherwise unrelated proteins encoded by each gene. Ataxin-3 (atx3), the protein associated with MJD, is a deubiquitinating enzyme (DUB) which mainly comprises a N-terminal catalytic domain, termed Josephin domain (JD), and a C-terminal tail that contains two or three ubiquitin-interacting motifs (UIMs) and the polyQ stretch. Though its concrete biological function remains to be elucidated, information regarding atx3 activity and molecular interactions points out some involvement with the ubiquitin-proteasome pathway (UPP) and with transcription regulation. To this date, the experimentally observed deubiquitinating activity has been reported as being very low, being admitted that, as with many other DUBs, atx3 activity may be closely regulated and thus require external factors to reach optimal levels.

The way expanded atx3 and every other polyQ-expanded proteins lead to the corresponding diseases also remains to be determined but, though it is admitted that there are some events common to all of the diseases (including formation of toxic protein aggregates), the different properties of each particular protein hosting the polyQ expansion are probably the cause of the different profiles exhibited by each polyQ expansion disease. One way by which this protein context may play a role in these neurodegenerative diseases is related to the differential susceptibility to be targeted by post-translational modifications (PTMs), a group of ubiquitous and frequently reversible mechanisms that regulate many protein properties, modulating their function, subcellular localization and interactions. Importantly, cell-specific differences in protein properties resulting from differential regulation by PTMs may explain the region-specific

degeneration observed in every polyQ expansion disease. Many PTMs targeting polyQ expansion proteins have been related with the regulation of some of their properties and sometimes seen as interfering with events involved in disease development. In the particular case of atx3, PTMs also constitute a promising mechanism for regulation of its deubiquitinating activity. Consequently, the goal of the current work was to identify novel PTMs of atx3.

We chose to focus our attention on phosphorylation and sumoylation, two PTMs that have been associated with regulation of both DUBs and polyQ expansion proteins. In order to identify new phosphorylation sites in atx3, we immunoprecipitated an atx3 fusion protein from transfected HEK 293FT cells and analyzed it recurring to a mass spectrometric strategy specially directed at the mapping of phosphorylation sites. This analysis determined for the first time that serine 12 of atx3 is phosphorylated. Usage of no artificial stimulus other than that of the phosphatase inhibitor okadaic acid (OA) prompts us to admit that this modification occurs normally in HEK 293FT cells. After analyzing the tertiary structure of the JD and observing the proximity of this amino acid to those of the active site of the protein, we speculate that this modification likely influences atx3 deubiquitinating activity. As for sumoylation, we submitted 6His-tagged JD purified from *E. coli* to an *in vitro* sumoylation assay and concluded that it conjugated with small ubiquitin-like modifier 2 (SUMO2). Though we were unable to confirm this result *in vivo*, it constitutes the first evidence for sumoylation of atx3 and indicates that the catalytic domain of the protein is a target for this modification.

Further study of the PTMs we identified and subsequent characterization of the way they regulate atx3 properties may help us increase our knowledge about atx3 function and the mechanisms responsible for MJD development.

Keywords: *Machado-Joseph disease*, *polyglutamine expansion diseases*, *ataxin-3*, *Josephin domain*, *deubiquitinating enzymes*, *phosphorylation*, *sumoylation*

Resumo

A doença de Machado-Joseph (MJD), a ataxia com hereditariedade autossómica dominante mais comum, pertence a um grupo de doenças neurodegenerativas que partilham o facto de serem causadas por expansões instáveis de repetições de codões CAG nos genes correspondentes. Estas desordens são colectivamente designadas doenças de poliglutaminas (polyQ), uma vez que a expansão é traduzida como uma sequência expandida de glutaminas nas proteínas codificadas por cada gene; essas proteínas não estão, de outro modo, relacionadas. A ataxina-3 (atx3), a proteína associada à MJD, é uma enzima com actividade de hidrolase de ubiquitinas (DUB) formada por um domínio catalítico presente no terminal amino, designado de domínio Josephin (JD), e por uma cauda presente no terminal carboxílico, que contém dois ou três motivos de interacção com ubiquitina (UIMs) e a sequência de polyQ. Embora a função fisiológica desta proteína permaneça ainda desconhecida, as informações existentes acerca da actividade da atx3 e das interacções moleculares que estabelece indiciam um certo envolvimento com a via da ubiquitina-proteossoma (UPP) e com a regulação da transcrição. Até à data, a actividade proteolítica detectada experimentalmente tem sido considerada muito baixa, admitindo-se que, tal como acontece com muitas outras DUBs, a actividade da atx3 deverá ser estritamente regulada e necessitará, portanto, de factores externos para atingir níveis fisiologicamente significativos.

A forma como a atx3 expandida e todas as outras proteínas com uma expansão de polyQ conduzem às doenças em questão também não foi ainda determinada, mas, embora se admita que certos acontecimentos são comuns a todas as patologias (incluindo a formação de agregados tóxicos de proteínas), reconhece-se que as diferentes propriedades de cada proteína que contém a expansão de polyQ são a causa dos diferentes perfis apresentados por cada doença de polyQ. Um modo através do qual este contexto proteico pode ser importante relaciona-se com a

diferente susceptibilidade para ser alvo de modificações pós-traducionais (PTMs), um grupo de mecanismos ubíquos e frequentemente reversíveis que regulam muitas propriedades das proteínas, modulando a sua função, a sua localização subcelular e as suas interacções. Um aspecto a ter em conta é a eventual possibilidade de se vir a explicar a degeneração específica de determinadas regiões do sistema nervoso que se observa em todas as doenças de expansão de polyQ como resultado de diferenças nas propriedades das proteínas que resultem de uma regulação diferencial por PTMs. Muitas PTMs de proteínas com expansão de polyQ têm sido relacionadas com algumas das suas propriedades e, por vezes, admitidas como interferindo em processos relacionados com o desenvolvimento das patologias. No caso particular da atx3, as PTMs poderão também constituir um mecanismo promissor de regulação da sua actividade de hidrolase de ubiquitina. Consequentemente, o objectivo deste trabalho era identificar novas PTMs da atx3.

Escolhemos concentrar a nossa atenção na fosforilação e na sumoilação, duas PTMs que têm sido associadas tanto com a regulação das DUBs como das proteínas com expansão de polyQ. De modo a identificar novos locais de fosforilação da atx3, imunoprecipitámos uma proteína de fusão da atx3 a partir de células HEK 293FT e analisámos a amostra recorrendo a um método de espectrometria de massa especialmente direccionado para o mapeamento de locais de fosforilação. Esta análise determinou pela primeira vez que a serina 12 da atx3 é fosforilada. O facto de não ter sido usado nenhum estímulo artificial à excepção do ácido okadaico, um inibidor de fosfatases, leva-nos a admitir que esta modificação ocorre normalmente em células HEK 293FT. A análise da estrutura terciária do JD e consequente observação da proximidade entre este aminoácido e os aminoácidos do local activo da proteína leva-nos a especular que é provável que esta modificação influencie a actividade proteolítica da atx3. No que diz respeito à sumoilação, submetemos 6His-JD purificado a partir de *E. coli* a um ensaio de sumoilação *in vitro* e concluímos que ele se conjugava com SUMO2. Embora não tenha sido possível confirmar este resultado *in*

vivo, esta é a primeira prova de sumoilação da atx3 e indica que o domínio catalítico da proteína é alvo desta modificação.

A continuação do estudo das PTMs que identificámos e subsequente caracterização do modo como regulam as propriedades da atx3 poderão ajudar-nos a compreender melhor a função da atx3 e os mecanismos responsáveis pela MJD.

Chapter 1 Introduction

1.1 – Machado-Joseph disease

1.1.1 – Machado-Joseph disease presentation, neurological features and genetics

Machado-Joseph disease (MJD), otherwise known as spinocerebellar ataxia type 3 (SCA3), is an inherited neurodegenerative disorder originally described in people of Portuguese Azorean descent (Rosenberg, 1992; Sudarsky & Coutinho, 1995), but nowadays admitted as the most common form of autosomal dominantly-inherited ataxia in the world (Ranum et al., 1995; Riess et al., 2008; Schöls et al., 2004). So far, apart from Portugal, the disease has been identified in Spain, Italy, Germany, China, Taiwan, Japan, Australia, Brazil, United States and Canada (Rosenberg, 1992; Riess et al., 2008; Sudarsky & Coutinho, 1995).

MJD neurodegeneration profile involves neuronal loss in selective regions of the nervous system, including the cerebellum (spinocerebellar pathways and dentate nucleus), the substantia nigra, nuclei from the pons, spinal cord and cranial nerves (including locus coeruleus and vestibular nuclei), as well as visual, auditory, vestibular, somatosensory and ingestion-related systems; cerebral and cerebellar cortexes, inferior olive and Purkinje cells are moderately preserved (Dürr et al., 1996; Maciel et al., 1995; Rosenberg, 1992; Rüb et al., 2008; Sudarsky & Coutinho, 1995). Regarding brain functionality, metabolism (assessed by glucose utilization) was shown to decrease in the cerebellum, brainstem, cerebral cortex, thalamus and putamen (Riess et al. 2008; Soong et al., 1997; Wüllner et al., 2005), and both dopaminergic and cholinergic neurotransmissions were reported to be negatively affected (Rüb et al., 2008; Wüllner et al., 2005; Yen et al., 2000). MJD pathogenesis results in a set of characteristic clinical symptoms, including the hallmark progressive ataxia, other general neuromuscular complications like dystonia, dysarthria, spasticity, rigidity, fasciculations, postural instability and proprioceptive loss, visual (nystagmus, eyelid

retraction, ophthalmoparesis, double vision) and speech (dysarthria) disorders, dysphagia, amyotrophy, corticospinal and autonomic nervous system dysfunctions and neuropathy (Riess et al., 2008; Rosenberg, 1992; Soong et al., 1997; Sudarsky & Coutinho, 1995).

The gene causatively associated with MJD is termed MJD1 (SCA3) and is located on the long arm of chromosome 14 (14q32.1) (Kawaguchi et al., 1994; Takiyama et al., 1993). The disease develops in people carrying an unstable expansion of CAG repeats, which encode for glutamines, in the coding region of that gene's sequence. This results in an expanded tract of repeated glutamines in the protein encoded by MJD1, named ataxin-3 (atx3) (Kawaguchi et al., 1994). In the healthy population, the number of CAG repeats lies between 10 and 51, with 55 to 87 CAG repeats being reported to associate with the disease (Cummings & Zoghbi, 2000; Maciel et al., 2001). Nonetheless, CAG repeat numbers between 45 and 51 seem to belong to an overlapping region of healthy and disease phenotypes, since, while the longest repeat number detected in healthy people was 51, some smaller repeats have also been identified as associated with the development of MJD, the smallest of then being 45 CAG repeats (Maciel et al., 2001; Riess et al., 2008).

1.1.2 – Polyglutamine expansion diseases

In accordance to what has been said above, MJD belongs to a wider group of neurodegenerative disorders, the polyglutamine (polyQ) expansion diseases, defined by the fact that they all result from the same kind of polyQ expansion of their associated proteins. This group also includes Huntington's disease (HD), dentatorubral-pallidoluysian atrophy (DRPLA), spinal and bulbar muscular atrophy (SBMA) and spinocerebellar ataxia types (SCA) 1, 2, 6, 7 and 17 (Gatchel & Zoghbi, 2005; Zoghbi & Orr, 2000) (Table I). All these diseases are characterized by a selective neuronal loss accompanied by a collection of associated physical and psychological

complications, though the particular features vary among the different diseases (Gatchel & Zoghbi, 2005). In fact, although the different polyQ diseases have several common traits, the proteins responsible for each of them share no homology outside the polyQ tract, being unrelated and having very different biological functions (Gatchel & Zoghbi, 2005; Zoghbi & Orr, 2000).

Table I: Biologic features of polyQ expansion diseases (adapted from Gatchel & Zoghbi, 2005 and Shao & Diamond, 2007)

Disease name	Mutated gene	Protein product	Putative function	CAG repeat size		Regions most affected
				Normal	Pathogenic	
HD	*HD*	huntingtin	Signalling, transport, transcription	6-34	36-121	Striatum, cerebral cortex
DRLPA	*DRPLA*	atrophin 1	Transcription	7-34	49-88	Cerebellum, cerebral cortex, basal ganglia, Luys body
SBMA	*AR*	androgen receptor	Steroid-hormone receptor	9-36	38-62	Anterior horn and bulbar neurons, dorsal root ganglia
SCA1	*SCA1*	ataxin-1	Transcription	6-39	40-82	Cerebellar Purkinje cells, dentate nucleus, brainstem
SCA2	*SCA2*	ataxin-2	RNA metabolism	15-24	32-200	Cerebellar Purkinje cells, brainstem, frontotemporal lobes
SCA3	*MJD/SCA3*	ataxin-3	Deubiquitinating activity and transcription regulation	10-51	55-87	Cerebellar dentate neurons, basal ganglia, brainstem, spinal cord
SCA6	*CACNA1A*	$CACNA1_A$	P/Q-type α1A calcium channel subunit	4-20	20-29	Cerebellar Purkinje cells, dentate nucleus, inferior olive
SCA7	*SCA7*	ataxin-7	Transcription	4-35	37-306	Cerebellum, brainstem, macula, visual cortex
SCA17	*SCA17*	TBP	Transcription	25-42	47-63	Cerebellar Purkinje cells, inferior olive

Abbreviations: HD, Huntington's disease; DRPLA, dentatorubral-pallidoluysian atrophy; SBMA, spinal and bulbar muscular atrophy; SCA, spinocerebellar ataxia; MJD, Machado-Joseph disease; *CACNA1A*, calcium channel, voltage dependent, P/Q type, α1A subunit; TBP, TATA box binding protein.

The mechanisms by which the expanded proteins lead to the stated pathologies are still not completely understood. One important common aspect to all polyQ

expansion diseases is the existence of a negative correlation between the age of onset and the number of CAG repeats, meaning that a greater number of such repeats results in an earlier development of the corresponding disease (Dürr et al., 1996; Maciel et al., 1995; Ranum et al., 1995; Riess et al., 2008; Zoghbi & Orr, 2000). Additionally, different repeat numbers have been associated with different clinical presentations; in the particular case of MJD, four subphenotypes have been defined according to the age of onset and the particular symptomatic profiles (Dürr et al., 1996; Maciel et al., 1995; Riess et al., 2008; Rosenberg, 1992). The facts that the polyQ expansion is a common causative aspect to all these diseases and that its size correlates negatively with the age of onset are indicative of the importance the expanded polyQ sequence has on the mechanisms leading to the diseases. The involvement of the polyQ expanded tracts has been explained in several ways, including: a) polyQ stretch-induced modifications of the host protein function; b) generation of toxic polyQ-containing fragments after auto-cleavage of the host protein; c) polyQ tract-induced conformational changes in the protein, leading to an aggregation-prone state and consequent generation of toxic oligomers; d) transcriptional changes, caused by interactions of the expanded protein with specific transcription factors; e) proteotoxic stress as a result of the disruption of the quality-control systems of the cell; f) mitochondrial dysfunction (Shao and Diamond, 2007). One characteristic hallmark of all polyQ expansion diseases is the formation of large macromolecular protein aggregates or inclusion bodies containing the expanded and misfolded disease protein, in the nucleus and/or cytoplasm of neurons (and glial cells, in rare cases) (Zoghbi & Orr, 2000). These cell-generated structures, distinct from the small aggregates or oligomers formed by the self-association of the proteins, have been related to the pathogenesis of the polyQ expansion diseases for a long time, but recently many authors have been supporting the idea that they actually result from end-stage protective cellular mechanisms moved against the toxicity of the misfolded expanded protein (Arrasate et al., 2004; Ross & Poirier, 2004; Slow et al., 2005, Shao

& Diamond, 2007). Notably, polyQ toxicity has been associated not only with the expanded proteins that are translated from the CAG-expanded genes, but also with the expanded CAG repeat-containing RNA, which has been shown to cause toxicity by itself (Li et al., 2008).

Although there are some common aspects to the different polyQ diseases that can be attended to in order to study possible common pathogenesis mechanisms, there are particular aspects to them as well. The different neurodegeneration and symptomatic profiles of each polyQ expansion disease may be explained as a result of the fact that the polyQ expansion is inserted into a different host protein in each different disease. The diverse properties of each of those proteins, which include their subcellular localization, abundance, activity and biological role, along with the way the polyQ expansion affects them, shall constitute the factors responsible for each disease's specificity (Gatchel & Zoghbi, 2005). The cellular mechanisms proposed to link atx3 polyQ expansion with MJD pathogenesis will be discussed in later sections.

1.2 – Ataxin-3

1.2.1 – Distribution and ubiquity

Atx3 is a protein of wide distribution among the different eukaryotic groups of living organisms, having been identified in protozoans, plants, fungi and animals, from nematodes to arthropods and vertebrates (National Center for Biotechnology Information - NCBI - protein database). In mice and humans, despite the localized neuronal degeneration observed in MJD patients, atx3 displays an ubiquitous expression among different body tissues and cell types (do Carmo Costa et al., 2004; Ichikawa et al., 2001; Paulson et al., 1997a; Schmidt et al., 1998; Trottier et al., 1998). It was found to be widely expressed throughout the brain, though different regions present varying expression levels (Trottier et al., 1998).

1.2.2 – Structure and domains

Atx3 is a protein essentially composed of a structured globular N-terminal domain, followed by a flexible C-terminal tail (Masino et al., 2003). The N-terminal domain, termed Josephin domain (JD), shows ubiquitin (Ub) protease activity, while the flexible tail presents two ubiquitin-interacting motifs (UIMs), followed by the variable length polyQ region, whose expansion beyond a certain threshold is responsible for MJD (Albrecht et al., 2004; Burnett et al., 2003; Nicastro et al., 2005) (Figure 1).

Four different isoforms probably resulting from alternative splicing have been extensively described, the longest having an approximate molecular weight of 42 kDa (Goto et al., 1997; Ichikawa et al., 2001; Trottier et al., 1998). Notably, one of these isoforms has an extra UIM localized in the C-terminal, downstream the polyQ domain. Interestingly though, a recent study identified a total of 56 human alternative splicing variants, expected to be translated into at least 20 isoforms, with varying predicted domain arquitecture (Bettencourt et al., 2009).

Figure 1 – Schematic illustration of atx3 domain structure. The N-terminal catalytic JD is followed by two UIMs that precede the polyQ region, of variable length (Q_n). One isoform has a third UIM at the C-terminal, downstream the polyQ tract.

1.2.3 – Function and biological role

1.2.3.1 – Ubiquitin binding and deubiquitinating activity: involvement with the ubiquitin-proteosome pathway

Despite the existent information about the domains and activity of atx3, the protein's concrete biological function and physiological role remain to be elucidated. There are, however, several clues that suggest an involvement with the ubiquitin-proteasome pathway (UPP), the main mechanism for the turnover of short-lived or damaged proteins (Doss-Pepe et al., 2003).

Ubiquitination, i e, the covalent attachment of a Ub molecule or a polyUb chain to one or several lysine residues of a target protein, constitutes a mechanism regulating many different cellular activities, including protein degradation through the well-known UPP, DNA repair, chromatin remodeling, cell cycle progression, subcellular localization and signaling pathways (Reyes-Turcu et al., 2009; Reyes-Turcu & Wilkinson et al., 2009; Weisman et al., 2001). Importantly, polyUb chains can have diverse topologies resulting from different linkage modes existing between Ub monomers, and the different Ub arrangements produced by different types of Ub attachment determine the way the target protein properties are regulated (Reyes-Turcu & Wilkinson et al., 2009; Weisman et al., 2001; Winborn et al., 2008). Atx3 is able to bind polyUb chains through the UIMs located at the C-terminal region, interacting with both lysine 48 and lysine 63-linked chains in a UIM-dependent manner, *in vitro* (Winborn et al. 2008). There is, however, a preference for chains of no less than four Ub monomers and, coincidently, lysine 48-linked polyUb chains of four or more monomers are the ones involved in the targeting of proteins for proteasomal degradation (Burnett et al., 2003; Mao et al., 2005; Schmitt et al., 2007). Atx3 has also been shown to be able to bind polyubiquitinated proteins in neural cells in a UIM-dependent way (Berke et al. 2005).

Furthermore, as previously mentioned, the JD shows Ub protease activity, being capable of promoting the cleavage of isopeptide bonds existing between Ub monomers. This was first predicted through an integrative bioinformatic analysis of atx3 sequence (Scheel et al., 2003) and later confirmed by biochemical experimentation using model substrates and by seeing that Ub-aldehyde, a specific Ub protease inhibitor, decreases atx3 activity (Burnett et al., 2003). Such activity established atx3 and other identified JD-containing proteins as deubiquitinating enzymes (DUBs) (Burnett et al., 2003; Scheel et al., 2003).

In cells, DUBs are a group of enzymes responsible for removing Ub or polyUb chains from a target protein, processing Ub preproteins and remodeling or disassembling bound or unbound polyUb chains, this way participating in the regulatory mechanisms that utilize ubiquitination signals (Reyes-Turcu et al., 2009; Reyes-Turcu & Wilkinson, 2009). There are five families of DUBs encoded by the eukaryotic genomes (one of which is constituted by atx3 and the other JD-containing proteins), and, despite lacking a great sequence or structural similarity, the catalytic cores and active site residues of four of them closely resemble the classical cysteine protease papain (Ha & Kim, 2008; Reyes-Turcu, 2009; Reyes-Turcu & Wilkinson, 2009). According to its tertiary structure, the JD of atx3 is mainly composed of two subdomains - a globular catalytic subdomain and a helical hairpin - with the catalytic site being located in the cleft that exists between them (Mao et al., 2005; Nicastro et al., 2005). Structural comparison between the JD and papain-like cysteine proteases shows that the JD shares great structural similarity with many members of this enzyme group, even greater than with other DUBs (Figure 2). Nonetheless, the amino acids of the catalytic triad, cysteine 14, histidine 119 and asparagine 134, show homology with the ones present in the Ub C-terminal hydrolases (UCH) and Ub-specific processing proteases (USP) families of DUBs (Mao et al., 2005; Nicastro et al., 2005; Scheel et al., 2003). As is common for DUBs and papain-like cysteine proteases in general, the active site cysteine is located on a α-helix, while the histidine is located on a β-strand.

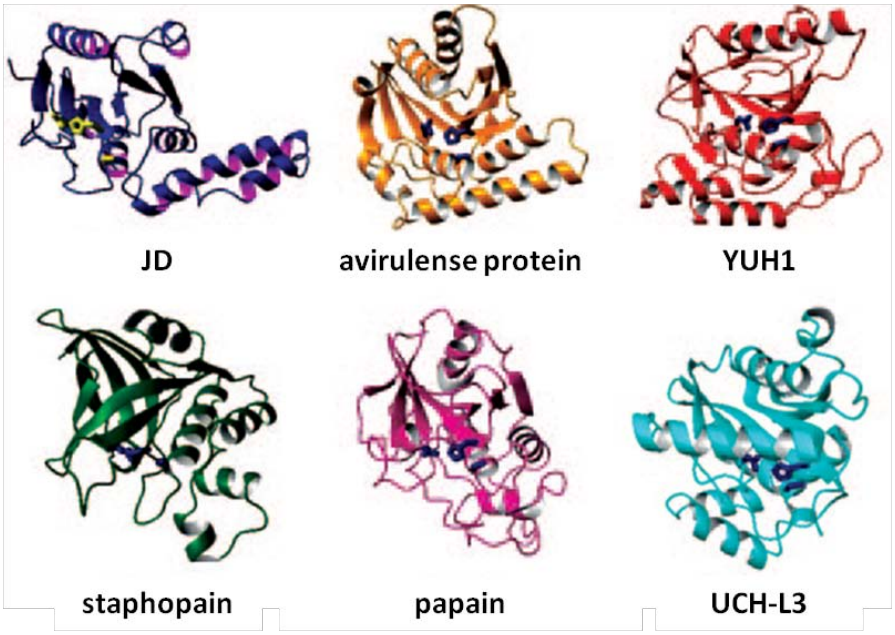

Figure 2 – Atx3 JD shares structural similarity with papain-like cystein proteases. Comparison between the atx3 JD structure (Protein Data Bank – PDB – ID code 1YZB) and those of the cystein proteases staphopain (PDB ID code 1CV8), avirulence protein (PDB ID code 1UKF), papain (PDB ID code 1PE6), yeast Ub C-terminal hidrolase 1 (YUH1; PDB ID code 1CMX) and Ub C-terminal esterase L3 (UCH-L3; PDB ID code 1UCH). YUH1 and UCH-L3 are both DUBs. The side chains of the catalytic triad are shown on each structure as sticks. (Adapted from Nicastro et al., 2005)

The precise physiological role of most DUBs remains undetermined, though, considering there is a great number of DUBs and a great variety in Ub and polyUb attachment arrangements, it is believed that different enzymes specifically recognize different kinds of ubiquitination signals. In the case of atx3, inhibition of the catalytic activity results in the increase of polyubiquitinated proteins (localized primarily in the nucleus), in a degree similar to what is observed when the proteasome is inhibited (Berke et al. 2005). This supports the idea of atx3 involvement with the polyubiquitinated proteins targeted for proteasomal degradation.

Results suggest that atx3 functions as a polyUb-editing protease, shortening polyUb chains, rather than favoring complete disassembly of polyUb chains, in order to

yield free Ub (Burnett et al., 2003; Burnett & Pittman, 2005; Winborn et al., 2008). UIMs may help to recruit the polyubiquitinated substrates and position the substrates relatively to the catalytic site in a way that allows for a sequential editing (Mao et al., 2005; Winborn et al., 2008). In fact, many DUBs have non-catalytical domains that contribute to recognition of different chain linkages (Reyes-Turcu & Wilkinson, 2009). The increase of polyubiquitinated proteins observed when atx3 catalytic activity is inhibited occurs only when the UIMs are intact, suggesting that they are important in the presentation of substrates to the JD (Berke et al. 2005). Importantly, Burnett and Pittman (2005) reported that atx3 is able to edit lysine 48-linked polyUb chains from a polyubiquitinated model protein (^{125}I-lysozyme) *in vitro*, at the same time blocking its proteasome-dependent degradation. Therefore, It has been proposed that atx3 partially deubiquitinates proteins and prevents their degradation by binding through the UIMs, while possibly maintaining their degradation signals. Nonetheless, Winborn et al. (2008) observed that atx3 preferentially cleaves lysine 63-linked chains and chains of mixed lysine 48 and lysine 63 linkage, suggesting that atx3 may function as a regulator of topologically complex polyUb chains. UIMs are important in determining this specificity, again supporting the idea that they may be important in positioning the polyUb chains for proteolytic cleavage (Winborn et al., 2008).

However, these considerations remain limited, taking into account that they are based only on *in vitro* observations. Importantly, to this date, atx3 steady state kinetic parameters have not been determined, though the proteolytic cleavage observed *in vitro* is very slow (Chow et al., 2004b; Winborn et al., 2008). This suggests that there must be some kind of external factor(s) required for optimal proteolysis (Chow et al., 2004b; Reyes-Turcu & Wilkinson, 2009). Moreover, as for many DUBs, the actual substrate(s) targeted by atx3 in the physiological context remains elusive, thus limiting our understanding of its function (Reyes-Turcu & Wilkinson, 2009). The low activity observed for atx3 may also be explained by the absence of the proper substrate, since the majority of DUBs has a suboptimal activity, determining that association with the

substrate(s) is required for a transition into an optimal catalytic-competent conformation (Reyes-Turcu et al., 2009).

The first *in vivo* clues to atx3 function as a DUB come from studies involving atx3 knockout (KO) mice (Schmitt et al., 2007). When compared to wild-type animals, atx3 KO mice showed no significant differences in terms of viability, fertility, health, emotionality and sensory or motor functions, nor have they revealed any morphological abnormalities. Noteworthy, however, is the observation that atx3 KO mice had increased levels of ubiquitinated proteins, a fact that substantiates atx3 role as a DUB *in vivo*. The absence of deleterious physiological consequences was suggested to be due to redundancy existing among DUBs (Schmitt et al., 2007).

Taken together, these results show that atx3 acts as a DUB likely associated with the UPP, though its precise biological role remains unclear. Nevertheless, the deubiquitinating activity may be important in a variety of other cellular processes, taking into account that ubiquitination, in all its alternative linkage forms, serves many different cellular functions, other than targeting proteins for proteasomal degradation (Todi, et al., 2007; Winborn et al., 2008). For instance, lysine 63-linked polyUb chains, shown to be bound and edited by atx3, serve functions in DNA repair mechanisms, translation, endocytosis and transport (Weissman, 2001; Winborn et al., 2008). The polyQ expansion of pathogenic atx3 may alter or block its function, and such incident may be relevant for the disease mechanisms of MJD.

1.2.3.2 – Ataxin-3 interactors and possible related functions

Several atx3 interacting partners have been identified so far, and the understanding of such interactions may provide clues into atx3 function.

Atx3 has been shown to interact with p97/valosin-containing protein (VCP) trough the C-terminal region (Boeddrich et al., 2006; Hirabayashi et al., 2001; Mao et al., 2005; Matsumoto et al., 2004; Zhong & Pittman, 2006) and with two human

homologs of the yeast DNA repair protein Rad23, HHR23A and HHR23B, through the JD (by the face opposite to the catalytic site) (Ferro et al., 2007; Nicastro et al., 2005; Wang et al., 2000). Both p97/VCP and HHR23A and B have been implicated in many different biological processes, including the UPP; they have both been linked to the shuttling of polyubiquitinated substrates to the proteasome for degradation and in particular to the endoplasmic reticulum-associated degradation (ERAD), i e, the system that mediates the ubiquitination of misfolded proteins or unassembled complex constituents present in the secretory pathway and their exportation to the cytosol for degradation by the proteasome (Albrecht et al., 2004; Burnett et al., 2003; Doss-Pepe et al., 2003; Wang et al., 2004; Wang et al., 2006). Atx3 interaction with these proteins further suggests its involvement in the UPP (Burnett et al., 2003; Mao et al., 2005), and, in effect, atx3 has been associated with the ERAD, though not with much consensus: there is dispute regarding whether atx3 promotes or decreases degradation by this pathway (Wang et al., 2006; Zhong & Pittman, 2006). Atx3 has even been found to associate with the proteasome itself through its N-terminal (Doss-Pepe et al., 2003), but a recent study showed that the interaction between them is not very strong or even direct (Todi et al., 2007). Functioning in articulation with these interactors, atx3 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 (Broeddrich et al., 2006; Wang et al., 2008).

Atx3 has also been shown to interact with neural precursor cell expressed developmentally downregulated gene 8 (NEDD8), a Ub-like protein, reportedly in a substrate-like manner (Ferro et al., 2007; Mori et al., 2005). NEDD8 can be conjugated with other proteins in a way reminiscent of ubiquitination and such attachment, referred to as neddylation, has been reported to regulate target protein's function. The

interaction between atx3 and NEDD8, along with a possibly observed deneddylase activity of atx3, may interfere with the neddylation of other substrates and thus influence the processes where attachment of NEDD8 is involved (Ferro et al., 2007).

Aggresomes are misfolded protein aggregates that are formed near the microtubule-organizing center (MTOC) when the proteasome isn't 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 cellular homeostasis (Markossian & Kurganov, 2004). Endogenous atx3 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 & Pittman, 2005). Atx3 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 & Pittman, 2005), and was recently shown to interact with tubulin and microtubule-associated protein 2 (MAP2), two other constituents of the cytoskeleton (Mazzuchelli et al., 2009). It has been proposed that atx3 may protect misfolded proteins before they reach the MTOC, or stabilize proteins involved in the transport (Burnett & Pittman, 2005).

1.2.3.3 – Involvement in transcription regulation

A different aspect of atx3 function concerns its role as a possible transcription regulator. In fact, atx3 has been shown to be able to regulate expression of a great number genes (Evert et al., 2003; Rodrigues et al., 2007). A microarray analysis using two different *Caenorhabditis elegans* strains KO for the worm ortholog of atx3 identified 290 genes (1.4% of the worm total genome) that are differentially expressed comparing to wild-type animals. 143 of these had unknown function, but the other 147 genes were grouped according to their proposed biological roles: cell structure and mobility components (50%), signal transduction (20%), the UPP (8%), and other cell processes

(22%), which include genes encoding enzymes, transporters, receptors and channels involved in diverse pathways. Of the 290 differentially expressed genes, 253 were up-regulated and 37 were down-regulated (Ferro et al., 2007; Rodrigues et al., 2007). Even though there was a clear transcriptional deregulation in the KO strains, in resemblance with the KO mice mentioned above, the animals were viable and had no overt phenotype, probably due to functional redundancy and adaptative modifications.

Atx3 function as a transcription regulator is also supported by some studies concerning its molecular interactions (Burnett et al., 2003; Li et al., 2002). Atx3 interacts with three transcription activators - cAMP response element-binding protein (CREB)-binding protein (CBP), p300 and p300/CBP-associated factor (PCAF), the major histone acetyltransferases - through its C-terminal region (Li et al., 2002), with two transcription repressors - histone deacetylase 3 (HDAC3) and nuclear receptor co-repressor (NCor) - in an interaction involving its UIMs (Evert et al., 2006a), and with histones, mainly through the N-terminal (Li et al., 2002). The same studies report that atx3 represses the transcription mechanisms mediated by these proteins through different processes. Histone acetylation is a means of controlling gene transcription: increased acetylation renders chromatin conformation sparser, enhancing its transcription, while decreased acetylation turns it denser, obstructing the access of important regulatory factors. CBP, p300 and PCAF are transcriptional co-activators that form complexes with other co-activators and proteins with acetyltransferase activity and acetylate histones; atx3 seems to repress transcription mediated by these co-activators through binding of these proteins through its C-terminal. Furthermore, binding of histones trough the N-terminal seems to repress acetylation by blocking the access to acetylation sites present in the histone proteins (Li et al., 2002; Nicastro et al., 2005). Atx3 interaction with HDAC3 and NCor was also reported to somehow lead to a decrease in histone acetylation and consequent transcription repression (Evert et al., 2006a).

Interestingly, there are some studies that expose an association between transcription regulation and the UPP, though there is no clear evidence for such connection in the case of atx3 (Burnett et al., 2003). It has been hypothesized, however, that atx3 deubiquitinating activity may help stabilize transcription regulators (Evert et al., 2006a; Rodrigues et al., 2007).

In a tentative approach to summarize what has been exposed about the possible biological roles of atx3, it can be said that current evidences connect its function to a) the UPP, with some studies pointing out an important participation in the ERAD and aggresome formation, and to b) transcriptional regulation.

1.2.4 – Intracellular localization and transport

In addition to the ubiquitous distribution of atx3 among tissues, the protein seems to be widely, though heterogeneously, distributed within the cells themselves, being found in the cytoplasm (mitochondria included) and the nucleus, with varying degrees of predominance depending on the cells in question (Macedo-Ribeiro et al., 2009; Paulson, et al., 1997b; Pozzi, et al. 2008; Tait et al., 1998; Trottier et al., 1998; Wang et al., 1997). In human brain cells observations, atx3 localized mainly in the perikarya, though, depending on the analyzed cells, it was also detected on proximal processes, axons and nuclei. This heterogeneity suggests that regulation of atx3 expression level and localization may be functionally important (Trottier et al., 1998).

Recent studies demonstrated that atx3 is actively transported across the nuclear envelop, being shuttled from the cytoplasm to the nucleus and vice-versa (Antony et al., 2009; Macedo-Ribeiro et al., 2009). Regarding nuclear importation, a functional nuclear localization signal (NLS) promoting atx3 transport to the cell nucleus has been detected immediately upstream the polyQ sequence (R282-R285) (Tait et al., 1998; Boeddrich et al., 2006; Macedo-Ribeiro et al., 2009). Nuclear exportation appears to integrate both chromosome region maintenance 1 (CRM1)-dependent and

independent mechanisms (Macedo-Ribeiro et al., 2009) and, as of yet, two apparently functional nuclear export signals (NES) have been reported (I77-Y99 and E141-E158), though without consensus (Antony et al., 2009; Macedo-Ribeiro et al., 2009).

1.3 – Expanded ataxin-3 and Machado-Joseph disease pathogenesis

To this date, the way through which polyQ expanded atx3 leads to MJD pathogenesis has not been clarified, though, as for every other polyQ disease, the unstable expansion is admittedly of causative importance. There are several observations supporting this idea: a) the polyQ disease proteins' sequence is dissimilar outside the polyQ tract; b) diseases' severity increases with glutamine repeat number; c) age of onset decreases with the increase of repeat number; d) several transgenic models expressing polyQ sequences outside the natural gene context present neurodegenerative phenotypes (Ikeda et al., 1996; Mangiarini et al., 1996; Ordway et al., 1999; Paulson et al., 1997b). As stated above, however, though the polyQ sequence expansion appears to be the triggering factor leading the development of MJD, atx3 regions outside the polyQ domain and the protein properties they determine are sure to define the development of the disease (Gatchel & Zoghbi, 2005; Shao and Diamond, 2007).

The different hypothesis and evidences regarding MJD pathogenesis are discussed below.

1.3.1 – Protein aggregates and nuclear inclusions

Studies suggest that expanded atx3 and every other polyQ expanded protein tend to form toxic aggregates, as a result of polyQ expansion-induced misfolding and consequent transition to aggregation-prone conformations (Bevivino & Loll, 2001; Chen et al., 2002b; Chow et al., 2004c; Jana & Nukina, 2004; Tanaka, et al., 2001).

Investigation has focused on a search to comprehend the mechanisms underlying atx3 aggregation, but, to this date, the understanding of such subject is still limited, with many mechanisms being proposed for the fibrillogenesis process (Chen et al., 2002b; Ellisdon et al., 2007; Gales et al., 2005). Curiously tough, even non-expanded atx-3 and the JD itself display tendency to aggregate (Masino et al., 2004; Gales et al., 2005). Aggregation may result from a monomeric thermodynamic nucleus formed by a single misfolded protein to which monomers are added, consequently leading to elongation and fibril formation; in the case of the expanded protein there is a second aggregation step that leads to the formation of more stable aggregates (Ellisdon et al., 2007) (Figure 3). In healthy people, the slow aggregation kinetics of the non-expanded atx3 and the high instability of the aggregates formed shall ensure an effective break down by the cell's intrinsic quality-control mechanisms. Conversely, in MJD patients, the enhanced kinetics of the expanded atx3 aggregation as well as the great stability of the aggregates formed shall lead to their aberrant accumulation inside the cells. This accumulation may increase with aging, as a result of the progressive failure of quality-control mechanisms (Ellisdon et al., 2006; Ellisdon et al., 2007; Gales et al., 2005; Masino et al., 2004).

As mentioned before, one common feature to all polyQ expansion diseases is the formation of large macromolecular aggregates containing the pathogenic protein. Even though wild-type atx3 displays a wide distribution within the cell, in MJD patients' brains, atx3 amasses in the form of nuclear inclusions (NIs) present only in neurons (Paulson et al., 1997b). Apart from the polyQ expanded atx3, NIs contain several other proteins, namely members of the cell quality-control systems, like proteasome constituents and molecular chaperones (Chai et al., 1999a; Chai et al., 1999b; Chow et al., 2004a; Muchowski et al., 2000; Schmidt et al., 2002; Zander et al., 2001). Intracellular inclusions have been often linked to the pathogenesis of polyQ diseases as a major means of polyQ toxicity because of their ability to sequester different molecules, provoking citotoxicity through several possible mechanisms: a) hindrance of

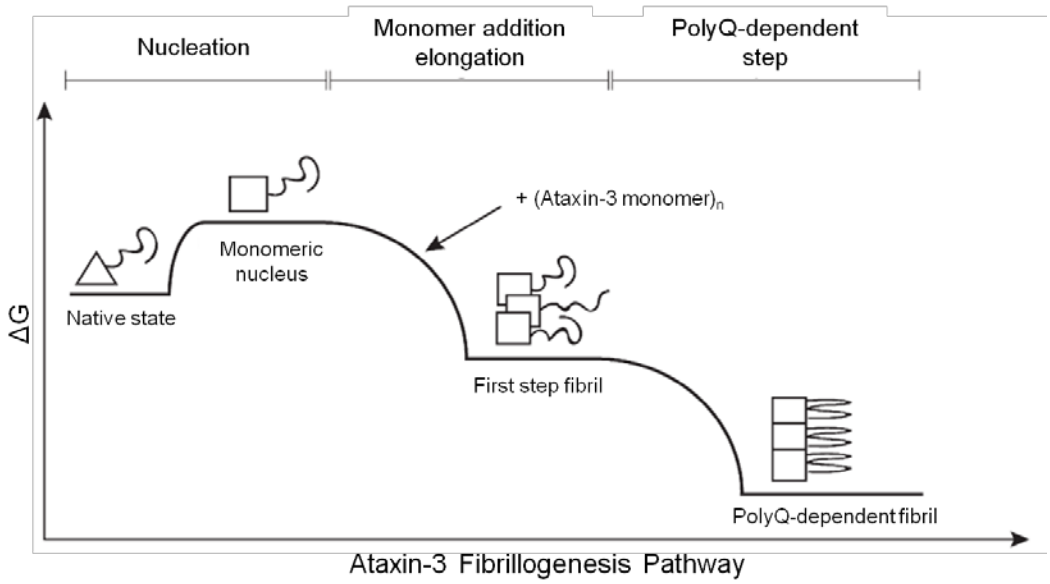

Figure 3 – Schematic representation of a possible atx3 fibrillogenesis pathway. When atx3 is in its native state, the JD and the UIMs are represented as a triangle and the polyQ tract as a predominantly random coil tail. Fibril formation is initiated by a monomeric thermodynamic nucleus formed as a result of structural changes occurring on the non-polyQ regions of the protein (represented by the square-shaped JD). This first-step of atx3 elongation occurs down an energy gradient by the addition of atx3 monomers to the growing fibril. PolyQ expanded atx3 undertakes a second aggregation stage, marked by the participation of the polyQ tract in the aggregation process and a large increase in size and stability of the amyloid-like fibrils. (Adapted from Ellisdon et al., 2007)

transcription, through sequestration of molecules involved in transcription regulation (Chai et al., 2002; McCampbell et al., 2000); b) a general disturbance of the quality-control systems of the cells, due to sequestration of proteasome constituents and molecular chaperons (Chai et al., 1999a; Chai et al., 1999b; Ferringo & Silver, 2000; Muchowski et al., 2000; Paulson, et al., 1997b; Schmidt et al., 2002; Warrick et al., 1999); c) hindering of axonal transport, resulting from motor protein titration and physical blocking (Gunawardena et al., 2003); d) other disturbs caused by recruitment

of Ub-binding proteins (since inclusions are heavily ubiquitinated) or other polyQ-containing proteins (Donaldson et al., 2003; Perez et al., 1998).

Nonetheless, the importance of these large macromolecular aggregates to polyQ disease pathogenesis is recently being disputed (Shao & Diamond, 2007). Many authors suggest that they are in fact the result of protective cellular mechanisms moved as a measure of the cell to cope with the toxicity of the expanded proteins, provoked by other agents. Studies concerning MJD demonstrate that there is no perfect overlap between the brain regions that degenerate and the regions presenting NIs (Evert et al., 2006b; Paulson et al., 1997b; Ross & Poirier, 2004; Trottier et al., 1998; Yamada et al., 2001). Furthermore, research into HD revealed that inclusion formation even associates with the survival of the corresponding cells (Slow et al., 2005; Arrasate et al., 2004).

Nowadays, in the matter of atx3 aggregation (and polyQ protein aggregation in general), the small aggregates or oligomers are the ones envisioned as the actual toxic species causing cytotoxicity. Importantly, to this date several atx3 oligomers and multimers have already been isolated (Gales et al., 2005). Atx3 aggregates are β-rich fibrillar structures of amyloid nature, reminiscent of the amyloid aggregates observed in Alzheimer's and prion diseases (Bevivino and Loll, 2001; Chen et al., 2002a). It is suggested that the soluble amyloid oligomers have an inherent toxicity caused by mechanisms common to all of them (Kayed et al., 2003), for example being able to destabilize the lipid bilayer, increasing its conductivity (Demuro et al., 2005; Kayed et al., 2004). Sequestering of quality-control system components and transcription factors have also been pointed out as possible causes of toxicity, as they lead to proteotoxic stress and transcriptional deregulation (Schaffar et al., 2004; Shao & Diamond, 2007) (Figure 4). The cell-mediated formation of NIs, though directed as an adaptive response, may instead take part in some kind of snowball effect, in which even more proteins are sequestered and more cellular systems become dysfunctional.

1.3.2 – Proteolytic cleavage and toxic fragment hypothesis

Several studies suggest that the proteolytic cleavage of polyQ disease proteins can contribute to their pathogenesis, the resulting fragments being of crucial importance for their aggregation and cytotoxicity; this idea is referred to as the toxic fragment hypothesis (Tarlac and Storey, 2003; Wellington et al., 1998) (Figure 4).

In effect, atx3 cleavage appears to increases its aggregation, with caspases and calcium-dependent calpain proteases being pointed as the enzymes possibly responsible for that process (Berke et al., 2004; Haacke et al., 2006; Haacke et al., 2007). Removal of the N-terminus of expanded atx3 was shown to be important for protein aggregation (Haacke et al., 2006). Cleavage appears to result in polyQ-containing C-terminal fragments that are toxic by themselves, and a peptide of this kind has been identified as more abundant in affected brain regions of MJD patients (Colomer Gould et al., 2007; Goti et al., 2004; Yoshizawa et al., 2000).

Contrastingly, recent studies demonstrated that, although all atx3 forms suffer some common proteolytic processes, expanded atx3 is cleaved to a lesser extent when compared with the normal atx3 (Pozzi et al., 2008).

1.3.3 – Ataxin-3 polyglutamine expansion-induced property changes

It is also possible that the polyQ expansion of atx3 interferes with the protein, producing changes in its function, interactions or localization that lead to cellular toxicity and play important roles in the development of MJD.

A first hypothesis could be that atx3 expansion leads to a loss of protein function and that this would be sufficiently significant to cause the disease. However, the mouse and *C. elegans* KOs seem to indicate that atx3 function is not crucial for the cells, as the animals present no overt phenotype (Rodrigues et al., 2007; Schmitt et al.,

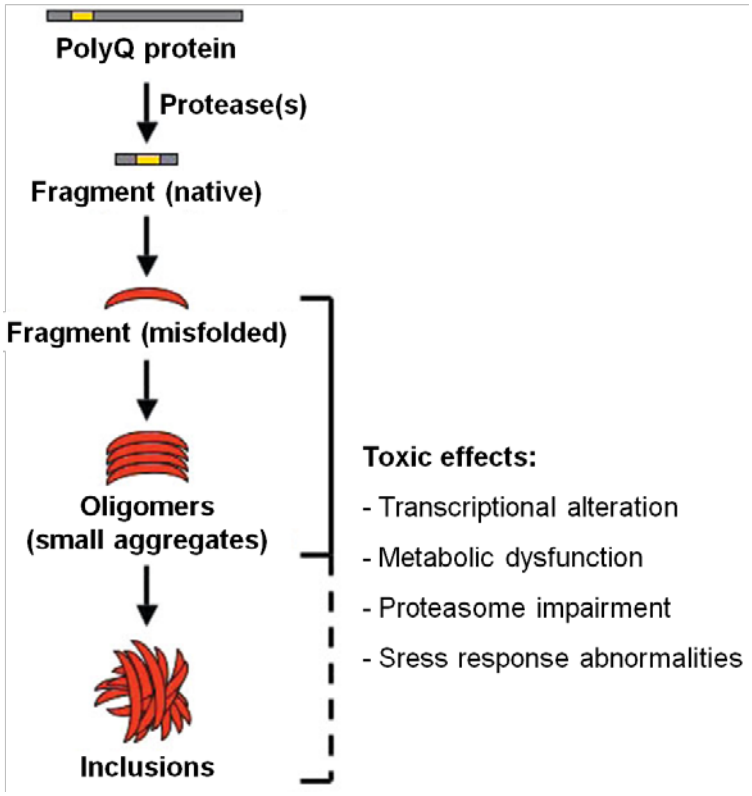

Figure 4 – Pathogenesis of polyQ expansion diseases. Many polyQ diseases appear to start from a proteolytic cleavage event that generates toxic fragments. The expanded polyQ sequence then allows transition into a misfolded conformation and the peptide may exert toxicity as a monomer or it may self-assemble and constitute toxic oligomers. Continued aggregation leads to deposition into larger macromolecular inclusions. Misfolded protein and aggregates may disrupt several cellular systems. (Adapted from Shao et al., 2007)

2007). Other hypothesis is that the expanded polyQ changes the protein's function in a way that it alters its cellular activity, actively causing cell death.

Atx3 has been linked with the UPP and, in line with what has been said above, disturbance of this pathway is a possible cause of toxicity, as it might interfere with the normal turnover and degradation of proteins. Studies have demonstrated that there are not much significant differences in the deubiquitinating activity between normal and expanded atx3 (Berke et al., 2004; Burnett et al., 2003). Expanded atx3 retains its

ability to bind polybiquitinated substrates in a UIM-dependent manner, with no global differences comparing to the normal protein (Berke et a., 2004). Nonetheless, the possibility that the two forms may bind differentially to particular substrates is not to be ruled out.

Atx3 participation in the UPP adds an interesting aspect to MJD: atx3 shall be presented to misfolded and aggregated proteins as part of its normal function, but possible stable interactions between them and the expanded protein may potentiate protein aggregation and constitute a cause for dysturbance of proteasomal function (Burnett et al., 2003; Burnett & Pittman, 2005). The presence of Ub and proteasome constituents in the protein NIs can partially be explained by the fact that atx3 physiologically interacts with them (Doss-Pepe et al., 2003).

Another aspect where an expanded polyQ tract may influence atx3 properties, possibly influencing its physiological role, is the interactions atx3 establishes. HHR23A and HHR23B binding is not affected by the polyQ expansion, but HHR23A co-localizes with expanded atx3-containing aggregates in cell lines (Wang et al., 2000). PolyQ tract expansion increases the extent of the interaction with p97/VCP, which also co-localizes with the NIs in MJD patients (Boeddrich et al., 2006). These interaction changes are likely to cause an altered distribution pattern of these interactors and influence their activity and other molecular interactions (Boeddrich et al., 2006; Higashiyama et al., 2002; Hirabayashi et al., 2001; Wang et al., 2000). Impairement of the ERAD caused by expansion of atx3 may induce endoplasmic reticulum proteotoxic stress that may contribute to the degenerative mechanisms (Wang et al., 2006; Zhong & Pittman, 2006).

PolyQ expansion may also influence atx3 proposed function as a transcription regulator. Expansion may alter atx3 binding properties and compromise its ability to establish transcription repressor complexes, leading to the increased expression of certain genes, some of which may contribute to the disease. The expanded form of atx3 appears to bind chromatin in different places and to aberrantly increase

transcription in cells (Evert et al., 2003; Evert et al., 2006a). Histone acetylation is increased in cells expressing expanded atx3 and human MJD pons, although both normal and expanded forms associate with HDAC3 and NCoR (Evert et al., 2006a). Expanded atx3 is also reported to bind transcriptional co-activators (CBP, p300 and PCAF) to a greater extent than the non-expanded form, though its inhibitory effect on transcription was similar (Li et al., 2002). Importantly, MJD patients' brains have been reported to show transcriptional deregulation of several genes (Evert et al., 2001; Evert et al., 2006b; Wen et al., 2003). A direct example of the negative effect of transcription comes from studies relating apoptosis with the cell loss observed in MJD, as expanded atx3 was reported to upregulate the Bax protein (a pro-apoptotic protein), while downregulating Bcl-xL levels (an anti-apoptotic protein), in cultured neurons (Chou et al., 2006).

1.3.4 – Ataxin-3: a protector against polyglutamine toxicity?

In resemblance with other polyQ diseases, it has been determined that, apart from expanded atx3, protein aggregates seen in MJD also present normal atx3 (Fujigasaki et al., 2000); this is possible because MJD and other polyQ diseases are autosomal dominant diseases, and so both normal and expanded forms can be present in the same cell. This situation can reflect the importance of regions outside the polyQ tract in the aggregation mechanism or result from conformational changes that the misfolded expanded atx3 induces in the non-expanded protein, leading to its recruitment to the forming aggregates (Chow et al., 2004a).

Another possibility, however, would be that atx3 is recruited to this aggregates because of the UIMs (inclusions are highly ubiquitinated) and/or because of its involvement in the UPP, being required by cellular mechanisms mobilized to deal with the aggregates (Burnett et al., 2003; Burnett & Pittman, 2005; Donaldson et al., 2003; Fujigasaki et al., 2001; Warrick et al., 2005). Consistently with this, a striking feature of

atx3 that should be considered when studying its biological role and MJD pathogenesis is that this protein co-localizes with aggregates formed in other polyQ expansion diseases (Uchihara et al., 2001) and in other neurodegenerative states (Burnett et al., 2003; Fujigasaki et al., 2000; Pountney et al., 2003; Seilhean et al., 2004; Takahashi et al., 2001).

Importantly, an interesting study by Warrick and co-workers (Warrick et al., 2005) strongly suggested that atx3 may have a protective role against polyQ toxicity. They reported that flies resulting from crosses between individuals expressing expanded atx3 or other expanded polyQ-disease proteins and individuals expressing non-expanded atx3 did not present the symptoms their diseased relatives had. This suggested that atx3 was able to mitigate toxicity in several *Drosophila* polyQ diseases models, including a MJD model. The protective activity may be linked to atx3 involvement in quality-control mechanisms such as the UPP, as it requires normal proteasome functionality and Ub-associated activities, including Ub protease activity and UIM functionality. These results may further explain why MJD patients with two pathogenic alleles have more severe phenotypes than heterozygotic patients (Lang et al., 1994); the phenotype of homozygotic patients may result not only from the two pathogenic alleles but also from a loss of that protective function (Warrick et al., 2005).

1.3.5 – The role of cellular and protein contexts

Although both normal and expanded forms of atx3 display an ubiquitous and similar expression throughout the body (particularly in the brain), like it has been said before, cell loss occurs only in selective parts of the nervous system (Paulson et al., 1997a; Trottier et al., 1998; Wang et al., 1997). The same thing has been reported for other polyQ expansion diseases, and such observations point out to a very important feature of these diseases' group: the role of cellular context. Pathogenic protein expression by itself is not sufficient to cause degeneration and so the whole

environment of the cell, and the way it interacts with the expanded protein, may be relevant for the pathogenic mechanisms. These facts relate to yet another, recurring, observation: although polyQ sequences are toxic by themselves, different diseases, with different associated proteins, have different disease phenotypes, explained by the importance of the other regions of the host protein, i e, the host protein context (Gatchel & Zoghbi, 2009; La Spada & Taylor, 2003). Taking this all together, the way a particular protein acts in the particular environment of each cell determines the specificities of the development of a particular polyQ disease and its distinctiveness from other diseases of this type. As such, properties like the protein's biological functions, interactions and localization, and the influence the expanded polyQ stretch has upon them, may conjugate with particular cellular contexts and lead to different outcomes, being crucial to pathogenesis.

In the case of MJD, many reports related with this subject focus the role of subcellular localization of atx3 in the development of the disease. Although nuclear localization of atx3 is not sufficient to cause the disease (Trottier et al., 1998), early studies reported that a non-expanded atx3 fragment targeted to the nucleus leads to aggregate formation, suggesting that the nuclear environment may be important in this event (Perez et al., 1998; Perez et al., 1999). Recent studies using transgenic mice demonstrated that targeting expanded atx3 to a nuclear localization aggravates the disease phenotype, while nuclear export reduces it (Bichelmeier et al., 2007). This implies that nuclear localization of atx3 is necessary for the manifestation of MJD symptoms *in vivo*.

1.4 – Post-translational modifications of ataxin-3

An emerging way by which the host protein context may be important in the development of polyQ expansion diseases relates to its potential susceptibility to suffer post-translational modifications (PTMs) (La Spada & Taylor, 2003; Pennuto et al.,

2009). PTMs consist of several mechanisms of enzymatic processing of proteins that occur during or after protein synthesis. They comprise a huge variety of processes, including proteolytic cleavage, addition or removal of amino acids, attachment of oligosaccharides or prosthetic groups, and addition of chemical groups or other proteins to certain amino acid residues. PTMs contribute to the formation of the mature protein and determine some of its properties, including subcellular localization, activity, interactions and stability. Some of them are reversible and play an important role in the cellular dynamics of the protein they modify, reversibly modulating some of its properties.

Logically, since PTMs constitute a group of key regulatory mechanisms modulating the activity of so many proteins, one can expect atx3 itself to be the target of some PTMs that alter its properties. The regulation of atx3 activity by PTMs is a very important aspect to consider when studying its possible functions, attending to the fact that DUB activity is highly regulated, since their protease activity must be correctly directed in order to ensure correct cleavage of the proper substrates (Reyes-Turcu, et al., 2009). Along with transcription, molecular interactions, degradation and proteolytic cleavage, PTMs are admitted as one possible mechanism regulating DUB activity, and to this date, many enzymes belonging to this group have been shown to be modulated by this kind of modifications (Nijman et al., 2005; Reyes-Turcu et al., 2009). PTMs can be predicted as mechanisms possibly responsible for enhancing atx3 activity in the proper cellular context; their absence may explain the low activity of atx3 reported in the experiments (Macedo-Ribeiro S., unpublished data). Furthermore, interaction of the DUBs with other proteins (scaffolds or adapters) has also been pointed out as an important mechanisms for DUB activation and the correct placement of substrate molecules relatively to the catalytic site, thus making up for the possible low affinity with the substrates (Reyes-Turcu, et al., 2009). PTMs may be important in regulating these molecular interactions, thus ultimately interfering with atx3 activity. PTMs may also regulate atx-3 subcellular localization, therefore explaining the diversity in intracellular

distribution observed both in cell lines and human brain cells (Macedo-Ribeiro et al., 2009; Paulson, et al., 1997b; Pozzi, et al. 2008; Tait et al., 1998; Trottier et al., 1998; Wang et al., 1997). This may constitute yet another indirect mechanism by which PTMs regulate atx3 function, considering that subcellular localization is another factor influencing DUBs activity (Reyes-Turcu et al., 2009).

Given the central role of PTMs in the cellular dynamics, it can be expected that these modifications modulate the mechanisms by which expanded polyQ proteins lead to the related diseases, in part participating in the processes by which different proteins determine the specificities of each polyQ disease (Pennuto et al., 2009). In fact, thus far there have been several PTMs associated with polyQ disease proteins, including proteolytic cleavage (referred above), phosporylation, acetylation, ubiquitination, sumoylation, palmitoylation and transglutamination (Figure 5). Concordantly, PTMs may provide clues to how expanded atx3 leads to MJD, through their influence in atx3 properties (Gales et al., 2005; La Spada & Taylor, 2003). Possible changes may again be related to atx3 enzymatic activity, interactions and localization, but also to its stability and propensity to form aggregates, among other things. As explained above, different levels of some PTMs between different cells may partly explain the regional-specificity of MJD. Finally, it can also be expected that some modifications important for the development of the disease may constitute possible targets for rational therapeutic interventions (La Spada & Taylor, 2003).

The following subsection will briefly analyze what is currently known about atx3 ubiquitination; since this PTM has been reported to be involved in atx3 degradation and activity, its study contributes to a better understanding of atx3 cellular functions and the possible effects PTMs have on them. The last two subsections will discuss what is currently known about the two modifications focused in the current work - phosphorylation and sumoylation - as regulators of several polyQ disease proteins. We will analyze what has been proposed as their physiological role in the different protein contexts and what is their potential influence in the polyQ expansion diseases

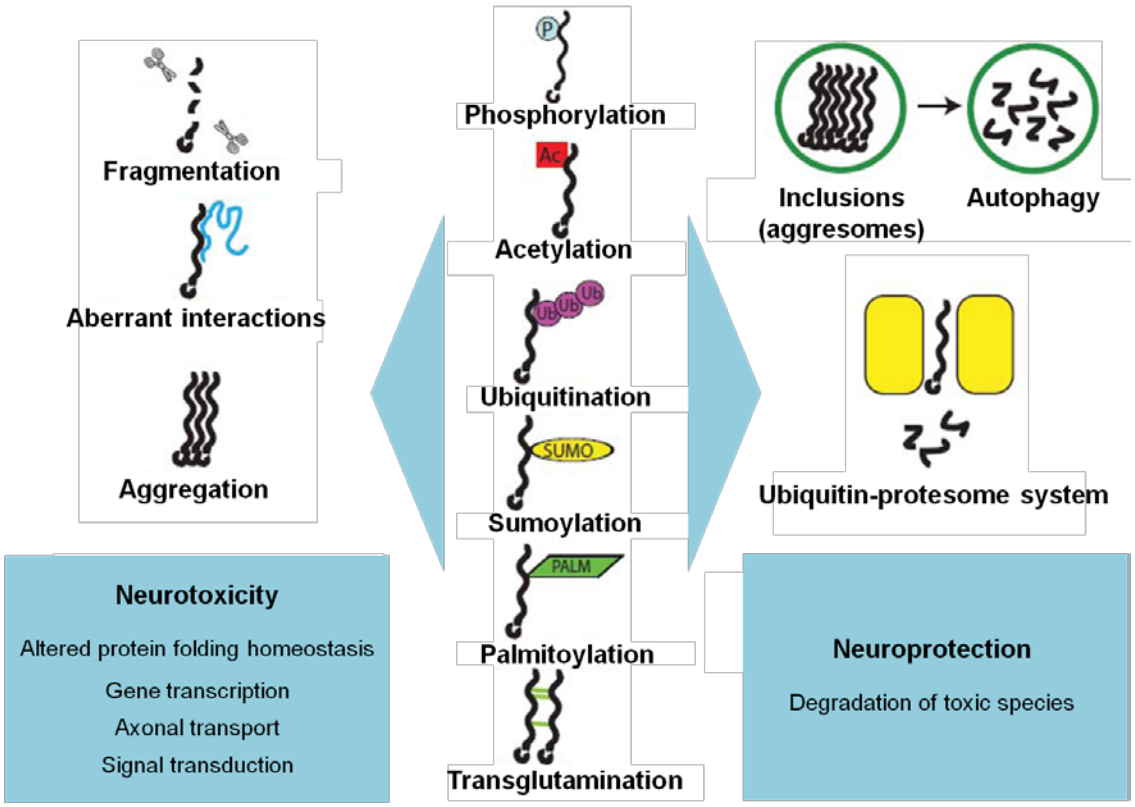

Figure 5 – PTMs of polyQ-containing proteins and their influence in pathogenesis. PolyQ proteins may be targeted by several PTMs, including phosphorylation, acetylation, ubiquitination, sumoylation, palmitoylation and transglutamination. The way these modifications affect protein properties and cellular dynamics can produce either neurotoxic or neuroprotective effects. (Pennuto et al., 2009)

pathogenesis mechanisms. Data regarding phosporylation and sumoylation of atx3 will be handled with particular detail, but as these studies are still very limited, reports about those other polyQ diseases' proteins shall be envisioned as sources of possible clues to the relevance of PTMs for atx3 function and MJD development.

1.4.1 – Ubiquitination and clues to regulation of ataxin-3 turnover and activity

Up until now, many authors have focused on the study of atx3 ubiquitination and its relation with the protein stability and degradation. As described above,

ubiquitination is a common PTM consisting in the covalent attachment of Ub molecules or polyUb chains to one or several lysine residues of another protein. A primary role of ubiquitination is the targeting of defective proteins or those with short half-lives for degradation in the proteasome (Schnell & Hicke, 2003; Weissman, 2001). Atx3 has been shown to be itself polyubiquitinated and to be (at least partially) degraded by the proteasome (Berke et al., 2005; Jana et al., 2005; Matsumoto et al., 2004; Todi et al., 2007; Tsai et al., 2003). One study demonstrated that the expanded protein is more stable than its non-expanded counterpart and that it is degraded by the proteasome less efficiently; this must contribute to the accumulation of the pathogenic protein and persistence of its toxic effects (Matsumoto et al., 2004). Interestingly, atx3 catalytic activity appears to somehow regulate its turnover, since catalytically inactive atx3 was reported to have higher steady state levels in cells, partially resulting from a reduction on degradation (Todi et al., 2007). Strikingly, however, in both cases and in several other studies (Berke et al., 2005; Miller et al., 2004), increased ubiqutination did not correlate with increased degradation. This may indicate that proteasomal degradation independently of ubiquitination may be important in these situations (Todi et al., 2007) or, in the case of the expanded protein, the differences observed may be due to a inefficient proteosomal recognition and degradation due to the expanded polyQ tract (Matsumoto et al., 2004).

As described, ubiquitination is not limited to targeting proteins for proteasomal degradadion. UIM-containing proteins are often subject of ubiquitination processes different from the classical lysine 48-linked polyubiquitination, suggesting that UIMs may mediate them (Miller et al., 2004). Berke and co-workers (Berke et al., 2005) demonstrated that atx3 is ubiquitinated under normal conditions, and concluded that the observed ubiquitination corresponded to mono or oligoubiquitination (likely short chains or several single Ub molecules attached to different lysine residues); this process was facilitated by functional UIMs. Importantly, there is some evidence that UIM-dependent ubiquitination inhibits the interaction between UIM-containing proteins

and other ubiquitinated proteins (Miller et al., 2004). The aforementioned study (Berke et al., 2005) also reported the presence of some lower molecular weight ubiquitinated species that were not abolished by UIM blockade and were suggested to correspond to monoubiquitination. Recent results by our group demonstrated that atx3 JD is monoubiquitinated *in vitro*, and such modification may account for those UIM-independent monoubiquitinated forms (Moreira, 2007). Endogenous atx3 was recently described to be monoubiquitinated in neural cells (Todi et al., 2009).

Though the possible function of these alternative forms of ubiquitination is not well understood, this same recent report demonstrated that atx3 ubiquitination increases its DUB activity, independently of potential cofactors or interactors (Todi et al., 2009). Interestingly, though the UIMs are important in determining cleaving preferences, this activity enhancement appears to be independent from the functionality of these motifs and from the length of the polyQ tract, and monoubiquitination was demonstrated to be enough to produce the effect. Further observing that several proteotoxic stressors enhanced atx3 ubiquitination, the authors suggested that the upregulation of atx3 activity by ubiquitination may serve as a negative feedback mechanism in the response to those kinds of cellular threats. Importantly, studies demonstrate that atx-3 is not targeted by its own DUB activity *in trans*, opposing the possibility of atx3 regulating its own activity through regulation of its ubiquitination status (Todi et al., 2007).

One other mechanism possibly related to atx3 ubiquitination is connected with its interaction with several E3 Ub ligases, the enzymes responsible for forming the isopeptide bonds between Ub molecules and the lysine residues of target proteins or other Ub molecules, forming polyUb chains. Atx3 (or polyQ expanded fragments of the protein) have been shown to interact with several Ub ligases - C-terminus of 70 KDa heat shock protein (Hsp70)-interacting protein (CHIP), parkin, E4B and Hrd1 – and these enzymes (the exception being Hrd1) were shown to promote atx3 ubiquitination and degradation (Jana et al., 2005; Matsumoto et al., 2004; Miller et al., 2005; Tsai et

al., 2003; Wang et al., 2006). These activities were further related with decrease of expanded atx3 aggregation and polyQ toxicity but, attending to what has been seen for one complex formed by a DUB and a E3 enzyme (Ub-specific protease 4 - Usp4 - and Rho52, respectively), one can speculate that atx3 and the Ub E3 ligases with which it interacts may transregulate each other as part of their normal function (Wada & Kamitani, 2006). The E3 enzymes may be responsible for regulating atx3 activity or targeting it to degradadion through promotion of its ubiquitination, while atx3 may edit polyUb chains added to other E3 ligase substrates and regulate the ubiquitination state of the E3 ligases themselves (Reyes-Turcu et al., 2009; Todi et al., 2009; Wada & Kamitani, 2006).

1.4.2 – Phosphorylation of polyglutamine disease proteins

Phosphorylation is a common PTM that regulates protein function, activity, molecular interactions and subcellular localization, among other properties, in an acute and reversible way. It consists in the addition of a phosphate group to the hydroxyl group of a serine, threonine or tyrosine residue of a protein; protein kinases catalyze the addition of such group, while protein phosphatases catalyze the reverse phenomenon, i e, dephosphorylation. The alterations phosphorylation causes are due to conformational changes induced by the presence of the negative-charged phosphate group.

Protein phosphorylation seems to play an important role in many different neurodegenerative diseases. Many proteins related to the pathogenesis of polyQ expansion diseases have been found to be phosphorylated by certain kinases, and such phenomenon seems to be of great importance for the mechanisms involved in the diseases (Table II).

Phosphorylation of serine 776 of ataxin-1, the protein responsible for SCA1, seems to have a critical role on the pathogenesis of this disease, enhancing

Table II: Phosphorylation of proteins involved in polyQ expansion diseases (based on the studies referenced in the text)

Disease name	Protein product	Phosphorylation sites	Kinase	Described Effects
HD	Huntingtin	S412	Akt, SGK	Rescues axonal transport and toxicity
		S434	Cdk5	Reduces cleavage by caspases and toxicity
		S531	---	Reduces cleavage by caspases and toxicity
		S1181 S1201	Cdk5	Reduces toxicity
		T3	---	Increases aggregation, but reduces toxicity
DRLPA	Atrophin-1	S734	JNK	---
SBMA	Androgen receptor	S215 S792	Akt	Reduces ligand binding, nuclear translocation, transcriptional activation, toxicity and aggregation; increases degradation
		S416	---	Increases toxicity of the expanded form
		S516	MAPK	Increases cleavage by caspases and toxicity of the expanded form
		S651	---	Promotes transcriptional activation and nuclear translocation
		Y267 Y363	Ack1	Promotes cancer cell proliferation
		Y534	SRC	Promotes transcriptional activation, nuclear translocation and proliferation of prostate cancer cells
SCA1	ataxin-1	S776	Akt	Decreases degradation
MJD/ SCA3	ataxin-3	S236 S340 S352	CK2	Increases nuclear transport, NI formation and gene repression
		S256	GSK3β	Reduces aggregation

Abbreviations: HD, Huntington's disease; DRPLA, dentatorubral-pallidoluysian atrophy; SBMA, spinal and bulbar muscular atrophy; SCA, spinocerebellar ataxina; MJD, Machado-Joseph disease; SGK, serum/glucocorticoid regulated kinase; Cdk5, cyclin-dependent kinase 5; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; SRC, steroid hormone receptor coactivator; CK2, casein kinase 2; GSK3β, glycogen synthase kinase 3. --- indicates absence of data.

aggregation of an expanded form of the protein (Emamian et al., 2003). Akt phosphorylation of this residue leads to the binding of 14-3-3 protein, which stabilizes ataxin-1 and slows down its degradation (Chen et al., 2003). It was further demonstrated that an expanded ataxin-1 phospho-dead mutant for that serine residue expressed in Purkinje cell nuclei of mice is less capable of inducing the disease; the mutation reduces its toxic effects and its accumulation into NIs (Emamian et al., 2003).

Huntingtin, the protein whose expanded form is responsible for HD, is already known to be phosphorylated in several residues: serines 421, 434, 536, 1181, 1201, 2076, 2653 and 2657 and threonine 3 (Aiken et al., 2009; Anne et al., 2007; Luo et al., 2005; Rangone et al., 2004; Schilling et al., 2007; Warby et al., 2005). Serine 412 is known to be phosphorylated by Akt and serum/glucocorticoid regulated kinase (SGK) (Rangone et al., 2004; Warby et al., 2005), while serines 434, 1181 and 1201 are phosphorylated by cyclin-dependent kinase 5 (Cdk5) (Anne et al., 2007; Luo et al., 2005). So far, studies concerning the role of huntingtin phosphosrylation point out to a protective function for this PTM. Initial studies demonstrated that Akt phosphorylation of huntingtin has a protective effect, reducing inclusion formation and cell death caused by expanded huntingtin (Humbert et al., 2002). Later, it was also demonstrated that phosphorylation of serine 421 of an expanded huntingtin fragment had a neuroprotective effect *in vivo*; calcineurin dephosphorylates that residue *in vitro* and in cells and inhibition of calcineurin prevented cell death of striatal neurons caused by expanded huntingtin (Ermak et al., 2009; Pardo et al., 2006). This protective effect may be related to the recently described ability of phosphorylated serine 421 to rescue the impairment of axonal transport caused by expanded huntingtin (Zala et al., 2008). SGK phosphorylation also has a protective effect against the toxicity induced by expanded huntingtin, in striatal neurons (Rangone et al., 2004). Phosphorylation of serine 434 reduces huntingtin cleavage by caspases, thus attenuating protein aggregation and toxicity (Luo et al., 2005), and the same was suggested for serine 536 (Schilling et al., 2007). Phosphorylation of threonine 3 and serines 1181 and 1201 was shown to

protect against expanded huntingtin (Aiken et al., 2009; Anne et al., 2007) and, additionally, lack of phosphorylation of these two serine residues confers toxicity even to the wild-type huntingtin (Anne et al., 2007). Deficiences regarding phosphorylation were also linked with disease phenotype in observations of HD animal models and patients brain tissue: Cdk5 activity is reduced in the brains of HD animal models (Luo et al., 2005), while, in human patients, Akt is altered (a shorter form appears) and the levels of calcineurin inhibitors decreased (Humbert et al., 2002). Finally, it was proposed that the observed differential levels of serine 421 phosphorylation between different parts of the brain (in both human and mouse brain, under normal conditions), along with the fact that cell culture models show reduced levels of endogenous phosphorylation of the disease protein, may explain the selective neuronal loss observed in HD (Warby et al., 2005).

Atrophin-1, the protein responsible for DRPLA, appears to be phosphorylated by c-Jun NH_2-terminal kinase (JNK) at serine 734, but the affinity of that enzyme is reduced when the polyQ expansion is present. The resulting slower phosphorylation may delay processes responsible for keeping neurons alive (Okamura-Oho et al., 2003).

Many phosphorylation sites have already been mapped in the androgen receptor (AR), the protein involved in SBMA: serines 16, 83, 96, 215, 258, 310, 426, 516, 651 and 792, and tyrosines 223, 267, 307, 346, 357, 362/363, 393, 534, 551, 915 (Gioeli et al., 2002; Guo et al., 2006; Palazzolo et al., 2007; Pennuto et al., 2009). In some cases, phosphorylation has been related to the functionality of the AR: phosporylation of serine 651 appears to modulate AR transcriptional activity by regulation of its nuclear exportation (Gioeli et al., 2006), while phosphorylation of tyrosine 534 (apparently phosphorylated by steroid hormone receptor coactivator - SRC) was shown to regulate not only AR transcriptional activity and nuclear translocation, but also proliferation of prostate cancer cells (prostate cancer is another disease related to the AR) (Guo et al., 2006). Tyrosines 267 and 363 are posphorylated

by Ack1 and also enhance proliferation of those cells (Mahajan et al., 2007). Concerning the effect of phosphorylation on polyQ toxicity, blockade of serine 516 phosphorylation (catalyzed by a mitogen-activated protein kinase - MAPK) was found to abrogate polyQ expanded AR-induced cell death and formation of caspase-3-derived cleavage products, which is a key event in SBMA pathogenesis (LaFevre-Bernt & Ellerby, 2003). Interestingly, however, phosphorylation of both serines 426 and 516 appear to have opposite effects, depending on the size of the polyQ sequence: blockade of these modifications, while diminishing expanded protein toxicity, turns non-expanded AR into a cytotoxic agent (Funderburk et al., 2008). Mimicking phosphorylation of serines 215 and 792, appointed as targets of Akt, by substitution with aspartate residues reduces AR ligand binding, ligand-dependent nuclear translocation, transcriptional activation and toxicity of the expanded form; co-expression of Akt had similar effects and blocked expanded AR-induced cell death (Palazzolo et al., 2007). Recently Akt phosphorylation of AR was further demonstrated to reduce its aggregation and increase its degradation by the UPP and Akt activation was shown to reduce the disease phenotype of a SBMA mouse model (Palazzolo et al., 2009).

Attending to all these results, phosphorylation of polyQ-containing proteins appears to have the two possible opposite effects concerning pathogenesis: it can either be protective or contribute to the pathological mechanisms and cell loss, depending on the protein involved and the phosphorylated site(s) in question. This is in accordance with the importance and ubiquitous nature of phosphorylation as a regulating effect. One can expect phosphorylation to be a PTM whose effect varies greatly between different diseases, as its effect depends on the protein it is modifying.

As of this date, few work has been done in order to understand the importance phosporylation has on atx3 properties and so as to establish whether (and how) it affects the mechanisms underlying MJD.

Through biochemical analysis of the phosphoamino acids present on ^{32}P-orthophosphate-radiolabled samples of atx3, Mueller and co-workers (Mueller et al., 2009) recently showed that, apparently, atx3 is phosphorylated only on serine residues, as the authors were unable to detect the presence of neither phosphothreonines nor phosphotyrosines. In fact, the reports currently available have only mapped serine phosphorylation sites, all belonging to the UIMs: serine 236, located on the first UIM, serines 256 and 260/261 (it is not clear which of the adjacent serine residues is phosphorylated), located on the second UIM, and serines 340 and 352 of the third UIM (present only in some atx3 isoforms) (Fei et al., 2007; Mueller et al., 2009).

Importantly, there are already some clues to the possible biological relevance of these modifications. Mimicking phosphorylation of serine residues 236, 340 and 352 leads to an increased nuclear localization of atx3 in a way independent from the NLS described, and to an enhanced repression of atx3-regulated transcription (Evert et al., 2006a; Macedo-Ribeiro et al., 2009; Mueller et al., 2009). “Phosphorylation” of residues 340 and 352 also correlated with increased stability and half-life (Mueller et al., 2009). Casein kinase 2 (CK2) was shown to interact with atx3 and phosphorylate its UIM-containing C-terminal region (Mueller et al., 2009; Tao et al., 2008). CK2 pharmacological inhibition reduced atx3 levels in the nucleus and NI formation and activated atx3-regulated gene transcription, indicating that CK2-dependent phosphorylation (either directly or indirectly) regulates these properties, probably by interference with the phosporylated state of the above three residues (Mueller et al., 2006). Concurrent mutation of every phosphorylation site does not result in any differences concerning atx3 molecular interactions (p97/VCP, Rad23B and HDAC3 were specifically tested), though simultaneous mutation of the phosphorylation sites present in the second UIM to aspartates increased the minimum size of the polyUb chains that are bound by the protein.

Some of the few available studies report that normal and expanded forms of atx3 are phosphorylated at similar levels, but there are some hypotheses that try to

explain possible links between phosphorylation and expanded atx3 toxicity (Mueller et al., 2009; Trottier et al., 1998). Phosphorylation of serines 236, 340 and 352 may participate in the nuclear translocation of expanded atx3 and, in the nucleus, the phosphorylated protein may lead to aberrant atx3-regulated gene expression (Evert et al., 2006a; Mueller et al., 2009). The increased stability may contribute to increased aggregation and NI formation. In fact, C-terminal-containing fragments of atx3 showed an increase of NI formation when serines 340 and 352 were mutated to mimic phosphorylation (Mueller et al., 2009). Furthermore, stress stimuli also induced atx3 nuclear translocation in a CK2-dependent manner, pointing out a mechanism through which proteotoxic stress may potentiate the expanded protein nuclear localization. Fei and co-workers (Fei et al., 2007) reported that serine 256 is phosphorylated by glycogen synthase kinase 3 β (GSK 3β), but observed a decreased phosphorylation of the expanded form of the protein. Contrary to those observations indicating a possible nefarious effect for atx3 phosphorylation, these authors observed that preventing phosphorylation of serine 256 in an expanded form of atx3, but not of a normal form of the protein, enhanced its aggregation ability. These results indicate that there may be some relation between the serine 256 phosphorylation and the formation of the toxic protein aggregates. Hsp70, a molecular chaperon that promotes protein folding and has a protective effect against polyQ diseases (Muchowski & Wacker, 2005), was able to disrupt aggregation of the non-phosphorylated expanded atx3, further evidencing that non-phosphorylated expanded atx3 is prone to form aggregates. As stated before, the effect of phosphorylation can be predicted to differ according to the different residues modified, thus conciliating these apparent conflicting observations.

More work will be necessary in order to increase our understanding of atx3 phosphorylation and of its role in the pathogenesis of MJD. A better conception of the biological roles of normal atx3 and the possible changes introduced by the polyQ expansion, as well as of the effects of phosphorylation in those contexts, will be needed. Importantly, phosphorylation has been described as one of the mechanisms

by which DUBs are regulated and the majority of these enzymes has already been demonstrated to be phosphorylated *in vivo*. Phosphorylation of DUBs may either increase or decrease their protease activity and there are many studies showing that this modification influences DUBs physiological roles in the overall cellular context (Nijman et al., 2005; Reyes-Turcu et al., 2009).

1.4.3 – Sumoylation of polyglutamine disease proteins

Sumoylation is a reversible PTM that shares many characteristics with ubiquitination and that has been recently shown to be widespread among the proteins of the cell. Like ubiquitination, it consists in the covalent attachment of a small protein, in this case small Ub-like modifiers (SUMO), to one or several lysine residues of another protein. As of yet, four SUMO proteins have been identified: SUMO1, 2, 3 and 4. The very cycle of sumoylation has a lot of common aspects with ubiquitination, with the enzymes involved in sumoylation, also named E1, E2 and E3, acting mainly in the same way as the ones involved in that other PTM. That is to say, the E1 activating enzyme (a heterodimer of SUMO1-activating enzyme subunit 1 - SAE1 - and SAE2) binds and activates SUMO through an ATP-dependent reaction, and the protein is then transferred to the E2 conjugating enzyme, which, together with a E3 ligase (there are several described), transfers it to the target lysine residue of the substrate protein, forming an isopeptide bond between them. However, contrary to ubiquitination, the E2 conjugating enzyme (termed Ub-conjugating enzyme 9 - Ubc9) has the ability to both recognize the target lysine residue and catalyze the transference of the SUMO protein by itself; E3 ligases mainly increase the efficiency of the process and help in determining the substrate specificity, being unnecessary in some of the cases (Anckar & Sistonen, 2007; Dorval & Fraser, 2007; Zhao, 2007). Sumoylation is highly specific in terms of the lysine residues to which SUMO is conjugated, not sharing with ubiquitination its certain degree of promiscuity when selecting the residues for

modification (Anckar & Sistonen, 2007). Again, similarly to ubiquitination, the constitution of polySUMO chains is possible, too (Dorval & Fraser, 2007). Removal of SUMO is termed desumoylation and is catalyzed by desumoylases, which are equivalent to (and sometimes considered as) DUBs (Reyes-Turcu et al., 2009).

Unlike ubiquitination, however, sumoylation is not associated with the targeting of proteins for degradation. So far, sumolylation has been implicated in changes in the substrate protein's function, activity, stability, subcellular localization, molecular interactions and susceptibility for other PTM processes, (Zhao, 2007). However, all of these vast changes can mainly be traced back to the modulation of molecular interactions, appointed as the primary effect of sumoylation (Geiss-Friedlander & Melchior, 2007; Zhao, 2007).

The role of sumoylation in these cellular activities has been shown to be significant in many disease contexts (Dorval & Fraser, 2007). Moreover, the relation between ubiquitination and sumoylation doesn't end in the similarities between the two processes; in fact, some of the target lysines are the same for both PTMs, suggesting the existence of a dynamic interplay between them (Dorval & Fraser, 2007; Wilson & Heaton, 2008). Taking all this into account, sumoylation must be considered a candidate for an important role regarding regulation of atx3 properties and MJD pathogenesis.

Several authors have associated sumoylation with numerous neurodegenerative disorders and a few studies have already tried to associate this PTM with polyQ expansion diseases (Figure 6). So far, SUMO proteins have been detected in the typical cellular inclusions seen in many neurodegenerative diseases and have been shown to target some of the proteins linked to their pathogenesis (Terashima et al., 2002; Steffan et al., 2004; Dorval & Fraser, 2007).

Neurons in affected regions of the brain of patients of HD, SCA1, DRPLA and MJD were shown to have a high immunoreactivity for SUMO-1 (Ueda et al., 2002). In the cerebellar cortex of MJD patients, Purkinje cells displayed granule-like signals in

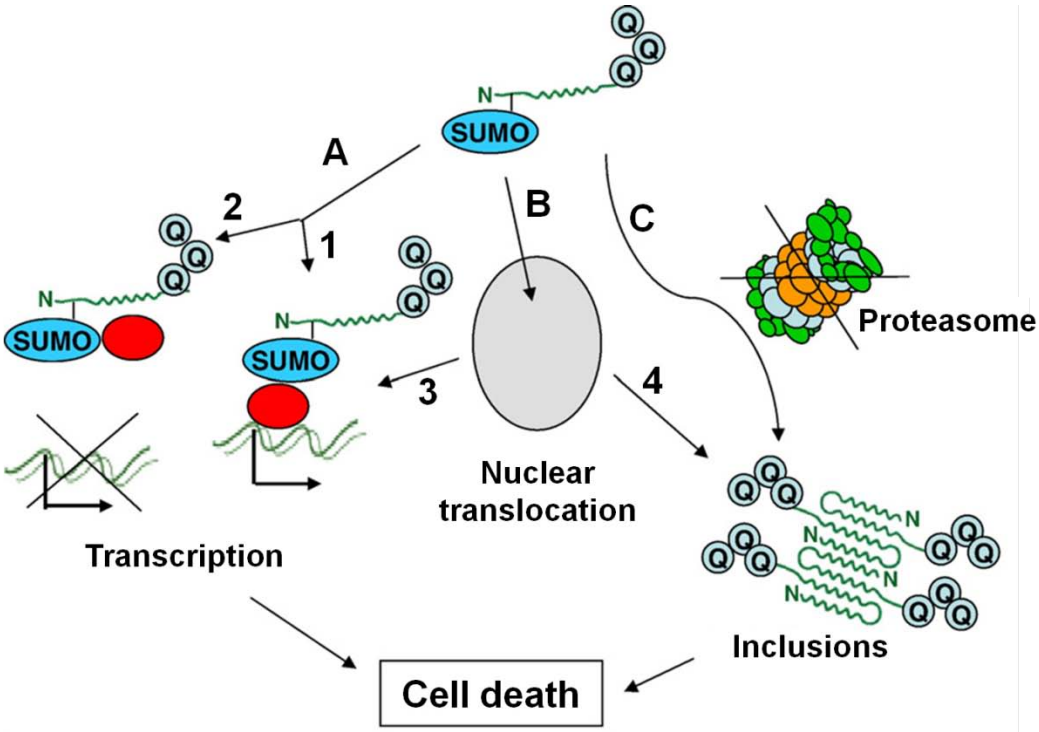

Figure 6 – Sumoylation effects on polyQ toxicity. (*A*) Sumoylation can directly alter transcriptional activity of the mutant polyQ protein (*1*) or promote sequestration of transcription factors/regulators (2). (*B*) Sumoylation can change subcellular localization, possibly affecting transcription (*3*) and/or formation of inclusions (*4*). (*C*) SUMO modification may also antagonize proteasome-mediated degradation of the mutant polyQ protein, possibly leading to protein accumulation, sequestration of other proteins and formation of inclusions. These toxic effects may contribute to cell loss in polyQ expansion diseases. (Adapted from Dorval & Frasier, 2007)

the cytoplasm while granule cells had a homogenous signal in the nucleus. Interestingly, although no definite co-localization between Ub (often observed in the NIs of MJD) and SUMO-1 was observed in any cell type, a positive correlation between their signals was detected in Purkinje cells (Ub stain in the nucleus was greater in high SUMO-1-signal cells), while in granule cells the relation was the opposite (Pountney et al., 2003). Though the SUMO-1 signal may also be related to proteins other than atx3 that also contribute to pathogenesis, this study revealed a cell-specific relationship

between ubiquitination and sumoylation, that may be relevant for the processes involved in MJD development.

Other authors reported the presence of SUMO-1 in the neuronal NIs of DRPLA patients' brain tissues and cells expressing expanded atrophin-1, showing an enhanced sumoylation of expanded atrophin-1-induced aggregates (Terashima et al., 2002). Additionally, abrogation of sumoylation in cells expressing expanded atrophin-1, through the co-expression of a sumoylation-defective SUMO-1 mutant, reduced aggregate formation and cell death, comparing with the co-expression of wild-type SUMO-1. These results seem to indicate a possible positive correlation between sumoylatation and disease.

Studies show that SUMO-1 is conjugated to lysines 386 and 520 of the AR and that this modification somehow interferes with its transcriptional activity (Poukka et al., 2000; Kaikkonen et al., 2009). Additionally, sumoylation appears to occur in a ligand-induced manner and SUMO-specific proteases 1 and 2 (SNP-1 and -2, respectively) are able to reverse the modification (Kaikkonen et al., 2009). Regarding the importance of sumoylation in SBMA pathogenesis, studies made in *Drosophila* expressing a fragment of an expanded form of the human AR reported that the normal degeneration caused by this protein is enhanced by the compromise of SUMO-1 conjugation (achieved by co-expressing an inactive form of an E1 enzyme). Such situation led to a degeneration phenotype very similar to the one resulting from the inhibition of the proteasome system or the molecular chaperon Hsp70. Moreover, although Hsp70 was able to rescue the disease phenotype caused by inhibition of the proteasome, that was not the case in respect to the inhibition of the E1 (Chan et al., 2002). Importantly, the same authors reportedly obtained similar results with a MJD model expressing expanded atx3. Together, these results suggest that sumoylation protective effect may be partly related with protein aggregation. A recent study went as far as demonstrating that enhanced sumoylation (apparently of the AR itself) in fact reduces polyQ expanded AR aggregation (Mukherjee et al., 2009).

The results of the two studies cited above demonstrate that sumoylation may be associated with the development and consequences of DRPLA, SBMA and MJD, the main focus of the present work. Although sumoylation occurrence seems to precipitate pathology of DRPLA, while somehow having a protective effect in SBMA and MJD, it should be considered that these studies refer to a general modification of sumoylation, at the level of the whole cell, and not only of the protein of interest. Thus, the most important aspect to be considered is that sumoylation seems to be important for the pathological mechanisms of these diseases. It is of great relevance to try to understand the effects of sumoylation of the disease protein itself.

Animal models expressing an expanded form of ataxin-1 have increased levels of sumoylated proteins in the cerebellar cortex (Ueda et al., 2002), though a later work showed that expanded ataxin-1 had a decreased sumoylation, when compared to the non-expanded form (Riley et al., 2005). It was also reported, however, that there are several factors influencing ataxin-1 sumoylation: inhibition of serine 776 phosphorylation restored sumoylation, showing a possible interplay between these two PTMs, and a functional nuclear localization signal is necessary for the occurrence of that same modification.

Sumoylation of a fragment of huntingtin *in vitro* was shown to increase its stability and accumulation, decrease its aggregation and enhance transcription repression (transcription repression is a deleterious effect observed in many polyQ diseases, including HD) (Steffan et al., 2004). The authors of this work also suggested that attachment of SUMO proteins to the expanded huntingtin could mask a cytoplasmic retention signal, targeting the protein to the nucleus, and that differences of such event between different cell types may explain the selective neuronal loss. Furthermore, experiments with a *Drosophila* animal model revealed that sumoylation of the disease protein enhances cell death. This may appear to be contradictory to the fact that it decreases aggregate formation, but this decreased aggregation can also indicate an increased amount of toxic soluble oligomers (this explanation was also

used to elucidate why phosporylation of threonine 3, while having a protective effect, enhances aggregation; Aiken et al., 2009). Researchers also showed that the effect of sumoylation is not only a result of a blockade of ubiquitination, a PTM that appears to be protective in this case. If that was the case, mutation of the target lysines would produce cytotoxicity; instead, cytotoxicity decreased, showing that the modification of these residues by sumoylation is more significant to pathogenesis (Steffan et al., 2004).

To this date, atx3 has never been shown to be sumoylated and, apart from what has been said above, no one has ever properly dealt with its possible influence in MJD. There are many ways by which sumoylation may contribute to the pathogenesis of this disease, though these potential mechanisms are just theoretical models suggested by what has been seen for other polyQ diseases. Through competition or disruption, sumoylation may block atx3 degradation by the proteasome system, leading to protein aggregation and inclusion formation; taking into account its importance as modulator of intermolecular interactions, sumoylation may also contribute to the sequestration of other molecules to the aggregates (Dorval & Fraser, 2007; Terashima et al., 2002; Ueda, et al., 2002). Given its role in the subcellular protein targeting, and in particular in nucleo-cytoplasmic shuttling, SUMO modification may also lead to the nuclear transport of atx3, which, in turn, leads to an increased formation of nuclear aggregates. The formation of aggregates and NIs and subsequent SUMO-mediated recruitment of important cellular systems may contribute to deleterious effects such as transcriptional changes. Another possibility may be the contribution of sumoylation to the formation of the toxic oligomers appointed to be decisively important (Dorval & Fraser, 2007; Steffan et al., 2004; Terashima et al., 2002).

Nonetheless, considering what was seen above, inhibition of sumoylation appears to have a negative effect, contributing to degeneration in a MJD model (Chan et al., 2002). These results could lead to the suspicion that, in fact, sumoylation has a protective role in MJD, but even though sumoylation seems to prevent some damage in

the work cited, one cannot conclude that this is due to sumoylation of atx3. The experiments were done trough a general disruption of sumoylation, which may have interfered with other cellular systems, aggravating the degeneration.

Further work directed to atx3 in particular must be done in order to establish if the protein is sumoylated, and in what conditions does this modification happen. It will then be important to determine if sumoylation, like phosphorylation and ubiquitination, is responsible for the regulation of atx3 properties, and evaluate how it interferes with the pathogenesis of MJD. Similarly to ubiquitination and phosphorylation, DUB activity has been shown to be regulated by sumoylation (Reyes-Turcu et al., 2009). For example, Ub-specific protease 25 (Usp25) was shown to be sumoylated in its two UIMs and such modification impaired binding to and hydrolysis of Ub chains (Meulmeester et al., 2008).

1.5 – Objectives

Considering what is currently known about regulation of DUB activity and pathogenesis of polyQ expansion diseases, further study into atx3 PTMs may be of key importance in increasing our understanding of the presently elusive function of this protein, and at the same time provide relevant clues concerning MJD pathogenesis. Consequently, the main aim of this experimental work was to try and describe novel PTMs of atx3 whose putative biological relevance may be later assessed, this way contributing to our comprehension of atx3 properties. Of all the existing possibilities, we directed our focus at two particular PTMs, phosphorylation and sumoylation, since they are known modulators of DUB function and polyQ toxicity (Reyes-Turcu et al., 2009). Phosphorylation is likely to also target amino acids other than the ones already described as being phosphorylated (and to different effects) and sumoylation has never been described to directly modify atx3. Accordingly, our objectives were to map new

phosphorylation sites of atx3 and determine whether this protein conjugates with SUMO.

Since previous studies concerning atx3 phosphorylation primarily focused on serine phosphorylation, we started by trying to detect tyrosine phosphorylation using a phospho-specific antibody. Then, in order to straightforwardly map phosphorylation sites, we used a mass spectrometry (MS) approach that allows the precise detection of phosphorylated residues. This kind of strategy already yielded comprehensive information regarding huntingtin and AR (Guo et al., 2006; Schilling et al., 2007). Protein samples used in these studies were to be obtained from human cell lines, since they are more likely to reflect what physiologically happens in cells within the human organism.

In order to try and determine if atx3 is a target for SUMO conjugation, we performed *in vitro* sumoylation assays on recombinant atx3 JD purified from bacteria. Then, with the intention of assessing the relevance of our *in vitro* results in the proper cell context, these assays were followed by cotransfection experiments performed on human cell lines.

Chapter 2 Materials and Methods

2.1 – Materials

Dulbecco's Modified Eagle Medium (D-MEM), Minimum Essential Medium (MEM), protease inhibitors chymostatin, pepstatin, antipain and leupeptin (CLAP) and brilliant blue R were acquired from Sigma-Aldrich Química S.A. (Sintra, Portugal). Fetal bovine serum (FBS), MEM Non-Essential Amino Acids (NEAA), geneticin G-418 sulphate, trypsin and Opti-MEM® were obtained from Gibco, as part of Invitrogen Life Technologies (Barcelone, Spain). Lipofectamine™ Reagent was purchased from Invitrogen Life Technologies (Barcelone, Spain). Phorbol 12-myrisate 13 acetate (PMA) and *in vitro* SUMOylation kit were acquired from Biomol, as part of Enzo Life Sciences (Lausen, Switzerland). Triton X-100 and bovine serum albumin (BSA) were purchased from Calbiochem, as part of Merck KGaA (Darmstadt, Germany). Pierce® BCA Protein Assay Kit was obtained from Thermo Scientific (Rockford, USA). Protein A Sepharose CL-4B, Enhanced Chemifluorecense substrate (ECF™) and Hiprep 26/60 were acquired from GE Healthcare (Carnaxide, Portugal). Low protein binding centrifugal filters (0,45 µ) were purchased from VWR (Carnaxide, Portugal). HisTrap HP column was purchased from Amersham Biosciences (Carnaxide, Portugal). Sodium dodecyl sulfate (SDS), SDS-polyacrilamide gel electrophoresis (SDS-PAGE) buffer (10x Tris/Glycine/SDS stock), acrylamide/Bis and acrylamide solutions were acquired from Bio-Rad (Amadora, Portugal). Page blue stain was obtained from Fermentas, distributed by Bioportugal (Porto, Portugal). Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore (Madrid, Spain). QuikChange® II XL Site-Directed Mutagenesis Kit was purchased from Stratagene (Cambridge, UK). Primers MJD1a-K166R_for and MJD1a-K166R_re were acquired from Bonsai Technology (Linda-a-Velha, Portugal); primers atx31_1n_S12D_for and atx31_1n_S12D_rev were purchased from Bonsai Technology, as part Fisher Scientific (Loures, Portugal). QIAprep® Spin Miniprep Kit and QIAGEN Plasmid Mini Kit were obtained from QIAGEN (QIAGEN GmbH, Hilden, Germany). All other reagents were obtained from Carlo Erba

Reagents (Rodano, Italy), José M. Vaz Pereira, S.A. (Benavente, Portugal), PanReac Química S.A.U. (Barcelona, Spain), Sigma-Aldrich (Sintra, Portugal) and Merck (Darmstadt, Germany).

All solutions were aqueous, expect for those of phenylmethanesulphonyl fluoride (PMSF) and PMA, which were prepared in dimethyl sulfoxide (DMSO).

2.2 – Expression plasmids

Eukaryotic expression pEGFP plasmid encoding human atx3 variant 1a (NCBI accession number: AAB33571.1) with 28 glutamines (GFP-atx3-Q_{28}) fused with green fluorescent protein (GFP) in the N-terminal was a kind gift from Dr. Henry Paulson, University of Iowa (Iowa City, USA; plasmids described in Chai et al., 1999b). pcDNA3 plasmids expressing hexahistidine (6His)-tagged human SUMO isoforms 1, 2 and 3 (6His-SUMO1 and 6His-SUMO2, respectively) were a kind offer from Dr. Paul E. Fraser, University of Toronto (Toronto, Canada; described in Dorval & Fraser, 2006 and sequenced for confirmation).

Bacterial expression pDEST17 plasmids encoding 6His-tagged human atx3 variant 1-1n (6His-atx3; NCBI accession numbers: NP_004984.2) and 6His-tagged JD (6His-JD) have been previously described by our group (Gales et al., 2005).

2.3 – Antibodies

Anti-GFP monoclonal antibody (mice) was acquired from Roche Applied Science (Carnaxide, Portugal). Anti-6His polyclonal antibody (rabbit) and anti-phosphotyrosine monoclonal antibody (mice) were purchased from Cell Signalling Technology (Boston, USA). Anti-SUMO2/3 polyclonal antibody (rabbit) accompanied the SUMOylation kit acquired from Biomol, as part of Enzo Life Sciences (Lausen, Switzerland). Alkalyne phosphatase-conjugated anti-mouse and anti-rabbit

immunoglobulin G (IgG) secondary antibodies were obtained from GE Healthcare (Carnaxide, Portugal). Pre-immune rabbit IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, USA).

2.4 – Protein sequence and structure analysis

Human atx3 variant 1a putative phosphorylation sites and kinases were determined by analysis of its amino acid sequence (NCBI accession number: AAB33571.1) using two phosphorylation prediction web-based softwares: Scansite 2.0 (http://scansite.mit.edu/) and NetPhosK 1.0 (http://www.cbs.dtu.dk/services/NetPhosK/). Prediction of possible sumoylation targets was made online using the SUMOplot™ Analysis Program (http://www.abgent.com/tools/sumoplot; Abgent, San Diego, California).

JD tertiary structure was obtained from the RCSB Protein Data Bank (http://www.pdb.org; Protein Data Bank - PDB - ID code 1YZB) and structural analysis was performed using the PyMOL Molecular Graphics System software (DeLano Scientific, San Carlos, USA).

2.5 – Mammalian cell culture and transfection

Human embryonic kidney (HEK) 293FT cells were grown in D-MEM, supplemented with 10% (v/v) FBS, 0,1 mM NEAA, 6 mM L-glutamine, 1mM sodium syruvate (in MEM) and 500 µg/ml geneticin. Cells were maintained at 37 ºC, in a humidified atmosphere containing 5% CO_2.

One day prior to transfection, fully confluent HEK 293FT cells were diluted 1:6 and then seeded onto 10 cm diameter culture plates. After 24 h of growth, transient transfection was carried out using Lipofectamine reagent with a modified version of the manufacturer's protocol, as follows: after substituting the culture medium for 6,4 ml

Opti-MEM supplemented with 10% FBS, lipid-DNA complexes obtained by mixing 10 µg of DNA plasmid with 6 µl of Lipofectamine reagent in 800 ml Opti-MEM were added to each cell plate; after overnight incubation under normal growth conditions, transfection medium was replaced for normal HEK 293FT cell medium and expression was left to occur for 24 h of cell growth. In the case of co-transfections, lipid-DNA complexes were generated using 10 µg of each desired plasmid.

2.6 – Chemical stimuli

Some transfected HEK 293FT cell cultures were subjected to chemical stimulation directed at enhancing phosphorylation with 20 nM OA for 16 h (Dorval & Fraser, 2006), 5 mM sodium orthovanadate (Na_3VO_4) for 30 min (Martin et al., 2006) or 200 nM PMA for 20 min (following the recommended of the manufacturer). All concentrations are final. These treatments were carried out in the final 24 h of cell culture, immediately before cell harvesting, at 37 ºC.

2.7 – Immunoprecipitation

Transfected HEK 293FT cell cultures were briefly washed with cold phosphate buffer saline (PBS; 137 mM NaCl, 2,7 KCl, 1,8 mM K_2PO_4, 10 mM $Na_2HPO_4 \cdot H_2O$, pH 7,4) and whole cell extracts were then prepared by adding 600 µl of IP buffer (IPB; 20 mM Tris pH 7,0, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 1 mM Na_3VO_4) supplemented with 1% Triton X-100, 1 µM OA and protease inhibitors (1 mM DTT, 0,1 mM PMSF, 10 µg/ml CLAP) and scraping on ice. After being sonicated on a probe sonicator for 1 min, lysates were cleared of their insoluble fraction by centrifugation (13.200xg, 10 min, 4 ºC) and protein concentration was assessed by the bicinchoninic acid (BCA) method using the BCA protein kit, according to the recommendations of the manufacturer. Input samples were prepared by mixing the extracts with 2x

concentrated SDS-PAGE sample buffer (125 mM Tris pH 6,8, 100mM glycine, 40% glycerol, 4% SDS, 200 mM DTT, 0,01% bromophenol blue) and boiling at 95 ºC for 5 min.

One mg of protein from each extract (diluted to 1 mg/ml, with the same buffer) was pre-cleared by incubation with 50 µl of a slurry of Protein A Sepharose beads (1:1 in IPB) for 1 h, followed by riddance of the beads by centrifugation (13.200xg, 5 min). Samples were then incubated overnight with 3 µg of anti-GFP or anti-phosphotyrosine antibody and precipitation of the immunocomplexes was performed by incubation with 100 µl of the Protein A Sepharose slurry for 2 h 30 min. The beads were then washed by centrifugation (13.200x*g*, 1 min), 2 times with IPB plus 1% Triton X-100, 3 times with IPB containing 500 mM NaCl and 1% Triton X-100 and 2 times with IPB. The above procedures were all carried out at 4ºC. Elution was finally achieved by adding 50µl of 2x concentrated SDS-PAGE sample buffer to the beads, boiling at 95 ºC for 5 min and spinning with centrifugal filters (13.000x*g*, 5 min). Resulting samples and inputs were analyzed by Western blot. IP control was made using pre-immune IgG instead of antibodies.

For MS analysis, 3 samples of each extract were subjected to this IP procedure, but collectively eluted in sequence by adding 2x concentrated SDS-PAGE sample buffer to the beads of one samples, boiling, spinning, adding the resulting eluent to another sample's beads and starting the procedure again.

2.8 – Heat shock stimulus and immobilized metal ion affinity chromatography

In the final 1 h of cell growth, some of the transfected HEK 293FT cell cultures cells were submitted to a heat shock stimulus, by transferring the respective plates to a 42 ºC hot bath. Control cells were kept at 37 ºC. Cultures were then momentarily washed with cold PBS and whole cell extracts were prepared by scrapping the plates in

600 µl of immobilized metal ion affinity chromatography (IMAC) buffer (8 M urea, 100 mM NaH_2PO_4 pH 8, 10 mM iodoacetamide, 10 mM β-mercaptoethanol, 1% Triton X-100, 5 mM imidazole) containing 100 µg/ml DTT, 0,1 mM PMSF, 10 µg/ml CLAP and 1 µM OA, on ice. Cells were sonicated on a probe sonicator for 30 s and the insoluble fraction of the lysates was then disposed of by centrifugation (13.200x*g*, 10 min, 4 ºC). Protein concentration was assessed by the BCA method using the BCA protein kit, according to the recommendations of the manufacturer. Input samples were prepared by mixing the extracts with 2x concentrated SDS-PAGE sample buffer and boiling at 95 ºC for 5 min.

One mg of protein from each extract (diluted to 1 mg/ml, with the same buffer) was incubated for 2 h with 50 µl of a slurry of Ni Sepharose beads (1:1 in IMAC buffer containing 20 mM imidazole). The beads were then washed by centrifugation (13.200x*g*, 1 min, 4 ºC) once with IMAC buffer containing 10mM imidazole and twice with 10 mM imidazole IMAC buffer, pH 6,4, in order to remove any nonspecifically-bound proteins. Until this point, every procedure was performed at 4ºC. Elution was finally performed by adding 50 µl of IMAC buffer containing 500 mM imidazole, pH 6,4, to the beads, incubating for 3 min and spinning with centrifugal filters (13.000x*g*, 5 min). After denaturation with SDS-PAGE sample buffer and boiling, resulting samples and inputs were analyzed by Western blot.

2.9 – Bacterial expression and purification of recombinant proteins

Escherichia coli BL21(DE3)-SI cells (Invitrogen Life Technologies, Barcelone, Spain) were transformed with the pDEST17 plasmids encoding 6His-atx3 and 6His-JD through heat shock. Cells were grown at 37 ºC in NaCl-free Luria broth medium (10% (w/v) triptone, 5% (w/v) yeast extract) with 100 µg/l ampicillin and 0,2% (w/v) glucose until the optical density (absorbance at 600 nm) reached 0,4-0,8, at which point expression was induced through addition of NaCl to a final concentration of 300 mM.

Expression was left to occur for 3 h, at 30 ºC. The cells were then collected by centrifugation (10.500x*g*, 20 min, 4ºC) and ressuspended in imidazole buffer (20 mM sodium phosphate pH 7,5, 0,5 M NaCl, 10 mM imidazole) containing lysozyme and kept at -20 °C. Before purification, serine protease inhibitor PMSF was added to a final concentration of 1 mM and the cells extracts were digested with 5 µg/ml DNAse I (in the presence of 10 mM $MgCl_2$) for 1 h, in ice, after which the samples were cleared of their insoluble fraction by centrifugation (17.400x*g*, 40 min, 4ºC).

The resulting supernatant was applied to a 5 ml HisTrap HP column (Amersham Biosciences, Carnaxide, Portugal) and eluted by rise of the imidazole concentration in three steps, through addition of imidazole buffer containing 50 mM, 100 mM and 500M imidazole. SDS-PAGE (12,5% (v/v) bis-acrylamide) followed by Page blue staining were carried out in order to determine which fractions were suitable for further purification, considering their enrichment in the protein of interest.

Appropriate fractions were pooled and then loaded to a Hiprep 26/60 (GE Healthcare; Carnaxide, Portugal) equilibrated in buffer A (20 mM HEPES pH 7,5, 200 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% (v/v) glycerol). Elution was carried out at 4 ºC with a flow rate of 0,5 ml/min and resulting fractions were analysed by SDS-PAGE followed by Page blue staining. The purest fractions were pooled and concentrated. Afterwards, samples were immediately frozen in liquid nitrogen and stored at -80ºC. Protein concentration was assessed by measurement of the absorbance at 280 nm using extinction coefficients of 36,160 $M^{-1}cm^{-1}$ and 34,760 $M^{-1}cm^{-1}$, for 6His-atx3 and 6His-JD, respectively.

2.10 – *In vitro* sumoylation assays

In vitro sumoylation assays were based on Biomol's SUMOylation Kit, using a modified version of the recommended procedure. 200 nM 6His-JD was incubated for 1 h, at 37 ºC, with SUMO E1, SUMO E2 and 6His-conjugated SUMO isoform 2 (6His-

SUMO2-aa), in the presence of Mg-ATP, 20 units/ml inorganic pyrophosphate (iPP), 1,5 mM DTT and the suitable reaction buffer (final reaction volume of 20 µl). Enzymes, 6His-SUMO2-aa, reaction buffer and Mg-ATP were supplied by the kit and added as recommended by the manufacturer. The 6His-SUMO2-aa designation is used in order to distinguish the kit-supplyed SUMO2 protein construct from the 6His-SUMO2 we expressed in HEK 293FT cells.

Positive controls were made replacing 6His-JD for Ras-related nuclear protein GTPase activating protein 1 (RanGAP1; Saitoh & Hinchey, 2000) according to the standard procedure. Negative controls were made excluding Mg-ATP and iPP. After incubation, reactions were stopped by quenching the assays with 2x concentrated SDS-PAGE loading buffer and denaturating by heating to 65 ºC for 5 min. Resulting samples were analysed by Western blot.

2.11 – SDS-PAGE and Western blot

Samples destined for Western blot analysis were electrophoresed in polyacrilamide gels until a suitable separation of the molecular weight standards was achieved. Depending on the situation, we used either a MiniPROTEAN® 3 system or a larger, PROTEN® II xi, system (Bio-Rad, Amadora, Portugal), that allowed loading of larger sample volumes; usage of this system is specified in the figure legends. Gel bis-acrylamide concentration was 10% (v/v) when using the first system and 7,5% (v/v) when using the other one. Loaded volumes of each input were determined so that the applied protein mass was the same for each lane of a single electrophoresis (40-90 µg); immunoprecipitant or IMAC-purificated samples' quantity was made constant by applying 20 µl of each of them, in the case of the MiniPROTEAN® 3 system, or the total obtained volume, in the case of the PROTEN® II xi system. Proteins were then electro-transferred (40 V) overnight, at 4 ºC, to PVDF membranes.

In the case of the samples resulting from the *in vitro* sumolylation assays, we electrophoresed 10 µl of each in 12,5% ((v/v) bis-acrylamide) gels (MiniPROTEAN® 3 system) and performed the electrotransference for 3 h and using nitrocellulose membranes instead.

Membranes were blocked by 1 h incubation with Tris-buffered saline (20 mM Tris-HCl, 137 mM NaCl, pH 7,6) containing 0,1% (v/v) Tween 20 (TBS-T) and 5% (w/v) low-fat dry milk. Incubation with primary antibodies diluted as recommended in TBS-T containing 0,5% (w/v) low-fat dry milk was carried out according to the manufacturer's instructions: anti-GFP and anti-SUMO2/3 antibodies were diluted 1:1.000 and incubated for 1 h at room temperature, while the anti-6His antibody, also diluted 1:1.000, was overnight at 4 ºC. Membranes were then washed with TBS-T containing 0,5% (w/v) low-fat dry milk (5 times, 5 min each) and subsequent incubation with suitable alkaline phosphatase-conjugated secondary antibody diluted 1:20.000 in the above solution was performed for 1 h at room temperature. Membranes were again washed and then resolved using ECF. Scanning was performed using Storm 860 Gel and Blot Imaging System (Amersham Biosciences, Buckinghamshire, UK). In the case of the anti-phosphotyrosine antibody (diluted 1:2.000 and incubated overnight at 4ºC), milk was substituted for 5% (w/v) BSA and washing was performed using TBS-T, in every step of the procedure. When reprobing was intended, membranes were stripped with 0,2 M NaOH for ~10 min and again blocked and marked as described.

2.12 – Mass spectrometry

For MS analysis, triple immunoprecipitants (~100-150µl) were alkylated by incubation with 1% (v/v) acrylamide for 20 min, at room temperature, and their protein content was separated by SDS-PAGE in 7,5% polyacrylamide gels using the PROTEAN® II xi system (100 µl of each was loaded into the gel). Gel fixation and staining were performed simultaneously by incubation with 45% (v/v) methanol, 10%

(v/v) acetic acid, 0,25% (w/v) Coomassie brilliant blue R for 8 min, and followed by destaining with 25% methanol, 5% acetic acid. In order to minimize possible contaminations with keratin, these solutions were prepared anew and every material contacting with the gel was washed with 1% SDS and mQ water. Polyacrilamide gel bands containing GFP-atx3 were excised using a clean scalpel, covered in mQ water and frozen.

Online liquid chromatography (LC) followed by ion spray MS (LC-ES-MS) analysis of the bands of interest was performed by the Proteomics Center at Children's Hospital Boston (Boston, USA). Gel-contained protein samples were subjected to an in-gel digestion with 12,5 ng/µl sequencing-grade trypsin (Promega, Madison, USA) in 100mM ammonium bicarbonate, overnight, at 37ºC, after which the resulting peptides were extracted with 100mM ammonium bicarbonate and acetonitrile and lyophilized. Samples were ressuspended in 5% acetonitrile, 5% formic acid and then directly injected into a LC/MS system encompassing a micro-autosampler, a Suvery HPLC pump and a Proteome X (LTQ) mass spectrometer (all acquired from Thermo Finnigan, San Jose, USA). The LC system featured a reversed phase column, packed in-house using C18 Magic packing material (3 mm, 200 Å; Michrom Bioresources, Auburn, USA), and PicoTip Emitters (New Objective, Woburn, USA). In the LC/MS system, peptides were eluted with 0,1% formic acid in acetronitrile, using a 30 min linear gradient of 8% to 34% acetonitrile, and then applied to the mass spectrometer by electrospray ionization. MS spectra were acquired by the Proteome X (LTQ) mass spectrometer in a data-dependent fashion, with the 6 top abundant species being selected for automated fragmentation. Phosphorylated peptides were detected by the characteristic mass shift of 80 or 98 *m/z* that corresponds to the neutral loss of a phosphate group. When the fragmentation of a MS spectrum resulted in an immediate neutral loss mimicking this mass shift in the MS^2, then a MS^3 fragmentation was performed on the resulting MS^2 peak. The mass spectrometric data obtained was searched against the human international protein index database (IPI human 336)

using the protein identification software Mascot (version 2.2.04; Matrix Sciences, London, UK) according to the appropriate search criteria necessary for the detection of phosphorylation. Spectra sequence identified by Mascot to be phosphorylated were validated manually to guarantee proper recognition of phospharylated amino acid position and to access overall quality of the data obtained.

2.13 – Site-directed mutagenesis

Mutagenesis was performed using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, Cambridge, UK), according to the procedure suggested by the manufacturer. Mutant 6His-atx3 S12D, with serine at amino acid residue 12 substituted for aspartate, was generated from the pDEST17 6His-atx3 DNA template using primers atx31_1n_S12D_for (5'-CAC GAG AAA CAA GAA GGC GAC CTT TGT GCT CAA CAT TGC CTG-3') and atx31_1n_S12D_rev (5'-CAG GCA ATG TTG AGC ACA AAG GTC GCC TTC TTG TTT CTC GTG-3'). Mutants 6His-atx3 K166R and 6His-JD K166R, with lysine at amino acid residue 166 substituted for arginine, were generated from the pDEST17 plasmids encoding 6His-atx3 and 6His-JD, respectively, using primers MJD1a-K166R_for (5'-GGT TAT TCT ATA TTT GTT GTT AGG GGT GAT CTG CCA GAT TGC G-3') and MJD1a-K166R_rev (5'- CGC AAT CTG GCA GAT CAC CCC TAA CAA CAA ATA TAG AAT AAC C-3'). Mutation of the expression plasmids was confirmed by automatic sequencing (STAB VIDA, Oeiras, Portugal).

Chapter 3 Results and Discussion

3.1 – Phosphorylation

3.1.1 – Amino acid sequence analysis of human ataxin-3 reveals 18 putative phosphorylation sites

As described above (see section 1.4.2), the only amino acid residues that are possibly subjected to phosphorylation are serines, threonines and tyrosines. Different kinases target residues according to the particular amino acid sequence in which they are inserted, and, taking into account the characteristics of these sequence motifs, it is possible to make a rough prediction of the potential phosphorylation sites of a protein, through an analysis of its primary sequence. Our analysis of the amino acid sequence of the human atx3 variant 1a (AAB33571.1) revealed 18 putative phosphorylation sites: 8 serines (S12, S29, S35, S256, S260, S265, S321 and S347), 5 threonines (T122, T138, T269, T274, T277) and 5 tyrosines (Y27, Y58, Y99, Y147 and Y160) (Table III). Some of these amino acids were revealed as possible targets of more than one kinase. As mentioned above, serines 256 and 260/261 have already been shown to be phophorylated (Fei et al., 2007; Mueller et al. 2009); serine 256 was however targeted by glycogen synthase kinase 3 β (GSK3β), an enzyme different than the one predicted (in the case of serine 260/261 it is not very clear whether it is phosphorylated by CK2). Importantly, serines 340 and 352, also described to be phosphorylated (Mueller et al. 2009), belong to the third UIM and are thus absent from this atx3 variant.

These prediction data allows the establishment of a starting point for the study of atx3 phosphorylation, considering that it points out a limited number of sequences whose characteristics make certain amino acids eligible for phosphorylation by various kinases. However, it should be noted that this is just a rough indication based on the protein primary sequence, hence experiments are necessary to determine which of the putative residues are actually modified.

Table III – Putative phosphorylation sites of human atx3 variant 1a (as predicted by Scansite 2.0; see Obenauer et al., 2003)

Amino acid residue	Sequence	Score	Prediction percentile	Surface accessibility	Kinase
Basophilic serine/threonine kinase group (Baso_ST_kin)					
S12	FHEKQEG**S**LCAQHCL	0,5329	4,786 %	0,235	PKCδ
T122	NYKEHWF**T**VRKLGKQ	0,4902	1,305 %	0,875	PKCα/β/γ
S256	LRRAIQL**S**MQGSSRN	0,4901	1,971 %	0,744	PKCδ
S260	IQLSMQG**S**SRNISQD	0,5514	4,175 %	2,307	PKCα/β/γ
S265	QGSSRNI**S**QDMTQTS	0,6980	3,310 %	1,029	Akt
S265	QGSSRNI**S**QDMTQTS	0,4923	0,550 %	1,029	CaMK2
S321	QQQQRDL**S**GQSSHPC	0,7189	4,131 %	0,971	Akt
DNA damage kinase group (DNA_dam_kin)					
S265	QGSSRNI**S**QDMTQTS	0,5021	0,542 %	1,029	DNA PK
S265	QGSSRNI**S**QDMTQTS	0,6252	4,260 %	1,029	ATM
T269	RNISQDM**T**QTSGTNL	0,5730	1,868 %	1,831	ATM
T269	RNISQDM**T**QTSGTNL	0,6078	2,840 %	1,831	DNA PK
T277	QTSGTNL**T**SEELRKR	0,6308	4,632 %	1,831	ATM
Acidophilic serine/threonine kinase group (Acid_ST_kin)					
S35	FSPVELS**S**IAHQLDE	0,6555	4,297 %	0,327	GSK3
T274	DMTQTSG**T**NLTSEEL	0,5018	3,275 %	0,872	CK1
Proline-dependent serine/threonine kinase group (Pro_ST_kin)					
S29	LLQGEYF**S**PVELSSI	0,6750	4,405 %	0,828	Cdc2
T138	FNLNSLL**T**GPELISD	0,6615	4,279 %	0,596	Erk1
S347	SDLGKAC**S**PFIMFAT	0,6750	4,405 %	0,156	Cdc2
Tyrosine kinase group (Y_kin)					
Y27	NNLLQGE**Y**FSPVELS	0,4998	3,292 %	1,104	EGFR
Y58	GGVTSED**Y**RTFLQQP	0,4927	3,745 %	2,542	PDGFR
Y58	GGVTSED**Y**RTFLQQP	0,7197	3,821 %	2,542	IR
Y99	ILFNSPE**Y**QRLRIDP	0,5101	3,952 %	2,690	EGFR
Y147	PELISDT**Y**LALFLAQ	0,5325	4,489 %	0,595	Fgr
Y160	AQLQQEG**Y**SIFVVKG	0,7244	4,099 %	0,501	IR

Score (0,0000-1,0000) represents the degree to which the sequence identifies with the quantified kinase preferences for each different amino acids in a motif, while percentile (0,000-100,000%) indicates the fraction of top fits in which the sequence is included, when compared to all the records of Vertebrate proteins from the Swiss-Prot database. So, in general terms, the predicted probability of a certain amino acid being phosphorylated increases with the increase of the score and the decrease of the percentile. Surface accessibility is calculated solely basing on the amino acid sequence. Bolded amino acid symbols represent the phosphorylation target of each sequence. The light blue shading signals the name of the different kinases groups analyzed, while the grey shading indicates sites that have already been described as being phosphorylated (Fei et al. 2007, Mueller et al., 2009). Kinase name abbreviations: PKC, protein kinase C; CaMK2, calmodullin-dependent kinase 2; DNA PK, DNA-dependent protein kinase; ATM, ataxia telangiectasia mutated kinase; GSK3, glycogen synthase kinase 3; CK1, casein kinase 1; Cdc2, cell division cycle 2 kinase; Erk1, extracellular signal-regulated kinase 1; EGFR, epidermal growth factor receptor kinase; PDGFR, platelet-derived growth factor receptor kinase; IR, insulin receptor kinase; Fgr, Gardner-Rasheed feline sarcoma viral oncogene homolog kinase.

3.1.2 – GFP-atx3 expression and purification

It was our goal to try and detect atx3 phosphorylation sites in purified protein samples obtained from a human cell line, since these expression conditions are much more likely to better reflect what happens in actual human cells, as far as post-translational processing of atx3 is concerned. This procedure doesn't allow for such a large scale of protein purification as other methods do, but, in fact, bacteria, though yielding large amounts of recombinant proteins, do not perform PTMs, and even for eukaryotic organisms, the more evolutionarily apart they are, the more unlikely are they to process their proteins in the same way – thus, using eukaryotic cell lines other than human is still not sufficient. Detection of phosphorylation in proteins derived from human cells gives a much stronger indication that the observed modification is biologically relevant. Additionally, since we aimed at detecting atx3 phosphorylation sites using MS techniques specially directed to the study of this PTM, two other main goals had to be reached: a) to obtain highly pure proteins samples, since any impurity might hinder or interfere with the extremely sensible MS analysis; b) to obtain a large protein quantity, so as to allow for the analysis of the greatest number of peptides possible (see section 3.1.4 for more details on this).

We thus expressed GFP-atx3 in HEK 239FT cells and purified it by IP using a monoclonal anti-GFP antibody. Evaluation of the efficacy of the procedure was done by Western blot analysis, through observation of the enrichment of the protein of interest in the inputs (both soluble and insoluble fractions) and the immunoprecipitant (Figure 7).

GFP-atx3 expression was highly effective, considering that a clear ~75 KDa band was detected in the transfected cells soluble input sample, but not in the non-transfected control (~75 KDa is the molecular mass expected for this fusion protein). Furthermore, the small amount of GFP-atx3 present in the insoluble fraction of the transfected cells (considering that the electrophoresed sample corresponds to 10x the

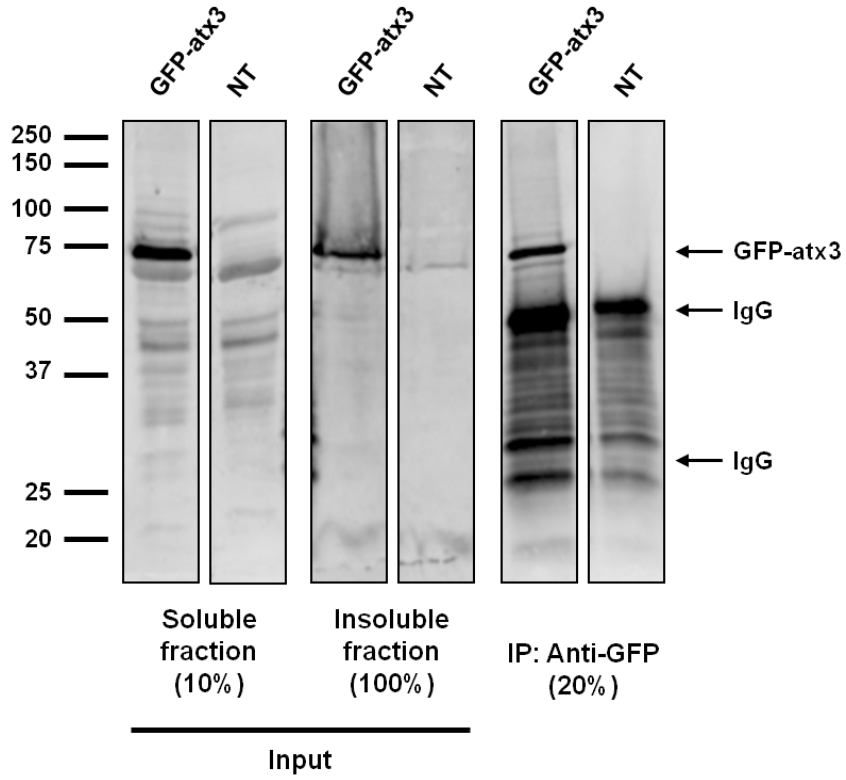

Figure 7 – Expression and IP of GFP-atx3. HEK 293FT cells were transiently transfected with GFP-atx3 and expression was left to occur for 24 h. The protein was purified by IP using a monoclonal anti-GFP antibody and a slurry of protein A Sepharose beads, and the resulting immunoprecipitant samples, along with the soluble and insoluble fractions of the input, were analyzed by Western Blot with the anti-GFP antibody. Observation of the GFP-atx3 signal in the input samples indicates that expression and solubilization of the protein were successfully achieved; the GFP-atx3 band present in the immunoprecipitant attests for the efficacy of the IP procedure. Relative protein amount contained in each electrophoresed sample is represented in brackets as a percentage of the total starting protein mass subjected to IP. NT refers to the non-transfected cells controls.

protein mass of the soluble inputs) indicates that our procedure efficiently solubilized GFP-atx3.

Analysis of the immunoprecipitants content revealed that GFP-atx3 IP was successfully achieved. Purity of the IP samples is further attested by the absence of

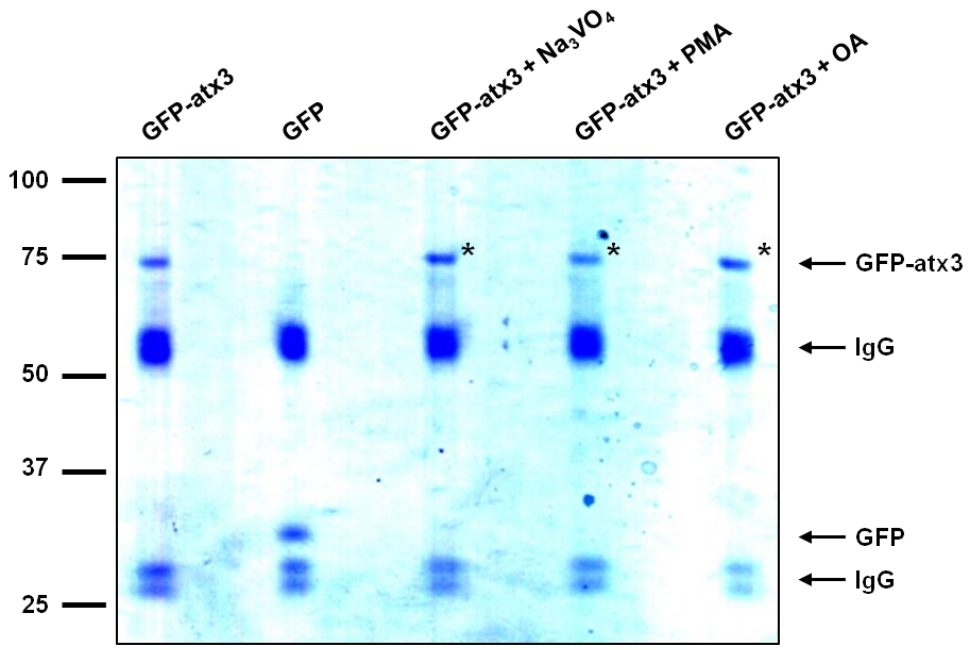

Figure 8 – GFP-atx3 SDS-PAGE protein bands subjected to MS analysis. HEK 293FT cells were transiently transfected with GFP-atx3 and expression was left to occur for 24 h, during which time period cells were stimulated with Na_3VO_4, PMA or OA, as described. GFP-atx3 was then purified by IP using a monoclonal anti-GFP antibody and a slurry of protein A Sepharose beads. The protein content of the immunoprecipitants was separated by SDS-PAGE (using a PROTEAN® II xi system) and the resulting gel was stained with Coomassie blue. Asterisk (*) represents bands subjected to MS analysis. Empty vector encoding GFP was used as negative control.

any significative protein bands in Coomassie stained SDS-PAGE gels, other than IgG chains and GFP-atx3 itself (Figure 8). As detailed above (see section 2.7), the amount of purified GFP-atx3 that was intended to be analyzed by MS was increased by triplicating the total protein mass that was subjected to IP, with no parallel increase of the final immunoprecipitant volume.

3.1.3 – Probing with a phosphotyrosine-specific antibody

To this date, though there are several tyrosine residues in atx3 putatively targeted for phosphorylation, studies so far have mainly focused on serine/threonine phosphorylation. Moreover, in their most recent work, Mueller and collaborators (2009) could not detect phosphotyrosines in the phosphoamino acid analysis of atx3.

As a first approach to determining the actual relevance of tyrosine phosphorylation in atx3, before MS experiments, we analyzed some IP-purified GFP-atx3 samples obtained from transfected HEK 293FT by Western blot with an anti-phosphotyrosine antibody (Figure 9). In order to enhance protein phosphorylation, cells were also stimulated with one of two phosphatase inhibitors, OA or Na_3VO_4 (which inhibit serine/threonine or tyrosine phosphatases, respectively), or the protein kinase C (PKC) stimulator PMA (selected for the fact that many putative phosphorylation sites are targeted by PKC). OA and PMA served as negative controls for the expected Na_3VO_4-enhanced tyrosine phosphorylation. Though the anti-phosphotyrosine antibody labeling revealed a protein band that seems to correspond to GFP-atx3 (Figure 10B), possibly indicating that the protein is phosphorylated in tyrosine residues under our experimental conditions, the labeling is in fact very weak and the Na_3VO_4 stimulus doesn't seem to significantly increase the signal we observed.

In order to clarify this situation, we also tried the reverse experiment, immunoprecipitating the tyrosine-phosphorylated proteins from transfected HEK 293FT cells stimulated with Na_3VO_4 using the anti-phosphotyrosine antibody and then seeing if we could detect GFP-atx3 in the immunoprecipitants (Figure 10). Though we were able to detect GFP-atx3 using this procedure, we also immunoprecipitated the protein with the pre-immune IgG control, which keeps us from being able to elaborate any conclusion about GFP-atx3 phosphorylation in tyrosines. This unspecific reaction may be due to the "sticky" nature of atx3, whose properties tend to make it aggregate with other molecules. Interestingly, however, the band we believe to correspond to GFP-

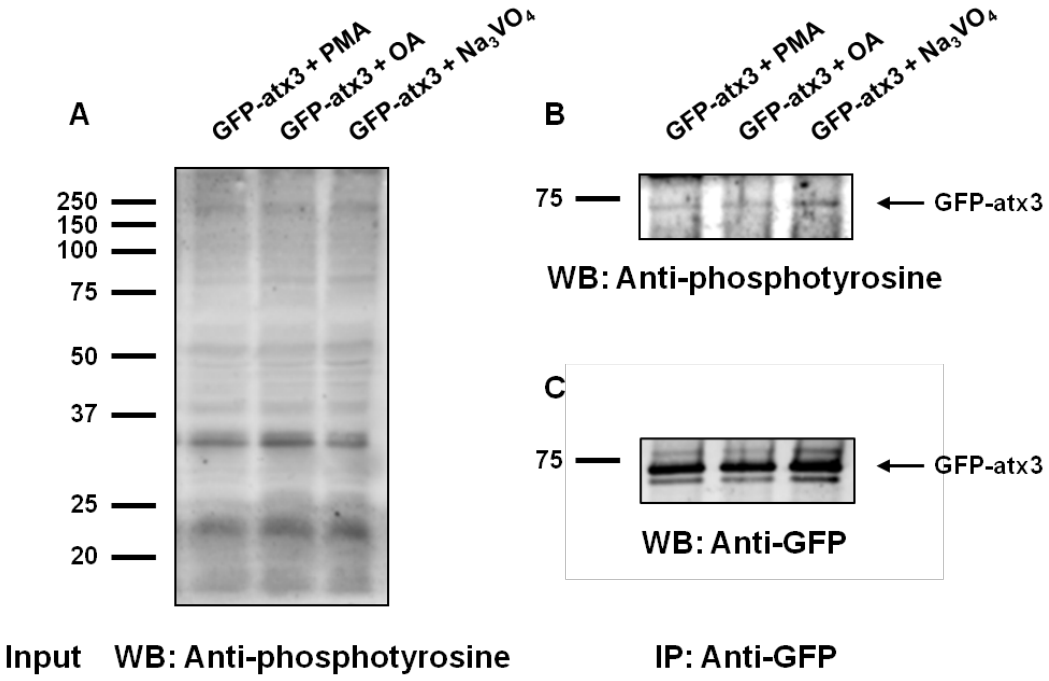

Figure 9 – Western blot analysis of GFP-atx3 with an anti-phosphotyrosine antibody. GFP-atx3 was expressed in HEK 293FT cells for 24 h, during which time period cells were stimulated with Na_3VO_4, PMA or OA, as described. (*A*) Input tyrosine-phosphorylated protein content was analyzed through Western with a monoclonal anti-phosphotyrosine antibody, yielding an indistinguishable smear. Samples resulting from IP with a monoclonal anti-GFP antibody were probed with both the (*B*) anti-phosphotyrosine antibody and the (*C*) anti-GFP antibody. The phospho-specific antibody revealed a very weak protein band that might correspond to GFP-atx3.

atx3 seems to be a little heavier than usual in the samples immunoprecipitated with the phospho-specific antibody, specially comparing to the pre-immune IgG control. The shift in the mobility of GFP-atx3 could eventually be due to the attachment of one (or more) phosphate group(s), supporting the idea of tyrosine phosphorylation of GFP-atx3.

Taken together, our observations do not allow us to formulate any decisive conclusion about tyrosine phosphorylation of atx3. Considering what has been

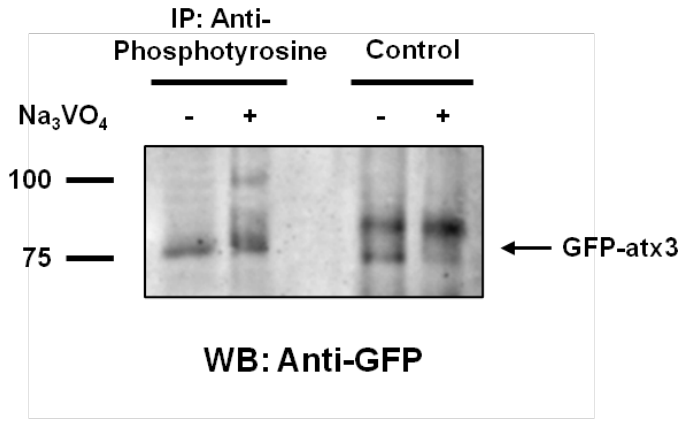

Figure 10 – IP of GFP-atx3 using an anti-phosphotyrosine antibody. GFP-atx3 was expressed in HEK 293FT cells for 24 h. After being stimulated with Na_3VO_4 as described, cells were harvested and then tyrosine-phosphorylated proteins were immunoprecipitated using a monoclonal anti-phosphotyrsorine antibody and a slurry of protein A Sepharose. The resulting immunoprecipitants were electrophoresed (in a PROTEAN® II xi system) and probed for the presence of GFP-atx3 with a monoclonal anti-GFP antibody. IP control was made by replacement of the anti-phosphotyrosine antibody with pre-immune IgGs. GFP-atx3 is present in both the anti-phosphotyrosine antibody immunoprecipitants and the negative controls, making any conclusion regarding GFP-atx3 tyrosine phosphorylation unfeasible.

previously observed by Mueller et al. (2009), we believe that this lack of clear evidence for tyrosine phosphorylation in our experiments may be indicative of low levels of tyrosine phosphorylation of atx3 (if they any at all).

3.1.4 – Mass spectrometric analysis of GFP-atx3 detects phosphorylation of serine 12

During the past years, MS has turned into a well-established method for the detection and identification of phosphorylated peptides, allowing the direct mapping of

particular phosphorylated amino acid residues (Macek et al., 2009). Notably, previous studies concerning other polyQ expansion diseases proteins (in particular huntingtin and the AR) have successfully employed MS in their search for phosphorylation sites (Gioeli et al., 2006; Guo et al., 2006; Schilling et al., 2007). The main principle behind the procedure is the fact that the attachment of a phosphate group alters the molecular mass/charge ratio of a peptide, a change that can be detected by the mass spectrometer. In one particular strategy, when phosphopeptides are subjected to molecular fragmentation during the MS procedure, they may release free phosphate groups. The mass spectrometer is able to detect this neutral loss of a phosphate group (which corresponds to a mass shift of 80 or 98 *m/z*) and at the same time identify the peptides, based on the time of flight of the several fragments produced (Macek et al., 2009). Analyzing the data altogether makes the exact mapping of phosphorylated sites possible.

Therefore, in conformity with our main objective, in order to straightforwardly identify phosphorylated residues in GFP-atx3, we subjected the protein to that kind of mass spectrometric analysis, using a LC-ES-MS system (as detailed in section 2.9 – MS analysis). For this analysis we used IP-purified GFP-atx3 obtained from HEK 293FT cells stimulated with OA, Na_3VO_4, or PMA, brought into play with the objective of enhancing possible protein phosphorylation. After separating the immunoprecipitant content by SDS-PAGE (Figure 8), GFP-atx3 protein bands were subjected to a tryptic digestion and the resulting peptides were thus processed and analyzed by the MS system for detection of phosphate group-yielding peptides and respective amino acid sequence identification.

Using this experimental approach, we were able to identify one phosphorylated peptide in the GFP-atx3 sample resulting from the OA-stimulated cells – $Q^{9}EG\mathbf{pS^{12}}LCAQHCLNNLLQGEYFSPVELSSIAHQLDEEER^{45}$ – determining that serine 12 is phosphorylated in GFP-atx3, under our experimental conditions (Figure 11). These results constitute strong evidence for serine 12 being a novel phosphorylation

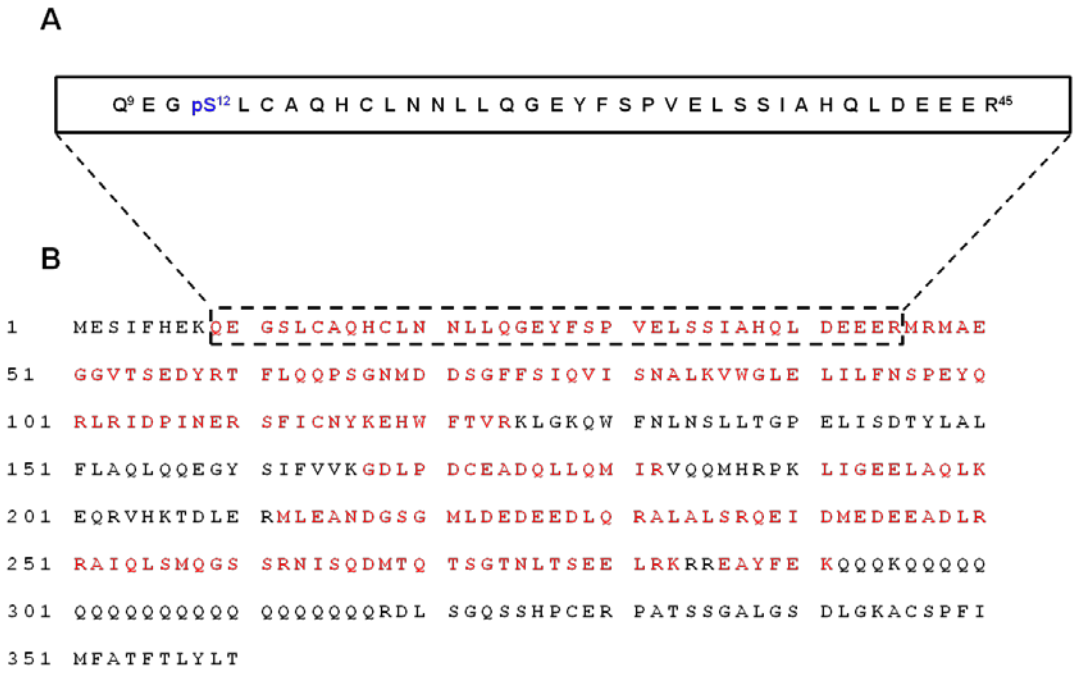

Figure 11 – LC-ES-MS detection of serine 12 phosphorylation in GFP-atx3. An IP-purified GFP-atx3 sample obtained from transfected HEK 239FT cells stimulated with OA was subjected to mass spectrometric analysis directed for the detection of phosphorylated peptides. (*A*) One monophosphorylated peptide was detected and the phosphorylation site mapped to the serine residue in position 12. (*B*) Human at3 variant 1a aminoacid sequence is represented with the peptides covered by the MS analysis colored in red.

site of atx3. Additionally, as our starting GFP-atx3 protein sample was obtained from cells stimulated with a serine/threonine phosphatase inhibitor, OA, and no other artificial stimuli, we admit that phosphorylation of serine 12 may happen natively under the cell culture conditions we used, with the phosphatase inhibition only eventually increasing the fraction of phosphorylated protein. This indicates that serine 12 phosphorylation may happen normally in cells and thus may have some kind of biological relevance.

Other GFP-atx3 samples didn't, however, yield any detectable phosphorylated peptides, and detection of the peptide referred above was somewhat weak, too. MS is

a technique for the detection of peptides, but because of the nature of the procedures and principles involded, only a fraction of the peptides of a sample may be expected to be detected. Therefore, the absence of detection must not be taken to mean that a named peptide (modified or not) does not exist in the starting sample; the only conclusion one must take is that detection was unsuccessful. In MS experiments, the more the protein amount analyzed, the better the peptide detection and the higher the percentage of amino acid sequence coverage. Additionally, it should be considered that only a relatively small fraction of a given protein shall be expected to be phosphorylated; this, coupled with possible dephosphorylation events during the IP procedure and an insufficiently high sample protein amount may explain the lack of detection of any additional phosphorylated peptides. This may also account for the lack of detection of peptides containing phosphorylated residues (serines 236, 256 and 260/261) already described by other authors (Fei et al., 2007; Mueller et al., 2009).

Everything considered, though subsequent experiments will be necessary to confirm our results (see section 4.2), MS analysis of GFP-atx3 was able to detect a new phosphorylation site of atx3, serine 12. The way we performed our experiments also points out for this modification to happen normally in cells.

Our observations contrast in part with the results obtained from Mueller et al. (2009), who were unable to detect phosphorylation of the N-terminal of atx3 using ^{32}P-orthophosphate-labeled atx3 fragments. Seeing as both studies were made using HEK 293 cells, this lack of detection by Mueller and coworkers may be due to low levels of serine 12 phosporylation in their samples (insufficiently high for the radio-labeling detection), comparing with our OA-stimulated samples. The strong signal they detected in the C-terminal region was conceivably due to the combination of the radioactive signals from the several phosphorylated sites present in the UIMs; notably, however, mutation of all these residues did not completely abolish full-length atx3 phosphorylation signal, indicating that other phosphorylated residues might be present.

On our part, stimulation of the cells with OA combined with the MS strategy presented above allowed us the detection of phosphorylated serine 12.

The phosphorylated amino acid prediction referred above presented serine 12 as being putatively phosphorylated by PKCδ (Table III). Using another bioinformatic tool for the comparison of human atx3 variant 1a amino acid sequence with the consensus sites usually selected by a number of different kinases, we were able to determine other kinases possibly involved in serine 12 phosphorylation (Table IV). Out of all the putative kinases, PKCδ (as predicted by Scansite) and PKA (predicted by NetPhosK) were the ones with higher theoretical probability of phosphorylating serine 12. As it will be discussed later, however, since these predictions are based only on the amino acid sequence preferences of the several kinases covered by these methods, more experiments are necessary for determining the kinase(s) responsible for the modification of serine 12 (see section 4.2).

Table IV – Kinases putatively responsible for the phosphorylation of serine 12 from human atx3 variant 1a (as predicted by NetPhosK 1.0; see Blom et al., 2004)

Kinase		Score
Protein kinase A	(PKA)	0,57
DNA-dendent protein kinase	(DNA PK)	0,48
Cell division cycle 2 kinase	(Cdc2)	0,46
Glycogen synthase kinase 3	(GSK3)	0,46
Calmodullin-dependent kinase 2	(CaMK2)	0,42
Casein kinase 1	(CKI)	0,38
Protein kinase C	(PKC)	0,32

Score values (0,00-1,00) define how well the amino acid sequence containing the phosphate-accepting residue conforms with the sequence preferences determined for each kinase.

3.1.5 – Serine 12 localizes close to the catalytic site and is accessible at the surface of the Josephin domain

Analysis of atx3 primary structure reveals that serine 12 is localized in the JD, very close to the cysteine residue of the active site (C14), and glutamine 9, another residue that is important for the catalytic activity of the protein (Masino et al., 2003) (Figure 12). Moreover, observation of the tertiary structure of the JD (Nicastro et al., 2005;) showed that serine 12 localizes not only very close to cysteine 14 and glutamine 9, but is also in the proximity of the amino acid residues that, along with cysteine 14, make up the catalytic triad - histidine 119 and asparagines 134 (Mao et al., 2005; Nicastro et al., 2005) (Figure 13). The JD is mainly composed of two subdomains, a globular catalytic subdomain and a helical hairpin, with the catalytic site being localized in the cleft that exists in the region of contact between them. Importantly, the overall structure does not allow for but a very limited movement between the two subdomains. Taking into consideration the proximity of serine 12 to the amino acids of the active site and the fact that it is localized in the region of contact between the two subdomains of the JD, it is reasonable to speculate that linkage of a phosphate group to that serine

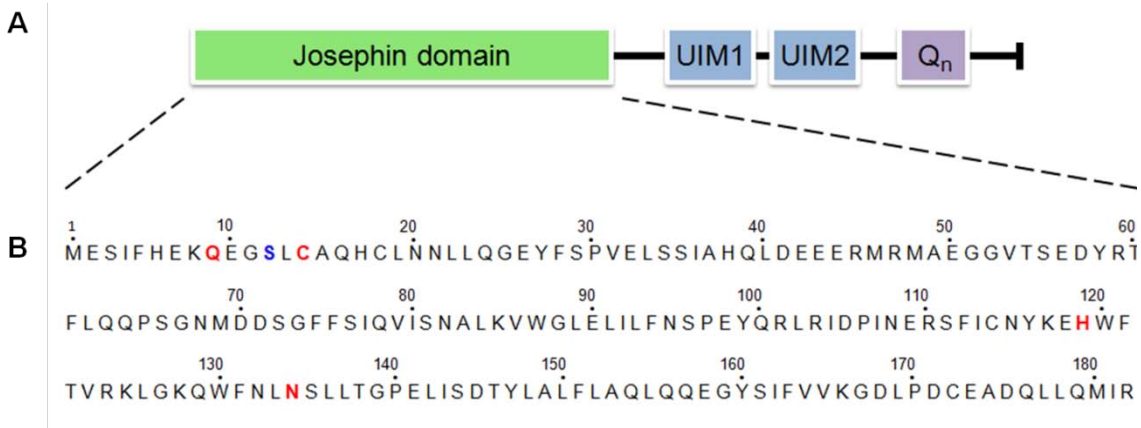

Figure 12 – Serine 12 is localized in the JD, close to the cystein residue of the active site. (*A*) Human atx3 variant 1a domain structure is schematically represented with (*B*) the JD aminoacid sequence displayed in detail. The active site residues are colored in red and serine 12 in blue.

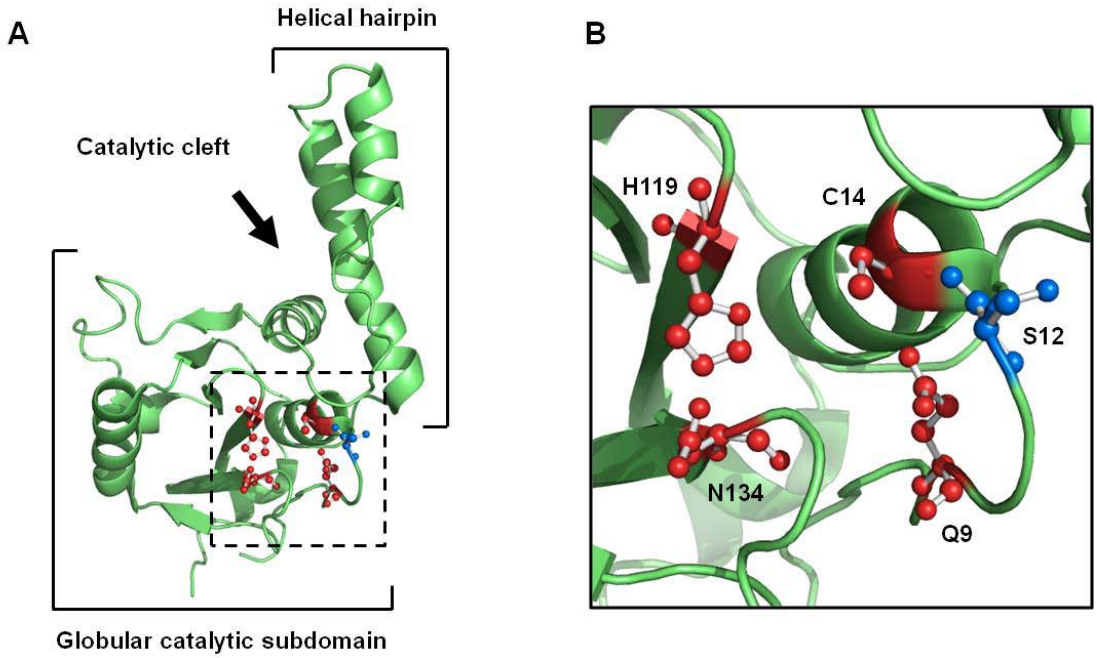

Figure 13 – Serine 12 localizes in the vicinity of the aminoacids of the active site. Tertiary structure of the JD of atx-3 (Nicastro et al., 2005; PDB ID code 1YZB) is represented as a ribbon diagram, with serine 12 (in blue) and the aminoacids of the active site (in red) displayed as ball-and-stick models. (*A*) Serine 12 localizes in the contact region between the two subdomains. The close distribution of the active site residues and serine 12 is highlighted and detailed in (*B*).

residue is likely to affect the proteolytic activity of the JD of atx3. The presence of a negatively-charged phosphate group conjugated with the serine 12 side chain may produce changes possibly affecting a) the catalytic site conformation, consequently changing its aptitude to cleave the isopeptide bonds existing between Ub molecules (whether increasing or decreasing it), b) the way the JD interacts with the substrate(s), and/or c) the access and alignment of the catalytic site to the isopeptide bonds to be cleaved.

In order to graphically observe the relative accessibility of the serine 12 residue to the kinase(s) possibly responsible for its phosphorylation, we looked at the JD surface and examined the location of that serine residue (Figure 14A). It is clear that when the JD is in its folded conformation, serine 12 localizes in the surface of the

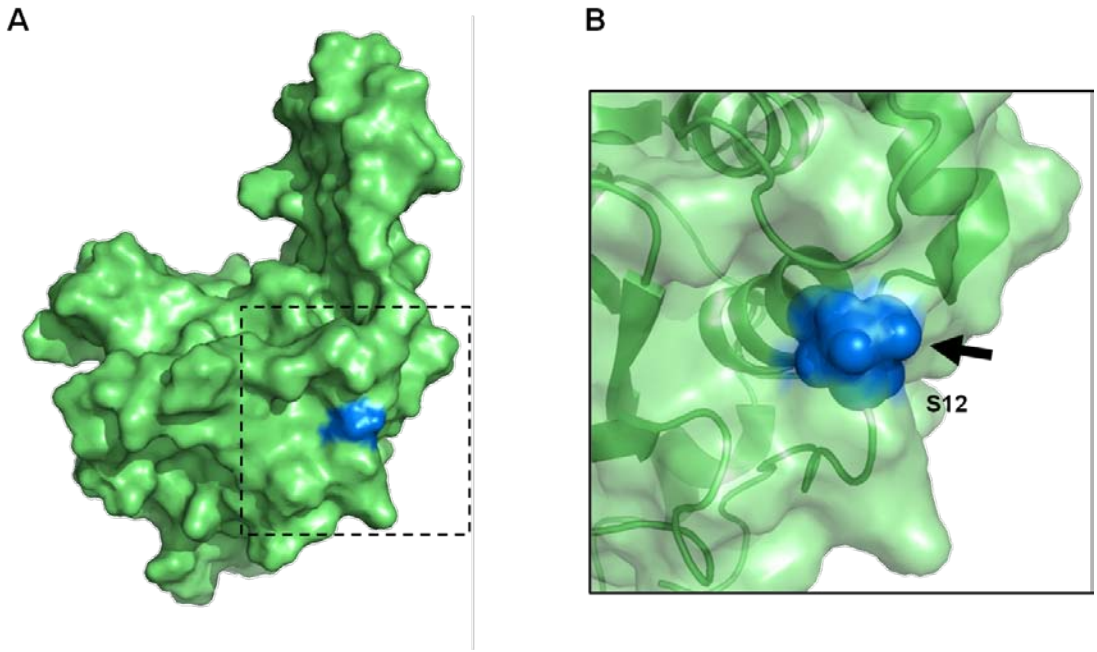

Figure 14 – Serine 12 distributes at the surface of the JD. (*A*) Surface of the JD of atx-3 (Nicastro et al., 2005; PDB ID code 1YZB) is represented with serine 12 colored in blue. (*B*) Tertiary structure in the close vicinity of serine 12 is displayed as a ribbon diagram under the transparent surface, while the serine residue itself is represented as spheres. Arrow indicates the phospho-accepting hidroxyl group of serine 12 side chain.

domain, with its hydroxyl group-containing side chain turned towards the solvent (Figure 14B). Serine 12 thus appears to be relatively accessible for the interaction with the enzyme(s) possibly catalyzing its phosphorylation and to the subsequent attachment of a phosphate group.

3.2 – Sumoylation

3.2.1 – Amino acid sequence analysis of human ataxin-3 reveals 4 putative sumoylation sites, three of them localized in the Josephin domain

In line with what has been said above for the prediction of possible phosphorylation sites, we analyzed the primary structure of human atx3 variant 1a, this

time searching only for amino acid sequences that somehow conformed to one particular tetrapeptide motif usually found in sumoylation targets: Ψ-K-X-D/E, where Ψ is a hydrophobic residue, K is the lysine residue that conjugates with SUMO, X is any amino acid and D/E is an aspartate or glutamate residue. This motif is viewed as the main sumoylation target sequence, taking into consideration its frequency in sumoylation targets and the fact that it is directly recognized by Ubc9, the only E2 enzyme of the SUMO conjugating pathway, being important in the positioning of this protein and the fine-tuning of substrate lysine specificity (Anckar & Sistonen, 2007). This search exposed 4 putative sumoylation sites: lysines 8, 125, 166 and 206, with lysine 166 presenting a very high associated score (Table V). Three of this putative sites (the exception being lysine 206) are contained in the JD, the catalytic domain of atx3 (amino acids 1-182; Masino et al., 2003); thus, it is plausible to think that, should any of them be an actual target for sumoylation, there could be some kind of regulatory function associated with their modification by SUMO.

Table V: Putative sumoylation sites of human ataxin-3 variant 1a (as predicted by the SUMOplot™ Analysis Program)

Amino acid residue	Sequence/group	Score
K8	ESIFH **EKQE** GSLCA	0,50
K125	HWFTV **RKLG** KQWFN	0,27
K166	YSIFV **VKGD** LPDCE	0,93
K206	KEQRV **HKTD** LERML	0,52

Score values (0,00-1,00) represent the probability for the recognized sequences to be targeted by sumoylation considering the degree of amino acid conformity with the Ψ-K-X-D/E sumoylation motif. Bold indicates the residues belonging to the recognized sequences, while underscore signals the actual putative lysine residue.

It should be noted, however, that although sumoylation is highly lysine-specific, not all Ψ-K-X-D/E tetrapeptides are in fact modified by SUMO. It has been suggested that other determinants may play important roles in defining the targeted lysines, with negative charges (either resulting from the presence of negatively-charged amino acids or phosphorylated amino acids) downstream the consensus site being pointed as possible additional signals; a positively-charged patch existing in the Ubc9 enzyme appears to be of significant importance in regulating sumoylation mediated by this determinants (Anckar & Sistonen, 2007; Mohideen et al., 2009). Moreover, recent developments have identified many SUMO-modified proteins lacking any known consensus site, consubstantiating the idea that, though the Ψ-K-X-D/E motif is frequently found in sumoylated protein, other as of yet unknown mechanisms completely independent of this tetrapeptide and probably involving more than a linear peptide motif may be important in targeting lysine residues for sumoylation (Blomster et al., 2009). As this prediction relies only on the comparison with the Ψ-K-X-D/E motif, it is excluding those other determinants and unknown factors regulating sumoylation. In accordance with what has been stated before, this kind of prediction is not sufficient to generate any conclusion regarding actual sumoylation of a particular lysine residue, but helps to direct the subsequent experiments, starting from the basis established by the protein sequence analysis tools.

3.2.2 – 6His-JD conjugates with SUMO2 under *in vitro* sumoylation reaction conditions

In order to determine if atx3 is a target for SUMO conjugation, we started with an *in vitro* approach, by performing sumoylation assays with an *in vitro* sumoylation kit (SUMOylation kit, Biomol). For these experiments we used 6His-JD instead of full length 6His-atx3, for two main reasons: a) the majority of the putative sumoylation sites are localized in the JD and b) should sumoylation be detected we would know that the

catalityc domain is a target for this modification. Since it was our goal to use a highly concentrated protein sample and we wished the protein was not post-translationally modified to begin with, we produced recombinant 6His-JD in *E. coli* and purified it by a series of chromatographic techniques using a protocol established by our group (Gales et al., 2005).

Previous results obtained from *in vitro* sumoylation assays performed by our group (Mazovec G. & Macedo-Ribeiro S., 2009, personal communication) suggested no significant involvement of SUMO1 and SUMO3, and so we focused our study on SUMO2 alone. We thus incubated 6His-JD with 6His-SUMO2-aa in the presence of SUMO E1 and SUMO E2 (Ubc2), under the optimal reaction conditions. Western blot analysis of the resulting samples with a anti-SUMO2/3 antibody revealed the presence of a protein species with a molecular weight of ~37 KDa, higher than 6His-SUMO2-aa molecular weight of ~16 KDa; considering that 6His-JD alone has ~24 KDa (Gales et al., 2005), it is plausible to admit that the observed band corresponds to 6His-JD conjugated with 6His-SUMO2-aa (Figure 15). After reprobing with an anti-6His antibody, a large amount of unconjugated 6His-JD was still observed and no 6His-JD band with that higher molecular weight was detected; we can thus admit that only a small fraction of the total 6His-JD seems to be sumoylated under these conditions. Interestingly enough, existing evidences state that the fraction of a certain protein targeted by sumoylation that is actually modified by this process is very low (Hay, 2005). Since we observed only that single band corresponding to sumoylated 6His-JD, we admit that our procedure just produced monosumoylated 6His-JD. This antibody did not detect 6His-SUMO2-aa probably because it is not sensible enough to detect the small amounts of 6His-SUMO2-aa used in the assay; in fact, though the protein is tagged with 6His, the manufacturer recommends usage of the SUMO2/3-specific antibody.

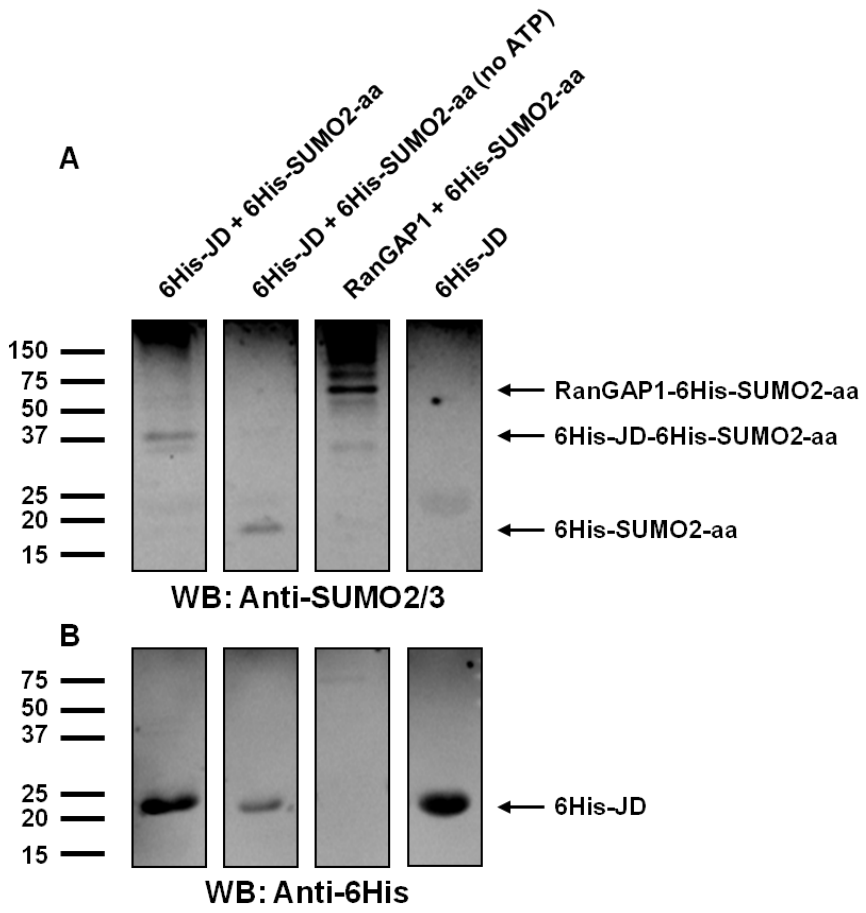

Figure 15 – *In vitro* sumoylation of 6His-JD. Recombinant 6His-JD expressed in *E. coli* and purified through a series of chromatographic steps was incubated for 1 h, at 37ºC, with 6His-SUMO2-aa and SUMO enzymes E1 and E2. The resulting sample and respective controls were analyzed by Western blot with (*A*) a polyclonal anti-SUMO2/3 antibody and (*B*) a polyclonal anti-6His antibody, revealing a protein band of ~37 kDa that is likely to correspond to 6His-JD conjugated with 6His-SUMO2-aa (6His-JD-6His-SUMO2-aa). This band is absent in the negative control, with no ATP supply. Positive control was made using RanGAP1, a protein known to be sumoylated (RanGAP1-6His-SUMO2-aa) (Saitoh & Hinchey, 2000).

These results strongly suggest that the catalytic domain of atx3 may be modified by conjugation with SUMO2, constituting the first plain evidence for atx3 sumoylation.

Out of the three major SUMO isoforms, SUMO2 and SUMO3 are the ones sharing the greater homology (~95%), with SUMO1 sequence identifying only by ~50% with those of the other two (Dorval & Fraser et al., 2007). Though these three isoforms may conjugate with the same substrates under certain conditions, SUMO1 and SUMO2/3 are functionally different, being regulated by the cells through different mechanisms (Dorval & Fraser, 2007; Saitoh & Hinchey, 2000). They are differently expressed depending on the cell type in question and display characteristic subcellular localizations; interestingly, SUMO2, like atx3, is found both in the cytoplasm and in the nucleus, though predominantly in the nucleus (Vertegaal et al., 2004). Importantly, a recent study showed that the majority of SUMO2 substrates' function is related to nucleic acid biology (Blomster et al., 2009); modification of atx3 by SUMO2 and possibly the interactions it mediates may influence the protein's role in transcription regulation. Moreover, seeing as atx3 nuclear localization is important in determining its toxicity (Bichelmeier et al., 2007), there can be some mechanisms through which SUMO2 conjugation with polyQ expanded atx3 in the nucleus influences the pathways leading to MJD.

Tough we observed no polysumoylation, SUMO2 and SUMO3 have been frequently related with the formation of polySUMO chains, which is mediated by the Ψ-K-X-D/E sumoylation motif they have (Dorval & Fraser, 2007; Vertegaal et al., 2004). Though there are no other reasons to speculate that atx3 is polysumoilated, it is also possible that our experimental conditions may not be enough for the constitution of polySUMO chains. What is more, though many recently unveiled SUMO2 substrates present the Ψ-K-X-D/E motif, a similar amount of protein lacked any known consensus sequence, keeping us from being able to anticipate which JD lysine(s) are more likely to conjugate with this SUMO isoform. Interestingly, recent evidences suggest that SUMO-2 targeting mechanism depends on the biological role of the particular substrate (Blomster et al., 2009).

Ubiquitination of the JD alone was shown to be sufficient to increase its catalytic activity (Todi et al. 2009). Considering the often observed interplay between sumoylation and ubiquitination, which sometimes includes the targeting of the same lysine residues by the two PTMs, it is plausible to hypothesize the existence of a putative mechanism for the regulation of atx3 activity, resulting from the interplay between JD ubiquitination and sumoylation. This interplay may also influence the protein stability, taking into account polyUb role in the UPP.

Be as it may, considering that these *in vitro* observations rely on strictly controlled and optimized conditions, other experiments are required in order to understand the authentic relevance of atx3 sumoylation by SUMO2 in the actual cellular context.

3.2.3 – Cotransfection experiments were inconclusive in determining whether ataxin-3 conjugates with SUMO2 in cells

In order to try and detect SUMO conjugation with atx3 in mammalian cells, we immunoprecipitated GFP-atx3 from HEK 293FT cells expressing both GFP-atx3 and 6His-SUMO proteins (6His-SUMO1, 2 and 3) with the anti-GFP antibody and analyzed the resulting samples for detection of 6His-SUMO by Western blot (Figure 16C, D). Although the probing with the anti-6His antibody yielded no detectable bands that might correspond to 6His-SUMO protein conjugated with GFP-atx3, it should be noted, however, that no bands corresponding to 6His-SUMO were observed in the inputs themselves (Figure 16A, B). This can be due to a very low expression of the His6-SUMO proteins or be the result of low sumoylation levels, considering that normally, in cells, only a small fraction of a named protein is eventually sumoylated (Hay, 2005).

In order to concentrate the 6His-SUMO proteins and the proteins that conjugated with them in the cells, we tried a reverse approach, by purifying the 6His-SUMO proteins using a Ni Sepharose resin (IMAC). This time, we also aimed at

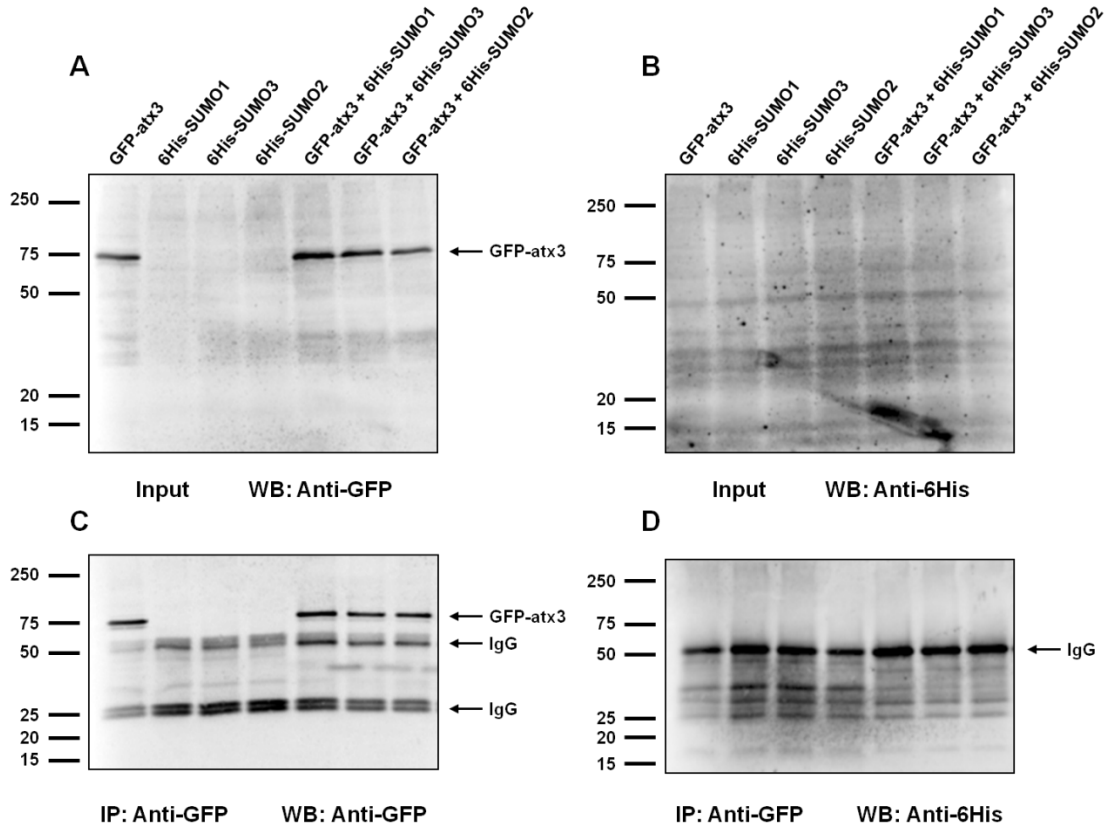

Figure 16 – IP experiments with cells cotransfected with GFP-atx3 and 6His-SUMO proteins. HEK 293FT cells were cotransfected with both GFP-atx3 and one 6His-SUMO isoform (6His-SUMO1, 2 or 3) and expression was left to occur for 24 h. Inputs (*A*, *B*) and samples resulting from IP with a monoclonal anti-GFP antibody (*C*, *D*) were analyzed by Western blot with both the monoclonal anti-GFP antibody and a polyclonal anti-6His antibody. Expression of the 6His-SUMO constructs may not have been successful, taking into consideration that no clear band corresponding to 6His-SUMO proteins (~20 KDa; Dorval and Fraser, 2007) was observed in the inputs.

increasing the sumoylation levels by submitting the cotransfected cells to a heat shock treatment (1h at 42 ºC), since it has been previously described that stress stimuli increase the attachment of SUMO2 to their target proteins (Blomster et al., 2009; Saitoh & Hinchey, 2000). Additionally, SUMO2 localizes predominantly in the nucleus (Vertegaal et al., 2004); taking that into consideration and seeing that heat stress

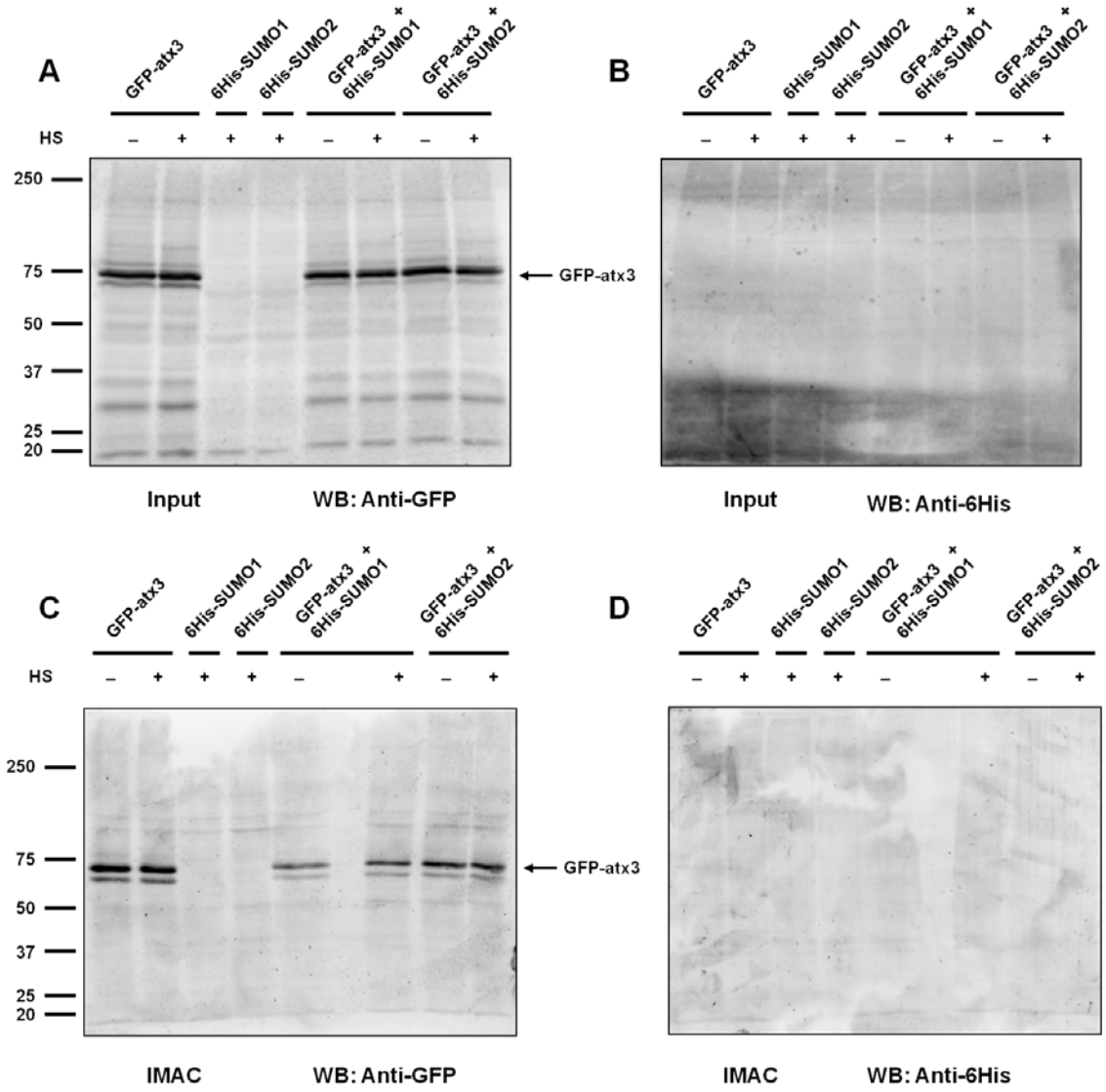

Figure 17 – IMAC purification of 6His-SUMO from cells cotransfected with GFP-atx3 and 6His-SUMO. HEK 293FT cells were cotransfected with both GFP-atx3 and 6His-SUMO isoforms 1 or 2 (6His-SUMO1 or 2) and expression was left to occur for 24 h. During the last 1 h of protein expression, cells were subjected to a heat shock stimulus (HS), through incubation at 42 ºC. Inputs (*A*, *B*) and samples resulting from IMAC with Ni resin (*C*, *D*) were analyzed by Western blot with both a monoclonal anti-GFP antibody and a polyclonal anti-6His antibody (IMAC-purified samples were electrophoresed using a PROTEAN® II xi system). Expression of the 6His-SUMO constructs was unsuccessful once again and purification procedure was flawed, since GFP-atx3 was found in the IMAC-purified samples transfected only with GFP-atx3.

increases atx3 nuclear distribution (Mueller et al., 2009), we thought this procedure could potentiate sumoylation of atx3 in particular. In these experiments we focused only on 6His-SUMO1 and 2, since we wanted to compare between the SUMO isoform we determined to conjugate with 6His-JD (SUMO2) and one SUMO isoform that is not involved in the modification of 6His-JD, under the *in vitro* reaction conditions (SUMO1) (Mazovec G. & S. Macedo-Ribeiro, personal communication).

Western blot analysis of the samples resulting from the Ni resin purification (Figure 17C, D) showed bands corresponding to GFP-atx3 with the same molecular weight as free GFP-atx3, and their detection was also possible in the case of the samples resulting from transfection with GFP-atx3 alone. As such, we admit that our experimental procedure was not effective at the Ni Sepharose purification level, but apparently may have also been ineffective during the very expression of the 6His-SUMO constructs, considering that, once again, we were unable to detect 6His-SUMO even in the inputs (Figure 17A, B). Once again, we cannot exclude the possibility of the sumoylation levels being very low under our experimental coditions; future studies shall also include a positive control, like RanGAP1, for instance, that will allow as to test this prospect (Saitoh & Hinchey, 2000).

Taking these points into consideration, though we were unable to detect conjugation between GFP-atx3 and 6His-SUMO in HEK 293FT cells using these experimental approaches, we can't rule out the possibility of atx3 being modified by SUMO *in vivo*. Further experiments will be necessary to correctly study the eventual conjugation between atx3 and SUMO in cells (see section 4.2).

Chapter 4 Closing remarks

4.1 – Conclusions

The main aim of the experimental work that has been presented here was to determine novel PTMs affecting atx3, the protein causatively involved in the development of MJD. PTMs affect a vast number of protein properties and may thus account for the regulation of atx3 localization, activity, stability and aggregation, possibly being important in the pathogenesis mechanisms of MJD. Throughout this work we focused mainly on two PTMs: phosphorylation and sumoylation.

Through mass spectrometric analysis of GFP-atx3 produced in HEK 293FT cells, we were able to detect phosphorylation of serine 12, a residue that is located at the surface of the JD and may therefore be exposed to this PTM. Additionally, serine 12 is in close proximity to the catalytic site (especially to cysteine 14), which leads us to speculate that addition of a phosphate group to this serine residue may somehow affect atx3 enzymatic activity, possibly constituting a regulatory mechanism of its biological function, in the overall cell context.

In vitro sumoylation assays pointed out to the fact that 6His-JD may conjugate with SUMO2, constituting the first concrete experimental evidence for sumoylation of atx3. Nonetheless, we were unable to confirm these results in mammalian cell models.

4.2 – Future perspectives

Having described two PTMs of atx3 that had never been identified before and considering the importance attributed to these modifications regarding protein function and polyQ disease mechanisms, this experimental work opens up a number of interesting possibilities for the study of atx3 biology and, consequently, MJD pathogenesis. Nonetheless, before the effect of the PTMs can be determined, more research will be necessary in order to confirm the modifications we identified and understand what are the conditions under which they are observed.

As it has been said before, mass spectrometric detection of the phosphorylated peptide was somehow weak, and so, in order to confirm our result, we will have to repeat the experiences we made. Besides, it will be very important to confirm this modification using another experimental approach, for instance by looking at the incorporation of (^{32}P)-ortophosphate in GFP-atx3, in cells, and see if mutation of serine 12 reduces the radioactive signal (Fei et al., 2007; Mueller et al., 2009). Importantly, however, since we just tried to increase GFP-atx3 phosphophorylation by generic inhibition of the serine/threonine phosphatases, we are inclined to admit that this modification happens normally in cells.

In the case of sumoylation, we will have to confirm our observations with further *in vitro* assays. We may also try to use this assays in order to see if the C-terminal is also targeted by sumoylation. Then, it will be crucial to clearly determine whether this modification happens normally in the actual cellular context, because only this way will we be able to know if it is biologically relevant. With this objective in mind, we may try an immunocytochemistry approach, in order to compare the distribution of either endogenous or recombinant atx3 and SUMO2 in cells, and see if they co-localize. Actual confirmation of atx3 (or JD) modification by SUMO2 may be achieved after optimization of our cotransfection experiments but, considering that the attachment of SUMO is generally very low, it would be important to try to enhance sumoylation by cotransfecting the cells with enzymes involved in the sumoylation pathway (for instance, the SUMO E2 enzyme, Ubc9; Kim et al., 2006) and avoid desumoylation through employment of specific SUMO hidrolase inhibitors. The purification procedures shall also suffer modifications directed at maximizing their specificity and efficiency. The observation of the profile of sumoylated atx3 or JD obtained from cells may also allow the detection of species corresponding to poly- or multisumoylation (that is, with several SUMO molecules attached to different lysine residues).

It will be essential to follow up our definite confirmation of the results with a deeper characterization of the PTMs. In this respect, it will be important to determine

which is the enzyme responsible for phosphorylation of serine 12. The putative kinase data presented above (Table III and IV) may help selecting the enzymes that are most likely to target the serine 12 residue and confirmation may be achieved by trying to phosphorylate atx3 *in vitro* through incubation with the suspected enzymes and radio-labeled orthophosphate and seeing if the mutation of the serine residue reduces the radioactive signal (Fei et al., 2007; Mueller et al., 2009).

In the case of sumoylation, it may be significant to determine to which residue(s) does SUMO2 binds. This identification may be done through *in vitro* assays or cotransfection experiments using mutants where some of the putative lysine are mutated to arginines and subsequent analysis of the result in order to see if the mutation actually reduces the sumoylated protein signal (Dorval & Fraser., 2006; Steffan et al., 2004).

After the characterization of the PTMs detected by the present work, it will be essential to carry on their study by trying to evaluate and portray their actual biological importance, by determining the effect they have on atx3 properties, similarly to what has been done with other atx3 PTMs (Mueller et al., 2009; Todi et al., 2009). In this context, it will be crucial to have the protein in the desired modified state. As far as phosphorylation is concerned, we have already generated a phosphomimetic serine 12 mutant, in which serine 12 of 6His-atx3 is replaced by an aspartate residue, which is similar to serine, but negatively-charged (6His-atx3 S12D). The characterization of the effect that serine 12 phosphorylation is predicted to have on atx3 enzymatic activity may then be assessed by performing *in vitro* activity assays and comparing the mutant atx3 performance with that of the wild-type protein. Usage of different substrates may allow different kinds of study: Ub fluorogenic substrates may help in establishing kinetic differences, since they permit a continuous product detection; differently-linked polyUb chains may help in understanding if there are any differences in Ub linkage type preference caused by serine 12 phosphorylation, comparing to what has been previously published (Winborn et al., 2008). Importantly, phosphorylation has also been

related with changes in atx3 subcellular localization (Mueller et al., 2009) and so, in order to determine if serine 12 is also implicated in the process, we may analyze differences in subcellular localization between GFP-atx3 and both phosphomimetic and phosphodead mutants (where phosporylation is impossible due to serine substitution by an alanine residue), through citochemistry or cell fractioning techniques. Immunocitochemistry or cell fractionating techniques coupled with raising of phosphospecifc antibodies recognizing phosphorylated serine 12 may help to determine the natively-modified protein distribution in neuronal cells. It will also be relevant to try to compare the phosphomimetic mutant transcriptional repressor activity with that of the wild-type atx3 and the phosphodead mutant; this kind of study may be done through reporter gene assays of the matrix metallopeptidase 2 (MMP-2), according to what has been previously described (Mueller et al., 2009). The effect of serine 12 phosphorylation on atx3 stability may be evaluated using thermal stability assays and its cellular turnover may be determined by pulse-chase experiments (Todi et al., 2009). Changes in molecular interactions may be investigated by comparing the ability to co-immunoprecipitate known atx3 interactors from cell extracts between wild-type atx3 and both the phosphomimetic and phosphodead mutants (Mueller et al., 2009).

Concerning atx3 sumoylation, we may try to analyze SUMO2-modifyed atx3 (or JD) activity using sumoylated protein samples obtained from *in vitro* sumoylation assays and subsequent enrichment of the modified species through chromatographic techniques. Importantly, this has already been done for another DUB (Muelmeester et al., 2008). The SUMO2-conjugated protein may then be used for the *in vitro* activity assays presented above, in order to determine whether this modification, similarly to ubiquitination (Todi et al., 2009), affects atx3 enzymatic activity. Studies looking for possible differences in subcellular distribution, transcriptional repressor activity, turnover and protein-protein interaction may instead require transfection of the cells with atx3 recombinant proteins mimicking the SUMO2 modification. In previous studies

concerning polyQ disease proteins sumoylation, this has been done by generating fusion proteins containing both the polyQ tract-containing protein and SUMO2, fused in frame (Steffan et al., 2004). Alternatively, those experiments may be performed using atx3 SUMO conjugation-deficient mutants, obtained by substituting the lysine residues involved in SUMO conjugation for arginine residues. In this respect, we have already generated atx3 and JD mutants where lysine 166, the putative residue with the highest associated score, is replaced by an arginine (6His-atx3 K166R and 6His-JD K166R, respectively).

The final step in the evaluation of the biological importance of serine 12 phosphorylation and JD sumoylation will be to determine if these PTMs are somehow involved in the mechanisms underlying MJD. It will then be necessary to try and see if the ratio of phosphorylated or sumoylated protein differs from normal and expanded atx3 and compare the functional effects of the modifications between both normal and expanded protein. The observations may then be interpreted according to the mechanisms admittedly related with MJD pathogenesis. Considering what has been speculated about the possible involvement of PTMs with polyQ expansion diseases' selective neurodegeneration profile (Pennuto et al., 2009), it will also be crucial to evaluate the distribution of the modified protein in the brain of animal models, and then see how the modified protein abundance overlaps or not with the brain regions that are reported to be dysfunctional in MJD. In the case of phosphorylation, this may be done using the phospho-specific antibodies referred above; as for sumoylation, Western blot comparison between different brain fractions is likely more appropriate.

Important clues may also be provided by the effects these modifications have on atx3 tendency to aggregate. In cells, this may be assessed by immunocitochemistry, by observing the tendency of phosphomimetic and phosphodead mutants to form aggregates or inclusions, or by SDS-PAGE and Western blot, directed at the detection of high molecular weight species resulting from atx3 aggregation (Fei et al., 2007). In the case of sumoylation, experiments may be done using the SUMO2-atx3 fusion

protein or the SUMO conjugation-deficient mutants (Mukherjee et al., 2009; Steffan et al., 2004).

Lastly, if any significant hypothesis arise from the previous observations, it will be important to understand the effects of these modifications *in vivo*. This may be done by generating animal models expressing phosphomimetic or phosphodead atx3 proteins, both expanded and non-expanded, and determining if any differences can be observed regarding phenotype, cell death and inclusion formation, always comparing the results with the observations from non-modified atx3-expressing animals. Regarding sumoylation, these *in vivo* studies may be done using sumoylation-deficient atx3 mutants, as previously done with huntingtin (Steffan et al., 2004).

Further understanding the biologic importance of the two PTMs described in the present work may prove relevant for the constitution of a solid body of knowledge regarding atx3 elusive function and the neurodegenerative disease it causes when its polyQ sequence is expanded.

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