

Identification and Characterization of ataxin-3 Molecular Interactors

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Aos meus Pais e ao César...

Preceitos Legais

De acordo com o disposto no nº 2 do artigo 8º do Decreto-lei nº 388/70, nesta dissertação foram utilizados os resultados dos trabalhos publicados ou em preparação abaixo indicados. No cumprimento do disposto no referido Decreto-lei, a autora desta dissertação declara que interveio na concepção e execução do trabalho experimental, a interpretação dos resultados e na redacção dos resultados publicados ou em preparação, sob o nome de **Ferro A.**:

Based on the nº 2 do artigo 8º do Decreto-lei nº 388/70, in this dissertation were used experimental results published or under preparation stated below. The author of this dissertation declares that participated in the planification and execution of the experimental work, in the data interpretation and in the preparation in the work stated below, under the name **Ferro A.**:

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Resumo

A doença de Machado-Joseph ou ataxia espinocerebelosa do tipo 3 (DMJ/SCA3) é uma doença de repetições trinucleotídicas, causada pela expansão de um segmento repetitivo CAG, instável e altamente polimórfico, localizado na região 3' codificante do gene *ATXN3*. O segmento repetitivo, que normalmente contém 12-41 CAG, encontra-se expandido de 51 a 86 CAGs nos doentes. A DMJ/SCA3 é uma doença neurodegenerativa, sendo a ataxia autossómica dominante mais frequente em todo o mundo.

O mecanismo subjacente às doenças de poliglutaminas (poliQ), como a DMJ/SCA3, não é completamente compreendido. Pensa-se que as doenças de poliQ resultam de um ganho de função tóxica, inerente às propriedades nocivas da região expandida nas proteínas mutadas. Embora estas proteínas se caracterizem por uma expressão ubíqua, somente algumas populações específicas de neurónios são afectados, originando um padrão de morte neuronal específico de cada doença.

O gene *ATXN3* codifica a proteína ataxina-3 (*ATXN3*), uma proteína poliglutamínica, intracelular de 42 kDa, com propriedades de ubiquitina hidrolase, *in vitro*. A existência de interacções proteicas diferenciais pode estar na base da morte neuronal selectiva, característica da DMJ/SCA3. O objectivo deste trabalho foi a identificação de interactores moleculares da ataxina-3.

Os primeiros interactores proteicos da *ATXN3* identificados foram duas proteínas humanas homólogas do RAD23, os HHR23A e HHR23B. Estas duas proteínas funcionam como moduladoras do sistema ubiquitina-proteassoma, e participam no mecanismo de reparação do ADN por excisão nucleotídica (NER), através da interacção molecular com a proteína XPC (*Xeroderma pigmentosum* group C). Neste estudo usámos ensaios de *yeast two-hybrid* e de imunofluorescência em células de mamífero, para mostrar que a *ATXN3* interactua e colocaliza com a proteína XPC. Em células irradiadas com raios ultra-violeta (UV) verificámos um aumento desta colocalização.

A possibilidade de realizar estudos funcionais num organismo multicelular simples, e a existência no laboratório de uma estirpe de *C. elegans* com expressão silenciada do gene da ataxina-3 (*atx-3*), levaram-nos e seguida a avaliar a conservação, no nemátode, das interacções proteicas observadas no humano, bem como a possível participação da *ATXN3* em vias de reparação do ADN. Neste trabalho, clonámos e expressámos pela primeira vez a proteína homóloga das HHR em *C. elegans*, a ZK20.3. Verificámos que a *atx-3* interactua com a ZK20.3, evidenciando a conservação evolutiva da base estrutural necessária para esta interacção. Além disso, constatámos que o transcrito ZK20.3 é altamente expresso no nemátode adulto, tal como o descrito para *atx-3*. Analisámos, por RT-PCR semi-quantitativa,

o efeito do mutagénico ENU (*N*-ethyl-*N*-nitrosourea) na expressão dos genes *atx-3*, *ZK20.3* e *xpc-1* em *C. elegans*. Neste estudo, mostrámos que a expressão do gene *atx-3* é indutível pelo ENU, o que sugere uma participação da *atx-3* na resposta do nemátode à genotoxicidade. Nos ensaios comportamentais, a diferença significativa verificada no número de descendentes dos animais mutantes, *atx-3*^{-/-}, face aos *wild-type*, quando tratados com 1 mM ENU, parece indicar uma maior resistência da estirpe mutante a condições genotóxicas; estes resultados conjuntamente com a indução de expressão de *atx-3* verificada na presença de ENU, apontam para uma regulação negativa da *atx-3* nos mecanismos de reparação do DNA.

Foi recentemente verificada a acumulação de NEDD8 (*neural precursor cell expressed developmentally down-regulated 8*), uma proteína ubiquitina-like, nas inclusões proteicas existentes nos cérebros de doentes DMJ/SCA3. Neste estudo, utilizando ensaios *in vitro* e de imunofluorescência, verificámos que a ATXN3 normal interaccua com a NEDD8. A interacção é independente dos domínios UIM (ubiquitin-interacting motif) da ATXN3, já que a presença do domínio josefina é suficiente e necessária para que a interacção ocorra. A conservação da interacção entre a ataxina-3 de *C. elegans* e a NEDD8, demonstra a sua importância biológica e funcional. Estudos de previsão estrutural, sugerem que a interacção da NEDD8 com o domínio josefina é do tipo substrato-enzima. Os resultados obtidos *in vitro* com um substrato fluorogénico, NEDD8-AMC confirmaram as previsões estruturais, uma vez que a ATXN3 possui actividade de desnedilase *in vitro*. A actividade de desnedilase é dependente da cisteína do centro catalítico, na posição 14 do domínio josefina, uma vez que, a mutação deste aminoácido elimina a actividade da proteína *in vitro*, embora não afecte a interacção com o NEDD8.

As diferentes observações obtidas com este trabalho poderão ajudar a compreender melhor a(s) função(ões) da ATXN3 e o seu papel nos mecanismos biológicos nos quais parece estar envolvida.

Abstract

Machado-Joseph disease or spinocerebellar ataxia type 3 (MJD/SCA3) is a trinucleotide repeat disease, caused by the expansion of an unstable and highly polymorphic CAG segment, localized in the 3'-coding region of *ATXN3* gene. The repeat segment, which normally consists of 12-41 CAG, is expanded from 51 up to 86 CAGs in patients. MJD/SCA3 is a neurodegenerative disorder and is the most frequent autosomal dominant ataxia worldwide. Although the mechanism underlying polyglutamine (polyQ) diseases, including MJD, is poorly understood, it is believed that they are caused by a gain of toxic function, inherent to the expanded polyQ tract in the mutated protein. Despite the ubiquitous expression of polyglutamine proteins, only a specific subset of neurons is affected in the disease, leading to a pattern of neuronal loss that is specific of each disease.

The *ATXN3* gene encodes ataxin-3 (ATXN3), a 42-KDa intracellular polyglutamine protein with deubiquitylating properties *in vitro*. The establishment of differential interactions by ATXN3 may be the basis of the selective neuronal loss characteristic of MJD/SCA3. Our goal was to identify the molecular partners of ATXN3. The first interactors of human ATXN3 identified were the human homologues of RAD23, HHR23A, and HHR23B. These proteins have been characterised as modulators of the ubiquitin-proteasome system (UPS) and as participants of the DNA damage recognition mechanism, in nucleotide excision repair (NER), through interaction with *Xeroderma pigmentosum* group C (XP-C) protein. Here, we used yeast two-hybrid assays and immunofluorescence analysis, in mammalian cells, to show that ATXN3 interacts and co-localizes with XPC protein. Using UV-radiation assays, we also show that their co-localization is enhanced in irradiated cells.

The possibility to carry out functional studies in a multicellular yet simpler organism, and the availability of a *C. elegans* knockout strain for *atx-3* in the lab, led us to proceed our studies in this model. We studied the conservation of the interactions observed with the human proteins, and the possible participation of ATXN3 in DNA repair. For the first time, we have cloned and expressed the *C. elegans* RAD23 protein homologue, ZK20.3. Moreover, we show that *atx-3* interacts with ZK20.3, suggesting evolutionary conservation of the structural determinants relevant for this novel interaction. ZK20.3 is highly expressed in worm adults, as previously observed for *atx-3*. Using semi-quantitative RT-PCR, we analysed the effect of *N*-ethyl-*N*-nitrosourea (ENU) upon the expression of *atx-3*, ZK20.3 and *xpc-1* in *C. elegans*. Here, we show that *atx-3* is an ENU-responsive gene, suggesting a potential role of *atx-3* in *C. elegans*' response to mutagenics. Behavioural assays showed a significant difference between wild-type animal and *atx-3*^{-/-} progeny, when exposed to 1 mM of ENU, suggesting that the absence of *atx-3* confers higher resistance of animals to DNA-damaging

conditions. Altogether, these results are compatible with the involvement of atx-3 as an inducible negative regulator of the DNA repair mechanisms.

Recently, the accumulation of the neural precursor cell expressed developmentally down-regulated 8 (NEDD8), an ubiquitin-like protein, in the inclusions of MJD brains was reported. Here, we report a new molecular interaction between wild-type ATXN3 and NEDD8, using *in vitro* and immunofluorescence assays. We show that this interaction is independent of the ubiquitin-interacting motifs in ATXN3, since the presence of the Josephin domain is sufficient and necessary for the interaction to occur. The conservation of the interaction between the *C. elegans* ataxin-3 homologue (atx-3) and NEDD8 suggests its biological and functional relevance. Our molecular docking studies of the NEDD8 molecule to the Josephin domain of ATXN3 suggest that NEDD8 interacts with ATXN3 in a substrate-like mode. In agreement, using NEDD8-AMC hydrolase assays we show that ATXN3 displays deneddylase activity, *in vitro*, against a fluorogenic NEDD8-linked substrate. The insertion of a mutation in the active site of ATXN3, C14A, though not affecting the interaction with NEDD8, abrogates ATXN3 catalytic activity towards this substrate.

The evidence generated in this work should contribute to a better understanding of the normal function(s) of ATXN3 and to elucidate its role in the pathways that it seems to be involved with.

Résumé

La Maladie de Machado-Joseph (MJD/SCA3) est une maladie de trinucleotides, dont la cause est l'expansion d'une répétition CAG instable et très polymorphique, dans la région 3' codante du gène *ATXN3*. Le segment répétitif, qui contient normalement 12-44 CAG, est expansé jusqu'à 61-87 CAG chez les patients. MJD/SCA3 est une maladie neurodégénérative autosomique dominante, et une des ataxies dominantes plus fréquentes dans le monde.

Même si le mécanisme pathogénique des maladies de polyglutamines est très peu compris, on croit que ces maladies sont causées par un gain de fonction toxique inhérent à la protéine mutante. Malgré l'expression ubiquë des protéines de polyglutamines, ces maladies sont caractérisées par des patrons spécifiques de mort neuronale.

Le gène *ATXN3* codifie la protéine ataxine-3 (ATXN3), une protéine intracellulaire de 42kDa avec activité de desubiquitination *in vitro*. L'établissement d'interactions différentielles par cette protéine peut expliquer pour la perte sélective de neurones qui caractérise la MJD, malgré l'expression généralisée de ATXN3. Le but de ce travail a été l'identification des interacteurs de ATXN3. Les premiers interacteurs moléculaires de ATXN3 identifiés ont été des homologues humaines de RAD23, HHR23A et HHR23B. Ces protéines ont été caractérisées comme modulateurs du système ubiquitine-proteasome (UPS) et participent au mécanisme de reconnaissance de lésions du DNA dans le système de réparation par excision (NER), par l'interaction avec la protéine *Xeroderma pigmentosum* group C (XP-C). En ce travail, on montre par le système y2h et par l'analyse d'immunofluorescence dans des cellules HEK293 que ATXN3 et XPC interagissent et co-localisent, et que la radiation UV augmente cette co-localisation.

En plus, on rapporte, pour la première fois, le clonage et analyse d'expression de l'homologue de RAD23 dans le *C. elegans*, ZK20.3, et on démontre que *atx-3* (l'homologue de ATXN3) interagit avec ZK20.3, ce qui suggère la conservation évolutive des motifs structuraux nécessaires à cette interaction. Le gène ZK20.3 s'exprime fortement dans les vers adultes, comme il a été observé pour l'ataxine-3. Par RT-PCR, on étudié l'effet de l'agent mutagénique ENU (N-ethyl-N-nitrosurée) dans l'expression des gènes *atx-3*, *ZK20.3* et *xpc-1* dans les vers. Une induction de l'expression de *atx-3* a été détectée chez les animaux traités à l'ENU, ce qui suggère un rôle pour l'*atx-3* dans la réponse à la genotoxicité. La différence dans le nombre de descendants de *C. elegans* du type sauvage et *knock-out* pour *atx-3* traités avec ENU suggère une résistance augmentée des mutants. Ces résultats semblent indiquer un rôle de l'ataxine-3 comme régulateur négatif de la réponse aux lésions de DNA.

Récemment, il a été démontré dans le cerveau de patients MJD/SCA3 que les inclusions intranucléaires ubiquitinées, caractéristiques des maladies de polyglutamines, contenaient aussi de la protéine NEDD8 (*neural precursor cell expressed developmentally down-regulated 8*), une protéine *ubiquitin-like* (UBL). En cet étude, on a vérifié, par des essais *in vitro* et d'immunofluorescence, que l'ATXN3 normale interagit avec NEDD8. Cette interaction ne dépend pas des domaines UIM (*ubiquitin-interaction motifs*), vu que le domaine Josephin (JD) est suffisant et nécessaire pour l'interaction. La conservation de l'interaction entre l'ataxine-3 de *C. elegans* et NEDD8 démontre son importance biologique et fonctionnelle. Nos études de prévision structurelle suggèrent que l'interaction de NEDD8 avec le JD est du type substrat-enzyme. Les résultats des études *in vitro* avec un substrat fluorescent NEDD8-AMC ont confirmé cette hypothèse, vu que ATXN3 démontre une activité deneddilase. Cette activité catalytique dépend de la cysteine 14 du centre actif, dans le JD, car la mutation de ce résidu élimine l'activité deneddilase de la protéine, même si elle ne modifie pas l'interaction entre ATXN3 et NEDD8.

Les résultats de ce travail peuvent aider à mieux comprendre les fonctions physiologiques d'ATXN3 et son rôle spécifique chez les mécanismes biologiques qu'elle intègre.

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Abbreviations

aka	also known as
AMC	7-amino-4-methylcoumarin
AR	androgen receptor
AT	amino-1,2,3 triazole
bp	base pair
CAG	cytosine-adenine-guanine
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
CBP	CREB-binding protein
cDNA	complementary DNA
CREB	cyclic AMP-response element binding protein
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
ECF	enhanced chemifluorescence
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetracetic acid
ENU	<i>N</i> -ethyl- <i>N</i> -nitrosourea
GFP	green fluorescent protein
HD	Huntington disease
HECT	homologous E6-AP carboxil-terminal
HEPES	<i>N</i> -(2-hidroxyethyl)-piperazine- <i>N</i> '-2-ethanesulphonic acid
HRP	horseradish peroxidase
HS	horse serum
Id	identity
IPTG	isopropyl- β -D-thiogalactopyranoside
JD	josephin domain
kDa	kilodalton
KO	knockout
L	litre
MEM	modified Eagle's medium
min	minute
mL	mililitre
mM	milimolar
n	number of experiments/samples
N2	Bristol laboratory <i>C. elegans</i> strain (wild-type strain)

NEDD8	neural precursor cell expressed developmentally down-regulated 8
NGM	nematode growth medium
OD	optical density
PAGE	polyacrylamide gel
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pDEST	destination vector
PolyQ	polyglutamine
RING	really interesting new gene
RNA	ribonucleic acid
rpm	rotation per minute
RT-PCR	reverse transcription-PCR
s	second
SCA	spinocerebellar ataxia
SCF	SKP1-Culin-1-F-box protein E3
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
<i>Taq</i>	<i>Thermus aquaticus</i>
TBS	tris-buffered saline
Ub	ubiquitin
UBL	ubiquitin-like
UIM	ubiquitin-interacting motif
UPS	ubiquitin-proteasome system
UV	ultraviolet (radiation)
wt	wild-type
XPC	xeroderma pigmentosum complementary group C
Y2H	yeast two-hybrid
μL	microlitre

Trinucleotide repeat disorders

The identification of expanded trinucleotide repeats in various genes has been found to be the ultimate cause of a growing number of neurodegenerative disorders. The trinucleotide repeats expansions are dynamic, changing in size upon transmission to the offspring. The repeats usually show meiotic and mitotic instability. Ensuing their intergenerational instability, affected families experience *anticipation* or earlier age of onset and increased severity of the disease.

To date, 16 neurological diseases are known to be caused by expansion of trinucleotide repeats (Orr and Zoghbi, 2007). Based on the repeat localization within the gene, the trinucleotide repeat disorders may be sub-divided in two classes: coding or noncoding.

Non-coding trinucleotide repeat diseases

The non-coding trinucleotide repeat diseases typically comprise large and highly variable repeat expansions, which result in multiple tissue dysfunction or degeneration. The diseases phenotypes are variable, possibly due to the pronounced degree of somatic heterogeneity. It has been discovered that the mutational event of this sub-class is based on the existence of pre-mutations, usually clinically silent intermediate size expansions from which the larger mutations are transmitted. Non-coding trinucleotide diseases may be caused by CGG, GCC, GAA, CTG and CAG repeats. Besides the sequence of the repeat, its location with respect to the gene, are determinant factors in the pathogenic mechanism underlying each disease. Non-coding trinucleotide diseases may result from the lost function of the respective proteins or acquired function of a toxic transcript. This group includes Fragile X syndrome (FRAXA), Fragile-XE syndrome (FRAXE), Friedreich ataxia (FA), myotonic dystrophy (DM), Fragile X-associated tremor ataxia syndrome (FXTAS), spinocerebellar ataxia type 8 (SCA8), spinocerebellar ataxia type 12 (SCA12) (Orr and Zoghbi, 2007) (**Table 1**).

Coding trinucleotide repeat diseases

The expansions associated with this sub-class are much smaller and, the repeat instability is a phenomenon less pronounced, but no less severe. This group includes diseases caused by the expansion of a repetitive segment of alanine (GCG) or glutamine (CAG).

Polyalanine diseases

Expansions of trinucleotide repeats (GCG) encoding polyalanine tracts are the cause of several diseases, predominantly congenital malformation syndromes, skeletal dysplasia and nervous system anomalies. Typically, the expanded repeats result from in-frame duplications or insertions within the nucleotide sequence encoding the alanine stretch; the expanded alleles are stable mitotically and meiotically, with the only exception being a further expansion of one trinucleotide repeat in the *PABPN1* gene involved in oculopharyngeal muscular dystrophy (OPMD). Other examples of such disorders are synpolydactyly syndrome (SPD), hand-foot-genital syndrome (HFGS), X-linked mental retardation and growth hormone deficit (XLMR+GHD) and X-linked mental retardation and abnormal genitalia (XLAG) (Amiel *et al.*, 2004).

Polyglutamine diseases

The identification of the causative mutation of SBMA (spinal and bulbar muscular atrophy) or Kennedy disease, back in 1991, by Fischbeck *et al.* (1991) defined a new class of neurodegenerative diseases. This emerging class of triplet diseases includes, so far, nine polyglutamine (polyQ) diseases: SBMA/Kennedy disease, Huntington disease (HD), and seven spinocerebellar ataxias - SCA1, dentatorubral-pallidoluysian atrophy (DRPLA), SCA2, Machado-Joseph disease/SCA3, SCA6, SCA7 and SCA17 (**Table 2**). The expansion of a repetitive tract of CAG (cytosine-adenine-guanine) is located in the coding region of the genes of interest, hence producing proteins with long polyglutamine tracts.

With the unique exception of SMBA, which is X-linked, all are autosomal dominant inherited disorders, with a late clinical onset characterized by progressive neuronal degeneration with loss of coordination, motor and, in some of these diseases, cognitive impairment (reviewed in Zoghbi and Orr, 2000). Clinically, the polyQ diseases often overlap and their strikingly similar mutations may suggest that a common pathogenic mechanism may underlie all polyQ diseases. The recent work of Lim *et al.* (2006), strengthened this close proximity between the different polyQ-associated ataxias, with the comprehensive definition of the “ataxia-ome”, where many ataxia-causing diseases were shown to share defined interaction clusters.

Table 1. Features of unstable repeat diseases caused by expanded, non-coding triplets.

Disease (acronym)	MIM	Main clinicopathology	Repeat unit/location	repeat intervals		Gene product
				Normal	Pathological	
Fragile X syndrome (FRAXA)	309550	Mental retardation, macroorchidism, connective tissue defects, behavioral abnormalities	(CGC) _n /5'UTR	6-60	>200 (full mutation)	FMRP
Fragile XE syndrome (FRAXE)	309548	Mental retardation	(CCG) _n /5' UTR	4-39	200-900	FMR2
Friedreich ataxia (FRDA)	229300	Sensory ataxia, cardiomyopathy, diabetes	(GAA) _n /intron 1	6-32	200, 1700	Frataxin
Myotonic dystrophy type 1 (DM1)	160900	Myotonia, weakness, cardiac conduction defects, insulin resistance, cataracts, testicular atrophy, and mental retardation in congenital form	(CTG) _n /3' UTR	5-37	50-10,000	DMPK (serine/threonine kinase)
Fragile X-associated tremor ataxia syndrome (FXTAS)	309550	Late onset ataxia, tremor, Parkinsonism, and dementia	(CGG) _n /5'UTR	6-60	60-200 (pre-mutation)	FMR1 RNA
Spinocerebellar ataxia type 8 (SCA8)	608768	Ataxia, dysarthria, nystagmus	(CTG) _n /3-prime terminal exon	16-34	>74	SCA8 RNA
Spinocerebellar ataxia type 12 (SCA12)	603516	Ataxia and seizures	(CAG) _n /5'UTR	7-45	55-78	PPP2R2B (regulatory subunit of phosphatase PP2A)
Huntington disease-like 2 (HDL2)	606438	Similar to HD	(CTG) _n /splice acceptor site of an alternatively spliced exon	7-28	66-78	Junctophilin

No physiological function was determined for the majority of the proteins involved in the polyQ diseases, with the exception of the androgen receptor in SBMA, the voltage-gated $\alpha 1A$ subunit of calcium channel in SCA6, the transcription activator atrophin-1 in DRPLA, and the TATA-binding protein mutated in SCA17 (Table1).

Apart from the existence of a polyQ tract, no other similar features were found, so far, among polyQ proteins, albeit the location of the polyQ tract within the protein differs from one disease gene to another. The expansion of the repetitive segment seems to confer toxic properties to the proteins, and evidence suggests the inherent misfolded state is linked with its toxicity and gain of function, leading to the abnormal protein-protein interactions and aggregation. Despite the fact that both normal and mutated polyQ proteins show ubiquitous expression, both in the CNS and in peripheral tissues, the diseases are mainly confined to the CNS (except in SBMA and in a juvenile-onset SCA7 case), where only specific sub-types of neurons are affected, leading to very selective patterns of neurodegeneration (Zoghbi and Orr, 2000).

The CAG repeats are highly polymorphic in the normal population, presenting somatic and germline instability; the expanded CAG repeats are particularly unstable, changing in size upon transmission (Richards, 2001). The repeat length correlates inversely with clinical age of onset and severity of the disease (Gusella and MacDonald, 2000); larger expansions are often associated with juvenile onset and more generalised neurodegeneration pattern (Holmberg *et al.*, 1998; Andresen *et al.*, 2007). The dynamic nature of these mutations, may in part justify the variability in expression, including the age-at-onset, and the severity of the disease. Moreover, due to this intergenerational repeat instability, affected families may display the phenomenon of anticipation, characterised by earlier age-at-onset, increased severity, and faster progression of the disease (reviewed in Orr and Zoghbi, 2007).

Pathogenic mechanisms underlying polyQ disease

The importance of polyQ domain

As mentioned above, apart from the polyQ segment, the proteins involved in polyglutamine diseases show virtually no similarity. This, and the fact that the age-at-onset and severity correlates with the polyQ length, suggests a major role for this domain itself in the pathogenesis. Based on this, most of the initial experiments

Table 2. Features of polyglutamines disease caused by expanded, coding CAG repeats.

Disease (acronym)	MIM	Main clinicopathology	Gene	CAG intervals		Protein product, MW	Function
				Normal	Pathological		
Dentatorubropallydollsian atrophy (DRPLA)	125370	ataxia, seizures, choreoathetosis, dementia	<i>ATN1</i>	6-34	49-88	atrophin-1 (ATN1), 125 kDa	Transcriptional regulator
Huntington disease (HD)	143100	chorea, dystonia, cognitive deficit, psychiatric alterations	<i>HD</i>	6-34	36-121	huntingtin (Htt), 350 kDa	Unknown
Spinal and bulbar muscular atrophy (SBMA; Kennedy disease)	313200	motor weakness, swallowing, gynaecomastia, decreased fertility	<i>AR</i>	9-36	38-62	androgen receptor, 100 kDa	Androgen receptor
Spinocerebellar ataxia type 1 (SCA1)	164400	ataxia, dysarthria, spasticity, cognitive impairment	<i>ATXN1</i>	6-38	39-82	ataxin-1 (ATXN1), 87 kDa	Unknown (transcription?)
Spinocerebellar ataxia type 2 (SCA2)	183090	ataxia, polyneuropathy, decreased reflexes, possible infantile variant with retinopathy	<i>ATXN2</i>	15-24	35-200	ataxin-2 (ATXN2), 90 kDa	Unknown (RNA metabolism?)
Machado-Joseph disease or Spinocerebellar ataxia type 3 (MJD/SCA3)	109150	ataxia, dysarthria parkinsonism, spasticity, myotrophy	<i>ATXN3/MJD1</i>	12-41	(51)55-86	ataxin-3 (ATXN3), 42 kDa	Deubiquitylating enzyme (?)
Spinocerebellar ataxia type 6 (SCA6)	183086	ataxia, dysarthria, nystagmus, tremors	<i>CACNA1A</i>	4-18	19-33	α_{1A} subunit of the neuronal P/Q-type voltage-gated calcium channel (CACN α_{1A}), 250 kDa	Ca ²⁺ channel
Spinocerebellar ataxia type 7 (SCA7)	164500	ataxia, blindness, possible infantile variant with cardiac failure	<i>ATXN7</i>	4-27	37- $\geq$ 200	ataxin-7 (ATXN7), 96 kDa	Unknown (transcription?)
Spinocerebellar ataxia type 17 (SCA17)	607136	ataxia, cognitive deficit, seizures, psychiatric alterations	<i>TBP</i>	25-42	45-63	TATA-binding protein (TBP), 42 kDa	Transcription factor

were centred in determining the biological importance of the polyQ tract in disease. Several lines of evidence have been strengthening the hypothesis that the polyQ diseases are due to a toxic gain of function attributable to the polyQ expansion: (1) the deletion of the endogenous mouse *HD* homologue gene, lead to embryonic lethality but not a HD-like disease (Zeitlin *et al.*, 1995); (2) absence of a functional *AR* gene causes male feminization and not SBMA (Zoppi *et al.*, 1993) while castration of male mice exhibiting SBMA reverted dramatically the phenotype, suggesting to be an hormone-dependent phenotype (Katsuno *et al.*, 2002); (3) nullizygous *ATXN1* mice displayed no cerebellar ataxia (Matilla *et al.*, 1998); (4) a conditional HD mouse model expressing mutated huntingtin (htt) under the regulation of a tet-on/off promoter system showed that expression of the mutant Htt led to the appearance of characteristic features of the disease and blockage of such expression ameliorated the inclusion formation as well as the progressive motor dysfunction (Yamamoto *et al.*, 2000); (5) the existence of homozygotes in HD (Squitieri *et al.*, 2003), MJD (Lang *et al.*, 1994) and SCA17 (Toyoshima *et al.*, 2004) typically exhibiting aggravated phenotype is evidence for a partial dominance effect.

The influence of the protein context

The idea that the expanded polyglutamines are toxic and responsible for pathology, led to the early experiments and the development of transgenic mouse models for HD (Mangiarini *et al.*, 1996; Mangiarini *et al.*, 1997) and MJD (Ikeda *et al.*, 1996). In both cases, mice expressing a truncated fragment of the protein containing an expanded polyQ tract could recapitulate most of the disease phenotypes, namely the intergenerational instability associated with the expanded alleles. Conversely, no disease phenotype was observed in mice expressing normal-sized polyQ tracts. However, the most critical biological feature, neuronal degeneration, could only be observed when a full-length mutant htt was expressed (Reddy *et al.*, 1998). With an age-dependent phenotype, the HD mouse model showed selective neuronal degeneration and evidence that the protein context, and not solely the polyQ tract, could mediate the disease progression and selective vulnerability associated with each different polyQ disease (Reddy *et al.*, 1998).

The importance of the protein context in polyQ proteins was reinforced by the identification of key amino acids or segments of the protein that could modulate the presentation and severity of disease. In SCA1, Skinner *et al.* (2002) identified an amino

acidic segment, external to polyQ, which could affect the disease progression. Later on, in complementary studies, Emamian *et al.* (2003) observed that a serine at position 776 in the protein (S776) and its inherent phosphorylation was associated with the formation of aggregates and neurodegeneration; Chen *et al.* (2003) found that, in a fly SCA1 model, the protein-protein interactions depended on S776 were modulated by its phosphorylated-state in a polyQ-dependent manner. More recently, it was demonstrated in a cell- and rat-based model, that the toxicity of mutant Htt could be inhibited by the AKT-dependent phosphorylation of Htt at S421 (Humbert *et al.*, 2002) or by cyclin-dependent kinase 5 (Cdk5)-mediated phosphorylation at S434 of Htt (Luo *et al.*, 2005) suggesting a neuroprotective role of phosphorylation in HD (Pardo *et al.*, 2006). Recently, both normal and mutant ATXN3 were shown to be substrates of glycogen synthase kinase 3 β (GSK3 β); furthermore, phosphorylation was seen to be dependent of S256 and to be inversely correlated with polyQ length; moreover, phosphorylation inhibited aggregation of mutant ATXN3, implying a critical role of phosphorylation, also in MJD (Fei *et al.*, 2007). Together, these observations suggest that post-translational modification may be determinant molecular events in the disease initiation and/or progression and that the polyQ expansion may be not sufficient to the pathology.

The increasing biological relevance of a region external to the polyQ region, may suggest that expression of the normal protein is important in the disease process, although by a mechanism that remains to be elucidated.

The aggregation phenomenon

The presence of aggregates containing the mutant protein is a hallmark of all polyQ diseases (Orr and Zoghbi, 2007). Analysis of patient's post-mortem material allowed the observation that the expansion of the polyQ segments above a given threshold led to neurological disease and to the formation of mutant protein-containing aggregates (DiFiglia *et al.*, 1997). This inherent characteristic of expanded polyQ proteins, rapidly gathered the interest of the scientific community and several theories emerged trying to explain the pathogenesis associated with these aggregation-prone conditions.

Polar zipper

The “polar zipper” theory presented by Perutz *et al.* hypothesized that expanded units of polyQ proteins could establish strong hydrogen bonds with each other above a threshold length leading to insoluble parallel β -sheet structures (Perutz *et al.*, 1994).

Later on, it was suggested an alternative model in which β -helices, composed of 20 residues per turn, could be considered as the precipitating unit underlying the aggregation phenomena (Perutz *et al.*, 2002a; Perutz *et al.*, 2002b).

Transglutaminases

The participation of transglutaminases, and their ability to cross-link the polyQ proteins, was also pointed out as a possible mechanism underlying the aggregation typical in these diseases (Kahlem *et al.*, 1996; Kahlem *et al.*, 1998).

Aberrant interactions

The establishment of new aberrant protein-protein interactions dependent of the expansion-promoted conformational changes may be important for the formation of aggregates. The inhibition and sequestration of cellular proteins as transcription factors or activators, which comprise also polyQ tracts, may increase and aggravate the aggregative phenomena (Chai *et al.*, 2001).

Toxic fragment

Based on the intrinsic properties of the polyQ tract, the hypothesis of the existence or the production of a toxic fragment, comprising the polyQ domain, also tries to explain the neurotoxicity underlying these disorders, attributing secondary importance to the protein context. *In vitro* experiments that showed full-length huntingtin, AR, atrophin-1 and ATXN3 as substrates of purified caspases (Goldberg *et al.*, 1996; Kobayashi *et al.*, 1998; Wellington *et al.*, 1998; Berke *et al.*, 2004), consolidated the existence of C-terminal fragments as putative by-products. The biological relevance of these fragments were recently shown both in MJD transgenic mice and MJD patients, where a fragment comprising the polyQ domain exerted cytotoxicity when expressed above a critical concentration (Goti *et al.*, 2004). Additionally, Haacke *et al.* (2006) showed that expanded ATXN3, upon N-terminal truncation, could initiate aggregation in a neuronal cell line; accordingly, they demonstrated that ATXN3 is a substrate of calcium-dependent calpain proteases (Haacke *et al.*, 2007), reinforcing the biological importance of ATXN3 fragmentation mainly at the beginning of the MJD pathology. Moreover, work from our group using a worm-based model (Teixeira-Castro *et al.* unpublished work) suggests that a C-terminal fragment of human ATXN3 sustains the

nucleation/seeding events that lead, in an aging-dependent manner to visible aggregation in the worm. These facts support the notion that proteolytic cleavage of polyQ proteins may contribute to onset or progression of the disease process.

Aggregates: a good or a bad solution?

The role of inclusions in the characteristic neuronal loss remains controversial. Whether they are, *per se*, toxic, neutral or a protective strategy of the cell to restrict the abnormal reactivity of misfolded polyQ proteins towards other cellular components still needs clarification.

The fact that inclusions are composed of proteasome subunits, ubiquitin, chaperones and even the normal-sized proteins, and not solely by “pure” polyQ proteins, supports the inherent toxicity of these structures (Paulson *et al.*, 1997b; Stenoien *et al.*, 1999; Chai *et al.*, 2001). In fact, over-expression of chaperones in animal and cell-based models of these disorders ameliorated the polyQ toxicity (Warrick *et al.*, 1999; Kobayashi *et al.*, 2000; Cummings *et al.*, 2001), further reinforcing that the presence of chaperones in the aggregates are failed attempts of the cell to eliminate or solubilize such toxic structures.

Yet, several evidences favour the hypothesis that these structures may be a way that cell found to “suppress” the disruptive presence of the mutant proteins by stacking them in aggregates. First, inclusions are not restricted to the sites where severe neuronal loss is observed (Holmberg *et al.*, 1998). In patients with SCA17, polyQ-positive aggregates are present in the neurons of the grey matter that do not die in the disease process; whereas, affected, “destined-to-die” Purkinje cells show no aggregation (Rolfs *et al.*, 2003). Moreover, experimental conditions that prompted a reduction in the formation of inclusions, led to enhanced toxicity of a fragment of mutant huntingtin in neuronal cultures (Saudou *et al.*, 1998), and in neurons of a SCA1 mouse model (Cummings *et al.*, 1999). Moreover, a SCA1 mouse model expressing the mutant ATXN1 without its dimerization domain, showed signs of the disease without neuronal inclusion (Klement *et al.*, 1998). Another evidence lays on the naturally occurring aggregates resulting of the metabolic aging neuron, as the Marinesco bodies found in the substantia nigra, which seems to be a strategy adopted by normal-aging neurons, throughout time (Fujigasaki *et al.*, 2000).

If the inclusions play a protective role, they must be doing so by avoiding inappropriate interactions within the cell. Thus, the study of the normal and mutant protein's interactions has also been a theme of great interest, in an attempt to establish the pathway(s) in which they participate and, ultimately, the mechanism(s) leading to the disease.

The apparently dissonant evidence regarding the importance of the protein context, the polyQ tract or the role of aggregates in the cell, may result from the different cell types and mouse models explored. The difference in the protein content of the cells used may account for some of the differences observed, as can the genetic background of the mice used to generate transgenic or KO mice, due to the possible existence of genetic modifiers, which are still poorly understood. The differential use of either full-length or truncated mutant variants of the polyQ protein may also contribute to some of the apparent differences reported.

The mechanism of pathogenesis polyQ proteins is intimately linked to at least two major cellular events, transcription regulation and the protein quality control.

Transcription (dys)regulation

The polyQ aggregates are both nuclear and/or cytosolic, but most are predominantly intranuclear inclusions, being found in cells affected by the disease, suggesting that the nucleus is the major site of the pathology (Orr and Zoghbi, 2007). The exceptions are SCA2 and SCA6, characterised *mainly* by the formation of cytosolic or perinuclear inclusions, respectively (Ishikawa *et al.*, 2001).

The translocation of polyQ proteins to the nucleus has been set as a prerequisite of their toxicity. It has been demonstrated that mutation of nuclear localisation signals (NLS) aborting the localization of mutant ATXN1 in the nucleus, led to no manifestation of the disease in mice (Klement *et al.*, 1998). In a cell-based model, with striatal neurons expressing a nuclear export signal (NES) in mutant fragment of huntingtin reduced its ability to cause cellular death (Saudou *et al.*, 1998), whereas, the presence of a NLS caused the opposite effect (Peters *et al.*, 1999). Recently, Bichelmeier *et al.* (2007), showed the first *in vivo* evidence that nuclear localization of ataxin-3 is required for the manifestation of symptoms, whereas the export of ataxin-3 out of the

nucleus prevents the manifestation of phenotype. Thus, the nucleus seems to be critical for the pathogenesis of polyQ diseases. Several evidence have supported the notion that major alterations in gene expression occur in the cell in polyQ-associated disorders (reviewed in Okazawa, 2003), namely HD (Wyttenbach *et al.*, 2001; Zuccato *et al.*, 2001; Luthi-Carter *et al.*, 2002), DRPLA (Wood *et al.*, 2000), MJD (Evert *et al.*, 2001; Evert *et al.*, 2006), SBMA (Lieberman *et al.*, 2002), SCA1 (Lin *et al.*, 2000) and SCA7 (La Spada *et al.*, 2001).

PolyQ-dependent transcription regulation/modulation may be explained by three mechanisms.

The entrapment hypothesis suggests that polyQ proteins may “trap” rate-limited transcriptional activators or co-activators. The entrapment of rate-limited, essential transcriptional modulators, into inclusions, may lead the cell to an unsustainable situation, causing cell death. Several reports have directly associated polyQ proteins with transcriptional modulators as TBP, TBP-interacting protein TAF_{II}130 and SP1 transcription activators (Dunah *et al.*, 2002). SP1 is ubiquitously expressed and is involved in the regulation of essential genes.

CBP (CREB-binding protein) is a transcriptional co-activator, typically found in inclusions of cell cultures, transgenic models and post-mortem tissues of HD and DRPLA patients (Steffan *et al.*, 2000; Nucifora *et al.*, 2001), SCA7 (Strom *et al.*, 2005), SBMA and MJD (McCampbell *et al.*, 2000). *In vivo* proof that the depletion of CBP may lead to neurological phenotypes resembling polyQ diseases, came from the KO mice of CREB (cAMP response element-binding), which showed neurodegeneration (Mantamadiotis *et al.*, 2002).

Alternatively, polyQ proteins may exert their toxic effect through direct *inhibition of acetyltransferase activity of transcription modulators*, leading to diminished histone acetylation and, concomitantly, gene transcription activation. CBP and PCAF (p300/CBP-associated factor) are transcription activators that regulate gene expression through their intrinsic histone acetylase activity (Ogryzko *et al.*, 1996). A mutant truncated fragment of huntingtin has been shown to inhibit transcription, by repressing the histone acetylase activity of CBP/p300/PCAF transcriptional co-activator (Steffan *et al.*, 2001). An active participation of polyQ proteins in transcription, as dual co-repressors, has been demonstrated for ATXN3; Li *et al.* (2002), observed that ATXN3 could interact through its C-terminal with CBP, p300 and PCAF, inhibiting CREB-

dependent gene transcription; and through its N-terminal, ATXN3 could inhibit transcription by direct binding to histones, masking their acetylation sites.

Besides the analysis of the indirect consequences of the polyQ expression in cells or animal models, the involvement of polyQ disorders in the *direct repression of the transcriptional machinery* as cause of neurodegeneration may be ascertained. Atrophin-1 (ATN1) is a transcriptional co-repressor; the expansion of its polyQ tract leads to DRPLA (Wood *et al.*, 2000). The direct interaction of ATN1 and ETO/MTG8, a nuclear protein participating in repressor complexes, unveiled a possible role of another polyQ protein in transcription regulation. Zhang *et al.* (2002) showed that both the human and fly homologues of ATN1 had transcription repressive activities *in vivo*, inversely correlating with the length of polyQ tract. As described before, both the androgen receptor (AR) involved in SBMA, and the TATA-binding protein (TBP), mutated in SCA17 are transcription factors (Nakamura *et al.*, 2001). TBP binds to TATA box of several genes, thus having a general effect upon transcription. Moreover, a direct interplay between the expression of different polyQ proteins has been reported: Schaffar *et al.* (2004) reported that soluble huntingtin was negatively modulating the activity of TBP, in a polyQ-dependent manner.

None of the hypotheses reviewed is mutually exclusive, and the observed interplay between the polyQ proteins suggests exactly the opposite. Either in a more specific (e.g. SP1 in HD) or broad manner (CBP has been referenced in a large set of polyQ disorders), the evidence gathered are consistent with the polyQ proteins involvement in transcription modulation.

Protein quality control

The proteasome is a large multicatalytic complex found in most prokaryotic and eukaryotic cells and is located in both the cytoplasm and the nucleus (Ellis, 2006). Working together with the proteasome there are the chaperones. Most chaperones are expressed constitutively, though some are heat and stress induced: the heat-shock proteins (HSPs). Wickner *et al.* (1999) showed that HSPs could indeed associate with proteasome and facilitate the degradation of unfolded proteins, as an alternative to the refolding pathway. For example, the discovery of two additional co-chaperones, CHIP and BAG-1, helped to elucidate the link between chaperones and proteasome. CHIP (C-terminus Hsp70 interacting protein) is a chaperone-dependent E3 ligase that binds to

Hsc/Hsp70, inhibits its chaperone activity, and promotes proteasomal degradation of its unfolded substrates. Moreover, BAG-1 helps Hsc/Hsp70 to deliver its cargo to the 26S proteasome by binding the proteasome through its UBL domain. Conversely if the Hsc-Hsp70 complex and its bound cargo interact with two other co-chaperones, Hip/Hop, the refolding pathway is favoured and the cargo is not destroyed in the proteasome. As a chaperone-dependent E3 ligase, CHIP establishes a close link between molecular chaperones and the ubiquitin/proteasome system in the protein quality control (Bregegere *et al.*, 2006). A tight equilibrium underlies the decision for protein refolding or degradation.

Protein quality control and neurodegeneration

Neuronal inclusions in MJD, HD, SCA7, SCA1 and SBMA are known to harbour components of the protein surveillance machinery, as ubiquitin, proteasome subunits, chaperones and ubiquitin-like proteins (Paulson *et al.*, 1997b; Cummings *et al.*, 1998; Chai *et al.*, 1999a; Chai *et al.*, 1999b; Stenoien *et al.*, 1999; Takahashi *et al.*, 2002; Chai *et al.*, 2004; Mori *et al.*, 2005). Such a strong presence of proteins involved in the protein quality control mechanisms leaves little doubts about the effort that the cell exposed to expanded polyQ endeavors to restore its protein homeostasis. In a fly model of MJD, overexpression of chaperones was able to reverse the polyQ aggregation observed (Warrick *et al.*, 1998). Moreover, several pieces of evidence demonstrated that chaperone overexpression led to increased solubility of polyQ proteins (Chan *et al.*, 2000; Muchowski *et al.*, 2000; Cummings *et al.*, 2001; Bailey *et al.*, 2002). The shuttling of misfolded proteins to the proteasome has been recently addressed by Jana *et al.* (2005) who demonstrated that interaction of CHIP with HSP70 facilitates the ubiquitylation of huntingtin and ATXN3.

The co-existence of proteasome subunits in the polyQ inclusions raised the possibility of proteasome impairment in polyQ disease. This was strengthened when Holmberg *et al.* (2004) showed, using live cell imaging, that polyQ aggregates could irreversibly sequester the proteasome; additionally, the aggregates could get trapped within the proteasome, leading to its inefficient activity. However, conflicting data demonstrated that soluble and normal expanded proteins could be efficiently degraded by 26S but the aggregates seemed to “overwhelm” the degradation motor of the cell, albeit not irreversibly inhibiting it (Yamamoto *et al.*, 2000; Michalik and Van Broeckhoven, 2004). Moreover, the pharmacological inhibition of the proteasome in a cell line expressing

mutant ATXN3 led to aggregation enhancement, suggesting an active role of the 26S complex in aggregate clearance in MJD pathology (Chai *et al.*, 1999b).

Other ubiquitin-proteasome system (UPS) components are present in polyQ inclusions. Typically, neuronal inclusions are ubiquitylated and increasing evidence links the UPS to the neurodegeneration. This was demonstrated with a mouse model expressing the mutated ATXN1 protein and lacking E3 ubiquitin-ligase E6-AP, which displayed accelerated neurodegeneration (Cummings *et al.*, 1999). The presence of frameshifted ubiquitin, Ub⁺1, in inclusions found in several neurodegenerative diseases, namely MJD, reinforced the idea that altered UPS may contribute to the pathology (van Leeuwen *et al.*, 1998; de Pril *et al.*, 2004). Mutated Ub⁺1 is incorporated in polyUb chains, but is resistant to deubiquitylation, and competes with normal ubiquitin inhibiting the proteasome (Lam *et al.*, 2000 1579), when present at a high concentration *in vivo* (van Tijn *et al.*, 2007). The authors suggested that this frameshifted Ub⁺1 molecule may, over time, be an aggravating factor/modifier in polyQ disease progression.

Ubiquitin-proteasome system

For a protein to be degraded by the UPS it is mandatory that it follows two steps: suffer conjugation with polyUb chain(s) and be efficiently translocated for the 20S catalytic chamber of the proteasome for degradation, with the concomitant release of reusable ubiquitin, a crucial step promoted by deubiquitylating enzymes (DUB) (reviewed in Glickman and Ciechanover, 2002). Several positive and negative modulators regulate protein ubiquitylation and, subsequently, the UPS, by balancing the refolding of the misfolded proteins with a close interplay of 19S proteasome cap, chaperones and co-chaperones and their actual degradation by the 20S catalytic core, with the necessary ubiquitin and amino acidic recycling.

Positive regulators

E4 multiubiquitin elongation factors are examples of positive regulators of UPS. These catalyse the elongation of the ubiquitin chains of substrates linked to E2-E3 complexes. CHIP, as mentioned before, is one of such factors and acts with HSP70 as shuttling substrate cargo to the 26S proteasome (Esser *et al.*, 2004); for instance, its association with parkin (an E3 ligase whose mutation causes autosomal recessive juvenile Parkinsonism) enhances Pael-R ubiquitylation, leading to its degradation (Imai *et al.*,

2002). Moreover, CHIP has been also implicated in the ubiquitylation of ATXN3 (polyQ length-dependent); CHIP overexpression reduces ATXN3 aggregation rate and cell death ensued by polyQ truncated or full-length protein (Jana *et al.*, 2005). Interestingly, mammalian E4B (UFD2a, ubiquitin fusion degradation protein-2) has also been implicated in mutant ATXN3 clearance, through enhancement of its ubiquitylation rate and degradation by the proteasome (Matsumoto *et al.*, 2004).

Negative regulators

Negative modulators of UPS include two classes of deubiquitylating enzymes (DUB): ubiquitin C-terminal hydrolase (UCHL) and ubiquitin-specific protease (USP); both have been implicated in neurodegenerative disorders. It is known that ATXN1 interacts with USP7, in a polyQ dependent manner (Hong *et al.*, 2002); mutations in UCH-L1 are linked to Parkinson's disease (PD); they cause a reduction in the enzyme's proteolytic activity and enhance aggregation (Ardley *et al.*, 2004), and to gracile axonal dystrophy (gad) in mice, an autosomal recessive disorder characterised by altered UPS. Moreover, the AR is able to establish an interaction with ARNIP (androgen receptor N-terminal interacting protein), an E3 ubiquitin ligase (Beitel *et al.*, 2002). The 19S regulatory base itself showed chaperone-like activity, refolding to their native conformation substrates that had been ubiquitin-targeted, preventing their translocation and degradation in the 20S chamber (Braun *et al.*, 1999), suggesting that unfolded proteins "kissed for death" and delivered at the proteasome, do not necessarily undergo 26S-dependent degradation (reviewed in Glickman and Ciechanover, 2002). Furthermore, several reports showed that normal ATXN3 binds polyUb chains with at least four molecules of Ub (Burnett *et al.*, 2003; Donaldson *et al.*, 2003; Doss-Pepe *et al.*, 2003) and shows deubiquitylating activity *in vitro* (Burnett *et al.*, 2003; Burnett and Pittman, 2005).

The UPS system is highly regulated, and a perturbation at any point of it may lead to deregulation of the cell's protein homeostasis, which, in time, may eventually lead to neurodegeneration.

Ubiquitylation

Two aspects increase the complexity of ubiquitylation in the cell: the number of Ub molecules conjugated to the substrate and the position of the lysine residue in Ub used for the attachment and the formation of the chain. Substrates can be mono-, oligo- or

polyubiquitylated. Moreover, some proteins can also be N-terminal ubiquitylated; in these cases, the first ubiquitin is attached to the free α -NH₂ terminus of the protein, rather than to an internal lysine (Glickman and Ciechanover, 2002).

In monoubiquitylation, the post-translational modification consists in the conjugation of a single ubiquitin to the substrate. Polyubiquitylation requires the attachment of a ubiquitin chain comprising four or more molecules of Ub. In oligoubiquitylation, the substrate may be monoubiquitylated in several different lysine (K) residues or modified by the covalent attachment to a single lysine of Ub chains containing less than four Ub molecules (Glickman and Ciechanover, 2002).

In oligo- and polyubiquitylation chains can be extended using one of the seven possible residues in Ub (K6, K11, K27, K29, K33, K48 and K63). Although the biological relevance of some polyUb conjugations is unknown, all seven lysine residues can be used for polyUb chain formation *in vivo* (reviewed in Woelk *et al.*, 2007).

Proteins conjugated with a K48-linked polyUb chain are typically sent for degradation at the proteasome, whereas monoubiquitylated or polyubiquitylated proteins using lysine other than K48 are involved in mediating the protein's subcellular localisation or its function, as in DNA repair, translation, endocytosis and vesicle transport (Glickman and Ciechanover, 2002; Welchman *et al.*, 2005).

A substantial intricacy appears to exist within the ubiquitylation pathway, as recently it was postulated that NEMO, an essential regulator of NF- κ B activation, protects its poly-Ub(K63)-conjugated substrate from degradation, by preventing the conversion of the conjugated ubiquitin chain into a K48-branched polyUb chain (Heyninck and Beyaert, 2005). Moreover, Saeki *et al.* (2004), reported that UFD2 is able to catalyse the linkage switch from K29, typically used in monoubiquitylation, to elongated K48-linked polyUb chains. This interchangeability of polyUb chains intensifies the complexity of post-translational modifications (Woelk *et al.*, 2007). In addition to this, several ubiquitin-like proteins (UBL) have been identified. The attachment of UBL proteins UBL is known to be involved in the regulation of several cellular functions and in target specificity.

Ubiquitin-like proteins

Like ubiquitin, and using a similar conjugation cascade, UBLs can be covalently attached through their C-terminal glycine to the ϵ -NH₂ group of lysine in the substrate. Ubiquitin like-proteins are sub-divided in two classes, depending on their primary sequence (**Table 3**) (Walters *et al.*, 2004). **Type I** proteins are similar to ubiquitin and exist as

free molecules, conjugatable to substrates upon processing; besides Ub, only SUMO-2 and SUMO-3 are able to constitute poly-UBL chains, but no function has been attributed to these yet (Welchman *et al.*, 2005). **Type II** proteins have at least one conjugatable, built-in ubiquitin-like domain (UBL), highly similar to Ub, and other ubiquitin-associated domains (UBA).

Ubiquitin-like protein	
Type I	Type II
NEDD8	HHR23A
ISG15	HHR23B
FUB1	NUB1
FAT10	parkin
SUMO-1	hPLIC-1
SUMO-2	hPLIC-2
SUMO-3	A1Up
Apg 8	BAG1
Apg 12	ubiquilin3
Urm1	elongin-B
UBL5	U7I3
Ufm1	S3A1
	BAT3
	Uch14

Table 3. Ubiquitin-like proteins: description of several ubiquitin family members. **Type I** group includes NEDD8, neural precursor cell expressed developmentally downregulated-8; SUMO1,-2, -3, small ubiquitin-related modifier-1, -2, -3; ISG15, interferon-stimulated gene-15; FUB1, fau ubiquitin-like protein; FAT10, F-adjacent transcript-10; Apg8 and 12, autophagy 8 and 12; Urm1, ubiquitin-related modifier-1; UBL5, ubiquitin-like protein-5; Ufm1, ubiquitin-fold modifier-1. **Type II** classe comprises HHR23a and -b, human homolog of RAD23-A and -B; hPLIC-1 and -2, human proteasome ligase interaction component-1 and -2; ubiquilin-3; A1Up, ataxin-1 ubiquitin-like interacting protein; NUB1, NEDD8 ultimate buster-1; BAG1, BCL-2 binding athanogene-1 protein; Parkin; Elongin-B; U7I3, ubiquitin conjugating enzyme 7 interacting protein 3; S3A1, splicing factor 3 subunit 1; BAT3, HLA-B-associated transcript; Uch14, ubiquitin carboxyl-terminal hydrolase 14.

Due to the presence of these domains, type II UBL proteins are able to bind directly to subunits of the proteasome, through the UBL domain, and to ubiquitin or polyUb chains, through their UBA domain (reviewed in Jentsch and Pyrowolakis, 2000; Glickman and Ciechanover, 2002; Welchman *et al.*, 2005). Of note is that, ATXN3 has been related to some of type I and type II proteins, as SUMO-1 (Shen *et al.*, 2006), HHR23 proteins (Wang *et al.*, 2000), parkin (Tsai *et al.*, 2003) and hPLIC-1 (Heir *et al.*, 2006). The interaction of ATXN3 and NEDD8 will be discussed later in this work (Chapter 2). Both

NEDD8 and SUMO have been implicated in neurodegenerative diseases (Kuazi *et al.*, 2003; Mori *et al.*, 2005).

NEDD8

NEDD8 (neural precursor cell expressed developmentally downregulated-8) is the UBL with the highest structural homology with Ub (Whitby *et al.*, 1998) and conjugates to target proteins in a cascade of events similar to ubiquitylation (Hochstrasser, 2000). The E1-activating enzyme APPB1-UBA3 determines the specificity for NEDD8 and E2-conjugating enzyme, UBC12 catalyzes the conjugation of NEDD8 to the target through an isopeptide bond (Osaka *et al.*, 1998). So far, NEDD8 has been reported to conjugate with a limited number of target proteins, including the cullin family members (Pan *et al.*, 2004), p53 and mdm2 (Xirodimas *et al.*, 2004) and more recently, synphilin-1, a major component of inclusion bodies found in the brains of patients with neurodegenerative α -synucleinopathies, including Parkinson's disease (Tanji *et al.*, 2006). Cullins are the molecular scaffolds for the assembly of several E3 ubiquitin ligases, and the best-known substrates for NEDD8 conjugation (Pan *et al.*, 2004). Neddylation upregulates the activities of cullin-organized E3 ubiquitin-ligases while conferring intrinsic instability onto cullins (Wu *et al.*, 2005), thereby outlining the close link that exists between the ubiquitin and the NEDD8 pathways. NEDD8 deconjugation is equally important for the cell and can be carried out by members of most of the deubiquitylating enzyme (DUB) families USP (USP21), JAMM group (CNS/COP9 signalosome) and the ULP family (NEDP1/SEN8/DEN1) (Parry and Estelle, 2004). NEDD8 has an essential role in cell cycle control and embryogenesis evidenced by gene knockout studies (Tateishi *et al.*, 2001; Kurz *et al.*, 2002). Recently, Oved *et al.* (2006) showed that NEDD8 is implicated also in the metabolism of membrane growth factor receptors. Conjugation of NEDD8 can also lead to direct target degradation in the proteasome. This is mediated by NEDD8 ultimate buster-1 (NUB1) (Tanji *et al.*, 2005) and NUB1 ligand (NUBL1) (Tanaka *et al.*, 2003), which bind the S5a subunit of the 19S regulatory particle. Hence, NEDD8 is primarily a regulator of ubiquitin E3 ligase activity but is also involved in proteasome degradation. This has been strengthened with the reported accumulation of NEDD8, in ubiquitylated inclusions in brain tissue from patients with various neurodegenerative disorders, namely MJD, which suggest the involvement of NEDD8 in the formation of inclusions via the UPS (Mori *et al.*, 2005).

DNA repair

The diversity of ubiquitylation possibilities mentioned before sets this post-transcriptional modification as an important mechanism involved in virtually all cellular processes. Genomic integrity enables the correct transmission of DNA information to the next generations. Through time, cells are exposed to several exogenous (e.g., ionizing radiation, ultraviolet light and natural/synthetic chemicals) and endogenous (e.g. oxidative generated species) genotoxic conditions. DNA lesions may range from single-stranded breaks (SSB), double stranded breaks (DSB) or formation of DNA adducts normally inducing large DNA-helix distorting (Huang and D'Andrea, 2006). These alterations may interfere with DNA replication, induce cell cycle arrest, or block transcription (reviewed in Hoeijmakers, 2001).

There are five main DNA repair pathways that cell may engage to cope with the DNA lesions, with overlapping features: nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR) all involved in SSB repair; homologous recombination (HR) or non-homologous end joining (NHEJ) repair leads to the excision of DSB lesions (Huang and D'Andrea, 2006).

Nucleotide excision repair

The most versatile DNA damage repair mechanism is nucleotide excision repair (NER), as it comprises two sub-pathways: transcription-coupled repair (TCR) and genome global repair (GGR). NER is responsible for the removal of single-stranded alterations and large DNA helix-distorting lesions induced by UV-light, as cyclobutane pyrimidine dimers (CPD) and pyrimidine(6-4)pyrimidone dimers (6-4PP) as well as intrastrand crosslinks and bulky adducts provoked by several chemical agents, and to some extent, reactive oxygen species (ROS) (de Laat *et al.*, 1999). TCR is specifically engaged in removing lesion from the coding strands of genes that are actively transcribed by RNA polymerase II; whereas GGR is responsible for the subtracting NER-inducible DNA lesions occurring throughout the entire genome (Hoeijmakers, 2001; Huang and D'Andrea, 2006).

NER associated diseases

Defective NER activity has been associated with several human genetic disorders showing severe sun-sensitivity, with high cancer-propensity, and severe neurological abnormalities as is the case of Xeroderma pigmentosum (XP) and Cockayne's syndrome

(CS). Seven NER-deficient genetic complementation groups of XP (XP-A to XP-G) and two groups of CS (CS-A and -B) have been identified. In Xeroderma pigmentosum complementation group C (XP-C) patients, only the GGR sub-pathway appears impaired, whereas TCR rate is normal (Bootsma *et al.*, 1997). The gene involved in XP-C encodes for the XPC protein, a 125 kDa protein, which forms *in vivo* a trimeric complex with HHR23B and centrin-2 (Masutani *et al.*, 1994; Araki *et al.*, 2001).

The mechanism

The NER pathway requires the formation of multiprotein complexes at the lesion sites and ~20 proteins acting cooperatively to achieve proficient nucleotide excision repair (Sancar *et al.*, 2004) (**Fig.1**). In the GGR sub-pathway, the XPC/HHR23B/CETN2 complex functions as a recognition complex with binding properties to the lesion site. Given the versatility of NER and the wide range of damaged substrates recognized by NER-specific proteins, the excision repair complex appears not to recognize specifically the chemical group involved in the lesion, but instead the *major structural conformations* induced by the lesions (Sancar *et al.*, 2004). The recognition and subsequent binding of the XPC/HHR23B/CETN2 recognition complex is thought to favour the sequential binding of other repair factors as TFIIH, XPA, RPA and two NER-specific endonucleases XPG and XPF (complexed with ERCC1), essential for an efficient NER reaction.

The XPC/HHR23B/CETN recognition complex shows low affinity to subtle-distortion lesions (e.g. CPD) (Kusumoto *et al.*, 2001). This raised the possibility that the DDB protein (Damaged DNA binding, aka XPE) could cooperate with the recognition complex in the identification of low-distorting DNA damages (Fitch *et al.*, 2003b). The DDB is a heterodimeric protein comprising 127 kDa (DDB1/p127) and 48 kDa (DDB2/p48) subunits (Costa *et al.*, 2003). Recently, Sugawara *et al.* (2005) showed that XPC and UV-DDB interact physically and that XPC is ubiquitinated upon UV irradiation, in a DDB-dependent manner. Interestingly, it was shown that p53 controls the expression of DDB2 (Hwang *et al.*, 1999) and XPC (Fitch *et al.*, 2003a; Wang *et al.*, 2003). The DDB2 subunit is also a substrate of CUL4A, which was shown to stimulate its ubiquitylation and proteasome degradation, establishing a direct link between the CUL4A expression, the down-regulation of DDB2 expression and therefore the GGR rate (Chen *et al.*, 2001). Both p53 and CUL4A are substrates of NEDD8 raising the possibility that deneddylases may participate in the regulation of the NER pathway.

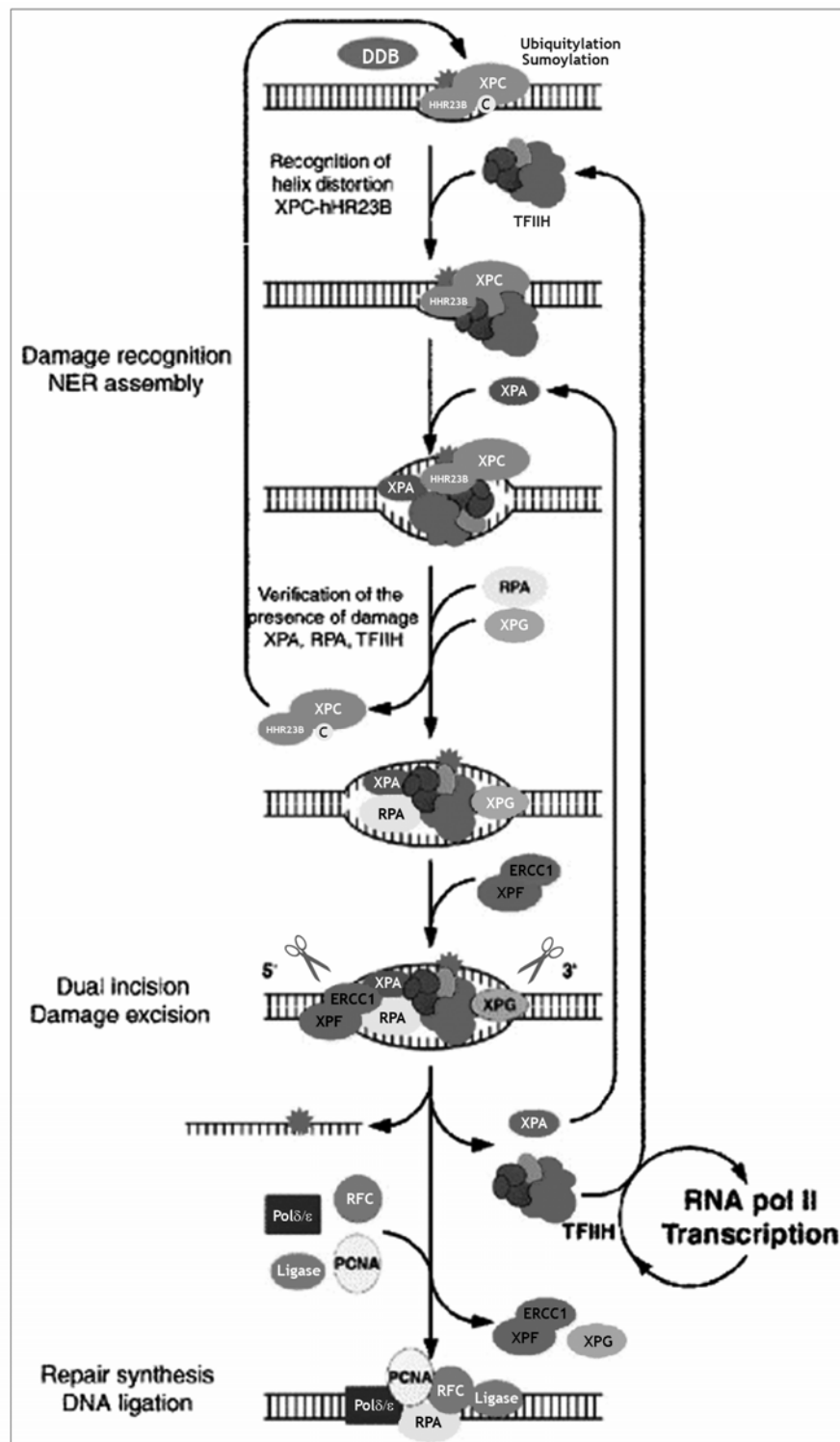


Figure 1. Schematic representation of global genome repair (GGR) sub-pathway. The association and dissociation steps of the different proteins involved in GGR-NER are represented by oriented arrows. The TFIIH complex denotes the strong interplay existing between DNA repair and transcription. Adapted from (Park and Choi, 2006).

Upon binding of XPC/HHR23B/CETN2, TFIIH recruitment is essential, as is the helicase activity of its XPB and XPD subunits which unwinds the DNA helix around the inflicted DNA damage site. Positioning of TFIIH at the damage site triggers the formation of the incision complex formed by RPA and XPA, plus the two endonucleases, XPG and XPF/ERCC1. Binding of the endonucleases occurs independently of each other, but the association of XPG to the complex is thought to be mediated by its interaction with TFIIH (Iyer *et al.*, 1996). The release of XPC/HHR23B/CETN2 complex is triggered by XPG association and binding of XPF/ERCC1 and XPA completes the incision complex; both binding of the endonuclease XPF/ERCC1 to the lesion site and the activation of bound XPG to perform 3' cleavage are XPA-oriented with the cooperation of RPA; the XPF/ERCC1 is responsible for the 5' DNA cleavage. Finally, the DNA portion containing the lesion is removed by dual excision (reviewed Volker *et al.*, 2001). The synthesis and ligation of new undamaged DNA may be carried out by either polymerase δ or polymerase ϵ , in the presence of the processivity factor PCNA (proliferating cell nuclear antigen) and its cofactor RCF (replication factor C). Ligation of newly synthesised DNA to the initial sequence is dependent of DNA ligase I (Costa *et al.*, 2003).

In TCR-NER, the DNA lesions are detected directly by RNA polymerase II that stops transcription at the lesion site; the CSA and CSB proteins and subsequent TFIIH binding at the lesion site displace the stalled polymerase II allowing further binding of other repair factors, as described above. Upon lesion detection and recruitment of TFIIH, both the TCR- and GGR-NER follow a common mechanism (reviewed in de Laat *et al.*, 1999; Park and Choi, 2006).

The DNA repair mechanisms must be tightly regulated, as at least three of the NER factors play crucial roles in other cellular processes as transcription (TFIIH), recombination (RPA) and replication (XPF/ERCC1).

Recently, Wang *et al.* (2005) showed that SUMO-1 may also modify XPC, upon UV irradiation. It is a modification dependent of DDB2 and XPA proteins and seems to be related with the stabilization and availability of XPC in cells.

DNA repair and neurodegeneration

Evidence showing DNA repair and neurodegeneration overlapping is growing. Many human disorders characterised with neurological deficits are associated with mutations affecting the cellular machinery participating in DNA repair. Two distinct groups of

defective DNA repair disorders associated with ataxia can be appointed: the first is characterised by diseases with defects in the double strand breaks (DSB) repair and maintenance mechanisms and includes ataxia telangiectasia (AT), AT-like disease and Nijmegen breakage syndrome (NBS), characterised also by a high susceptibility to cancer. The second group is that of purely neurological disorders, associated with an abnormal cellular response to single-strand breaks (SSB) and includes ataxia oculomotor apraxia type 1 (AOA1), AOA2 and spinocerebellar ataxia with axonal neuropathy-1 (SCAN1) (reviewed in Paulson and Miller, 2005). All AT, AT-like, AOA1 and AOA2 are autosomal recessive spinocerebellar ataxias with oculomotor apraxia. Mutations in *ATM* gene causes ataxia telangiectasia; ATM protein is the ultimate controller of the cellular responses to DSB repair and is closely related to the MRN complex that comprises MRE11/RAD50/NBS1(nibrin) proteins. Mutations in the *MRE11* gene are responsible for AT-like disease, whereas defective NBS1 protein is associated with the Nijmegen breakage syndrome.

Ataxia due to defects in SSB can be caused by mutations in gene encoding tyrosyl phosphodiesterase (TDP1) leading to SCAN1, in aprataxin (APXT) causing AOA1 (Moreira *et al.*, 2001), and an inactivating mutation in senataxin (SETX) leads to AOA2; senataxin has also been implicated in an autosomal dominant form of juvenile amyotrophic lateral sclerosis (ALS). Interestingly, oxidative stress (a known cause of DNA damage) was shown to be associated with the recessive forms of ALS (Ferrante *et al.*, 1997; Manfredi and Xu, 2005). Both DSB and SSB cellular responses can be triggered by ROS or ionizing radiation (Taroni and DiDonato, 2004).

Terminally differentiated cells are naturally more sensitive to any kind of stress. But why can neurons be particularly susceptible? Due to the metabolic and energy demands, neurons may be exposed to continued, high levels of ROS species produced from neuronal metabolism. Thus, oxidative stress may be, throughout time, responsible for the accumulated DNA damage. In fact, direct involvement of oxidative stress has been reported in several neurodegenerative diseases, apart from the mentioned above: PD (Moore *et al.*, 2005), AD (Smith *et al.*, 2000), HD (Firdaus *et al.*, 2006), SCA2 (Giuliano *et al.*, 2003). Thus, neurons that are more prone to the formation of ROS due to their O₂ and energy requirements, may be more susceptible to DNA damage, for example, oxidation, than other cells with less energy requirements and that are capable to “dilute” and repair upon cell division, accumulated toxic species. In fact, mutant ATXN3 was shown to confer a higher susceptibility to post-mitotic cell death, as its N-terminal

truncated expanded enhanced cytotoxicity in G0/G1 phase-arrested cells subjected to starvation (Yoshizawa *et al.*, 2000), evidencing that differentiated neurons may be potentially more susceptible to the stress induced by polyQ and aggravated by their expansion.

Neurons might either be more susceptible to accumulated DNA damage than other cell or have a lower threshold for DNA damage-prone conditions leading more easily to neuronal death (Paulson and Miller, 2005).

Machado-Joseph disease

This disease was first described in North-American families of Azorean ancestry, later in the mainland and then in non-Portuguese families from all continents (Sequeiros and Coutinho, 1981; Sequeiros and Coutinho, 1993). Nakano *et al.* (1972) reported the first cases, in the Machado family from S. Miguel, in 1972; the disease presented as an autosomal dominant ataxia, and was named by the authors, Machado disease. Woods *et al.* (1972) observed the Thomas family, also with Portuguese-Azorean ancestry; although resembling that reported by Nakano *et al.* (1972), some clinical particularities led the authors to define it as a new clinical entity, they named nigro-spino-dentatal degeneration. A few years later, Rosenberg *et al.* (1976) defined a new hereditary ataxia by observation of the Joseph family, originated from Flores Island, which they named as Joseph disease. In 1978, Coutinho and Andrade reached a milestone in the disease history, with the *re-definition* of Machado-Joseph disease as a unique, well-defined single pathology. With families described all over the world, Machado-Joseph disease (MIM #109150), also known as spinocerebellar ataxia type 3, is the most common form of dominantly inherited ataxia (Schols *et al.*, 2004; Sequeiros *et al.*, 2007).

Clinico-pathology

MJD is a progressive neurodegenerative disease, affecting mainly the motor function. Patients usually present with ataxia, pyramidal and extrapyramidal signs, peripheral amyotrophies, external progressive ophthalmoplegia, intention fasciculation-like movements of facial and lingual muscles, rigidity, and bulging eyes (Coutinho and Andrade, 1978; Lima and Coutinho, 1980; Barbeau *et al.*, 1984; Coutinho, 1992). Importantly, no cognitive impairment is observed in MJD patients; this may be a key

criterium in the differential diagnosis with other spinocerebellar ataxias including alterations of the intellect (Coutinho, 1992).

The high phenotypic variability associated with MJD, led to the organization of the disease into four sub-types, sorted by the age of clinical onset and major symptoms of the disorder (Lima and Coutinho, 1980). In sub-type 1 are classified the MJD forms with early age of onset, a faster progression, and more intense pyramidal and extra-pyramidal signs. Sub-type 2 is the most frequent, and is characterised by intermediate age-of-onset and progression, with patients exhibiting the usual ataxia and ophthalmoplegia and the possible evolution to either sub-type 1. In sub-type 3, MJD patients show the latest age at onset, slowed disease progression and more peripheral signs. A fourth sub-type was added by Rosenberg (1983); it is the rarest and comprehends MJD patients exhibiting Parkinsonic symptoms associated with the more typical MJD symptoms (Rosenberg, 1983; Margolis, 2002).

Anatomopathology

The neuropathological features show also a large variability, but most frequently MJD patients exhibited spinocerebellar degeneration and neuronal loss of the dentate nucleus. A progressive neuronal loss and gliosis of the *striatum*, *substantia nigra* (compacta and reticulate), pons and of the medulla anterior horn and motor cranial nuclei is also reported (Coutinho *et al.*, 1982; Coutinho, 1992; Ross, 1995).

Inheritance

Machado-Joseph disease is transmitted in an autosomal dominant manner, implying that one copy of the mutated gene is sufficient for the disease to be transmitted to the offspring; some rare cases of *skipped generations* are known (Sequeiros and Coutinho, 1981). In MJD, intergenerational instability in the transmission of the mutated allele is very common. Often, the expanded alleles in paternal transmission are less stable than in maternal transmission, originating larger repeat expansions in the offspring (Maciel *et al.*, 1995; Maruyama *et al.*, 1995; Igarashi *et al.*, 1996). The affected offspring often manifest the disease earlier in life, with faster progression and aggravated symptomatology. This phenomenon of *anticipation* is common in MJD families (Sequeiros and Coutinho, 1981; Sequeiros and Coutinho, 1993) and there is an inverse correlation

between the CAG size and the beginning or course of the disease (Maciel *et al.*, 1995; Takiyama *et al.*, 1998; Maciel *et al.*, 1999), though with no clinical predictive value.

The *MJD1/ATXN3* gene

In 1994, the mapping of the *MJD locus* to chromosome 14q was confirmed in Azorean families (Sequeiros *et al.*, 1994). Based on the novel characteristics of the genes identified for SBMA, HD, SCA1, SCA2 and DRPLA, Kawaguchi *et al.* (1994) soon identified and mapped *MJD1* to the long arm of chromosome 14 (14q24.3-32.1). *MJD1* was then confirmed as the mutated gene in MJD, since an expanded CAG tract was present only in clinically confirmed patients. The molecular diagnosis of MJD then became possible, and the mutation was then confirmed in families of diverse origins (Maciel *et al.*, 1995; Higgins *et al.*, 1996; Lopes-Cendes *et al.*, 1996; Gaspar *et al.*, 2001).

MJD is caused by the expansion of a CAG repeat in the 3' coding portion of the gene *ATXN3* (formerly *MJD1*). The repeat is highly polymorphic, ranging from 12-41 repeats in normal-sized alleles, and 55-86 in the expanded allele of MJD patients (Maciel *et al.*, 2001).

ATXN3 is evolutionary conserved and found in genomes from fungi to humans. The human *ATXN3* gene spans for 48240 bp and comprises 11 exons; the CAG repeat is located in exon 10.

Northern blot analysis revealed ubiquitous *ATXN3* expression and the existence of four different transcripts (1.4, 1.8, 4.5 and 7.5 Kb), thought to result from alternative splicing and polyadenylation events, occurring in exon 10 or exon 11 (Ichikawa *et al.*, 2001). So far, five *ATXN3* cDNA variants were described: MJD1a, MJD1-1 and MJD5-1, MJD2-1 and H2 (Kawaguchi *et al.*, 1994; Goto *et al.*, 1997); the latter lacks exon 2, but preserves the open reading frame, identical to variant MJD1-1 (Ichikawa *et al.*, 2001).

The *ATXN3* protein(s)

The human *ATXN3* gene product, ataxin-3, is a 42 KDa ubiquitously expressed protein, with a polyglutamine (polyQ) tract located in the C-terminal region (Kawaguchi *et al.*, 1994; Schmitt *et al.*, 1997). Ataxin-3 is the smallest of the known polyglutamine-containing proteins. It comprises an N-terminal domain, the Josephin domain (JD), two to three ubiquitin interacting domains (UIM), depending on the isoform, and the polyQ region (**Fig.2**). Ataxin-3 has at least three protein isoforms reported. *ATXN3* isoform 1

(ATXN3 var2.1, SwissProt no P54252) is encoded by the MJD2.1 cDNA variant and is the longest ATXN3 variant, spanning 364 aa, contains only two UIMs located before the polyQ region, and has an hydrophobic C-terminal (Goto *et al.*, 1997). Isoform 2 (ATXN3 var1.1, SwissProt no P54252-2 VSP_002784) is a splicing variant encoded by the cDNA variants MJD1.1 and MJD5.1, has 361 amino acids, possesses three UIMs and has a hydrophilic C-terminal; the MJD1a encodes a truncated variant of isoform 1 (16 aa shorter, 348 aa), due to premature stop codon (ATXN3 var1a, SwissProt no P54252 VAR_013690) (Kawaguchi *et al.*, 1994). Isoform 3 (SwissProt no P54252-3 VSP_002783, VSP_002784) is 55 aa shorter than isoform 2 due to the absence of exon 2 in the encoding cDNA variant H2, what makes it the only ATXN3 variant lacking the C14 residue in the JD (Ichikawa *et al.*, 2001).

The JD domain (Pfam02099) comprises the first 198 aa and is the most highly conserved domain of the protein (**Fig.3**). Using a bioinformatic-integrated approach Scheel *et al.* (2003) predicted that ATXN3 could be an ubiquitin-specific cysteinic-protease (USP), based on the conservation of the predictive catalytic triad (C14-H119-N134), typically found in these proteins as well as ubiquitin C-terminal hydrolase (UCH).

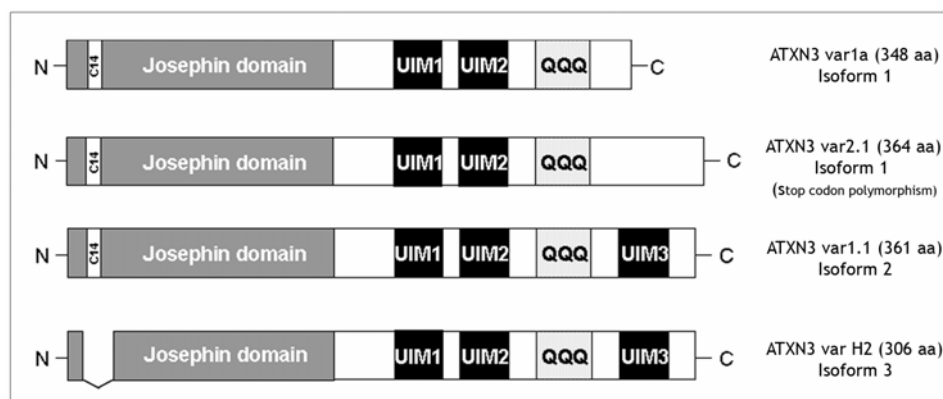


Figure 2. Schematic representation of the protein domains in ataxin-3 isoforms. ATXN3 is a 42-kDa protein comprising three splice variants. ATXN3 isoforms may comprise two-to-three ubiquitin-interacting motifs (UIM). The repetitive polyQ segment is located at the C-terminal region of the protein. Of note is absence of the C14 residue in the ATXN3 varH2.

Moreover, further evidence from the work of Donaldson *et al.* (2003) who, using a bioinformatic analysis, suggested also ATXN3 to be an ubiquitin-binding protein, localizing to polyQ aggregates in a UIM-dependent manner, with partial modulation of

the JD. Soon, *in vitro* assays validated these observations and a binding preference of ATXN3 for K48-linked polyubiquitylated proteins containing chains of at least four ubiquitin molecules was shown, a property dependent on the integrity of ATXN3's UIMs (Burnett *et al.*, 2003; Chai *et al.*, 2004). Moreover, in independent studies ATXN3 showed a C14-dependent deubiquitylating (DUB) activity *in vitro* (Burnett *et al.*, 2003; Chow *et al.*, 2004). Over-expression of inactive C14A-mutated protein in neural cell lines led to an evident accumulation of ubiquitylated proteins, comparable only to proteasome-inhibited conditions. The authors also suggested that ATXN3 is able of self-ubiquitylation (dependent of the UIMs) and self-deubiquitylating processing, unveiling a possible auto-regulation of its participation in the protein quality control pathways (Berke and Paulson, 2003).

Little information has been reported on the structure of full-length ATXN3. A first “draft” of the structure was obtained using limited proteolysis and NMR assays that revealed the existence of a compact 20 kDa region resistant to proteolysis, comprising a region later defined as the JD, and a flexible C-terminal that includes the polyQ domain (Masino *et al.*, 2003).

More recent structural studies, using NMR on JD alone, confirmed that this is the only constitutively folded region in ATXN3, rich in α -helices and adopting an open semi-elongated L-structure, compatible with its cysteinic protease activity (Nicastro *et al.*, 2005; Nicastro *et al.*, 2006).

The ubiquitin interacting motif (UIM) is a highly conserved amino acidic sequence first assigned to proteasome S5a subunit, but then extended to proteins participating in pathways as diverse as ubiquitylation, ubiquitin metabolism and endocytosis (Young *et al.*, 1998; Hofmann and Falquet, 2001; Miller *et al.*, 2007). A dual function has been ascertained to UIMs: binding of ubiquitin or ubiquitylated proteins and promotion of ubiquitylation (mono-, oligo- and polyubiquitylation) (Miller *et al.*, 2004; Regan-Klapisz *et al.*, 2005).

ATXN3 has also been found to bind ubiquitylated proteins in a way modulated by its UIM domains. Recently, Miller *et al.*, showed that the UIMs of ATXN3, on their own can inhibit the aggregation of mutant Htt (Miller *et al.*, 2007).

ATXN3 homologues

In the human genome, there are two *loci* for ATXN3 on chromosomes 14q32 and Xp22.2. The latter is known as ATXN3L (*like*) gene (NR_003585) and it encodes a putative

ATXN3-like protein (SwissProt no Q9H3M9) though no biological proof for this protein has been reported. ATXN3L has 355 aa and comprises a JD, two UIM and the polyQ domain; ATXN3L is similar to isoform 1 of ATXN3 (**Fig.2**) and shows 80% identity at the amino acid level, with this isoform. The lack of introns, known functionality and the high homology displayed by *ATXN3L* sets it as a pseudogene of *ATXN3*.

ATXN3 is evolutionarily conserved and homologous proteins are found in several animal and plant species. Namely, ATXN3 was found in rodents, *Rattus norvegicus* (NP_067734, 88% identity (Id)) (Schmitt *et al.*, 1997), *Mus musculus* (NP_083981, 88% Id) (do Carmo Costa *et al.*, 2004), chicken (*Gallus gallus*, NP_989688, 82% Id) (Linhartova *et al.*, 1999), *Caenorhabditis elegans* (NP_506873, 38% Id) (Rodrigues *et al.*, 2007) and plants, *Arabidopsis thaliana* (NP_190981, 36% Id) (**Fig.3**).

Studies of the *ATXN3* rat homologue were restricted to gene sequence and transcription analysis. The rat sequence was found to be identical to the human sequence. Expression analysis revealed an ubiquitous expression of a larger transcript (6 Kb), whereas a smaller variant (1.3 Kb) was exclusively found in the testis (Schmitt *et al.*, 1997), as proposed for the human gene (Ichikawa *et al.*, 2001).

An exhaustive study of the mouse homologue gene carried out by Costa *et al.* (2004), led to the determination of a genomic structure similar to that of the human gene (Schmitt *et al.*, 2003); characterisation of the promoter also revealed a TATA-less structure, with several conserved putative transcription factor binding regions, namely SP1 and MyoD. The murine protein is ubiquitously expressed throughout development as in adult tissues, showing a particularly elevated expression in muscle, ciliated epithelial cells and brain regions affected in MJD; as in the rat, differentially expressed protein isoforms were found in the mouse heart, skeletal muscle and testis, denoting a tissue-specificity of *ATXN3* expression that warrants further investigation.

More recently, the gene structure, mRNA and protein expression of the *C. elegans* homologue were also reported (Rodrigues *et al.*, 2007). The *atx-3* gene is expressed throughout the *C. elegans* development and mainly in adulthood. Sub-cellular expression analysis showed a cytoplasmatic but also nuclear localization; *in vitro* assays showed that the worm homologue is, like the human protein, a cysteinic-deubiquitylating enzyme. This work suggests that *atx-3* is the true ortholog of *ATXN3* in the worm. Moreover, using a genome-wide expression approach, the authors showed that the absence of *atx-3* gene in KO animals leads to a major deregulation of genes involved in UPS, structure/motility and signal transduction (Rodrigues *et al.*, 2007).

It is interesting to observe in **Fig.3** that there is no conservation of the polyQ region in the homologous proteins, indicating that the polyQ domain may not have a major functional relevance in normal ataxin-3.

ATXN3 expression

The first evidence regarding human ATXN3's expression was obtained from brain tissue and lymphoblastoid cells lines of MJD patients and healthy individuals (Paulson *et al.*, 1997a; Wang *et al.*, 1997). Expression of both normal and expanded ATXN3 was observed in the CNS, including in areas usually spared by the disease, and the PNS; moreover, ATXN3 showed mainly cytoplasmatic staining. A restricted expression pattern was reported for some brain regions, namely the striatum, where only a subset of neurons showed ATXN3 expression (Paulson *et al.*, 1997a). Later, Paulson *et al.* (1997b), reported that the mutant protein accumulates in the nucleus as ubiquitylated inclusions solely in the neurons of affected MJD regions. However, conflicting evidence regarding ATXN3 localization started to emerge. Wang *et al.* (1997), showed both cytoplasmatic and nuclear staining of ATXN3 in MJD brains. Trottier *et al.* (1998) reported a cytosolic expression of ATXN3 in HeLa; although in subcellular fractionation, ATXN3 co-fractioned in both the nuclear and mitochondrial fractions; conversely, in brains of MJD patients, most neurons of the areas analysed showed cytosolic expression. Mammalian cell line assays revealed that ATXN3 was found mainly, though not exclusively, in the nucleus, where it was associated with the inner nuclear membrane (Tait *et al.*, 1998). The growing evidence of the nuclear localization of normal ATXN3, and the finding of a nuclear localisation signal (NLS) led to the conclusion that normal ATXN3 may translocate into the nucleus, independently of an expanded polyQ domain (Tait *et al.*, 1998).

It is possible that the conflicting results regarding sub-cellular localization are consequence of the use of different antibodies and tissue processing methods, which in our experience may alter the results.

ATXN3 known interactome

Although much has been learned about ATXN3 in the last decade, its physiological function remains elusive.

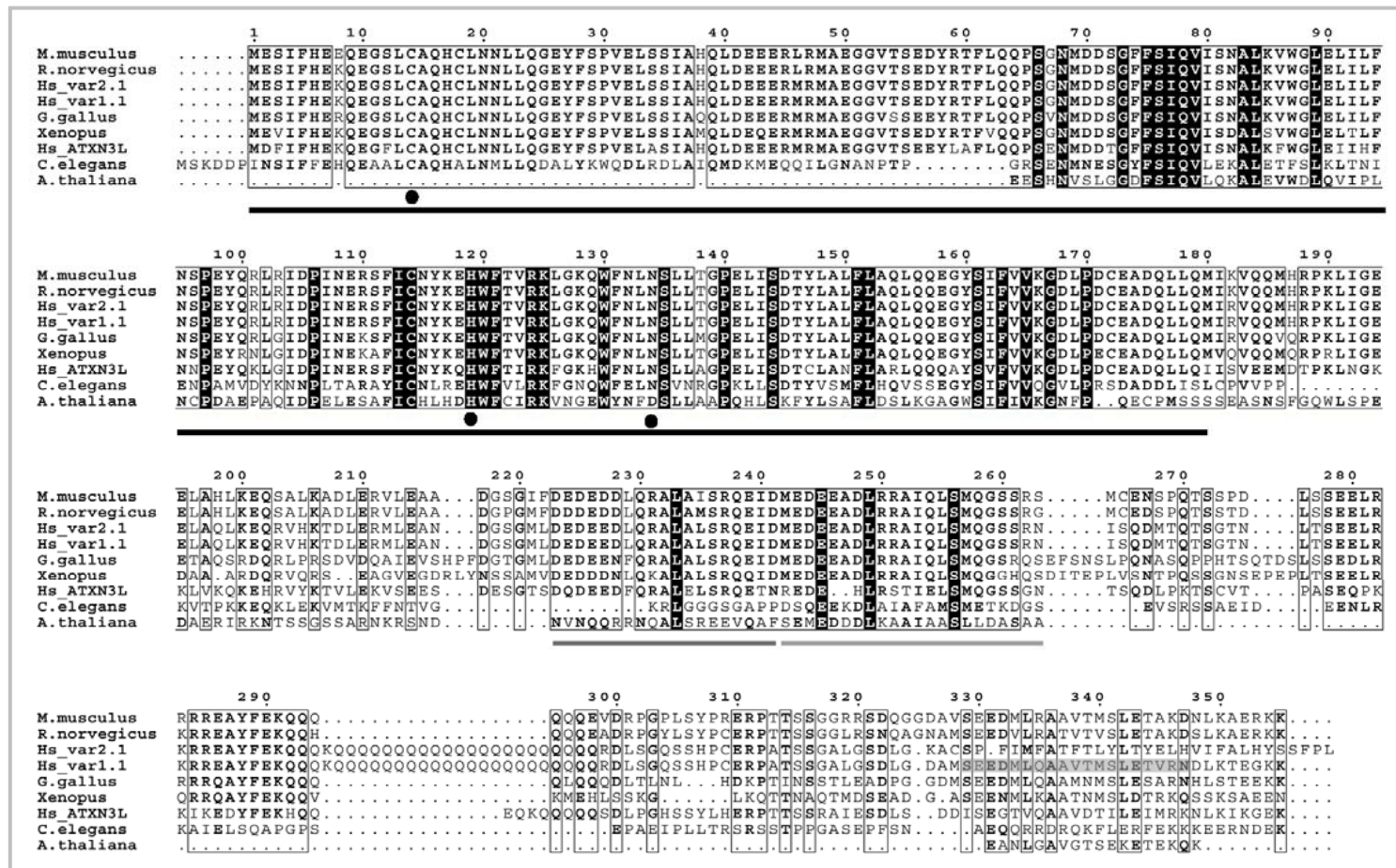


Figure 3. Multiple alignment of human (*Hs_var2.1*, *Hs_var1.1* and *Hs_ATXN3L*), rodent (*M.musculus* and *R.norvegicus*), chicken (*G.gallus*), frog (*Xenopus*), worm (*C.elegans*) and plant (*A.thaliana*) ataxin-3 homologous proteins. Columns frames show a global similarity score over 60 %, calculated from all group, completely conserved amino acids are black-countershaded and similar amino acid are in bold. In the picture, the black lines depict the Josephin domain, the dark grey and light grey lines represent UIM1 and UIM2, respectively; the horizontal light grey square highlights the UIM3 in ATXN3 var1.1; the black dots mark the predicted catalytic triad C14-H119-N134. Sequence alignments and colouring were performed with the T-Coffee (Notredame *et al.*, 2000) and ESPrit (Gouet *et al.*, 1999) programs, respectively. Numbers represent the amino acid scale bar.

The absence of *atx-3* expression produced no obvious phenotype in *atx-3^{-/-}* KO worm animals (Rodrigues *et al.*, 2007). Moreover, Costa, MC (unpublished results) showed that the knockdown expression of the murine homologue of ATXN3 did not affect the viability of mouse cell lines, further supporting the notion that the ATXN3 expression is not essential for cells or, perhaps, the existence of genetic redundancy. Without an obvious pathway affected by the abrogation of ATXN3 expression in these genetic approaches, the discovery of novel interactors may help to gain insight into the molecular mechanisms in which ATXN3 is participating. The first direct interaction of ATXN3 was reported by Wang *et al.* (2000), who demonstrated that ataxin-3 binds the two human homologues of the yeast DNA repair protein RAD23, HHR23A and HHR23B; the interaction described was mediated by the JD domain of ATXN3 and the N-terminal domain ubiquitin-like (UBL) domain of HHR23 proteins. Both HHR23A and HHR23B proteins have been defined as two important participants of the global genome NER sub-pathway (Li *et al.*, 1997; Sugasawa *et al.*, 1997; Hsieh *et al.*, 2005). In addition to its function in DNA repair, HHR23B was shown to be a modulator of UPS, as it participates in the translocation of substrates targeted for degradation at the proteasome (Hiyama *et al.*, 1999; Doss-Pepe *et al.*, 2003).

In vitro and *in vivo* assays demonstrated that both normal and expanded ATXN3 also interact with the cAMP-response element (CREB)-binding protein (CBP), p300 and p300/CBP associated factor (PCAF), inhibiting the transcription of CREB-dependent genes; moreover, it was showed that ATXN3 is able to bind directly the acetylation site in the histones, acting as a dual transcriptional repressor (Li *et al.*, 2002). More recently, the involvement of ATXN3 in the regulation of transcription has been strengthened, as normal ATXN3 binds to target DNA sequences in specific chromatin regions of the matrix metalloproteinase-2 (MMP-2) gene promoter. The recruitment of histone deacetylase 3 (HDAC3), nuclear receptor corepressor (NCoR) and the deacetylation of histones bound to the promoter underlies the mechanism of the transcription repression (Evert *et al.*, 2006).

ATXN3 is also known to interact with VCP (valosin-containing protein, and yeast cdc48), in a polyQ dependent manner; VCP is a molecular chaperone involved in the translocation of polyubiquitylated substrates to 26S proteasome, together with ATXN3 and HHR23B (Dai and Li, 2000; Kobayashi *et al.*, 2002; Doss-Pepe *et al.*, 2003). Moreover, this interaction is able to modulate the fibrillogenesis of mutant ATXN3 *in vitro*, in a concentration-dependent manner (Boeddrich *et al.*, 2006).

A binding preference of ataxin-3 for K48-linked polyubiquitylated proteins containing chains of at least four ubiquitin molecules has been also demonstrated *in vivo* and *in vitro* (Burnett *et al.*, 2003; Donaldson *et al.*, 2003). The interaction between ATXN3 and polyubiquitylated proteins is evolutionarily conserved, as both the *C. elegans* and mouse ataxin-3 homologues also exhibit deubiquitylating activity *in vitro*, also dependent of the cysteine residue (Costa MC *et al.* unpublished data and Rodrigues *et al.*, 2007).

Interestingly, ATXN3 also showed self-interaction, mediated by its JD (Gales *et al.*, 2005), reinforcing the results of Berke *et al.* (2005) that pointed out ATXN3 as a substrate of itself.

Recently, in a genetic screening, five new interactions of ATXN3 were identified using mutant ATXN3, in an yeast two-hybrid human brain cDNA library and colocalisation studies; of note are the SUMO-1 (small ubiquitin-like modifier-1), human rhodopsin guanosine diphosphate dissociation inhibitor alpha and human neuronal amiloride-sensitive cation channel 2 (Shen *et al.*, 2006). The interaction between ATXN3 and SUMO-1 was reported as being mediated through the N-terminal region of ATXN3, suggesting some independency of the polyQ domain. Conversely, Miller *et al.* (2007), reported that the new interaction between SUMO-1 and ATXN3 was established through its UIM regions, at C-terminal portion of ATXN3. Further clarification on this interaction is need, as SUMO-1 may play an important role in the post-transcriptional modification of ATXN3, namely affecting its cellular localisation.

ATXN3 has been also reported to interact with UFD2a (ubiquitin fusion degradation protein-2) (Matsumoto *et al.*, 2004) and with CHIP (Jana *et al.*, 2005), two ubiquitin ligases, strengthening the hypothesis of ATXN3 participation in UPS.

ATXN3 is involved in several pathways

The evidence gathered about ATXN3 subcellular localisation and interactions support several possibilities for the biological function of ATXN3 in cells:

- (1) ATXN3 may be a **transcriptional repressor** by interacting directly with transcription factors and histone deacetylases, enhancing its activity (Li *et al.*, 2002) (Evert *et al.*, 2006).

- (2) ATXN3 may participate in **cellular DNA damage response**, specifically in GGR-NER due to the interaction of ATXN3 with the HHR23 proteins (Wang *et al.*, 2000), though no biological proof of this has been reported.
- (3) ATXN3 may also be participant of the **protein surveillance mechanisms** since it:
- (i) interacts specifically with the HHR23 proteins, two known proteasome-binding factors;
 - (ii) interacts with VCP, a chaperone involved in the retro-translocation of misfolded protein from the endoplasmic reticulum to the 26S proteasome, broadening its participation in the cell quality control systems (Hirabayashi *et al.*, 2001; Kobayashi *et al.*, 2002; Doss-Pepe *et al.*, 2003; Nakayama, 2003; Boeddrich *et al.*, 2006; Zhong and Pittman, 2006);
 - (iii) it is associated with several E3-ligases such as parkin (Tsai *et al.*, 2003), CHIP (Jana *et al.*, 2005), p300 and PCAF, the p300-CBP-associated factor (Li *et al.*, 2002; Linares *et al.*, 2007).
 - (iv) shows deubiquitylating activity towards polyUb chains *in vitro* (Burnett *et al.*, 2003; Chai *et al.*, 2004; Berke *et al.*, 2005; Mao *et al.*, 2005; Rodrigues *et al.*, 2007);
 - (v) was shown to suppress the polyQ toxicity in a *Drosophila* model of MJD, and such neuroprotective role was dependent of the deubiquitylating activity of normal ATXN3 and of the proteasome (Warrick *et al.*, 2005).

However, a definite conclusion about the ATXN3 function remains elusive.

Objectives

Little was known about the ATXN3 protein itself or its physiological function(s), when this experimental work was started. Given this unexplored area, our objective was set in the identification and further characterisation of the molecular partners of ATXN3, gathering insight about the normal function(s) of ATXN3.

We initiated our work by performing a functional screening of a human brain cDNA library, using the yeast two-hybrid system, in order to identify novel ATXN3 interactors.

However, the results obtained with this strategy, for technical reasons, were not sufficient (Appendix 1). For this, we aimed at:

1. studying potential direct atxn3 protein-protein interactions, assessing strong candidates for the ATXN3 interactome (Chapter 1 and Chapter 2);
2. evaluating the potential evolutionary conservation of the interactions identified, verifying if the interaction pairs identified in human were maintained for the ATXN3 *C. elegans* homologue, atx-3 (Chapter 1 and Chapter 2).

DNA techniques

DNA quantification was determined on a spectrophotometer and stock concentrations was calculated accordingly to the formula: $C_{\text{stock}} = A_{260} \times \text{dilution} \times 50 \mu\text{g/mL}$. Restriction digestions and ligation reactions were performed by standard cloning techniques (Sambrook and Russel, 2001). Polymerase chain reactions (PCR), in this work, were carried out using regular *Taq* (Fermentas), or with proofreading properties as Expand High Fidelity and Expand Long Template (Roche), following the manufacturer's instructions; Pfu polymerase (Stratagene) was used for the site-directed mutagenesis reactions.

Plasmid DNA isolation from *E. coli*

In general, plasmid DNA was extracted from bacteria, using procedures from commercial available kits (Qiagen), or using the standard alkaline method. Briefly, overnight cultures were harvested by centrifugation, at 3250 rpm, for 5 min. Pellet was resuspended in maxiprep solution (10 mM EDTA, 25 mM Tris-Cl pH 8, 1% glucose); fresh made lysis NAHO/SDS buffer (0.2 M NaHO/1% SDS) was added and the mixture was incubated on ice for 15 min. Proteins were precipitated with 3 M NaCH_3COO pH 5.5, and separated, at 16400 rpm, for 10 min. The supernatant was transferred to new microtubes; DNA was precipitated with ethanol absolute, mixed, and then centrifuged at 16400 rpm, for 20 min. The pellet was washed with 70% ethanol, air-dried and resuspended in H_2O or TE (100 mM Tris-HCl pH 8.0, 10 mM EDTA). Mix 1 μL RNase (DNase-free, 0.5 $\mu\text{g}/\mu\text{L}$) per 50 μL DNA and incubated one hour, at 37 °C.

Protein techniques

Quantification of proteins was performed by Bradford's method (Bradford, 1976), according to the manufacturer's instructions (Sigma). Absorbance was measured at 595 nm, on a spectrophotometer, using 1:1000 dilutions; protein concentrations were extrapolated from a standard curve obtained with a bovine serum albumin (BSA) dilutions set. Proteins were separated in SDS-PAGE gels, according to the expected size of the protein. Proteins were either stained with blue coomassie or electro-transferred into PVDF or nitrocellulose membranes, according to standard methods. Proteins were visualized with Ponceau S staining and/or immunoblotted.

SDS-PAGE electrophoresis

SDS-PAGE separation was performed as described (Laemmli, 1970), using a 40% acrylamide solution (37.5:1 acrylamide/bis-acrylamide), or 30% acrylamide solution (29:1 acrylamide/bis-acrylamide). The protein samples were diluted sample buffer 1X (62.5 mM Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 200 mM DTT, 0.02% bromophenol blue), denatured at 95 °C, for 5 min, and loaded onto the SDS-PAGE with a prestained protein marker. Gels were run at 130 V (constant voltage), using SDS-PAGE running buffer 1X (25 mM Tris-base pH 8.3, 192 mM glycine, 1% SDS).

***C. elegans* strains**

The nematode N2 Bristol (wild-type) and *atx-3* (knockout for the *atx-3* gene) strains were obtained from *Caenorhabditis* Genetic Consortium (CGC). The *atx-3* KO strain contains a 366 bp deletion, comprising 287 bp of the promoter and 79 bp of *atx-3* exon 1. The strain was back-crossed twice in our lab, in order to eliminate random mutations due to the original trimethyl psolaren/UV mutagenesis. The strains were maintained at 20-21 °C, in NGM plates [3 g/L NaCl, 2,5 g/L bacto-peptona, 20 g/L agar, 1 mM CaCl₂, 1 mM MgSO₄, 5 µg/ml cholesterol, 19 mM potassium phosphate (pH6.0)], seeded with the *E. coli* NA22 strain.

Worm synchronized cultures

Synchronously growing plates for total RNA extraction were prepared by the alkaline hypochlorite method. For large quantities of worms, 9-cm enriched peptone plates [3 g/L NaCl, 20 g/L bacto-peptona, 20 g/L agar, 1 mM MgSO₄, 5 µg/ml cholesterol, 19 mM potassium phosphate (pH 6.0)], seeded with NA22, were chunked from growth plates. Plates were grown for two days, until gravid adult worms were the majority of the population. The worms from two to three grown plates were collected to a conical screw-cap tube, washed twice with 10 mL sterile M9 buffer (6 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 5 g/L NaCl, 1 mM MgSO₄) with brief spun (1000 rpm, 1 min) in-between. The bleaching of the worm pellet was performed with 7-10 mL of alkaline hypochlorite solution (2 mL fresh bleach, 5 mL 1 M NaHO), for 10 min, with careful vortexing every 2 min. After bleaching, the released eggs were spun (1000 rpm, 1 min), washed twice with 10 mL of sterile M9 buffer and collected by centrifugation. The pellets were then resuspended in 500 µL of sterile M9 buffer, transferred to the necessary fresh enriched

peptone plates with a glass Pasteur pipette, and grown to the desired larval stage at 20 °C.

ENU mutagenesis

For the *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis experiments, the working solutions of ENU were always prepared in sterile M9 buffer, from a stock solution of 100 mM of ENU (in 100% ethanol) with no more than one week old at -20 °C as described (De Stasio and Dorman, 2001). The stock solutions were prepared from the same ENU powder stock (Sigma). Five or six synchronized 9-cm enriched peptone plates of N2 or *atx-3* L4 larval-stage worms were collected in a conical screw-cap tube. The animals were washed twice, with 20 mL of sterile M9 buffer. For the mutagenesis experiments, the control worm pellet was resuspended with 4 mL of M9 buffer, whereas the mutagenized worms were resuspended in 4 mL of ENU working solutions. The worms were incubated in the dark, in a rotary shaker (40 rpm) for four hours, at 20 °C. The worms were then spun (1000 rpm, 1 min) and washed two to three times with sterile M9 buffer. The worm pellet was then processed as necessary (see below).

Protein extraction from *C. elegans*

Protein extracts of *C. elegans* were prepared, generally from five L4 larval-stage synchronized 9-cm enriched peptone plates. Worms were collected with 10 mL of M9 to a tube, briefly centrifuged, and washed two to three times with M9 to wash-off bacteria. To obtain protein extracts from ENU-treated worms, the animals were first treated as described in “ENU mutagenesis” protocol or processed as follows. Proteins were extracted from the worm pellets by adding 1V of worm lysis buffer [WLB, 100 mM Tris pH6.8, 2% SDS, 15% glycerol plus 1X complete protease cocktail inhibitors (Roche)]. Samples were then boiled for 5-10 min in a water bath, with occasional shaking. Proteins were spinned at 3200 rpm, for 20 min at 4 °C. The supernatants were collected and protein concentration measured by Bradford method. Protein stocks were stored at -80 °C.

Total RNA extraction

Extraction of total RNA from *C. elegans* was carried out from synchronized 9-cm enriched peptone plates of larval-stage worm cultures. Mutagenised (previous section)

or control *C. elegans* were collected, and total RNA was extracted using Trizol (Invitrogen), as described (<http://cmgm.stanford.edu/~kimlab/germline>). Briefly, 1 mL Trizol/500 μ L worm pellet was added, mixtures vortexed, and immediately frozen at -80 °C; then, 6-8 to eight cycles of -80 °C/37 °C incubations were carried out to burst the worms. Total RNA extraction was carried out according the manufacturer's instructions.

Brood size test

Synchronized cultures of worms were obtained by picking 20 gravid adults to a single 6-cm seeded NGM plate, which were left to lay eggs for four hours. The gravid adults were then removed, and the eggs were grown synchronously until L4-larval stage. These synchronized cultures were collected and washed twice with 10 mL of sterile M9 buffer. For the mutagenesis assays, the pellet of each 5-cm plate were incubated with 4 mL of ENU working solution at the desired concentration for four hours, in the dark, at 20 °C, while the control animals (one plate per each ENU concentration tested) were incubated with 4 mL of sterile modified M9. Afterwards, the animals were washed two to three times with sterile M9 buffer, transferred to a fresh 9-cm seeded NGM plate, and left to recover overnight, at 20 °C. The next day, 15 alive and moving animals (P0) of each condition were individually plated in a 6-cm seeded NGM plate, and its progeny scored in the following six days. The P0 animals were transferred to a new seeded plate every day, and the progeny in each plate was scored at L4-young adult stage.

Chapter 1

ATXN3 and DNA repair

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In preparation

Abstract

Machado-Joseph Disease (MJD/SCA3) is an autosomal dominant neurodegenerative disorder caused by the expansion of a CAG segment in the coding portion of the gene. In humans, the *ATXN3* gene encodes for ataxin-3 (ATXN3), a 42-KDa intracellular protein with deubiquitylating properties. The establishment of differential interactions by ATXN3 may be the basis of the selective neuronal loss characteristic of MJD albeit the ubiquitous expression of ATXN3. The first molecular interactors of human ATXN3 identified were the human homologues of RAD23, HHR23A, and HHR23B. These proteins have been characterised as modulators of the ubiquitin/proteasome system (UPS) and as participants of the DNA damage recognition mechanism in nucleotide excision repair (NER), through interaction with *Xeroderma pigmentosum* group C (XP-C) protein. Here, we show by immunofluorescence analysis in HEK293 cells that ATXN3 and XPC proteins are co-localized and that UV radiation enhances this co-localization in the exposed cells. In addition, we report, for the first time, the cloning and expression of the *C. elegans* RAD23 protein homologue 2-like, ZK20.3, and we show that *atx-3* interacts with ZK20.3 suggesting evolutionary conservation of the structural determinants relevant for this novel interaction. ZK20.3 is highly expressed in worm adults, as previously observed for *atx-3*. Using semi-quantitative RT-PCR, we analysed the effect of *N*-ethyl-*N*-nitrosourea (ENU) upon the expression of *atx-3*, ZK20.3 and *xpc-1* in *C. elegans*. We then demonstrated that *atx-3* is upregulated in ENU-mutagenized worms, suggesting a potential role of *atx-3* in *C. elegans*' response to mutagenesis. Behavioural assays showed no effect of ENU upon N2 or KO's progeny when compared to controls, though a significant difference was observed between the two genotypes.

Introduction

There are nine polyglutamine diseases reported to date. Machado-Joseph disease (MJD/SCA3) is the most common form of these dominantly-inherited ataxia worldwide (Schols *et al.*, 2004). Affected individuals present an abnormal form of the ATX3 gene product comprising 55 to 87 glutamines in the C-terminal region instead of the usual 12 to 41 glutamines found in normal individuals (Maciel *et al.*, 2001). This is due to a

dynamic mutation caused by the expansion of a trinucleotide repeat (CAG) in the 3'-coding region of *ATXN3* (Kawaguchi *et al.*, 1994). Despite, the ubiquitous expression of *ATXN3* (Paulson *et al.*, 1997a), only specific brain regions are affected in MJD. Expanded *ATXN3* accumulates in the MJD affected regions as ubiquitylated neuronal inclusions containing, also, proteasomal subunits and molecular chaperones (Paulson *et al.*, 1997b; Schmidt *et al.*, 2002; Berke and Paulson, 2003; Chai *et al.*, 2004). Intraneuronal aggregation is a common feature of all polyglutamine diseases, but whether these proteinaceous structures are a cell's attempt to suppress toxicity, or if they are *per se*, the cause of neuronal dysfunction leading to disease, remains controversial.

Recent reports have described *ATXN3* as a polyubiquitin binding protein, with a preference for chains with at least four ubiquitin-moieties (Donaldson *et al.*, 2003; Doss-Pepe *et al.*, 2003; Chai *et al.*, 2004), and to possess deubiquitylating (DUB) activity *in vitro* (Burnett *et al.*, 2003), dependent on its catalytic centre cysteine residue (C14) (Berke *et al.*, 2005; Burnett and Pittman, 2005).

As described in the "General Introduction" the normal function of *ATXN3* is not fully understood and the study of its interacting partners is therefore expected to help in this elucidation. Wang *et al.* (2000) reported the interaction of *ATXN3* and HHR23A/B proteins, the human homologues of RAD23 protein. In addition to their role in DNA repair (van der Spek *et al.*, 1996; Li *et al.*, 1997; Sugasawa *et al.*, 1997; Hsieh *et al.*, 2005; Chen and Madura, 2006), these proteins were shown to act as shuttling factors, acting in conjugation with *ATXN3*, p97/VCP (valosin-containing protein) and proteasomal subunits (Walters *et al.*, 2003) for the delivery of polyubiquitylated proteins to the proteasome (Dai and Li, 2000; Doss-Pepe *et al.*, 2003; Boeddrich *et al.*, 2006). However, the importance of *ATXN3* and HHR23A/B interactions for DNA nucleotide excision repair (NER) mechanism has remained unexplored.

NER eliminates bulky helix-distorting DNA adducts caused by genotoxic lesions, such as UV-induced cyclobutane pyrimidine dimers (CPD), pyrimidine-pyrimidone (6-4) photoproducts (6-4 PP), caused by several chemical agents, including oxidative DNA damage (Reardon *et al.*, 1997; Costa *et al.*, 2003). Efficient repair is vital for the maintenance of genomic integrity (Friedberg, 2001). XPC-HHR23B is one of the complexes known to be involved in the recognition of the DNA lesion, the earliest step of GGR-NER (Huang and D'Andrea, 2006). XPC is responsible for the physical binding of

the complex to damaged DNA, but HHR23B stimulates XPC action at early stages of damage recognition and modulates the complex displacement afterwards (You *et al.*, 2003). It was shown that XPC also interacts with centrin-2 (CETN2), forming a trimeric complex *in vivo*, XPC/HHR23B/CETN2. Centrin-2, together with HHR23B, stabilizes XPC, hence stimulating the repair activity of the complex (Araki *et al.*, 2001).

How does this correlate with polyglutamine diseases? Neurons are particularly susceptible to many kinds of stress, and polyglutamine protein accumulation imposes a great deal of cellular stress. Either by depletion of important factors, impairment of the proteasome by misfolded protein overloading, or other mechanisms not yet fully understood, it might lead neuronal cells to significant dysfunction and death.

Evidence shows that, cellular models of HD expressing expanded full-length huntingtin (Wyttenbach *et al.*, 2002) or its pathogenic N-terminal portion (encoded by exon 1 of *HD*), produced increased levels of ROS, in a CAG length-dependent manner (Firdaus *et al.*, 2006). It has been reported that mitochondrial dysfunction and excitotoxic mechanisms promote oxidative damage in the brain of HD patients (Tabrizi *et al.*, 1999). Moreover, experiments with lymphoblastoid cell culture of patients homozygous for HD mutation revealed severe ultra-structural and functional mitochondrial alterations (Squitieri *et al.*, 2006). Accumulation of oxidative damage products, such as 8-hydroxydeoxyguanosine (OH8dG) in areas degenerated in HD (Browne *et al.*, 1999), and in a HD mouse model overexpressing huntingtin (Bogdanov *et al.*, 2001), provided further evidence that oxidative stress may be involved in HD pathogenesis. Moreover, Giuliano *et al.*, reported accumulation of ROS in HD and SCA2 patients, due to polyglutamine activation of ATM/ATR-dependent DNA damage response (Giuliano *et al.*, 2003). ROS/oxidative DNA damage may cause neurodegeneration by, e.g., impairing the transcription of neural genes, due to progressive impairment of DNA repair.

NER repairs DNA damage induced by UV and chemical agents, and, to some extent, DNA oxidative lesions produced by ROS (Reardon *et al.*, 1997; Brooks *et al.*, 2000).

Recently, a study conducted in cultured CNS neurons showed that expanded ATXN3 induces neurodegeneration in specific neuronal subpopulations, normally affected in the disease, by activating a mitochondria-dependent apoptotic cascade and by promoting the release of apoptogenic proteins (Chou *et al.*, 2006). The mitochondrial damage seen in cultured neurons normally affected in the disease may be only the tip of the iceberg. In vivo, this type of damage may go on for decades, with progressive mitochondrial

impairment leading to cellular energy imbalance and marked production of ROS. The depletion of the ATP supply in the neuron will interfere with removal of proteins by the ubiquitin-proteasome system, due to the 26S absolute energy requirement. As shown before, proteasome impairment causes accumulation of polyubiquitylated proteins, and its diminished catalytic activity may influence NER negatively (Wang *et al.*, 2005). DNA damage, mitochondria shutdown, ROS production, transcription deregulation or protein quality control impairments are phenomena affecting all kinds of cells. Neurons, however, as non-replaceable, highly metabolic cells, with large energy requirements will, overtime, suffer any disturbance more dramatically, and this may be translated in irreversible cellular damage.

Apart from its role in the protein quality control system, ATXN3 was shown to be also a transcription regulator (Li *et al.*, 2002). ATXN3 has been reported as a dual factor, either binding histones directly, inhibiting their acetylation and transcription, or as a transcription co-repressor of CREB-dependent genes, by interacting with acetyltransferases like CBP, p300 and P/CAF, inhibiting their histone acetyltransferase activity.

This link of ATXN3 to chromatin remodelling, may well be linked to a putative role in DNA repair. Histones may be modified by methylation, acetylation, ubiquitylation or sumoylation, modifications, which generally favour, through specific mechanisms, chromatin relaxation and nucleosome unwinding, allowing replication, transcription, and/or DNA repair (Peterson and Laniel, 2004). Recently, it was reported that UV radiation-stimulated histone acetylation, in yeast, is independent of Rad4 or Rad14 (XPC and XPA human homologues, respectively) (Yu *et al.*, 2005). ATXN3 interacts with NER components known to be involved in the initial/recognition events, participating in GGR-NER on naked chromatin (de Laat *et al.*, 1999), namely HHR23 proteins (Wang *et al.*, 2000). Hence, the transcriptional co-repressor capacity of ATXN3 (Li *et al.*, 2002) does not exclude the possibility of ATXN3 participation in NER or recruitment of DNA damage recognition factors to lesion sites, but may potentially reflect different functions of ATXN3 upon hetero- or euchromatin.

The HHR23 proteins have been characterized both as modulators of the ubiquitin/proteasome system (UPS) and as participants of the DNA damage recognition mechanism in nucleotide excision repair (NER), through interaction with *xeroderma pigmentosum* group C (XPC, NP_004619) and centrin-2 (CETN2, NP_004335) proteins.

These proteins form a trimeric complex and the direct interaction of HHR23B and CETN2 with XPC stabilizes it (Araki *et al.*, 2001; Popescu *et al.*, 2003; Nishi *et al.*, 2005).

The interaction of ATXN3 with HHR23 proteins led us to raise the hypothesis that ATXN3 could also interact with the other proteins of the trimeric complex, XPC and CETN2, and be modulating the damage recognition complex activity.

These hypotheses were studied in both human cell and *C. elegans* systems. In human cell system (**see section 1A**), we focused on the interactions between the human proteins and how they might be modified by DNA damage-inducing conditions. Due to its easy manipulation, the possibility to perform functional assays and the availability of a knockout strain for *atx-3*, we also used *C. elegans* in our studies (**see section 1B**). We analysed the conservation of the interactions, determined the effect of genotoxic stress in the homologue genes of *C. elegans*, and assessed the importance of ataxin-3 in worm's response to genotoxic insult.

Chapter 1: Section 1A

Human ATXN3 and the NER pathway

Introduction

The reported interaction between ATXN3 and the two proteins HHR23A and HHR23B (Wang *et al.*, 2000), known to be involved in DNA repair, namely in the global genome repair branch of the nucleotide excision repair system (GGR-NER), instigated us to study the consequences of this interaction, and explore the possible interaction of ATXN3 with other proteins, involved in GGR-NER, as XPC and centrin-2 (CETN2).

For this we analysed these putative interactions using *in vitro* and *in vivo* approaches, as the yeast two-hybrid system (Fields and Song, 1989), GST pull-down and immunofluorescence assays. Moreover, we assessed the effect of DNA damage-inducing condition upon these interactions, in a cell-based system.

Material and Methods

Antibodies

A novel primary antibody against the ATXN3 variant MJD2.1, anti-MJD2.1 antibody, was synthesized externally in Davids Biotechnologie by immunization of rabbits. The antibody anti-MJD2.1 was raised against the recombinant full-length ATXN3 var2.1. Competition assays were performed to assess the specificity of the antibody (**Fig.A5** of Appendix 2).

The anti-MJD1.1 antibody against the human ATXN3 var1.1 is described in chapter 2, whereas the anti-303JSP antibody specific for the atx-3 protein was described before (Rodrigues *et al.*, 2007); the anti-mjd antibody was kindly provided by Dr Henry Paulson (Paulson *et al.*, 1997a).

Yeast expression plasmids

The pAS1/MJD2.1(17Q), pAS1/2.1N, pAS1_G/MJD1.1(14Q) and pAS1_G/JD plasmids and the empty vectors pAS1_G and pACT2_G were previously described (chapter 2). To obtain the pACT2_G/HHR23A and pACT2_G/HHR23B plasmids, the cDNA sequence encoding HHR23A and HHR23B were reverse transcribed from total RNA of HeLa cells, and then amplified

using attB1-HHR23A, attB2-HHR23A and attB1-HHR23B, attB2-HHR23B primers, respectively. To obtain the pACT2_G/XPC-D1 encoding the N-terminal fragment of XPC protein, and pACT2_G/XPC-D2 construct for its C-terminal, the cDNA sequence encoding XPC-D1 and XPC-D2 were reverse transcribed from total RNA of HeLa cells, and then amplified using attB1-XPC-D1, attB2.1-XPC-D1 and attB1.2-XPC-D2, attB2-XPC-D2 primers, respectively. The cDNA encoding human centrin-2 (CETN2) was obtained from total RNA of HeLa cells as described (Araki *et al.*, 2001) but the second amplification was carried out using attB1-CETN2 and attB2-CETN2, in order to clone the cDNA fragment into pACT2_G using the Gateway system (Hartley *et al.*, 2000). All aforementioned PCR products were first cloned into pDONR201 or pDONR207 Gateway vectors, and then transferred to pACT2_G or pAS1_G; the HHR23A and HHR23B attB-PCR products were first cloned into pGEM-T-Easy vector (Promega), and then sub-cloned to yeast expression vectors using classical cloning techniques (Sambrook and Russel, 2001). All clones were verified by automated sequencing. For primers sequence, refer to **Table A3** of Appendix 2.

Bacterial expression plasmids

To obtain the plasmids expressing the GST-HHR23A, GST-HHR23B and GST-CETN2, the attB-PCR products were transferred from the specific pDONRs vectors to pDEST15, using the Gateway technology (Hartley *et al.*, 2000). All clones were verified by automated sequencing. The plasmids expressing 6His-ATXN3 var1.1, 6His-JD (6His tagged-Josephin domain) and 6His-D1 (comprising the JD plus ubiquitin interacting motifs UIM1 and UIM2) were described before (Gales *et al.*, 2005).

Yeast two-hybrid assay

The yeast strains Y190 (ATCC, 96400) and Y187 (ATCC, 96399) were transformed using the lithium acetate method, as described (Gietz *et al.*, 1995). All transformations with pAS1_G-JD, pAS1_G-ATXN3 var1.1 and pAS1-2.1N were grown on SC-WLH supplemented with 130, 140 or 170 mM of AT (amino-1,2,3 triazole), respectively; the remaining were grown on SC-WLH with 20 mM AT. The interactions between TSCII (hamartin) and TSCI (tuberin) (van Slegtenhorst *et al.*, 1998), or SNF4 and SNF1 (Fields and Song, 1989), were used as positive controls in the assays. Incubations were performed at 30 °C, for

five to ten days. Colony-lift β -galactosidase filter assay was performed according to the manufacturer's instructions (Clontech).

Expression of recombinant proteins

Expression and purification of 6His-tagged ATXN3 var1.1 and 6His-JD was described before (Gales *et al.*, 2005). Expression of GST-HHR23A, GST-HHR23B and GST-CETN2 were carried out in BL21-SI *E.coli* strain (Invitrogen) with 300 mM NaCl. Generally, protein expression was induced for three hours. Harvested cells were resuspended in 1/10 volume of resuspension buffer (TBS 1X, 10 μ g/mL lysozyme, 10 mM EDTA pH8, PMSF 0.5 mM), supplemented with EDTA-free protease inhibitors cocktail (Roche). After lysis, the supernatant was cleared by centrifugation, and the GST-tagged proteins were purified with glutathione-sepharose™ 4B beads (Amersham Biosciences); elution was carried out with 10 mM reduced glutathione in 50 mM Tris-Cl pH 8.

GST pull-down assay

The purified GST or GST-tagged proteins were incubated for one hour, at 4 °C, with 50 μ l of glutathione-sepharose™ 4B beads, in 500 μ l of in vitro binding buffer (IBB, 50 mM Tris-Cl pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) (Otera *et al.*, 2000). The 6His-JD or 6His-ATXN3-var1.1 were added and incubated for 1-2 hours, at 4 °C, on a rotator; 1% bovine serum albumin (BSA) was used for blocking in 6His-ATXN3-var1.1 reactions. Bound fractions were washed with IBB without glycerol and BSA, eluted by boiling in 50 μ l of 2X SDS sample buffer, and then analyzed by SDS-PAGE (12%). Immunoblottings were performed using goat anti-GST (1:7500, GE Healthcare) or rabbit anti-MJD1.1 (1:2000) with overnight incubation. Secondary HRP-coupled anti-goat antibody (Sc-2020, Santa Cruz Biotechnology) was diluted at 1:20000, and anti-rabbit (401393, Calbiochem) at 1:20000. All antibodies were diluted in 3% SM/TBST (3% skimmed milk, Tris-buffered saline (TBS) 1X, 0.05% Tween 20). Detection was carried out with ECL (GE Healthcare).

Immunofluorescence analysis

For immunolocalization of endogenous ATXN3 and XPC, HEK293 cells were grown on glass coverslips, for 48h, on DMEM (D5648, Sigma). Cells were fixed and permeabilized, as described before (Correia *et al.*, 2003). Briefly, cells were fixed with 4%

paraformaldehyde/4% saccharose. After washing twice with PBS, permeabilisation was carried out using PBS/0.3% Triton X-100, and blocking was performed, for one hour, with PBS/0.1% Tween 20/0.2% gelatin (Sigma) (PBST-0.2%G). The endogenous XPC and ATXN3 proteins were detected with anti-XPC monoclonal antibody (ab-6264, Abcam) at 1:50, and with polyclonal anti-mjd antibody (Paulson *et al.*, 1997a) at 1:10000, respectively, both in PBST-0.1%G. AlexaFluor 488-coupled goat anti-rabbit and AlexaFluor 568-coupled goat anti-mouse (Molecular probes) secondary antibodies were used simultaneously at 1:1000 in PBST-G. Coverslips were mounted in VectorShield with DAPI, and analyzed using a x63/1.40 NA oil objective (Leica) in a laser scanning confocal microscope (SP2 AOBS SE; Leica Microsystems), at room temperature, using LCS software (Leica Microsystems).

UV radiation

To perform UV radiation assays, the HEK293 cells were grown on glass coverslips, for 48h. Immediately before irradiation, cells were washed in pre-warmed PBS and then UV irradiated (254 nm) from above, with 20 J/m², using a UV crosslinker device (GE Healthcare). Afterwards, cells were either processed for immunocytochemistry, or maintained in the initial medium (preserved during the assay), to allow repair before the immunofluorescent staining (described above).

Results

Human ATXN3 binds more than one component of the XPC DNA damage recognition complex

ATXN3 interacts with XPC

We prepared for the yeast two-hybrid assays the following constructs: bait encoding normal full-length ATXN3 var1.1 (Gal4BD-ATXN3(14) var1.1), bait encoding a truncated variant of the protein comprising the JD plus the first UIM (ubiquitin-interacting motif) (Gal4BD-2.1N) and the preys encoding human HHR23A, HHR23B and CETN2. Western blot confirmed their expression in the yeast (**Fig.A4** in Appendix 2).

The NER DNA damage recognition complex comprises XPC, HHR23B and CETN2 (Araki *et al.*, 2001). The complex is stabilised by the direct interaction of HHR23B and CETN2 with XPC. To analyse if ATXN3 could bind the other components of the NER complex, XPC and CETN2, we prepared the constructs encoding the N-terminal or the C-terminal of the protein, Gal4AD-XPC-D1 and Gal4AD-XPC-D2, respectively, since we were unable to obtain a full-length clone for the human XPC protein (**Fig.4A**).

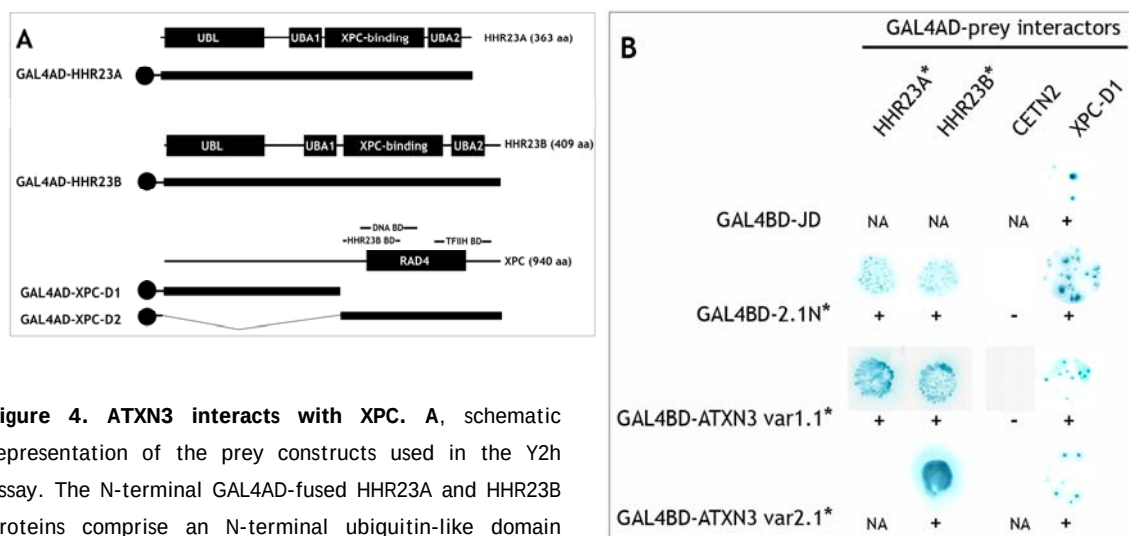


Figure 4. ATXN3 interacts with XPC. **A**, schematic representation of the prey constructs used in the Y2h assay. The N-terminal GAL4AD-fused HHR23A and HHR23B proteins comprise an N-terminal ubiquitin-like domain (UBL), two ubiquitin associated domains (UBA), and a xeroderma pigmentosum complementary group C (XPC)-binding domain. Two fragments of the full-length XPC protein were defined: XPC-D1 is an N-terminal fragment; XPC-D2 is the C-terminal fragment, which comprises the known HHR23B, DNA and TFIIH binding sites, as depicted in the figure. Schematic drawings are not at scale. **B**, the yeast two-hybrid assays between ATXN3 and NER proteins revealed positive interaction with XPC-D1. Two ATXN3 variants were used: ATXN3 var1.1 comprises three UIMs and ATXN3 var2.1, which has only two UIMs before the polyQ domain. Two C-terminal truncations were also assayed, GAL4BD-2.1N, which includes the JD plus the functional UIM1, and GAL4JD comprises the JD alone. Yeast co-transformations were plated in high stringency plates, and an interaction was considered positive (+) when growth on nutritional selection plates was observed and colonies stained blue, when tested for β -galactosidase expression (blue dots); asterisks correspond to protein-protein interactions reported before (Wang *et al.*, 2000). NA, interaction not assessed.

The results showed that human ATXN3, independently of the cDNA variant expressed, is able to bind XPC by its N-terminal region. Moreover, the presence of ATXN3 JD is sufficient for the interaction to occur, as positive interactions were observed between Gal4BD-2.1N or Gal4BD-JD and Gal4AD-XPC-D1 (**Fig.4B**); a weaker interaction was also detected with the C-terminal fragment of XPC, Gal4AD-XPC-D2 (data not shown). In

contrast, no direct interaction was observed between ATXN3 and CETN2. The positive interactions found were specific, as the baits did not interact with Gal4AD alone, neither the preys with the Gal4BD alone.

GST pull down assays were performed, which further confirmed the Y2H results, regarding ATXN3 and CETN2 results (**Fig.5**). We were unable to clone XPC full-length and to produced the GST-XPC protein; thus we could not confirm this interaction using GST pull-down assays.

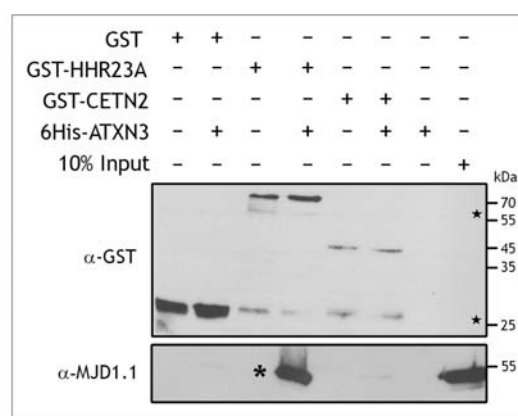


Figure 5. ATXN3 does not interact with CETN2. A, purified GST-HHR23A and GST-CETN2 were incubated with 6His-ATXN3. The described interaction of ATXN3 and HHR23A (Wang *et al.*, 2000) was observed in the conditions used (asterisk). Inputs lanes represent 10% of 6His-ATXN3 used in the assays. No interaction between ATXN3 and GST-CETN2 was observed *in vitro*. GST alone showed no relevant binding in the assays. Black stars indicate protein degradation fragments.

UV exposure enhances ATXN3 and XPC co-localization in HEK293 cells

We analysed the expression and localization of both endogenous ATXN3 and XPC proteins in HEK293 cells. Using double-immunostaining, we determined if ATXN3 and XPC could co-localise in this mammalian cell line. Cells were fixed, permeabilised and then immunoastained with monoclonal anti-XPC and polyclonal anti-mjd antibodies. Both proteins exhibited a diffuse distribution in the cell (**Fig.6, (a)**). Nonetheless, merged images allowed us to observe that ATXN3 and XPC co-localised primarily within the nucleus (depicted by yellow areas).

Next, we assessed the distribution of ATXN3 and XPC in irradiated cells that were allowed to recover and repair the DNA for either 5 min (**Fig.6, (b)**) or one hour (c).

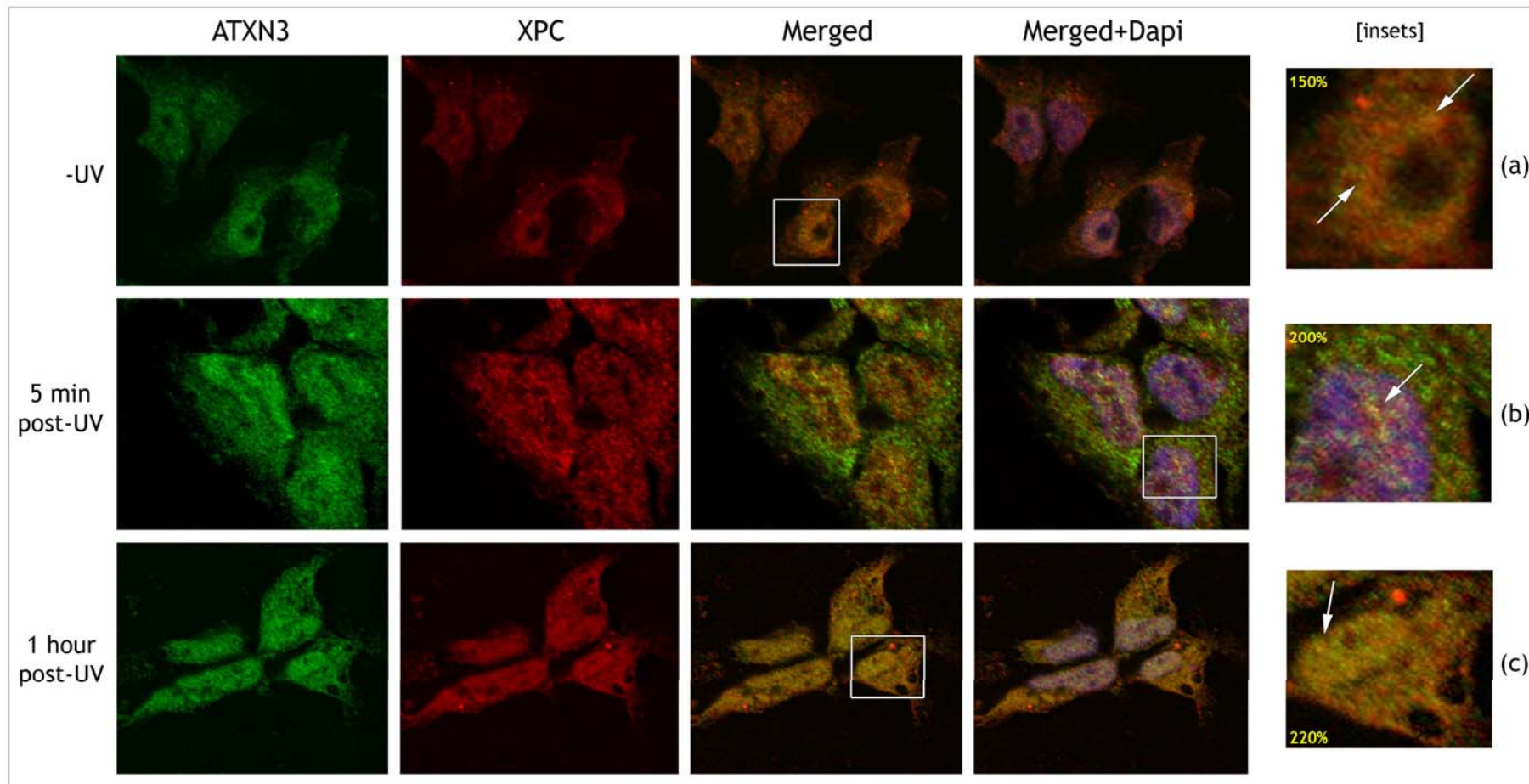


Figure 6. Endogenous ATXN3 and XPC colocalise in HEK293 cells. HEK293 cells were mocked treated (-UV, a) or were irradiated with 20 J/m² UV (b, c) and then allowed to recover for 5 min (b), or 1 hour (c) before fixation. Cells were processed for fluorescence microscopy using anti-mjd and anti-XPC antibodies, followed by secondary antibodies conjugated to anti-rabbit AlexaFluor488 and anti-mouse AlexaFluor568: ATXN3 (green) and XPC (red); nuclei were stained with Dapi; merged images depict colocalisation of ATXN3 and XPC. The insets are from the delimited areas where the nuclei and nucleoli are better seen (magnifications are indicated); arrows depict areas of colocalisation.

Interestingly, we observed that nuclear immunostaining of both proteins increased, as well as their co-localization.

After 5 min of recovery, the immunostaining showed a higher co-localization signal (depicted in yellow) within the nucleus of HEK293 cells. The immunostaining appears to be more punctated than in non-irradiated cells, possibly indicating the ATXN3 may be localising in the same damage spots, to which XPC has been recruited. Another group of cells was also allowed to recover for one hour (**Fig.6, c**), after the UV exposure. Here, the immunostaining showed a more diffuse expression of both proteins, in comparison to 5 min recovered cells period. Nevertheless, the co-localization of ATXN3 and XPC is notorious not only in the nucleus, but also in the cytoplasm. These observations, together with the Y2H results are compatible with the possibility that ATXN3 interacts, *in vivo*, with XPC in HEK293 cells.

Discussion

Genomic integrity is crucial for the proper transmission of genetic information. From prokaryotes to eukaryotes nucleotide excision repair (NER) is a multistep process responsible for removing DNA lesions (cyclobutane pyrimidine dimers (CPD) and (6-4) photoproducts (6-4PP)) produced by the shortwave UV component of sunlight and bulky chemical adducts. In mammals, two major sub-pathways of NER are defined: global genome NER (GGR), which repairs lesions over the entire genome, and transcription-coupled NER (TCR), which repairs transcription-blocking lesions present in actively transcribing DNA strands (Wood, 1997; de Laat *et al.*, 1999)

The HHR23B protein is a key component in the DNA damage recognition complex of the NER-GGR. Together with XPC and centrin-2, HHR23B forms the trimeric-recognition complex, in which it is responsible for the stabilisation of XPC to the site of repair (Araki *et al.*, 2001). The interaction of ATXN3 and HHR23B has been previously explored in the perspective that both proteins may be active participants in the ubiquitin-proteasome system (UPS) for protein degradation. Here, we worked on the possibility of ATXN3-HHR23 interactions as clues to other physiological roles of ataxin-3, namely in

NER. We first investigated whether ATXN3 could interact with the other components of the DNA damage recognition complex, the XPC and CETN2 proteins.

Interestingly, we observed that ATXN3 is able to bind XPC. The Y2H results showed a positive interaction between ATXN3 and the fragment of XPC, XPC-D1 (**Fig.4**). Only the interaction with the N-terminal fragment of XPC, XPC-D1 was expected, as the C-terminal fragment, XPC-D2, comprises the TFIIH, DNA, and HHR23B binding domains. The sequential nature of the events (**Fig.1**, page 23 of “*General Introduction*”) that take place at the DNA damage spot, the formation of the recognition complex and the subsequent binding of the repair proteins, makes it possible that ATXN3 may also bind the XPC protein through its C-terminal. However, due to the known interaction of ATXN3 and HHR23B, it is reasonable to hypothesise that, the interaction with XPC-D2 results of an indirect, “bridging” effect between the latter and the endogenous RAD23 protein of yeast. The presence of the JD of ATXN3 (Gal4BD-JD) was sufficient for the interaction with XPC-D1 to occur.

The co-immunoprecipitation of endogenous ATXN3 and XPC from cellular extracts is crucial to further validate this novel interaction. The confirmation of mapping of the interaction by direct testing of XPC fragments and ATXN3 by, for example, GST pull-down assay is also mandatory and will dissipate any doubts regarding the XPC region through which the interaction is actually occurring.

The interaction between ATXN3 and CETN2 was assessed but both the Y2H and the GST pull-down assays revealed no positive interaction between the two proteins (**Fig.4B** and **Fig.5**). A transient interaction between these two proteins, which we could not detect with the methodology chosen, cannot be excluded.

Nevertheless, we believe that these results broaden the participation of ATXN3 in the NER recognition complex and suggest that this protein may be participating in the DNA repair process, not only in the stabilization of the complex through its interaction with HHR23B, but also by interacting with the XPC, which is responsible for the “docking” of the complex to damage DNA.

These preliminary observations open new possibilities for the normal function(s) of ATXN3. Recently, normal ATXN3 has been described as binding to promoter regions of the MMP-2 gene and repressing the transcription of GATA-2-dependent target genes; its

ability to bind the histones' acetylation sites, the direct interaction with HDAC3 and chromatin are consistent with ATXN3 acting as a transcription repressor (Li *et al.*, 2002; Evert *et al.*, 2006), but may be also as a component of DNA repair complexes.

In the UPS, ATXN3 may participate in the substrate delivery to the proteasome, by acting in conjugation with HHR23B and VCP (Hirabayashi *et al.*, 2001; Kobayashi *et al.*, 2002; Doss-Pepe *et al.*, 2003; Richly *et al.*, 2005); concomitantly, ATXN3 has been assigned as a DUB enzyme with *in vitro* activity (Burnett *et al.*, 2003; Berke *et al.*, 2005; Nicastro *et al.*, 2005). The HHR23B is known to stabilise, through its ubiquitin-associated (UBA) domain, the XPC protein at the damage site (Ng *et al.*, 2003). Intriguingly, polyubiquitylation of XPC is not intended for degradation but rather for further stabilization of XPC, promoting its availability in case of DNA damage (Sugasawa *et al.*, 2005). Though the nature of the polyUb chain conjugated to XPC was unexplored, Sugasawa *et al.*, proposed that the ubiquitylated XPC is protected against proteasome-dependent degradation through its interaction with HHR23B (Sugasawa *et al.*, 2005). Others have proposed that UBA domains can inhibit elongation of polyubiquitin chains by capping conjugated ubiquitin (Ortolan *et al.*, 2000; Chen *et al.*, 2001). Recently Heessen *et al.*, proposed the UBA2 domain as key intrinsic stabilizer, and hypothesized that through its interaction with polyUb chains, it could allow HHR23s to interact with the proteasome while escaping destruction (Heessen *et al.*, 2005). It could be that the association of HHR23B's UBA2 domain with the polyUb conjugated to XPC may hinder the delivery of this substrate to the proteasome. On the other hand, the nature of the polyUb chain itself may be protective of degradation: if XPC is conjugated to K63-linked chains, for example, it would not be eligible for destruction. Long, K48-linked polyUb chains escaping proteasome degradation, although unexpected, also exist. This is also the case of yeast Met4 protein, a bZIP (basic leucine zipper) family transcriptional activator that can be ubiquitylated, but remains stable. Kaiser *et al.*, showed that yeast Met4 can be polyubiquitylated with K48-linked chains, though maintaining its stability. This evidence and the fact that XPC is stabilised by HHR23B, a cargo-shuttle of ubiquitylated substrates and a proteasome-interacting protein, is compatible with the possibility that K48-polyUb chains might conjugate XPC (Kaiser *et al.*, 2006).

There are three possibilities: (1) ATXN3 may be forming a quaternary complex with the HHR23B/XPC/CETN2; (2) it also may be competing with HHR23B for XPC, and the fact that both interactions occur through the JD supports this last notion; (3) the possibility that XPC may be a substrate of ATXN3 DUB activity should also not be ruled out. *In vivo*,

most of the HHR23B protein is complexed with XPC, to form the damage recognition complex (Ortolan *et al.*, 2004). The direct binding of ATXN3 to either XPC or HHR23B may disrupt the complex stabilisation equilibrium, inducing the shuttling of the complex for degradation in the proteasome, allowing rapid elimination of HHR23B/XPC/CETN2 when unnecessary. Alternatively, the interaction with ATXN3 and its DUB activity, may allow the trimming of the polyUb chains, facilitating the delivery and degradation of K48-linked polyubiquitylated XPC in the proteasome. The *in vitro* ubiquitylation of XPC and the analysis of the effect of the addition of recombinant ATXN3 in its catalytically active and inactive form (ATXN3C14A) could show if XPC is a substrate of ATXN3. It would also be interesting to evaluate if ATXN3 modulates the availability of XPC for efficient DNA repair, *in vivo*.

Although very preliminary, our results could suggest that ATXN3, either as a direct interactor of XPC or as an isopeptidase affecting its polyubiquitylation rate might be (1) directly modulating the availability of XPC (or HHR23B) or (2) its readiness to act as caretaker of the genome, and, thus, ATXN3 could be globally acting as negative regulator of the GGR-NER pathway.

Experiments to address these questions still need to be performed. Using, for example, gap repair activity assays (Krishna *et al.*, 2005) in cellular extracts expressing either normal ATXN3, its catalytic inactive form [ATXN3(C14A)] or with knockdown of ATXN3's expression (RNAi), we could analyse the effect of the protein in cell's capacity to perform DNA repair. The unscheduled DNA synthesis assay (UDS) (Sanes and Okun, 1972) could also be used to monitor in a cell culture the extent of DNA synthesis, in the presence of fully active ATXN3 or not (either by expressing ATXN3(C14A) or fully knock-down ATXN3's expression), after exposure to DNA damaging conditions. This would offer an indirect evidence of the *in vivo* consequences of ATXN3 expression upon on the cell's ability to repair DNA.

As a start, we chose to use a simpler animal model, *C. elegans*, to pursue the biological relevance of these findings (Section 1B).

Chapter1: Section 1B

*Atx-3 and ZK20.3, a conserved interaction
suggesting a role in the DNA damage response*

Introduction

Atx-3, the homologue protein of ATXN3 in *C. elegans*, is 38% identical to its human counterpart. The primary structure of atx-3 is conserved, as the Josephin domain (JD) and two ubiquitin-interacting motifs (UIMs) are present. This led us to study the possible conservation of some of the interactions known for ATXN3. The possibility to carry out functional studies in a multicellular yet simpler organism, and the availability of a knockout strain for *atx-3* in the lab, led us to proceed our studies in *C. elegans*.

In this section, it will be described and discussed how the expression of *atx-3* in wild-type animals is affected, when these are subjected to genotoxic stress, namely to ENU treatment. The mRNA expression of two transcripts, conceptually involved in DNA repair in the nematode, *ZK20.3* and *xpc-1*, was analysed. The role of *atx-3* in the worm's response to DNA damage-inducing conditions was also addressed.

Material and Methods

Antibody

A novel primary antibody against the RAD23 homologue 2 protein of *C. elegans*, *ZK20.3*, was obtained externally, in Davids Biotechnologie, by immunization of rabbits. The anti-*ZK20.3* antibody was prepared against a specific peptide (IDNVDEDGNDDLN), located in the C-terminal region of the *ZK20.3* protein. Competition assays were performed, and the specificity of anti-*ZK20.3* antibody was proven (**Fig.A5** of Appendix).

Yeast expression plasmids

The constructs expressing normal human ATXN3 (pAS1/MJD2.1(17Q), and pAS1_G/MJD1.1(14Q)), or expressing a truncated variant of the protein (pAS1/2.1N), as well as the empty vectors, pAS1_G and pACT2_G, are described in Chapter 2. The plasmids encoding GAL4AD-HHR23A, GAL4AD-HHR23B and GAL4AD-CETN2 are described before, in **section 1A** of this chapter. The cDNA encoding *ZK20.3* was obtained from total RNA extracted with Trizol reagent (Invitrogen), from N2, wild-type *C. elegans*. This cDNA fragment was first amplified using SL1 and attB2-*ZK20.3* primers, and then with attB1.2-

ZK20.3 and attB2-ZK20.3; the cDNA fragment isolated encodes a protein lacking its first 50 amino acids. The cDNA fragment encoding the full-length atx-3 protein was obtained as described before (Gales *et al.*, 2005). Subsequently, the atx-3 cDNA was re-amplified with the primers attB1-atx-3 and attB2-atx-3. The PCR products were first cloned into pDONR207 Gateway vector, and then transferred to pACT2_G, or pAS1_G. The constructs were verified by automated sequencing. For primer sequence, refer to **Table A3** of Appendix.

Bacterial expression plasmids

To obtain the plasmid expressing GST-ZK20.3, the attB-PCR product was transferred to pDEST15 using the Gateway technology, and then verified by automated sequencing. The plasmids expressing the GST-HHR23A and GST-HHR23B are described in **section 1A** of this chapter. The expression and purification conditions of 6His-ATX-3 and 6His-ATXN3 var1.1 were described before (Gales *et al.*, 2005).

Yeast two-hybrid assay

The yeast strains Y190 (ATCC, 96400) and Y187 (ATCC, 96399) were transformed using the lithium acetate method, as described (Gietz *et al.*, 1995), using a bait:prey plasmid DNA ratio of 3:1. Transformations with pAS1_G-ATXN3 var1.1 or pAS1-2.1N were grown on SC-WLH supplemented with 140 or 170 mM of AT (amino-1,2,3 triazole), respectively; the remaining were grown on plates with 20 mM AT. We used as positive controls the interactions between TSCII (hamartin) and TSCI (tuberin) (van Slegtenhorst *et al.*, 1998), or SNF4 and SNF1 (Fields and Song, 1989). Incubations were performed, at 30 °C, for five to ten days. Colony-lift β-galactosidase filter assay was performed according the manufacturer's instructions (Clontech).

Expression of recombinant proteins

The expression of GST-ZK20.3 was carried out overnight, at 30 °C, in BL21-SI *E.coli* strain (Invitrogen) with 300 mM NaCl. Harvested cells were resuspended in 1/10 volume of resuspension buffer (TBS 1X, 10 µg/mL lysozyme, 10 mM EDTA pH8, PMSF 0.5 mM), supplemented with EDTA-free protease inhibitors cocktail (Roche). After lysis, the supernatant was cleared by centrifugation, and the GST-tagged proteins were purified

with glutathione-sepharose™ 4B beads (Amersham Biosciences); elution was carried out with 10 mM reduced glutathione in 50 mM Tris-Cl pH 8.

GST pull-down assay

The assays were carried out as described in the previous section 2A, of this chapter (Otera *et al.*, 2000). Briefly, the purified GST or GST-tagged proteins were incubated for one hour, at 4 °C, with 50 µl of glutathione-sepharose™ 4B beads, in 500 µl of in vitro binding buffer (IBB). The 6His-ATX-3 was added in IBB without BSA, and incubated for 1-2 hours, at 4 °C, on a rotator. Bound fractions were washed with IBB without glycerol and BSA, eluted by boiling in 50 µl of 2X SDS sample buffer, and then resolved by SDS-PAGE (12%). Immunoblottings were performed using goat anti-GST (1:7500, GE Healthcare), mouse anti-6His (1:2000, GE Healthcare) or rabbit anti-303JSP (1:2000). Secondary HRP-coupled anti-goat antibody (Sc-2020, Santa Cruz Biotechnology) was diluted at 1:20000, anti-mouse (Santa Cruz Biotechnology Calbiochem), at 1:10000, and anti-rabbit (401393, Calbiochem) at 1:20000. The anti-goat and anti-rabbit dilutions were prepared in 3% SM/TBST (3% skimmed milk, Tris-buffered saline (TBS) 1X, 0.05% Tween 20), whereas anti-mouse antibody was diluted in 1% SM/TBST. Detection was carried out with ECL (GE Healthcare).

***C. elegans* mutagenesis**

Wild-type (Bristol N2) and *atx-3*^{-/-} knockout (VC346, *atx-3(gk193)V.*) strains from Consortium Gene Center (CGC) were maintained using standard procedures (Brenner, 1974). The *atx-3*^{-/-} strain was backcrossed two times. Synchronized L4 stage worm cultures were mutagenized with *N*-ethyl-*N*-nitrosourea (ENU), with incubations on ENU solutions at the appropriate concentration, or in modified M9 for control animals, according to the conditions defined by (De Stasio and Dorman, 2001). Afterwards, animals were plated, overnight, in seeded NGM plates to allow recovery. For the brood size analysis, P0 animals were singly plated and scored as described below.

Brood size test

The analysis of the progeny of mutagenized relative to control animals was performed as described previously (De Stasio and Dorman, 2001), with some modifications: briefly, the P0 animals were transferred singly to a new seeded NGM plate each day, up to six

days post-mutagenesis. Scoring of the progeny laid in each plate was done at L2/L3 larval stages.

Determination of *C. elegans* ZK20.3, *xpc-1* and *atx-3* expression by semi-quantitative RT-PCR

Total RNA was obtained from synchronized cultures. Mutagenized or control *C. elegans* were collected, and total RNA was extracted using Trizol (Invitrogen), as described <http://cmgm.stanford.edu/~kimlab/germline>. Three micrograms of total RNA were used in the first-strand synthesis with Superscript II (Invitrogen), accordingly to manufacturer's instructions. Amplification of the selected genes was carried out simultaneously with co-amplification of the *actin-1* transcript (NM_073418), used as internal standard gene. Definition of the appropriate cycle number for both genes was defined empirically. The ZK20.3 amplification was carried out with ZK20.3-F1 and ZK20.3-R1 primers, as follows: 5 min at 95 °C, 20 cycles of 1 min at 95 °C, 1 min at 56 °C, and 2 min at 72 °C. Amplification of *xpc-1* was performed with xpc-F1 and xpc-R1 primers in the following conditions: 5 min at 95 °C, 29 cycles of 1 min at 95 °C, 1 min at 53 °C, and 2 min at 72 °C. For *atx-3* gene, we used atx-3-F1 and atx-3-R1 primers as follows: 5 min at 95 °C, 23 cycles of 1 min at 95 °C, 1 min at 60 °C, and 1 min at 72 °C. All the PCR reactions were set with a final extension of 5 min at 72 °C, and the amplification of the *actin-1* was carried out with ceact-1F and ceact-1R primers. PCR products were loaded onto 2% agarose gel, and then stained with ethidium bromide. Densitometry analysis was determined as band volume, using the Image Quantification software program (Amersham). The relative expression for each gene target represents the ratio of the target gene and *actin-1* expressions. Primer sequences in **Table A1** of Appendix 2.

Protein extraction from *C. elegans*

Protein extracts of *C. elegans* were prepared from L4 larval-stage synchronized cultures. Worms were collected with M9, and washed two to three times to wash-off bacteria (for mutagenesis assays, the worms were first treated as described using ENU solution at 1 mM). Proteins were then extracted from the worm pellets, by adding an equal volume of worm lysis buffer [WLB, 100 mM Tris pH6.8, 2% SDS, 15% glycerol, 1X complete protease cocktail inhibitors (Roche)], boiled for 5-10 min in a water bath, with

occasional shaking, and then spun at 3200 rpm, for 20 min, at 4 °C. The supernatants were collected and protein concentration determined by Bradford method (Bradford, 1976).

Data Analysis

Progeny ($n=15$), semi-quantitative RT-PCR ($n=5$ or 6) are expressed as arithmetic means $\pm$ S.D. n , number of independent experiments performed. The statistical difference between the groups and condition tested was analysed by Student's t test for independent samples. In a 95% of confidence interval (CI), a p value of <0.05 was considered significant.

Results

The interaction of ATXN3 and HHR23 proteins is conserved in C. elegans

Atx-3 interacts with *C. elegans* ZK20.3 protein, the homologue of HHR23 proteins

We initially cloned the ZK20.3 protein in order to perform yeast two-hybrid assays. Experimental attempts showed us that cloning ZK20.3 using the compiled sequences information at the Wormbase (www.wormbase.org/db/gene, WBGene00013924) and the theoretical ATG of the sequence posted was not possible. Comparative analysis of the primary sequence of several RAD23 homologues (**Fig.7A**) led us to re-design the cloning strategy in order to obtain M50 as the first amino acid in the ZK20.3 protein.

The expressed *C. elegans* ZK20.3 protein contains, as predicted, an ubiquitin-like domain (UBL), two ubiquitin associated domains (UBA1 and UBA2) and one stress-inducible phosphoprotein (STI1) binding domain (**Fig.7B**).

We initially determined if the interaction observed between human ATXN3 and HHR23 proteins, could be conserved in *C. elegans*. The yeast constructs expressing atx-3 and ZK20.3, the protein homologues of ATXN3 and HHR23 were prepared, evaluated for auto-activation of the Y2H system reporter genes and for toxicity.

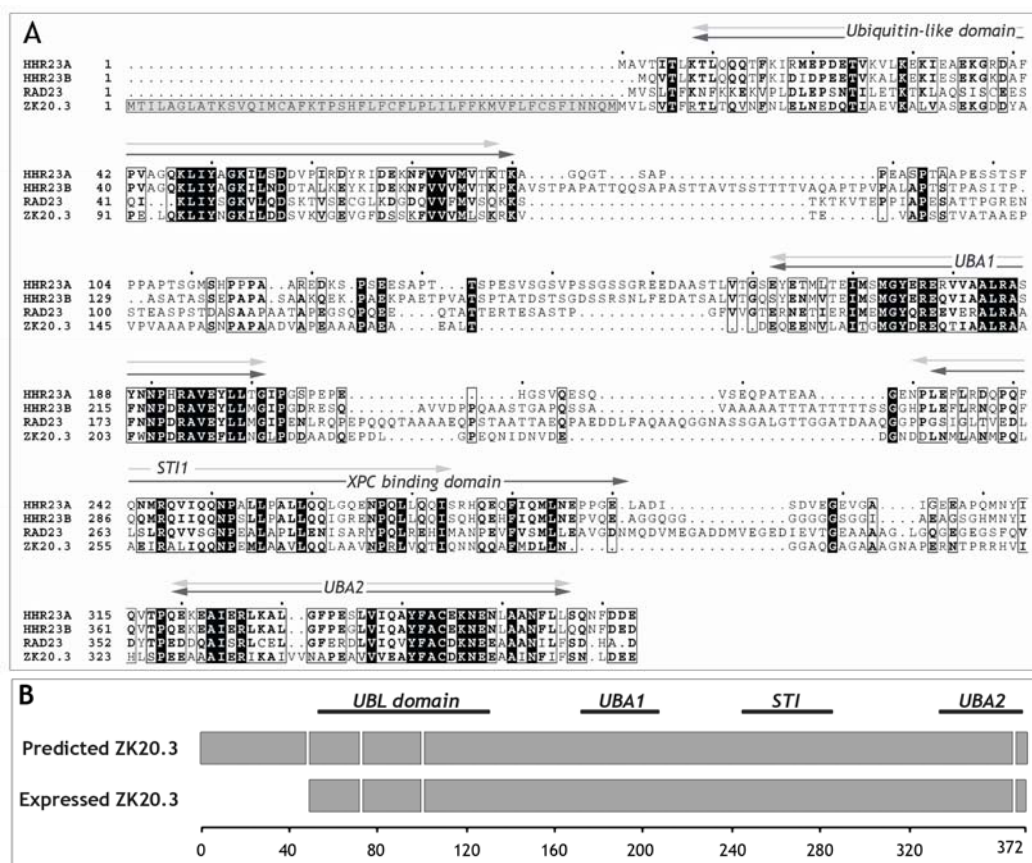


Figure 7. Multiple alignments of HHR23A and HHR23B, worm homologue of RAD23 protein (ZK20.3) and yeast RAD23 protein. A, columns showing a global similarity score over 60 %, calculated from all groups are framed in grey, and amino acid positions fully conserved in all species are black-shaded, whereas identical amino acid are written in bold. The dark grey and light grey lines highlight the domains of human and *C. elegans* proteins, respectively: UBL, UBA1 and UBA2; a XPC-binding domain is present in human HR23 proteins, whereas a STI1 motif is defined in the *C. elegans* counterpart. In the worm, ZK20.3 is predicted to possess an N-terminal region that shows no similarity with the other proteins (light grey-shade). Sequence alignments and colouring were performed with T-Coffee (Notredame *et al.*, 2000) and ESPrit (Gouet *et al.*, 1999) programs, respectively. The dots above the aligned sequences represent groups of ten amino acids each, considering the first sequence, HHR23A. **B,** a schematic drawing depicts UBL, UBA1, UBA2 and STI domains in the predicted and expressed ZK20.3 protein, discussed in this work (herein, the ZK20.3 protein consider is the expressed ZK20.3); amino acid scale in the bottom.

The results showed that atx-3 interacts with ZK20.3, supporting the biological importance of this interaction. Interestingly, human ATXN3 was also able to interact with ZK20.3, independently of the ATXN3 variant expressed (data not shown), denoting conservation of this protein's structure in the nematode. Moreover, we observed that ATXN3 was able to bind ZK20.3 through its N-terminal, since a positive interaction was observed with Gal4BD-2.1N protein, suggesting that the most N-terminal portion of ATXN3 is sufficient for the interaction.

C. elegans atx-3 was also able to bind the human HR23 proteins, HHR23A and HHR23B, in the Y2H system. Our results thus suggest that atx-3 structure is conserved in the nematode (**Fig.8**). However, no interaction of atx-3 and human XPC-D1 fragment and CETN2 was observed in the Y2H assays performed.

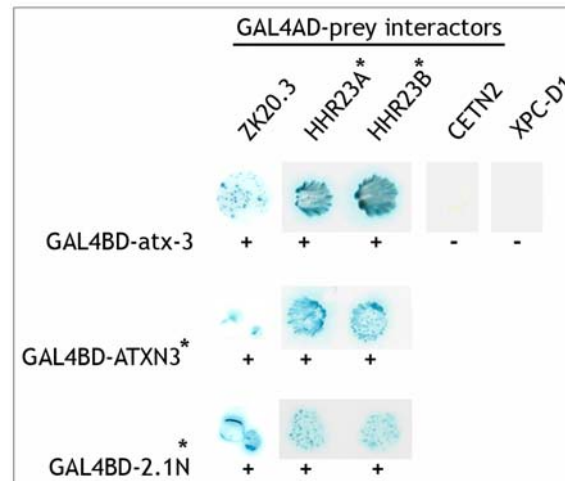


Figure 8. The atx-3 of *C. elegans* interacts with HHR23 proteins and with their homologue in the worm, the ZK20.3 protein. Gal4BD-ATXN3 construct corresponds to the human cDNA variant encoding normal ATXN3 var1.1, comprising three UIMs. A construct comprising a fragment of ATXN3, pAS1-2.1N, was also tested, which encodes for JD plus the functional UIM1. Gal4BD-atx-3 corresponds to the bait fusion of *C. elegans* homologue of ATXN3 that contains only two UIMs and two glutamines in the repetitive segment of this protein. Gal4AD-tagged constructs encoding human HHR23A, HHR23B, CETN2 and XPC-D1 were used as prey interactors. The Gal4AD-ZK20.3 corresponds to the prey fusion of *C. elegans* RAD23 protein. Yeast co-transformations were plated in high stringency plates; an interaction was considered positive (+) when growth on nutritional selection plates was observed, and colonies stained blue, when tested for β -galactosidase expression (blue dots); asterisks correspond to protein-protein interactions reported before (Wang *et al.*, 2000).

The results obtained with the Y2H system were confirmed by *in vitro* GST pull-down assays (**Fig.9**). Binding of 6His-ATXN3 and 6His-atx-3 to different GST-tagged proteins was evaluated. The interactions between ATXN3 and GST-HHR23A or GST-HHR23B were tested and replicated (Wang *et al.*, 2000) under the conditions used, serving as positive controls (**Fig. 9**). The *in vitro* results showed that atx-3 is able to interact with ZK20.3, indicating that this is a direct interaction. Interestingly, HHR23 fusion proteins, GST-HHR23A and GSTHHR23B also pulled-down 6His-atx-3 in the assay, validating the interaction also observed in the Y2H. It is, therefore, highly probable that atx-3 maintains the same 3D-structure as its human counterpart, enabling the conservation of

the interactions in two different methods. No direct interaction was observed between atx-3 and human CETN2, supporting the results obtained in the Y2H assays.

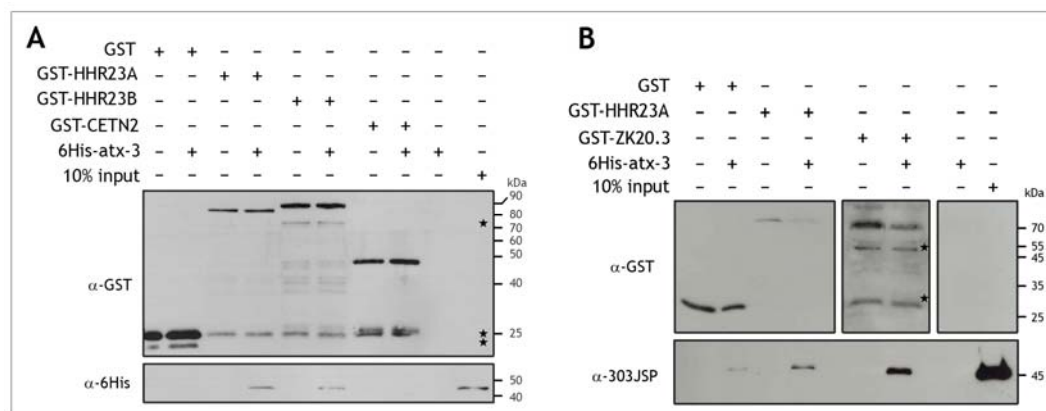


Figure 9. Atx-3 interacts with ZK20.3, the *C. elegans* homologue of HHR23B protein. The GST pull-down assays showed direct interaction of atx-3 with the *C. elegans* homologue of RAD23 protein, ZK20.3, as well as HHR23A and HHR23B, its human homologues. Inputs lanes represent 10% of 6His-atx-3 used in the assays. The *C. elegans* homologue, atx-3, was pulled-down by both HHR23 proteins, HHR23A (A, B) and HHR23B (A), as well as by its *C. elegans* homologue, ZK20.3 (B). No interactions between atx-3 with GST-CETN2 was observed *in vitro*. GST alone showed no binding in the assays. Black stars indicate protein degradation fragments.

ZK20.3 expression throughout *C. elegans* development

Since we have cloned ZK20.3 for the first time, we assessed its expression during *C. elegans* development. ZK20.3 is expressed in all developmental stages of the worm (**Fig.10**).

Semi-quantitative RT-PCR analysis showed a higher expression of ZK20.3 in eggs and in worm adults. Interestingly, the same pattern has been described for atx-3 (Rodrigues *et al.*, 2007), further strengthening the hypothesis that these proteins may be biological partners. The highest levels of ZK20.3 are observed in the egg-laying adults (with 126h post-hatching), probably due to a cumulative effect of the ZK20.3 expression specific of eggs and adult before the egg-laying (76h post-hatching). The dauer, a stall stage induced under stressing conditions, also showed high expression levels of ZK20.3, significantly different from the pre-dauer (L2) and post-dauer (L3/L4) larvae stages.

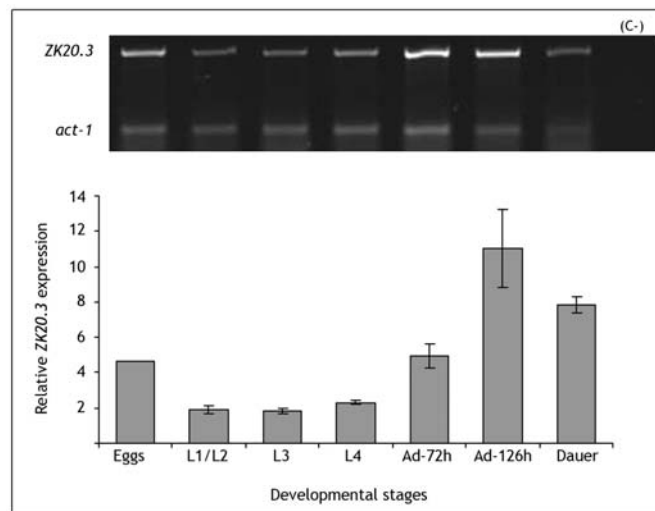


Figure 10. ZK20.3 is expressed throughout *C. elegans* development. Semi-quantitative RT-PCR analysis showed expression of ZK20.3 in all developmental stages, more significantly in egg-laying adults (Ad-126h) and dauer larval-stage. The gel image is representative of two RT-PCR and the graph shows normalized ratios of *atx-3* expression, using *act-1* as internal standard gene. (C-), negative control.

KO *atx-3*^{-/-} animals are more resistant to ENU

The conservation of the *atx-3* and ZK20.3 interaction in *C. elegans*, the ability of *atx-3* to interact with human HHR23 proteins, and vice-versa, led us to perform functional assays to further evaluate the biological relevance of the interaction, namely the role of *atx-3* in the nematode when subjected to DNA damage-inducing conditions.

In order to analyse the putative role of ATXN3 in DNA repair, namely in NER, we analysed the biological effect of a genotoxic chemical, the alkylating agent ENU (*N*-ethyl-*N*-nitrosourea), upon the brood size of wild-type (N2) and *atx-3*^{-/-} nematodes. ENU promotes the formation of bulky adducts in the DNA, inducing the NER response in cells (Costa *et al.*, 2003). The conditions of optimized ENU mutagenesis in wild-type *C. elegans* have been defined (De Stasio and Dorman, 2001). In order to determine the concentration of ENU to be used in the *atx-3*^{-/-} strain with minimum toxicity, but maintaining a significant rate of mutagenicity, we assayed three ENU concentrations (0.3, 0.6 and 1 mM), and scored the progeny of up to 15 animals for each genotype (N2 and *atx-3*^{-/-}) (**Fig.11A**).

The results showed a progressive reduction of the progeny of both worm genotypes, in the presence of ENU, when comparing with the untreated controls. Nonetheless, no significant statistical difference was observed in the progeny of N2 or *atx-3*^{-/-} when

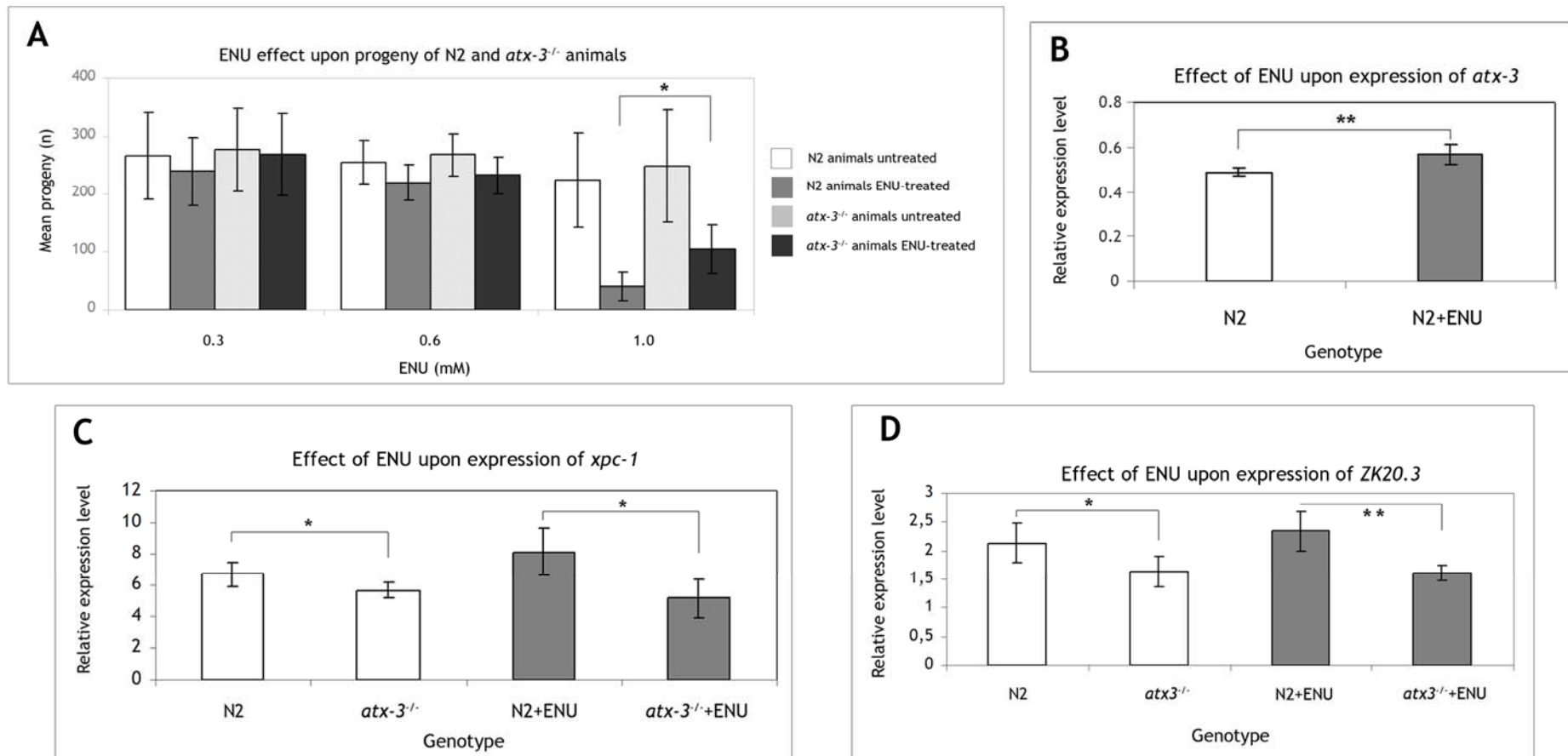


Figure 11. Effect of alkylating mutagen ENU upon wild-type (N2) and *atx-3^{-/-}* animals. A, effect of ENU exposure upon the progeny of wild-type and *atx-3^{-/-}* animals; three different concentrations of ENU were tested (0.3, 0.6 and 1 mM), in 15 independent animals (P0) of each genotype; standard deviation bars are derived from statistical analysis of duplicate experiments. Semi-quantitative RT-PCR analysis of *atx-3* (B), ZK20.3 (C) and *xpc-1* (D) expression in wild-type and *atx-3^{-/-}* animals in the presence or absence of the 1 mM of mutagen (+ENU); standard deviation bars were obtained from statistical analysis of *n* independent experiments: *xpc-1* (n=6), ZK20.3 (n=6) and *atx-3* (n=5). *, $p < 0.05$ and **, $p < 0.01$.

mutated with 0.3 mM or 0.6 mM ENU, relatively to the control animals. A concentration of 1 mM ENU exerted the strongest effect, provoking a notorious reduction of brood sizes for both genotypes (**Fig.11A**). Although both N2 and *atx-3*^{-/-} genotypes were drastically affected, we observed that mutagenized *atx-3*^{-/-} animals showed higher brood size than mutagenized wild-type (N2) worms. Statistical analysis of the data conferred significance to the experimental difference observed in brood size ($p < 0.05$). Surprisingly, the absence of *atx-3* seemed to confer a higher resistance of worms to the detrimental effects of ENU, producing more viable progeny.

To explore the role of *atx-3*, *ZK20.3* and *xpc-1* in DNA repair, we analysed the expression of these transcripts in normal and in DNA damage-promoting conditions by semi-quantitative RT-PCR. Synchronised L4 larvae-stage worm cultures were prepared and total RNA was extracted using the Trizol method. The cDNA synthesis was carried out using Superscript II (Invitrogen).

Expression of *atx-3* is induced in worms exposed to ENU

The analysis of *atx-3* in mutagenised wild-type animals (1 mM ENU) showed a significant induction (16.6% more) of *atx-3* expression ($p < 0.01$), when compared to non-treated, control animals. This may indicate a potential role of *atx-3* in the biological responses to mutagenesis engaged by *C. elegans*, such as DNA repair (**Fig.11B**).

Expression of *xpc-1* and *ZK20.3* is reduced in the absence of *atx-3*

The analysis of *xpc-1* expression in N2 and *atx-3*^{-/-} animals showed an overall significant interaction between ENU and the genotype in *xpc-1* expression (ANOVA, $p < 0.05$). The results showed a significant difference in *xpc-1* expression, between wild-type and untreated *atx-3*^{-/-} animals ($p = 0.02$); in basal conditions, *atx-3*^{-/-} worms seem to express less *xpc-1* than wild-type (15.0% less); *xpc-1* expression in *atx-3*^{-/-} worms, was even more significantly reduced (36.6% less) comparing to the wild-type, when the animals were subjected to ENU treatment ($p = 0.004$). Hence, there is a significant difference between the genotypes, in the absence or presence of the mutagenic agent, ENU (**Fig.11C**).

ZK20.3 expression analysis in N2 and *atx-3*^{-/-} also showed us a significant effect of genotype upon this transcript ($p < 0.05$). In the presence or absence of ENU (1 mM), the absence of *atx-3* affects significantly *ZK20.3* expression ($p < 0.05$), when compared to the

wild-type. In non-mutagenised animals, the *atx-3*^{-/-} showed a 23.4% decreased expression of ZK20.3; whereas, in the presence of ENU, even less ZK20.3 expression was detectable in *atx-3*^{-/-} animals, a 31.2% reduction was observed when comparing to ENU-treated wild-type animals (**Fig.11D**).

No significant differences in *xpc-1* and ZK20.3 expression were observed for either genotype (N2 or *atx-3*^{-/-}) when comparing ENU-treated (+ENU,1mM) to untreated control animals (both, $p>0.05$), indicating that, counterintuitively, the presence of ENU seems to have no significant effect upon *xpc-1* and ZK20.3 mRNA expression in animals, although they were subjected to DNA damaging condition (**Fig.11C, D**). Though a 21.6% expression increase of *xpc-1* was observed, when wild-type animals were treated with ENU, no statistical significance was found in this variation ($p=0.06$) (**Fig.11D**).

ENU produces no overall alteration of the ubiquitylation pattern in *C. elegans*

Total protein extracts of wild-type and *atx-3*^{-/-} animals were obtained and further analysed to determine if the ubiquitylation and neddylation patterns of these were altered when the worms were subjected to DNA damage-inducing conditions (1 mM ENU). No apparent differences were observed in the ubiquitylation pattern of KO animals (*atx-3*^{-/-}) when comparing to wild-type; likewise, no differences were identified in the ubiquitylation pattern of the protein expression profile of mutagenised animals (**Fig.12A**). The absence of *atx-3* did not seem to modify the overall level of protein ubiquitylation.

Differentially neddylated proteins in wild-type worms

We also analysed the global neddylation pattern in these animals, upon the conditions described. Although no overall difference was observed in protein neddylation when comparing untreated and ENU-treated animals, the analysis revealed the presence of four neddylated proteins, which are less abundant in the neddylation profiles of *atx-3*^{-/-} protein extracts (**Fig.12B**, arrowheads). No obvious difference was observed in these neddylation targets when worms were subjected to ENU alkylating mutagenic agent.

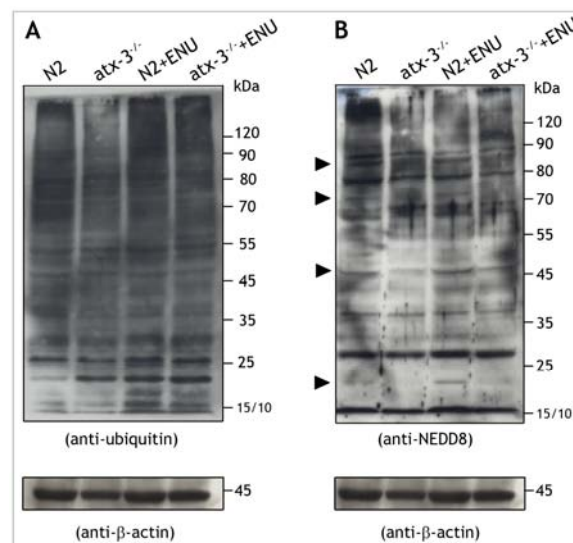


Figure 12. Analysis of neddylation pattern revealed four neddylation targets less abundant in *atx-3*^{-/-} animals. Synchronised worm cultures of wild-type (N2) and *atx-3*^{-/-} were grown in the absence or presence of 1 mM ENU. The protein extracts were subjected to SDS-PAGE and visualized with Western blot analysis using (A) anti-ubiquitin antibody (BIOMOL, PW8810) to detect ubiquitylated proteins or (B) anti-Nedd8 antibody (BIOMOL, PW9340) for proteins modified by neddylation; anti-β-actin (A, B) was used as loading control.

Discussion

Our group has recently reported the first molecular characterization of *atx-3*, the *C. elegans* homologue protein of ATXN3 (Rodrigues *et al.*, 2007). The relatively high sequence conservation of *atx-3* and the fact that it showed deubiquitylating activity *in vitro* as its human counterpart, does led us to hypothesise that this protein should maintain similar protein-protein interactions, and have the same or similar cellular function(s).

Our results showed a positive direct interaction between *atx-3* and ZK20.3, the *C. elegans* homologues of ATXN3 and HHR23. The conservation of this interaction throughout evolution denotes its high importance and, putatively, the biochemical pathway in which the two proteins are participating as interacting partners. Importantly, the conserved interaction with the *C. elegans* proteins allows us to conclude that *atx-3* has conserved the relevant biological structure, crucial for the interactions. This hypothesis was reinforced, when we observed that *atx-3* of *C. elegans*

binds HHR23A and HHR23B and, conversely, that ATXN3 binds ZK20.3, namely through the N-terminal region of the human protein.

Although no interaction was observed between human ATXN3 and CETN2, these negative results obtained from the Y2H and *in vitro* assays, do not allow us to exclude unequivocally the possibility of this interaction is occurring in the worm. The interaction with centrin, the *C. elegans* homologue of human CETN2, needs to be tested, as well as the hypothesis of the conservation of the xpc-1/centrin/ZK20.3/atx-3 complex in *C. elegans*.

The study of the worm protein interactions, led us to clone, for the first time, the coding sequence of the ZK20.3 protein, the *C. elegans* homologue of HHR23 proteins.

The ZK20.3 expression analysis throughout the *C. elegans* development showed that this message is highly represented in all stages of the animal. Interestingly, ZK20.3 is expressed, abundantly, in the same stages (adults) as *atx-3* (Rodrigues *et al.*, 2007), which reinforces the possibility of an interaction between the encoded proteins, *atx-3* and ZK20.3, in the nematode.

Of notice is the higher ZK20.3 expression observed in the dauer stage. There is no direct evidence of the participation of ZK20.3 in DNA repair. However, this possibility was strengthened recently, as the direct interaction between ZK20.3 and the *C. elegans* homologue of XPC, *xpc-1*, was reported as possible in a genome-wide predictive analysis of the *C. elegans* interactions (Zhong and Sternberg, 2006).

During the *C. elegans* development and whenever the condition are adverse to life (lack of food, low humidity, temperature alterations, animal overcrowding, etc), the animal may endure such harsh environmental conditions by switching to a facultative reversible stall stage called dauer (**Fig.13**). In this “lethargus” stage, the feeding and the locomotion are arrested indefinitely, and morphological modifications in animals are visible, as shedding of cuticle (Cassada and Russell, 1975). Increased oxygen uptake during this stall stage has been ascribed to an increase in metabolic activity during this period, perhaps associated with the developmental changes observed during the transition to the dauer stage. Due to its higher metabolic rate, dauer-larvae may produce more reactive oxygen species, hence be more susceptible to oxidative stress and, ultimately, to endogenous DNA damage. Interestingly, it is in this stall stage that

we observed the highest ZK20.3 expression. The maintenance of cell and genome integrity in dauer stage is crucial for animal's survival upon its re-entering in the normal life cycle. Therefore, the efficient DNA repair in this metabolic stage is determinant, and the high expression of ZK20.3, may indicate an active role of this protein in genome maintenance.

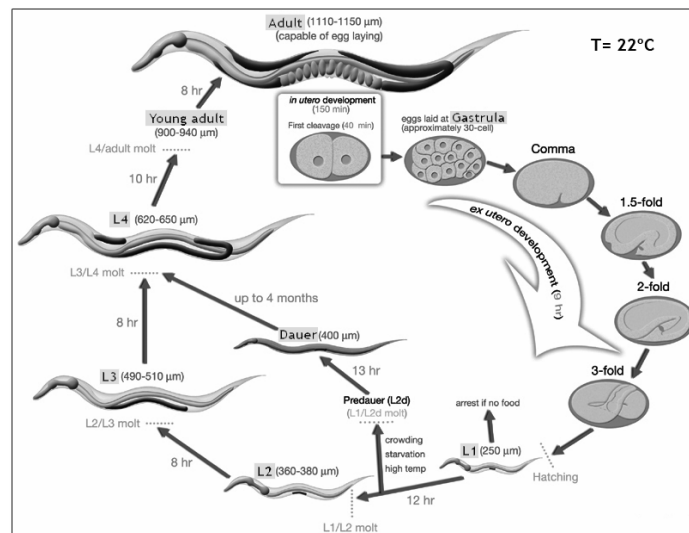


Figure 13. Life cycle of *C. elegans*. The life cycle of *C. elegans* comprises the embryonic stage, four larval stages (L1-L4) and adulthood (names of the stages are shaded in light grey). At 22°C, it takes approximately 45-50 hrs from the hatching until the reproductive adult stage. The duration of each developmental stage (defined in hours) varies. Upon environmental stress, the worm may enter a reversible, life-sustained stage (dauer stage). As dauer, the life of the animal may be extended up to months, until the conditions are normalised.

(Adapted from www.wormatlas.org/handbook/anatomyintro/anatomyintro.htm).

With this work we have shown that (1) the atx-3 and ZK20.3 interaction is conserved in *C. elegans*; (2) atx-3 is able to cross-interact with human HHR23 proteins, suggesting structural conservation of the interaction determinants and (3) we observed that atx3^{-/-} animals express less ZK20.3 and xpc-1 (4) are able to produce significantly more progeny, than wild-type when exposed to 1 mM ENU. (5) Atx-3 expression is induced upon DNA damaging conditions. At this point, we do not have experimental evidence supporting the existence of an interaction between worm atx-3 and xpc-1. Nevertheless, the prediction of the interaction between ZK20.3 and xpc-1 (Zhong and Sternberg, 2006), supports the xpc-1/centrin/ZK20.3/atx-3 complex possibility in the nematode.

We analysed the role of some of these proteins when the nematodes were subject to DNA damage-inducing conditions.

The expression analysis showed that when subjected to mutagenic agent ENU, the *C. elegans* increases *atx-3* expression. Previously, Jelinsky *et al.*, showed that several yeast genes involved in DNA repair and in ubiquitin/proteasome system were induced in response to DNA damage caused by MMS (methyl methanesulfonate) (Jelinsky *et al.*, 2000). Indeed, we observed that *atx-3* is an ENU-responsive gene, thus suggesting a direct link between its expression and the response to DNA damage. Whether *atx-3* increased expression is due to (1) a direct participation in the DNA repair response driven under such conditions, (2) as a modulator of the availability of DNA repair effectors such as *xpc-1* and *ZK20.3* or (3) due to its participation in UPS as a deubiquitylating enzyme needs further clarification.

We and others have pointed out that ATXN3 interacts with HHR23 proteins (Wang *et al.*, 2000) and XPC. Indeed, this instigated us to characterise the *xpc-1* and *ZK20.3* expression, under ENU-induced genotoxic conditions, in wild type (N2) and *atx-3^{-/-}* (KO) animals. To our surprise, we did not find a significant induction of the *xpc-1* and *ZK20.3* expression rates when wild-type animals were subjected to ENU-genotoxic stress (**Fig.11C, D**). Although the results seem paradoxical, we believe that effective mutagenesis assays, hence DNA damaging conditions, were performed (we followed the *C. elegans* ENU mutagenesis protocol established by (De Stasio and Dorman, 2001)). Indeed, in the progeny assay, using 1 mM ENU (the concentration later on established for all expression analysis) we observed a significant reduction in the number of viable descendents, both in wild-type (N2) and *atx-3^{-/-}* strains. Although indirectly, these results support the effectiveness of the protocol used. Despite the statistical non-significance of the results obtained herein, we still observed an increase in the expression of *xpc-1* and *ZK20.3* when the animals were mutagenised, comparing to the controls, which we cannot assume, peremptorily, has no biological relevance.

One possible reason for the apparent absence of a strong increase in the *xpc-1* and *ZK2.3* transcripts is perhaps we have “looked” for the expression of these transcripts, too late in time. The mutagenesis assay takes an incubation period of four hours, with the ENU solution. This is a crucial step as the thick, resistant *C. elegans*’ cuticle demands longer periods of exposure than other systems, for the treatments to be effective. Thus, the rather quick response that is known to occur in eukaryotic cell cultures may have been lost, and may be we have just analysed the rebound phase of a

potentially stronger response to the ENU genotoxic stress. Assuming this, *atx-3* may represent a later response, then still detectable.

We also characterised the *xpc-1* and *ZK20.3* expressions in wild-type (N2) and *atx-3*^{-/-} animals, in normal or ENU-treated conditions.

The analysis showed a significant reduction of the transcripts' expression in *atx-3*^{-/-} animals under normal growth conditions, when comparing to wild-type. Such difference was more apparent when the animals were subjected to DNA damage conditions. The XPC protein is stabilised by HHR23B, in mammalian cells (Ng *et al.*, 2003). Based on the evidence that the majority of XPC is complexed with HHR23B in eukaryotic cells, once a reduction of *ZK20.3* was observed, we were expecting a similar variation in the *xpc-1* expression levels, in *C. elegans*.

The *C. elegans* *xpc-1* and *ZK20.3* proteins may have similar biochemical functions to their human counterparts because (1) we show here that *atx-3* is able to interact also with *ZK20.3* and (2) the interaction between *xpc-1* and *ZK20.3* proteins is conserved in the worm (Zhong and Sternberg, 2006).

The loss of *atx-3* caused a reduction in *xpc-1* and *ZK20.3* expression; this is consistent with the hypothesis raised before that ATXN3 could be acting as a deubiquitylating enzyme (DUB), hence a negative regulator of the GGR-NER sub-pathway. Acting as a DUB, ATXN3 may degrade the XPC-conjugated polyUb chain, diminishing its stability. The results obtained in *C. elegans* reinforce this possibility: in the absence of the DUB *atx-3* and its potential isopeptidase activity, the *xpc-1* is more stable, hence less expression of *xpc-1* and *ZK20.3* mRNA is necessary.

It would be interesting to evaluate the effect of the overexpression of the endogenous *atx-3* or its human counterpart encoding normal ATXN3, in the expression of these transcripts. Will the differences observed invert? Or will they accentuate? And the expression of a catalytic inactive *atx-3*(C20) or ATXN3(C14) protein will sustain these observations? These questions must be address in the future.

Selective degradation of abnormal proteins is essential for cellular functions. The ubiquitin conjugating system provides a mechanism that enables the distinction of these abnormal proteins from correctly folded and modified proteins. The functional equilibrium between the UPP and molecular chaperones ensures the efficiency of the

protein quality control systems, avoiding the highly deleterious accumulation of damaged proteins.

ATXN3 is known to possess *in vitro* hydrolase activity either for ubiquitin (Burnett *et al.*, 2003; Berke *et al.*, 2005) or NEDD8 (Chapter 2); the *C. elegans* counterpart, atx-3, also exhibits *in vitro* isopeptidase activity against ubiquitin and interacts with NEDD8 (Chapter 2). We analysed the ubiquitylation and neddylation patterns in basal and DNA damaging conditions, in *atx-3*^{-/-} and wild-type. In the conditions tested, no overall difference was observed in the ubiquitylation pattern. Though apparently, the loss of *atx-3* and/or the presence of ENU do not disturb the overall ability of *C. elegans* to ubiquitylate/deubiquitylate proteins, we may not state definitely, that a variation in the protein degradation signalling is indeed absent, nor can we exclude an alteration in the ubiquitylation pattern of specific proteins. Interestingly, the absence of *atx-3* revealed some differences in the neddylation pattern observed, namely four proteins were reduced or absent in neddylation pattern of *atx-3*^{-/-}, when comparing to wild-type. We showed that the human ATXN3 may interact with NEDD8, and exerts deneddyase activity, *in vitro*. Further testing is required to assess the deneddyase activity of *C. elegans* homologue. The differences observed in the endogenous neddylated proteins must be analysed; but this *in vivo* evidence seems to strengthen the participation of ATXN3 in the modulation of post-translational modifications in the cellular environment.

Together with *atx-3*, *C. elegans* has another Josephin-related protein ([WP:CE32996](#)). This evidence makes us wonder if the modest effects observed in the experiments we undertook may not be a result of redundancy between the two proteins. Thus, we believe that all these questions must also be addressed in the *atx-3*^{-/-}/*josephin*^{-/-} double-knockout animals.

Chapter 2

NEDD8: a new ataxin-3 interactor

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Abstract

Machado-Joseph disease (MJD/SCA3) is an autosomal dominant neurodegenerative disease caused by the expansion of a CAG tract in the coding portion of the *ATXN3* gene. The presence of ubiquitin-positive aggregates of the defective protein in affected neurons is characteristic of this and most of the polyglutamine disorders. Recently, the accumulation of the neural precursor cell expressed developmentally down-regulated 8 (NEDD8), a ubiquitin-like protein, in the inclusions of MJD brains was reported. Here, we report a new molecular interaction between wild-type ataxin-3 and NEDD8, using in vitro and in situ approaches. Furthermore, we show that this interaction is not dependent on the ubiquitin-interacting motifs in ataxin-3, since the presence of the Josephin domain is sufficient for the interaction to occur. The conservation of the interaction between the *Caenorhabditis elegans* ataxin-3 homologue (atx-3) and NEDD8 suggests its biological and functional relevance. Molecular docking studies of the NEDD8 molecule to the Josephin domain of ataxin-3 suggest that NEDD8 interacts with ataxin-3 in a substrate-like mode. In agreement, ataxin-3 displays deneddylase activity against a fluorogenic NEDD8 substrate.

Introduction

Machado-Joseph disease (MJD/SCA3) is the most common dominantly inherited cerebellar ataxia worldwide (Schols *et al.*, 2004). The disease is caused by the expansion of a CAG repeat in the coding region of the gene *ATXN3*. This gene encodes ataxin-3 (ATXN3), a 42 kDa ubiquitously expressed protein, which has the polyglutamine tract located in the C-terminal region (Kawaguchi *et al.*, 1994; Schmidt *et al.*, 1998). The repeat segment that normally consists of 12-44 CAG is expanded from 61 up to 87 in patients (Maciel *et al.*, 2001). A striking feature of the polyglutamine diseases is the presence of neuronal inclusions in the affected brain regions; in MJD, the inclusions are both nuclear and cytoplasmic and typically ubiquitylated (Paulson *et al.*, 1997). These intracellular inclusions contain proteasome subunits and chaperones, which suggests an attempt of the cell to either reduce the amount of non-native protein and restore

protein homeostasis or eliminate the inclusions (Schmidt *et al.*, 2002). The physiological role of ATXN3 is not yet established.

Recently, it was noticed that the Josephin domain of ATXN3 (JD) contains conserved amino acids reminiscent of the catalytic domain of a member of the deubiquitylating cysteine protease UCH (ubiquitin C-terminal hydrolases) and USP (ubiquitin-specific protease) families (Scheel *et al.*, 2003) and later, in vitro assays showed that ATXN3 displays Ub hydrolase activity (Burnett *et al.*, 2003; Berke *et al.*, 2005). Strikingly, it was found that ATXN3 suppresses polyglutamine neurodegeneration in *Drosophila*, and this suppressing activity is dependent on its interaction with ubiquitin and on its protease activity (Warrick *et al.*, 2005). Recent data showed the presence of neural precursor cell expressed developmentally down-regulated gene 8 (NEDD8), an ubiquitin-like protein (UBL), in Ub-reactive neuronal and glial inclusions of several neurodegenerative diseases, namely MJD (Mori *et al.*, 2005). An active role was suggested for NEDD8 in the formation of the ubiquitylated inclusions through the ubiquitin-proteasome system (UPS).

NEDD8 is an 81 amino acid polypeptide, weighting 8 kDa, and shows the highest identity with Ub (58%) of all UBL proteins (Kamitani *et al.*, 1997). Like most UBL, *de novo* NEDD8 is synthesised as a pre-protein; in the processing of NEDD8, a short C-terminal peptide is cleaved, generating the mature UBL with a reactive G76 at its most C-terminal, essential for the conjugation reactions. The UCH-L3 enzyme was identified as capable of both ubiquitin and NEDD8 processing (Wada *et al.*, 1998), an evolutionarily conserved property (Frickel *et al.*, 2007). In a process known as neddylation, the mature NEDD8 is covalently linked to substrates, by the establishment of an isopeptide bond between its glycine at the C-terminal and the ϵ -amide group of the substrate's lysine (Kamitani *et al.*, 1997). In a process similar to ubiquitylation, a heterodimeric E1-like activating, complex comprising the amyloid precursor protein-binding protein (APP-BP1) and UBA3 protein, initiates the neddylation, whereas UBC12 (an E2-like conjugating enzyme) promotes NEDD8 conjugation to substrates (Osaka *et al.*, 1998). Transfer of NEDD8 to substrates is mediated by an E3-like ligase, but NEDD8-specific E3 enzymes are now starting to be unveiled.

Cullins were the first NEDD8 substrates to be discovered. Cullins are evolutionarily conserved from yeast to mammals (Petroski and Deshaies, 2005); several cullins (CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5 and CUL7) and cullin-like proteins (PARC and APC2)

are expressed in human cells (Petroski and Deshaies, 2005). Cullins are components of SCF-like complexes; these are E3-based ubiquitin ligases that regulate cullin-dependent ubiquitylation. These cullin-based E3 ligases are multimeric complexes comprising several subunits: a cullin or cullin-like protein, a E3 ligase protein, an adaptor protein and a substrate receptor. The specificity of the cullin-based complexes is conferred by the adaptors and substrate receptors bound to the cullin. The multitude of combinations between adaptors, substrate receptors bound to all possible cullin denotes the complexity of this system (Petroski and Deshaies, 2005; Hermand, 2006; Kerscher *et al.*, 2006). The E3-ligases proteins can be of two different mechanistically types: RING (really interesting new gene) finger proteins and HECT (homologous to E6-AP carboxyl terminus) proteins. In multimeric RING finger complexes, the RING finger protein binds the E2-conjugating protein, while the substrate receptors together with the adaptor bind the substrate to the complex. The HECT-domain E3s are unique in that Ub is transferred to a conserved cysteine residue of the E3 in a thiolester linkage, and then the E3 transfers the Ub to the substrate, also, directly bound to the E3 ligase. This function as a covalent intermediary in the transfer of Ub is not found in other classes of E3 proteins (reviewed in (Chiba and Tanaka, 2004)).

Several deubiquitylating enzymes can catalyse deneddylation, the removal of conjugated NEDD8 from substrates: USP21 (Gong *et al.*, 2000), the COP9 signalosome (CNS), through the metalloprotease JAMM motif of CSN5 subunit (Cope *et al.*, 2002) and DEN1 (aka NEDP1 or SENP8) (Parry and Estelle, 2004). CAND1 (aka, p120) is a cullin interacting protein, with specific affinity for deneddylated cullins (Liu *et al.*, 2002); CAND1 seems to regulate negatively the cell's cullin-based ubiquitylation by rendering cullins to an inactive state. The UBC12-mediated covalent attachment of NEDD8 to the C-terminal of cullins promotes E3 ligase activity of the complex by mediating the total displacement of CAND1 and enabling binding of the adaptor proteins (e.g. Skp1 binding to CUL1 in SCF complex) and substrates recognition subunits (e.g. F-box loaded with specific substrate in SCF complex), further activating cullins, recruiting ubiquitin-loaded E2s and consequently the proper substrate ubiquitylation. It is the cycling process of neddylation/deneddylation of cullins that is thought to be essential in the regulation of ubiquitylation mediated by cullin-E3 ligases (Pintard *et al.*, 2003; Petroski and Deshaies, 2005). Moreover, unless neddyated cullins are recycled by the CSN-promoted deneddylation, they undergo degradation, suggesting that this “on-off” state of neddyated/deneddyated cullins enables the maintenance of intracellular free NEDD8

and cullin pool. As importantly, this tight equilibrium prevents cell's excessive ubiquitylation (Wu *et al.*, 2005).

NEDD8 is highly conserved and it has been demonstrated to be essential for mice embryonic development (Tateishi *et al.*, 2001) and *C. elegans* larvae-viability (Kurz *et al.*, 2002). Interestingly, neddylation of cul-3 has been shown essential for the proper axonal arborisation and dendritization in *Drosophila* (Perez *et al.*, 2005). This might suggest a role of neddylation in neuroplasticity, with potential relevance in neurodegenerative diseases, namely in the mechanisms that the brain may adopt to circumvent its inherent cell death.

Another target for neddylation is the cell cycle regulator, p53. Xirodimas *et al.*, demonstrate that neddylation of p53 is dependent of mdm2 activity (Hdm2 in humans), an E3 RING-finger ubiquitin ligase with the capability of self-neddylation (Xirodimas *et al.*, 2004). Neddylation of p53 was shown to be determinant in the inhibition of transcriptional activity of p53 (Xirodimas *et al.*, 2004). Moreover, both mdm2-mediated neddylation and ubiquitylation of p53 modifications was dependent of the same cysteine residue of the E3 RING-type ligase (mdm2), unveiling that mdm2 could function also as an E3-NEDD8 ligase. This dual activity of E3-ubiquitin ligases had been reported before by Morimoto *et al.*, whom suggested that ROC1 RING protein (aka RBX1, HRT1) could behave as an E3-NEDD8 ligase, by promoting CUL1 neddylation (Morimoto *et al.*, 2003). Recently, another neddylation target has been identified. Oved *et al.* reported that neddylation of epidermal growth factor receptor (EGFR) mediated its lysosomal degradation (Oved *et al.*, 2006); ubiquitin and NEDD8 may share the c-Cbl RING-containing E3 ligase involved and UIM-containing proteins (e.g. Epsin15), at least in the c-Cbl- and ligand-dependent degradation process (Oved *et al.*, 2006). The synphilin-1, a major component of inclusion bodies found in the brains of patients with neurodegenerative α -synucleinopathies, including Parkinson's disease, was also reported to be a target of NEDD8 modification (Tanji *et al.*, 2006). All together, these results suggest high functional diversity for RING finger E3 ligases that may either behave as E3-ubiquitin and E3-NEDD8 ligases and a tight regulation of both neddylation/ubiquitylation in the protein degradation pathways. Surely, neddylation and its ensuing regulation play no minor role in the cellular protein homeostasis.

The fact that ATXN3 interacts with the UBL domain of HHR23 proteins (Wang *et al.*, 2000) and the recent finding that NEDD8 is present in ubiquitylated inclusions in the

brain of MJD patients, led us to investigate whether ATXN3 could interact with NEDD8. In this study, we demonstrate in vitro and in situ that ATXN3 is able to establish a direct interaction with NEDD8, through its Josephin domain (JD). Interestingly, the *Caenorhabditis elegans* homologue of ATXN3, atx-3, also interacts with NEDD8, indicating that this protein interaction is evolutionarily conserved and, thus, of functional relevance. Furthermore, ATXN3 is able to cleave a fluorogenic substrate, NEDD8-AMC, which indicates that ATXN3 also has deneddylase activity.

Material and Methods

Antibodies

Primary antibody against ataxin-3 variant MJD1-1 (Swiss Prot no. P54252-2 VSP_002784) was raised in rabbits, by immunization with normal full-length recombinant 6His-ATXN3-var1.1, anti-MJD1.1, (David Biotechnologies). Competition assays were performed to verify the antibody specificity (**Fig.21**).

Yeast two-hybrid assays

The yeast strain Y190 (96400) was purchased from ATCC. The original vectors pAS1-2 and pACT2 (kindly provided by Dr S. Elledge, Baylor College of Medicine) were converted into yeast two-hybrid Gateway[®] vectors pAS1_G and pACT2_G, respectively, following the manufacturer's instructions (Invitrogen). The pAS1_G-ATXN3 var1.1 coding for normal ATXN3, pAS1_G-JD, pACT2_G-JD, pAS1_G-NEDD8, pACT2_G-NEDD8 plasmids were generated using the Gateway cloning system[®]. The pAS1-ATXN3 var2.1 (17Q) (Swiss-Prot no. P54252 VAR_013690) and pAS1-ATXN3-2.1N were prepared using classical cloning techniques (Sambrook and Russel, 2001). The yeast transformations were performed using the lithium acetate method (Gietz *et al.*, 1995). Transformants of pAS1_G-JD, pAS1_G-ATXN3 var1.1 or pAS1-ATXN3-2.1N were grown on SC-WLH supplemented with 130, 140 or 170 mM of amino-1,2,3 triazole (AT), respectively; the remaining were grown on plates with 20 mM AT. The interaction between SNF1 and SNF4 encoded by pSE1112 and pSE1111, respectively, was used as positive control (kindly provided by Dr S. Elledge, Baylor College of Medicine). Incubations were performed at 30 °C for five

days. Colony-lift β -galactosidase filter assays were performed according to the manufacturer's instructions (Clontech).

Expression plasmids and recombinant proteins

GST-tagged proteins- For generation of GST-NEDD8 protein, NEDD8 cDNA was amplified from a human brain cDNA library using attB1-NEDD8 and attB2-NEDD8 primers. GST-Ub expression construct was obtained from the IMAGE di-ubiquitin (UBB) cDNA clone (4126622) by amplification of the monomeric Ub cDNA using attB1-UB and attB2-UB primers. GST-221cATXN3 expression construct was obtained using pAS1_G-ATXN3 var1.1 plasmid as template, attB1-221cATXN3 forward primer, and attB2-MJD1.1 reverse primer. The cDNA for human HHR23A was reverse transcribed from total RNA of HeLa cells extracted with Trizol (Invitrogen) using attB1-HHR23A and attB2-HHR23A primers; gene-specific sequences are underlined. RT-PCR reactions were performed with Superscript RT kit (Invitrogen) according to the manufacturer's instructions. The aforementioned PCR products were first cloned into pDONR201 or pDONR207 vectors and then transferred to pDEST15 using the Gateway cloning system (Invitrogen) (Hartley *et al.*, 2000) according to the supplier's instructions.

6His-tagged proteins- The bacterial constructs expressing 6His-tagged ATXN3-var1.1, 6His-atx-3 and 6His-tagged Josephin domain (JD) were obtained and expressed as described (Gales *et al.*, 2005). The 6His-D1 construct (Fig. 2(a)) was obtained by introducing a stop codon at position 787 in ATXN3 cDNA using the QuikChange site-directed mutagenesis kit (Stratagene) and protein expression was carried out as described (Gales *et al.*, 2005). All clones were confirmed by automated sequencing. The eukaryotic 6His-Ub plasmid and the 6His-NEDD8 constructs were gifts from Dr D. Bohmann, Univ. of Rochester and Dr R. Hay, Univ. of St Andrews (Xirodimas *et al.*, 2004), respectively.

GFP-tagged constructs- The eukaryotic pEGFP-ATXN3 (28Q) encoding normal ATXN3 in fusion with GFP (N-terminal) was a gift of Dr Henry Paulson, Univ. of Iowa. To obtain the mammalian pEGFP-JD plasmid encoding the fusion protein GFP-JD, a stop codon was introduced at position 548 in ATXN3 cDNA of the pEGFP-ATXN3 (28Q) using the QuikChange site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene). All clones were confirmed by automated sequencing.

Purification of recombinant proteins

The expression of GST, GST-NEDD8 and GST-Ub was induced in *Escherichia coli* strain BL21(DE3) (Invitrogen), whereas GST-221cATXN3 was expressed in BL21(DE3)pLysS (Invitrogen), all with 1 mM IPTG. GST-HHR23A was expressed in BL21(DE3)-SI cells (Invitrogen) using 300 mM NaCl. The bacterial protein expressions were carried out for three hours at 30 °C. All GST-tagged proteins were purified with glutathione-sepharose™ 4B beads (GE Healthcare) according to the manufacturer's instructions and eluted with 10 mM reduced glutathione in 50 mM Tris-Cl pH 8.

GST pull-down assay

The purified GST or GST-tagged proteins (2 µg) were incubated rotating for one hour at 4 °C with 50 µl of glutathione-sepharose™ 4B beads in 500 µl of in vitro binding buffer (IBB) (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 1 mM PMSF (Otera *et al.*, 2000)). The 6His-J1, 6His-D1 or 6His-ATXN3-var1.1 were added and incubated for 1-2 hours at 4 °C on a rotator. Incubations of 6His-ATXN3-var1.1, 6His-NEDD8 (UW9225, BioMol) or 6His-atx-3 were performed with 1% or 3% of BSA, respectively. Bound fractions were washed with IBB without glycerol and BSA, eluted by boiling in 50 µl of 2X SDS sample buffer and then were analysed by SDS-PAGE. Immunoblottings were performed using goat anti-GST (1:7500, GE Healthcare), mouse anti-6His (1:2000, Qiagen), rabbit anti-MJD1.1 (1:25000) or rabbit anti-NEDD8 (1:200) antibodies, all diluted in 1% SM/TBS-T (1% skimmed milk in Tris-buffered saline 1X (TBS), 0.05% Tween 20), or with rabbit anti-303JSP (1:2000) antibody (Rodrigues *et al.*, 2007), diluted in 3% SM/TBS-T. Secondary HRP-coupled antibody incubations were as follows: anti-goat 1:30000, anti-mouse (Santa Cruz Biotechnology) both at 1:10000 1% SM/TBS-T and anti-rabbit (Santa Cruz Biotechnology) 1:25000 1% SM/TBS-T for ATXN3-var1.1 or 1:20000 3% SM/TBS-T for atx-3. Detection was carried out with ECL (GE Healthcare).

Cell culture and transfection

HeLa cells were grown (37 °C, 5% CO₂) in DMEM (10938, GIBCO) supplemented with 10% of FBS, 100 U/ml penicillin and 500 µg/ml streptomycin (Gibco BRL). Cells were transfected at 70% cell confluence (5 µg of each expression plasmid) using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions and rinsed

with PBS eight hours later; the DNA concentration was measured by spectrophotometer. Forty-eight hours after transfection whole cell protein extracts were prepared in 250 μ l of lysis buffer [Buffer A (50 mM Tris-HCl pH7.5, 150 mM NaCl, 0.5% Tween 20, 1 mM PMSF) plus 1 mM imidazole] supplemented with EDTA-free protease inhibitors cocktail (Roche). After sonication lysates were cleared by centrifugation and incubated with 50 μ l of Ni-NTA agarose beads in lysis buffer. Beads were washed three times with washing buffer (Buffer A plus 30 mM imidazole) and bound fractions were eluted with 50 μ l of elution buffer (Buffer A plus 500 mM imidazole). Eluted proteins were subjected to SDS-PAGE and immunoblotted with rabbit anti-GFP antibody at 1:2000 1% SM/TBS-T overnight, 4 °C. Detection was carried out with ECF detection kit.

Immunofluorescence and immunoprecipitation

For immunolocalization of endogenous ATXN3 and NEDD8, HEK293 cells were grown on coverslips and were fixed for 20 min in freshly prepared 4% p-formaldehyde (Sigma) in phosphate-buffered saline (PBS), permeabilized with PBS/0.1% Triton X-100 for 5-7 min, washed in PBS and blocked with PBS/10% FBS. ATXN3 was detected with anti-SCA3 (1H9 clone, Chemicon) at 1:500 and NEDD8 was detected with rabbit anti-NEDD8 (PW9340, BioMol) at 1:50. Fluorescent secondary antibodies (AlexaFluor 488-coupled goat anti-rabbit and AlexaFluor 568-coupled goat anti-mouse, Molecular probes) were used at 1:1500. DNA was stained with Dapi (Sigma). Images captured were then prepared and analysed using Photoshop 5.0LE (Adobe). For immunoprecipitation, HEK293 cells (CRL-1573, ATCC) were transfected with 10 μ g of GFP-NEDD8 using Lipofectamine2000 reagent (Gibco BRL) following the manufacturer's instructions; cells were collected 48 h after transfection, lysed in buffer A plus protease inhibitors (Roche)), briefly sonicated and cleared by centrifuged. The lysates were then pre-cleared with Protein-G-Sepharose (GE Healthcare) for 1 h at 4 °C and then incubated with 9 μ l of polyclonal anti-NEDD8 antibody (PW9340) overnight at 4 °C. The immuno-complexes were precipitated with Protein-G-Sepharose at 4 °C for 2 h; the beads were washed two times with buffer A, minus the detergent, and finally eluted with 2X SDS sample buffer. The eluted proteins were analysed by western blot with anti-SCA3 (1H9) or anti-GFP.

Molecular docking prediction

Models of the JD-NEDD8 and of JD-ubiquitin complexes were built by superimposing the structures of the NED1-NEDD8 complex (PDB ID 1xt9 (Reverter *et al.*, 2005)) and of the UCH-L3 ubiquitin vinylmethylester complex (PDB ID 1xd3 (Misaghi *et al.*, 2005)), respectively, with the JD domain (PDB ID 1yzb (Nicastro *et al.*, 2005)) using the DALI server (Holm and Park, 2000). The structures superpose with JD with Z-scores of 1.8 and 4.5, and rmsds of 3.4 and 2.9 Å, respectively. The interface between the two proteins in the docked complexes was further refined by geometry optimization with CNS (Brunger *et al.*, 1998). All images were made with PyMOL (<http://pymol.sourceforge.net/>).

NEDD8-AMC cleavage assays

The putative deneddylase activity of the proteins was evaluated using the fluorogenic substrate NEDD8-AMC, a reagent that allows determination of NEDD8 c-terminal hydrolase activity, according to a previously described protocol (Burnett and Pittman, 2005). Briefly, 300 µl of assay buffer (50 mM HEPES, 0.5 mM EDTA, pH 7.5, with 0.1 mg/ml BSA and 1 mM DTT) containing 10 µg of ATXN3 or ATXN3C14A, purified as described (Gales *et al.*, 2005), was incubated at 37 °C for 30 minutes. The reaction was initiated by adding the substrate to a final concentration of 0.5 µM and the proteins' hydrolytic activities were measured as the release of AMC, after cleavage, for 9 minutes using a Perkin Elmer LS 50B fluorimeter.

Results and Discussion

ATXN3 interacts with NEDD8

In order to evaluate if ATXN3 interacts with NEDD8, we used the yeast two-hybrid system (Y2H) and GST pull-down assays.

For this study, several Y2H constructs of ATXN3 were made, coding for the entire protein (Gal4BD-ATXN3 var1.1), the JD (Gal4BD-JD) or the JD plus the first UIM

(ubiquitin-interacting motif) (Gal4BD-2.1N). A second variant of ATXN3, ATXN3 var2.1, in which UIM3 is absent, was also tested (**Fig.14**).

The results showed that ATXN3 interacts with NEDD8, in the Y2H assay (**Fig.15**). No difference was observed when isoform ATXN3 var1.1 or ATXN3 var2.1 were tested, indicating that the third UIM in ATXN3 does not influence its ability to interact with NEDD8.

To pinpoint the region in ATXN3 responsible for the interaction with NEDD8, two truncated fragments of ATXN3 were tested (**Fig.15**). The results obtained with the different fragments of ATXN3 showed that the JD is sufficient to establish the interaction with NEDD8. We tested also the reverse interaction between GAL4BD-NEDD8 and GAL4AD-JD, and a positive interaction was observed. Co-transformations of the bait or prey constructs with the pACT2 and pAS1 Y2H empty vectors, respectively, showed no positive interactions (**Fig.15**).

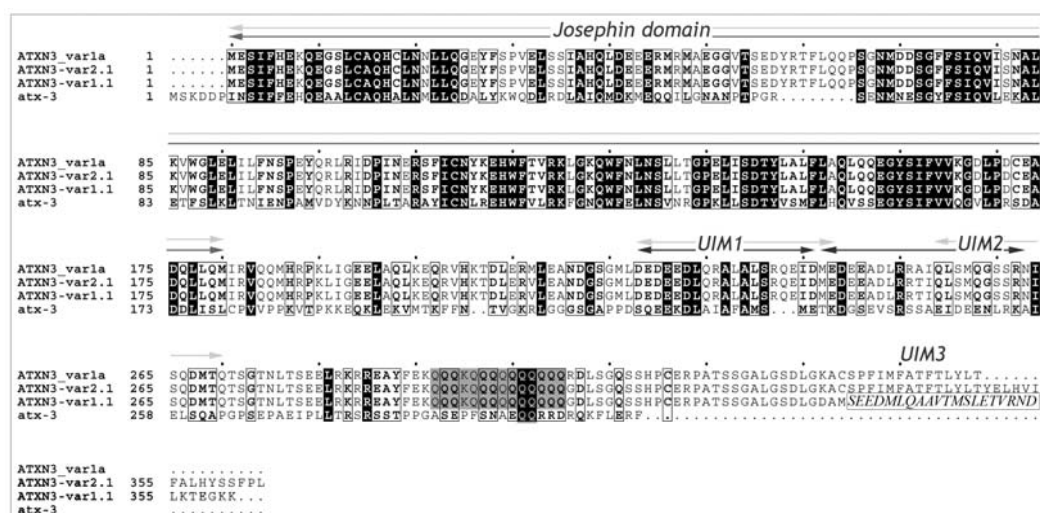


Figure 14. Sequence alignment of the human and *Caenorhabditis elegans* ataxin-3 proteins. Three isoforms of non-expanded human ataxin-3 have been studied: ATXN3 var2.1, ATXN3 var1.1 and ATXN3 var1a. The Machado-Joseph disease-like protein of *C. elegans* is here referred as atx-3. The Josephin domain (JD) and the UIM (ubiquitin-interacting motifs) of all proteins are delimited by two-head arrows (dark grey for the human isoforms and light grey for atx-3). The human isoforms ATXN3 var2.1 and ATXN3 var1a contain only two UIM1 and UIM2 (dark grey arrows) located before the repetitive segment of glutamines (shaded in medium grey). The MJD1.1 isoform comprises another UIM (UIM3, italicized). The atx-3 protein contains UIM1 and UIM2 (light grey arrows). Notice that atx-3 contains only two glutamine residues. Sequence alignments and colouring were performed with Clustal W (1.83) and ESPrit (Gouet *et al.*, 1999).

The results obtained with the Y2H analysis were confirmed using in vitro pull-down assays, by monitoring the binding of GST-NEDD8 to several 6His-tagged constructs of

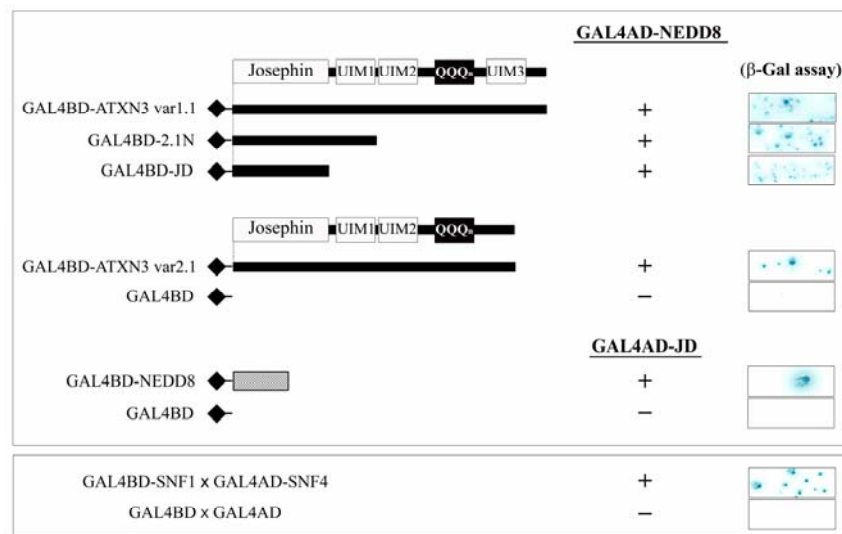


Figure 15. ATXN3 interacts with NEDD8 in the yeast two-hybrid system. The schematic representation depicts all yeast expression plasmids used. Two cDNA variants encoding normal ataxin-3 were used: GAL4BD-ATXN3 var1.1 and GAL4BD-ATXN3 var2.1, which lacks UIM3. Two constructs comprising fragments of ATXN3 were also tested: pAS1_G-JD encodes GAL4BD fusion to Josephin domain and pAS1-2.1N encodes JD plus functional UIM1. The reverse interaction between GAL4BD-NEDD8 (crossed rectangle) and GAL4AD-JD was also tested; the filled rhombus represents the GAL4BD tag. An interaction was considered positive (+) when growth on nutritional selection plates was observed and colonies stained blue when tested for β-galactosidase expression (β-Gal assay).

ATXN3 (**Fig.16(a)**). The direct interaction between ATXN3 and Ub was also tested, given the high identity between NEDD8 and Ub, and the fact that ATXN3 has been previously described to bind polyubiquitin (Burnett *et al.*, 2003; Chai *et al.*, 2004). As shown in **Figure 16(b)**, full-length ATXN3 interacts in vitro with NEDD8, further indicating that ATXN3 can bind NEDD8 directly.

In order to evaluate whether the interaction between ATXN3 and NEDD8 is evolutionarily conserved, we tested if the *C. elegans* atx-3 could also interact with human NEDD8. Interestingly, we observed a positive interaction between human NEDD8 and atx-3 protein (**Fig.16(c)**). The high degree of JD conservation, despite an overall identity of only 36% between atx-3 and human ATXN3, may explain this direct interaction (**Fig.14**). In *C. elegans*, ned-8 has been shown to be crucial for nematode development. The absence of ned-8 resulted in embryonic lethality or severe developmental abnormalities (Jones and Candido, 2000). The conservation of the atx-3/NEDD8 interaction throughout evolution suggests a biological and functional relevance for this protein pair.

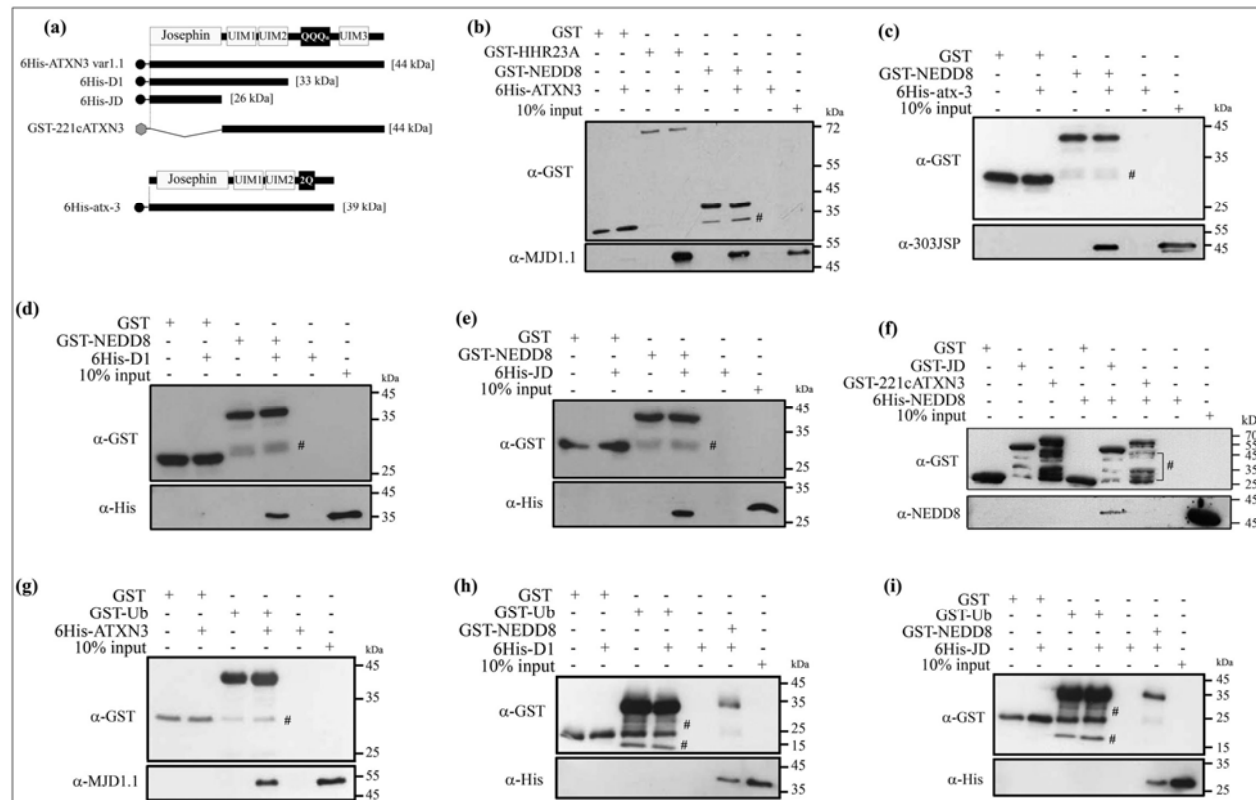


Figure 16. ATXN3 interacts with NEDD8 in vitro. The GST pull-down assays show direct interaction of ATXN3 with NEDD8 and ubiquitin. (a) Schematic representation of recombinant proteins used in the assays: 6His-ATXN3 var1.1 represents the full-length of ataxin-3 variant 1.1 with three UIMs; 6His-D1, comprises the JD plus two UIMs; 6His-JD comprises only the JD; GST-221cATXN3 comprises all domains of ATXN3 var1.1 except JD, and 6His-atx-3 represents the ataxin-3 of *C. elegans*. Expected molecular weights for all proteins are stated (brackets). Purified GST-NEDD8 and GST-Ub were incubated with ATXN3 full-length (b), 6His-D1 ((d), (h)), 6His-JD ((e), (f), (i)) or atx-3 (c); GST-221cATXN3 was incubated with 6His-NEDD8 ((f)). Interaction between GST-HHR23A and 6His-ATXN3 was observed under the conditions established ((b)). Input lanes show 10% of the 6His-tagged protein used. GST-NEDD8 pulled-down ATXN3 (b), its fragments 6His-D1 (d) and 6His-JD ((e), (f)) and atx-3 (c), but failed to interact with GST-221cATXN3 (f). The monomeric Ub showed interaction only with ATXN3 full-length ((g)). GST alone showed no binding in any assay. Bands identified with cardinals (#) are protein degradation products.

As a control experiment, we tested the interaction between ATXN3 and HHR23A (**Fig.16(b)**), and under the conditions used, we were able to replicate the interaction reported before (Wang *et al.*, 2000). Our results also suggest that ATXN3 is able to interact more efficiently with NEDD8 and HHR23A, than with monomeric Ub, since a higher amount of GST-Ub was needed to pull-down a quantity of ATXN3 similar to those obtained with NEDD8 and HHR23A (**Fig.16(b), (g)**).

We also mapped the minimum domain required for ATXN3 to interact with NEDD8, using the pull-down assays. Truncated fragments of ATXN3 corresponding to the JD plus UIM1 and UIM2 (6His-D1), the JD alone (6His-JD) or a C-terminal fragment comprising all ATXN3 domains except JD (GST-221cATXN3) were produced and assayed for binding to GST-NEDD8 or to GST-Ub. We did observe interaction between GST-NEDD8 and 6His-D1 or 6His-JD proteins (**Fig.16(d), (e)**), but no interaction was observed between GST-221cATXN3 and 6His-NEDD8 (**Fig.16(f)**). No interactions between the D1 or JD fragments of ATXN3 and monomeric Ub were observed in these in vitro studies (**Fig.16(h), (i)**).

Thus, we conclude, that the JD of ATXN3 is sufficient, and necessary, for the interaction with NEDD8, in agreement with the results obtained in the Y2H assay (Fig. 2). In control experiments we could not detect any binding of ATXN3 or its truncated forms to the beads alone, or to beads containing GST, indicating that the interactions observed with NEDD8 or monomeric Ub are specific (**Fig.16(b-i)**).

Taken together, the results obtained, using the Y2H system and GST pull-down assays, indicate that ATXN3 interacts with NEDD8 through its JD.

ATXN3 and NEDD8 co-localise and interact in mammalian cells

The subcellular expression of endogenous ATXN3 and NEDD8 proteins was determined in HeLa cells, using immunofluorescence analysis. Cells were fixed, permeabilized and then immunolabelled with anti-SCA3 (1H9) and anti-NEDD8 (PW9340) antibodies. Both proteins have nuclear and cytoplasmic diffuse distribution (**Fig.17(a)**). Incubations with secondary antibodies alone did not produce specific staining (data not shown). Merged images show co-localization of ATXN3 and NEDD8, both in the nucleus and cytoplasm, which is compatible with the possibility that the two proteins are interacting partners.

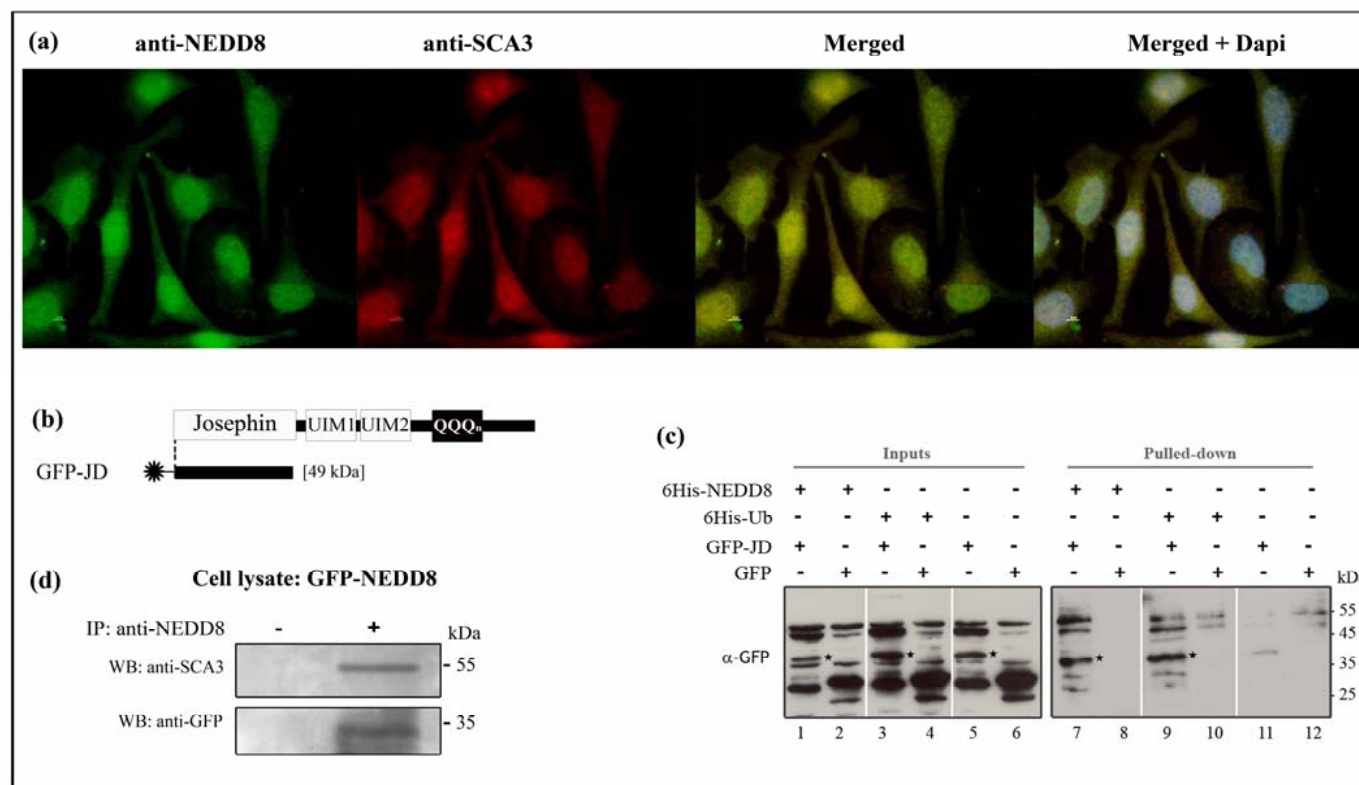


Figure 17. Ataxin-3 interacts and colocalize with NEDD8 in eukaryotic cells. (a) Immunofluorescence analysis showed nuclear and cytoplasmic colocalization of endogenous ATXN3 and NEDD8. ATXN3 was detected with anti-SCA3 (1H9) and probed with AlexaFluor 568-coupled goat anti-mouse antibody (red); NEDD8 was detected with polyclonal anti-NEDD8 antibody revealed by AlexaFluor 488-coupled goat anti-rabbit fluorescent antibody (green); nuclei were stained with Dapi (Sigma). (b) Schematic representation of GFP-JD used in mammalian cell transfection; the expected molecular weight is stated (brackets); filled star represents the GFP tag. (c) ATXN3 interacts with NEDD8 in mammalian cells. The 6His-NEDD8 or 6His-Ub proteins were co-expressed with GFP-JD (lanes 7, 9) or empty vector (lanes 8, 10) in HeLa cells. Lanes 1-6 were loaded with 5% of the total protein input of each assay. GFP-JD pulled-down (black star) by 6His-NEDD8 or 6His-Ub were detected by western blotting using the anti-GFP antibody (SC-8334). (d) Endogenous ATXN3 is immunoprecipitated by GFP-tagged NEDD8. Supernatants obtained from cells expressing GFP-NEDD8 were incubated overnight with anti-NEDD8 antibody (+) or without the antibody (-); immunocomplexes were subsequently separated by SDS-PAGE and immunoblotted with anti-SCA3 or anti-GFP antibodies.

In order to investigate whether ATXN3 can bind NEDD8 or Ub in the context of a mammalian cell, the interactions were analysed in transfected HeLa cells. Briefly, 6His-NEDD8 or 6His-Ub was co-expressed either with GFP-JD or GFP alone (**Fig.17(b)**). His-tagged proteins were purified under non-denaturing conditions, and the purified samples were subsequently immunoblotted with an anti-GFP antibody (**Fig.17(c)**).

Cells transfected only with pEGFP-JD or pEGFP were used as controls. The interaction of 6His-NEDD8 with the JD (**Fig.17(c)**, lane 7) was detected in this assay, supporting the previous results. No binding of GFP alone to NEDD8 was detected (lane 8), indicating that JD fusion protein was specifically co-purified with NEDD8. It should be noted that, the purified GFP-JD protein detected using the anti-GFP antibody has the molecular size corresponding to unmodified GFP-JD, suggesting that this domain is not neddylated, but rather that it interacts with NEDD8.

The interactions between 6His-Ub and GFP-JD were also tested. GFP alone showed no binding to monomeric Ub in this assay (**Fig.17(c)**, lane 10). When 6His-Ub was co-expressed with GFP-JD, the anti-GFP antibody detected a protein corresponding in size to the GFP-tagged JD (lane 9). The fact that this interaction could not be detected in the GST pull-down assay (**Fig.16(i)**) suggests that the interaction occurs with polyubiquitylated proteins.

To further confirm the occurrence of this new interaction *in vivo*, HEK293 cells were transiently transfected with a construct expressing GFP-NEDD8. When the cell lysates were incubated with polyclonal anti-NEDD8 we were able to co-immunoprecipitate the endogenous ATXN3 (**Fig.17(d)**). No ATXN3 was detected in the negative control (omission of the anti-NEDD8 antibody). An immunoblot assay with anti-GFP confirmed that GFP-NEDD8 protein was efficiently immunoprecipitated. Altogether, these results suggest that endogenous ATXN3 specifically interacts with NEDD8.

Costa *et al.*, has reported the expression pattern of murine ATXN3, which is expressed ubiquitously since early embryonic stages, at least in E11.5 (do Carmo Costa *et al.*, 2004). Interestingly, previous mRNA studies showed that the highest expression of the *NEDD8* occurs at the E11 stage; moreover, Kamitani *et al.*, (Kamitani *et al.*, 1997) have proposed that proteins expressed in the heart and skeletal muscle or in early development would be good candidates for NEDD8 interaction. Strikingly, mouse ATXN3 shows prominent expression in these particular tissues (do Carmo Costa *et al.*, 2004). All

these data support a biological relevance for the ATXN3/NEDD8 interaction, not only in differentiated tissues, but also since early stages of development.

NEDD8 interacts with ATXN3 in a substrate-like mode

Recently, the structure of JD of ATXN3 has been determined by NMR (Mao *et al.*, 2005; Nicastro *et al.*, 2005), revealing that it shares a significant structural homology with the DUB enzymes, human UCH-L3 (Misaghi *et al.*, 2005) and yeast YUH1 (Johnston *et al.*, 1999). Those studies allowed mapping of the active site (Q9, C14, H119, N134) and revealed that the binding site for the N-terminal UBL domain of HHR23B is comprised of an hydrophobic surface located opposite to the catalytic site of the JD (Nicastro *et al.*, 2005). The binding site of Ub as a substrate has been proposed to be located just above the active site, in a cleft bordered by the central catalytic subdomain and by a flexible helical hairpin that is likely to move to better accommodate the substrate (Mao *et al.*, 2005; Nicastro *et al.*, 2005). Our data shows that ATXN3 interacts specifically with NEDD8, and that the interaction site involves its catalytic JD. The high sequence and structural similarity between NEDD8, Ub and the UBL domain of HHR23B raises two possible scenarios: i) NEDD8 could interact with ATXN3 JD via its exposed hydrophobic surface used for the HHR23B interaction (Nicastro *et al.*, 2005) or ii) NEDD8 could interact with ATXN3 in a substrate-like mode, meaning that ATXN3 could have deneddylase activity.

We have analysed the surface distribution of conserved and divergent residues between the UBL domain of HHR23B and NEDD8, and between Ub and NEDD8, in an effort to identify the structural basis of their differential binding to ATXN3. The hydrophobic HHR23B-UBL surface relevant for the interaction with the JD is not strictly conserved in NEDD8 and we can predict that the NEDD8 interaction site on the JD is likely to be different. We predict that NEDD8 interacts with the JD in a substrate-like manner.

Our docking model of Ub and NEDD8 at the putative substrate-binding site of the JD was built based on the structural homology between Ub/NEDD8 hydrolases and JD. For both complexes the C-terminus -GG76 motif, common to NEDD8 and Ub, is bound deep in the narrow catalytic canyon, close to the catalytic C14 of ATXN3 (**Fig.18**). Accommodation of either NEDD8 or Ub involves small structural changes both in the JD and in the docked molecules (data not shown), in regions that have been shown, by chemical shift

perturbation, to be relevant for the JD-Ubiquitin interaction (Mao *et al.*, 2005). In these models, the negative electrostatic surface potential of the JD is complemented by the positive charge of the docked molecules (**Fig.18(a)** and **(b)**) and docking is further aided by surface complementarity.

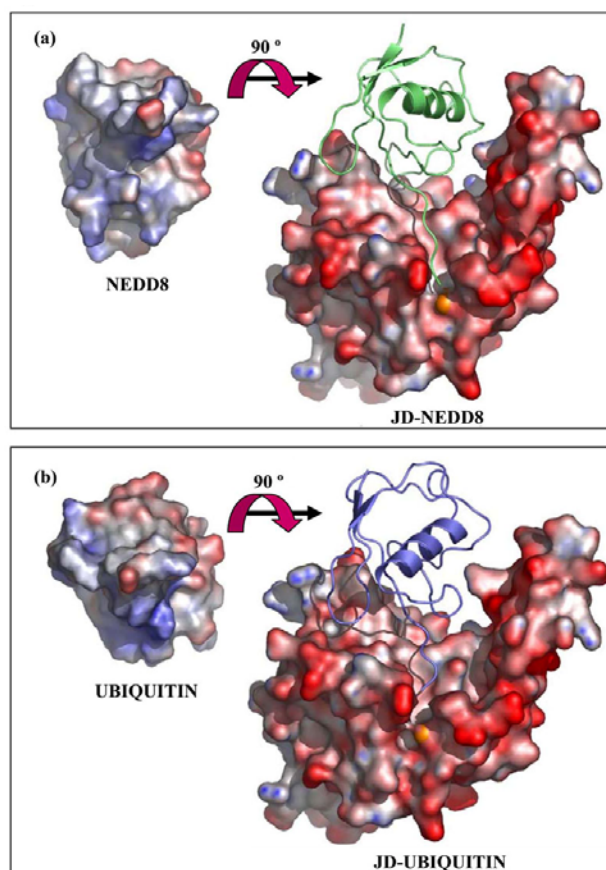


Figure 18. NEDD8 and ubiquitin share the same binding site on ATXN3 catalytic domain. Solid surface representation of the JD, NEDD8 and Ub coloured according to the electrostatic potential (blue for positive, red for negative). JD is complexed (a) with NEDD8 (green) and (b) with Ub (violet). The pictures on the left (a) and (b) figures show solid surface representations of NEDD8 and Ub rotated -90° along the horizontal axis in the figure plane, in comparison with their position in the docked complex. The docking surfaces of NEDD8 and Ub are predominantly basic and complement the acidic character of the substrate-binding cleft of the JD. The overall surface complementarity is notorious in both complexes.

Our modelling studies clearly show that both NEDD8 and Ub should bind in a substrate-like mode. In agreement, ATXN3 has NEDD8 C-terminal hydrolase activity against the fluorogenic substrate NEDD8-AMC (7-amino-4-methylcoumarin) (**Fig.19**).

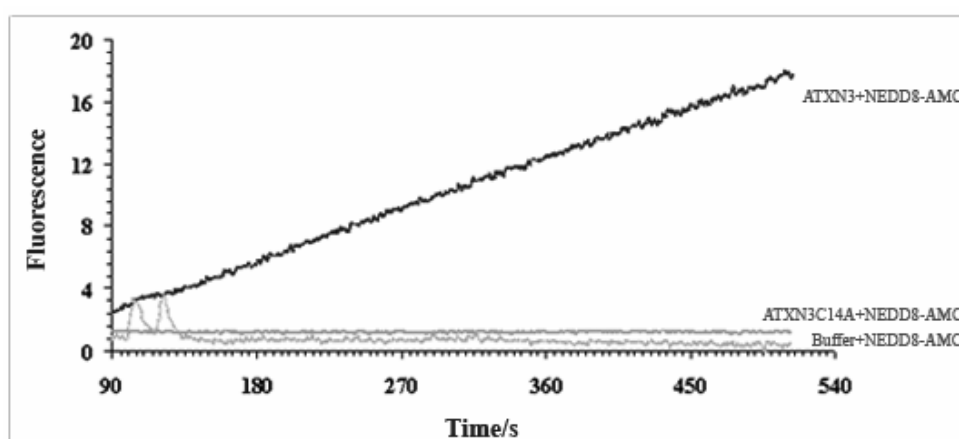


Figure 19. Hydrolysis of NEDD8-AMC by ATXN3 requires the C14 residue. ATXN3 displays C-terminal hydrolase activity against NEDD8-AMC (Boston Biochemicals). Recombinant ATXN3 or mutant ATXN3 (ATXN3C14A) were incubated with NEDD8-AMC (7-amino-4-methylcoumarin) for a total of 9 minutes, and their proteolytic activities measured from the AMC release extent, after cleavage. No cleavage was observed when only the assay buffer was present (grey line) or the mutant ATXN3C14A (orange line). However, when ATXN3 was present in the reaction a time-dependent release of AMC could be observed (blue line) indicating the hydrolytic activity of ATXN3 on NEDD8-AMC. The slope for the ATXN3 and ATXN3C14A activities were 0.037 s^{-1} and 0.0032 s^{-1} , respectively.

Furthermore, our results clearly show that the deneddylase activity of ATXN3 is dependent on the cysteine at position 14. The mutant ATXN3 (C14A) does not show deneddylase activity against NEDD8-AMC (**Fig.19**). However, this activity loss is not due to loss of interaction between these proteins since NEDD8 is still able to interact with mutant ATXN3 JD(C14A) (**Fig.20**).

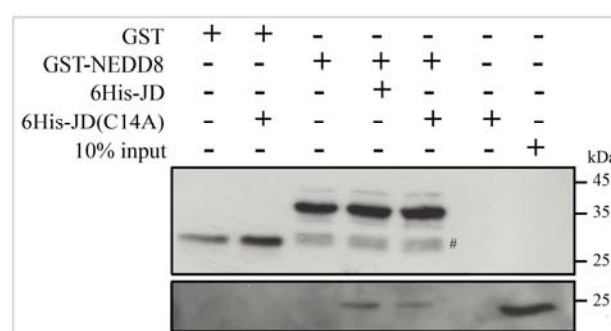


Figure 20. NEDD8 interacts with mutated ATXN3 (JD(C14A)) in vitro. The 6His-JD(C14A) construct was obtained by introducing a substitution of cysteine (C) by an alanine (A) at position 42 using the QuikChange site-directed mutagenesis kit (Stratagene). Protein expression was carried out as described (Gales *et al.*, 2005). The GST pull-down assay was performed as described ("Material and Methods" section). Bands identified with cardinals (#) are protein degradation products.

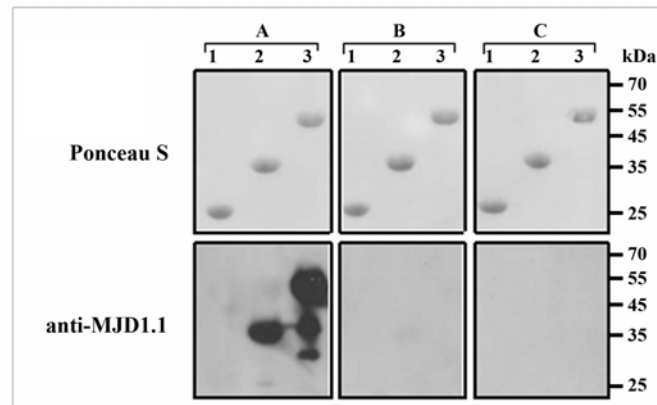


Figure 21. Characterization of a new rabbit polyclonal antibody produced against normal full-length recombinant 6His-ATXN3-var1.1, anti-MJD1.1. Five micrograms of the following 6His-tagged proteins were separated on a 12% SDS-PAGE gel: 1, JD, josphin domain alone; 2, JD plus UIM1 and UIM2; 3, full length ATXN3 var1.1. Proteins were transferred to a nitrocellulose membrane, stained with Ponceau S and subsequently immunoblotted as follows: A, anti-MJD1.1 at 1:20000; in B, anti-MJD1.1 was previously pre-incubated with 60 μ g of 6His-ATXN-3 var1.1 for one hour at 37 $^{\circ}$ C and then used at 1:20000 dilution; C, secondary antibody alone at 1:30000. These immunoblots against different recombinant 6His-tagged protein demonstrated that the anti-MJD1.1 antibody detects specifically ATXN3 full length as well as, its fragments comprising the JD plus UIM1 and UIM2 (A, 2) as it fails to detect recombinant 6His-JD (A, 1).

In conclusion, in this study, we identified a novel molecular partner of ATXN3, NEDD8. We demonstrated that the interaction between NEDD8 and ATXN3 is specific and independent of the presence of the UIM domains and the polyglutamine tract, since it occurs through its JD. The specific interaction between the JD and NEDD8 prompted us to build a theoretical model of the complex, which shows, that monomeric Ub and NEDD8 could share the same binding site. Moreover, we show that ATXN3 has a deneddylase activity dependent on the cysteine residue at position 14.

The NEDD8 protein is essential for the activity of SCF-like ubiquitin ligase complexes. These complexes determine the degradation of several substrates and consequently pace the pathways in which they participate. Our group has recently reported a transcriptomic analysis of *atx-3* knockout *C. elegans* strains (Rodrigues *et al.*, 2007) where we showed down-regulation of several genes encoding subunits of the SCF complex. Additionally, a recent analysis of genetic interactions in *C. elegans* by Zhong and Sternberg (Zhong and Sternberg, 2006), pinpointed several genes encoding UPS-related proteins and components of SCF-like complexes as genetic interactors of *atx-3* in the worm. Together with these results, the identification of NEDD8 as a new ATXN3

interacting protein adds a new perspective to the possible physiological role of ATXN3 modulating the activity of the UPS.

Further studies will be needed to explore the nature and specificity determinants of this newly identified interaction, to clarify its physiological role and the biological relevance of the putative deneddylase activity of ATXN3.

Discussion and Conclusions

The function of most of disease-causing polyglutamine proteins is largely unknown. Apart from the polyQ domain, there is virtually no sequence homology between polyQ proteins. In this work, we focused on the normal function of ATXN3, the smallest of all polyQ proteins.

ATXN3 role(s) in the cell

Evidence gathered about ATXN3 subcellular localisation and interactions raised several possibilities for the biological function(s) of ATXN3 in the cell, though a definite conclusion remains elusive.

ATXN3 may be involved in transcriptional regulation by interacting directly with transcription factors, CBP, p300 and pCAF, or indirectly, by inhibiting histones modifications (Li *et al.*, 2002). Moreover, ATXN3 was also shown to interact with HDAC3 and NCoR, leading to an increased histone deacetylase activity, dependent of ATXN3's intact UIMs (Evert *et al.*, 2006), and thus reinforcing its role as a transcriptional repressor.

ATXN3 may also participate in the cellular DNA damage response, specifically in NER-GGR. The interaction of ataxin-3 with the HHR23 proteins (Wang *et al.*, 2000), and with XPC (our work, Chapter 2), further strengthens this hypothesis. Apart from our initial work presented in here, however, there is no other biological evidence that ataxin-3 is directly involved in DNA repair.

The larger body of evidence until known suggest that ATXN3 participates in the protein surveillance mechanisms. It has been shown that it functions as a polyubiquitin chain hydrolase (Burnett *et al.*, 2003; Chai *et al.*, 2004; Berke *et al.*, 2005; Mao *et al.*, 2005), in a C14 residue-dependent manner. The interaction between ATXN3 and VCP, a chaperone involved in the translocation of misfolded protein to the 26S proteasome, is dependent on polyQ length and reinforces its participation in the cell quality control systems (Hirabayashi *et al.*, 2001; Kobayashi *et al.*, 2002; Doss-Pepe *et al.*, 2003; Nakayama, 2003; Boeddrich *et al.*, 2006; Zhong and Pittman, 2006). The modulation of this interaction the polyQ domain of ATXN3 may affect its deubiquitylating activity (Mao *et al.*, 2005). In fact, normal ATXN3 is recruited into the aggregates of several polyQ diseases, through interaction with its mutant counterpart or with other polyQ proteins (Perez *et al.*, 1998). Moreover, overexpression of normal ATXN3, in a *Drosophila* model of MJD, suppressed polyQ toxicity. Such neuroprotective role was dependent of the deubiquitylating activity of ATXN3 and of the proteasome (Warrick *et al.*, 2005).

Given the uncertainty about the physiological role of ATXN3, we decided to approach this question through the identification of ATXN3's molecular interactors.

Library screening

The initial strategy set for this work, a large genetic screening for molecular interactors of normal ATXN3 in a brain-cDNA library, did not produce the results that could have been expected. Though the quality of the library screened should be considered the major drawback in the success of this strategy, we still feel that the power of screening should have been higher. If more independent clones had been screened, we would definitely maximize our probabilities of finding a true positive interactor, and perhaps overcome the redundancy of the tested library. In light of our current experience, we also think that, some of the seven candidates selected from that screening should be cloned in the correct frame, and further tested, independently of the final validation outcome. Nevertheless, with this work we implemented the yeast two-hybrid system routinely in our lab, which is now being used to study interactions of the *C. elegans* and mouse orthologues of ATXN3 and we prepared, and characterised, new molecular tools for continuing the study of protein-protein interactions, either by designing a new library screening, or by analysing direct protein interactions.

Conversely, the direct protein-protein interaction analysis turned out to be a successful strategy. We could prove that normal ATXN3 interacts with XPC, a repair factor crucial in the early steps of GGR-NER, and NEDD8, an ubiquitin-like protein, involved in the cullin-dependent ubiquitylation system. The specific interaction between the JD and the HHR23 proteins (Chapter1), which in addition to their role in DNA repair are also, proteasome-binding factors, reinforces the role of ataxin-3 as an active participant in protein surveillance mechanisms of the cell, as is the interaction observed between normal ATXN3 and NEDD8; the clues obtained *in vitro* regarding its deneddylating activity may sustained the participation of ATXN3 in the regulation of UPS activity (Chapter 2).

ATXN3 and DNA repair

Back in 2000, the discovery of the interaction between ATXN3 and the HHR23A and HHR23B proteins (Wang *et al.*, 2000) led to significant research regarding the role of this interaction, in the protein quality control systems of the cell (Hirabayashi *et al.*, 2001; Kobayashi *et al.*, 2002; Doss-Pepe *et al.*, 2003; Nakayama, 2003; Boeddrich *et al.*, 2006; Zhong and Pittman, 2006). Yet, we set as our objective to define another functional context for this interaction. The interaction of ATXN3 and the HHR23 proteins raised the possibility that this interaction could also have some relevance for DNA repair, namely the GGR-NER pathway.

As discussed in chapter 2, the HHR23 proteins bind and function as stabilizers of XPC to the damage recognition complex (Sugasawa *et al.*, 1996; Li *et al.*, 1997; Sugawara *et al.*, 1997; Ng *et al.*, 2003; Sugawara *et al.*, 2005; Park and Choi, 2006). Moreover, XPC polyubiquitylation stabilizes this complex and promotes its availability to the damage site (Sugasawa *et al.*, 2005). The mechanism through which XPC stabilisation occurs remains elusive, but the intrinsic characteristics of the interactor HHR23 seem to play a pivotal role (Ortolan *et al.*, 2000; Chen *et al.*, 2001; Madura, 2002; Wang *et al.*, 2003; Heessen *et al.*, 2005; Sugawara *et al.*, 2005).

This new interaction between ATXN3 and XPC has to be further validated, and its structural determinants and functional significance must be further studied. The preliminary immunofluorescence experiments with irradiated HEK293 cells are promising. The observed induced colocalisation of ATXN3 and XPC that occurs post-UV irradiation strengthens the possibility that ATXN3 may be participating in GGR-NER *in vivo*. The significant induction of the expression of atx-3 in ENU-treated worms is compatible with this assumption. Though we think that this is strong evidence, worthy of attention, we may only speculate, at this point, about the possible role(s) of ATXN3 in this pathway.

We have raised three hypotheses: ATXN3 may either be (1) binding the HHR23B/XPC/CETN2 ternary complex and acting directly in GGR-NER. or (2) it may be competing with HHR23B for XPC (and the fact that both interactions occur through the JD supports this last notion); the direct binding of ATXN3 to either XPC or HHR23B would disrupt the complex stabilisation equilibrium, inducing the shuttling of the complex for degradation in the proteasome, and allowing rapid elimination of HHR23B/XPC/CETN2, when unnecessary. (3) The possibility that XPC may be a substrate of ATXN3, as a

deubiquitylating enzyme (DUB), must also be considered. The nature of the polyUb conjugated to XPC is still unknown. Although K63-linked polyUb chains are known to be involved in DNA repair (and this has not been ruled out for XPC), the fact that XPC is complexed with a proteasome-interacting protein (HHR23B) raises the possibility that it may be conjugated to K48-linked polyUb chains. Nevertheless, and independently of the nature of the polyUb chain conjugated to XPC, it is a possible substrate of ATXN3, as ATXN3 has *in vitro* DUB activity upon both K63- and K48-linked chains {Burnett, 2003 #813; Chai, 2004 #818 and H. Paulson, personal communication}.

As for its nature, little is known about the size of the XPC-conjugated ubiquitin chain optimal for efficient GGR-NER. The interaction with ATXN3 may allow the “proofreading” of the XPC polyUb chain to optimal sizing, or it may be acting as a negative regulator of GGR-NER, by destabilising XPC, by degrading the polyUb below the putative optimal size and addressing it to proteasome degradation.

The results obtained with *C. elegans*, in the progeny assay, sustain the latter hypothesis. This assay showed that, in the absence of *atx-3* expression (*atx-3*^{-/-}), the knockout animals were able to produce significantly more viable progeny than the wild-type strain, when treated with ENU. It seems that, upon DNA damage-inducing conditions, the absence of *atx-3* expression confers a higher resistance to genotoxic stress, either enabling the animals to repair more efficiently the damages induced. This and the remaining observations, such as the significant differences observed in the *xpc-1* and *ZK20.3* expressions, between wild-type and *atx-3*^{-/-} animals, upon basal and ENU-treated conditions, seem to support the hypothesis that ATXN3, or its worm counterpart, may be acting as a negative regulator of GGR-NER.

Recently, Wang *et al.* (2005) showed that, upon UV irradiation, XPC is modified by SUMO-1 in a XPA and DDB2-dependent manner. Sumoylation seems to enhance stabilization of XPC, as yeast studies showed that *ubc9-1Δ* strain (with deletion of Ubc9, the specific SUMO-1 E2 conjugating enzyme) had low RAD4 levels and increased UV sensitivity, in comparison to the normal strains {Ramsey, 2004 #988}. Sumoylation can also alter target's interactions (Johnson, 2004) and it may be modulating XPC's interactions throughout the NER reaction, for example, sensing the moment of its dissociation of repair complex. Interestingly, SUMO-1 has been reported as an ATXN3 interactor (Shen *et al.*, 2006), another evidence that may potentially link ATXN3 to XPC and, ultimately, to NER.

In summary, we gathered more evidence supporting the putative involvement of ATXN3 in GGR-NER, through a participation in XPC complex-remodelling activity that may modulate the rate or efficiency of this repair system *in vivo*.

The isopeptidase activity of ATXN3

Depending on their catalytic mechanism, proteases may be divided into five classes: aspartic, metallo, serine, threonine, and cysteine proteases. DUBs are cysteinic or metalloproteases. Furthermore, the cysteinic DUBs, depending of the active catalytic center, may be sub-divided into four classes: ubiquitin-specific proteases (USP), ubiquitin C-terminal hydrolases (UCH), ovarian tumor-related proteases (OTU), and Machado-Joseph disease proteases (MJD). The metalloproteases have a JAMM (JAB1/MPN/Mov34 metalloenzyme) motif at their catalytic centre (Amerik and Hochstrasser, 2004; Nijman *et al.*, 2005). DUBs catalyse the cleavage between the C-terminal glycine-76 of ubiquitin and the substrate.

Though DUB's function is not fully understood, they may be responsible for (1) the processing of the ubiquitin and UBL precursors, (2) the proofreading of the conjugated polyUb chains, either from mistakenly ubiquitylated proteins, or (3) from specific polyubiquitylated substrates, (4) retrieving polyUb chains before the substrate's proteolysis in the proteasome, (5) disassemble the ubiquitin oligomers (unanchored polyubiquitin chains), or (6) deubiquitylating of membrane proteins tagged for lysosomal/vacuole-dependent degradation or trafficking (Amerik and Hochstrasser, 2004). DUBs are essential for the maintenance of the ubiquitin cycle of conjugated and non-conjugated ubiquitin, sustaining the pool of free ubiquitin, necessary for new ubiquitylation reactions.

ATXN3, namely its JD domain, contains conserved amino acids reminiscent of the catalytic domain of a member of the deubiquitylating cysteine protease UCH and USP family (Scheel *et al.*, 2003; Amerik and Hochstrasser, 2004; Mao *et al.*, 2005; Nicastro *et al.*, 2005; Nicastro *et al.*, 2006), displays Ub hydrolase activity, and binds ubiquitin-aldehyde *in vitro* (Burnett *et al.*, 2003; Berke *et al.*, 2005).

The protein atx-3 is the homologue of ATXN3 in *C. elegans*. Similarly to its human counterpart, it conserves the catalytic triad characteristic of cysteinic proteases and has deubiquitylating activity *in vitro*. The knockout animal for atx-3 (*atx-3^{-/-}*) showed no obvious phenotypic alterations, compared to the wild-type (Rodrigues *et al.*, 2007). This

suggests that atx-3 is not an essential protein and, as a putative DUB, atx-3 may not be contributing significantly for the essential free ubiquitin pool within the cell; rather, it may be acting upon specific substrates, without significant repercussions to the cell. The same result was obtained for another *C. elegans* UCH (R10E11.3), UCH46 in humans, the knockdown expression of which led to a normal phenotype. Based on several pieces of evidence, Nijman *et al.* suggested that most DUB target specific substrates (Nijman *et al.*, 2005).

The biochemical characterisation of atx-3 as an enzyme is very incomplete. Yet, given the similarity between the homologues and the known *in vitro* preference of human ATXN3 for polyUb chains containing four or more Ub moieties, it is licit to speculate that these two proteins are also not involved in the processivity of ubiquitin or UBL proteins precursors, reinforcing their role in the regulation of ubiquitylation pattern of specific substrates. The results of ubiquitylation pattern analysis corroborate this hypothesis. No obvious difference was observed in the global ubiquitylation pattern of protein extracts from *atx-3*^{-/-} animals, when compared to the wild-type and, as seen before (Santos, 2005), nor even upon heat-shock (Rodrigues, A.J. personal communication) or ENU-treatment.

ATXN3 seems to be a weak DUB *in vitro* (K. Wilkinson, personal communication), which anticipates the difficulties that should be encountered in the *in vivo* validation of its activity and, concomitantly, in the identification of its substrates. Nevertheless, the identification of specific targets of this polyQ protein is mandatory.

Nevertheless, much has been done in the last decade, and a rather intricate net of experimental and bioinformatic evidence has been obtained for ATXN3. Identifying its biological target(s) will shed some light upon the biological pathways which ATXN3 is acting in, and, hopefully, will help to elucidate its normal function(s). With this work, we propose a novel putative target of ATXN3 activity, the XPC protein. Undoubtedly, further studies must be carried out, to better understand to what extent the polyubiquitylation of XPC and, extensively, the GGR-NER system are modulated by ATXN3 or any other DUB.

“DUBling” the chances: ubiquitin or NEDD8?

Several pieces of evidence showed that some predicted DUBs might also be active toward ubiquitin-like proteins. USP21 and UCH-L3 cleave both Ub and NEDD8; USP18 has been proposed to specifically cleave ISG15, another UBL (Wada *et al.*, 1998; Gong *et al.*, 2000; Malakhov *et al.*, 2002). Additionally, SENP8/NEDP1/DEN1, a cysteinic protease known to be involved in the proteolysis of SUMO, has also been reported to be a NEDD8-specific protease (Gan-Erdene *et al.*, 2003; Wu *et al.*, 2003).

As described and discussed in chapter 2, we have contributed to the identification and characterisation of a novel molecular interactor of ATXN3, NEDD8. Hence, we identified ATXN3 as another cysteinic DUB that displays affinity for NEDD8, *in vitro* and *in vivo*. This interaction is dependent on the JD domain of ATXN3, and modelling studies led us to predict that NEDD8 interacts with the JD in a substrate-like manner. In agreement, cleavage activity assays confirmed the structural hypothesis, as ATXN3 exhibits catalytic properties against NEDD8-AMC, a fluorogenic substrate; Costa *et al.* (unpublished data) verified the evolutionarily conservation of the deneddylase activity, since the murine homologue of ATXN3 is also able to cleave NEDD8-AMC, *in vitro*. The ATXN3's deneddylase activity is dependent of the cysteine residue (Cys14) at the catalytic triad in the JD, as the mutant protein, ATXN3C14A, displays no deneddylase activity against the fluorogenic substrate. Importantly, the activity loss is not due to the abrogation of the interaction, as JD(C14A) is still able to interact with NEDD8. The C14 residue is, crucial for the catalytic function, itself, of the active site of ATXN3; the importance of this residue, as a potential modifier for the disease, still needs to be evaluated. It is known that the catalytically inactive form of ATXN3 leads to the accumulation of ubiquitylated proteins in the cell (Berke *et al.*, 2005). It would be interesting to evaluate if an alteration at this position, and/or at any other of the catalytic triad, has any clinical relevance, for example, for the age-at-onset of the MJD.

The presence of NEDD8 in neuronal and glial inclusions reactive to ubiquitin has been reported for several neurodegenerative diseases, including MJD. Whether the presence of NEDD8 is a consequence of its interaction with ATXN3, or if it indicates the presence of neddylated proteins, remains unknown. The new animal model for MJD that is being developed in our lab (Costa *et al.*, in preparation) will allow, for example, the analysis of the composition of ubiquitylated and neddylated inclusions aiming at the identification of possible targets of ATXN3, either as DUB or deneddylase. The analysis

of the protein expression patterns from the *C. elegans atx-3^{-/-}* knockout strain or from a ATXN3 knockdowned cell line, may also be used to identify ATXN3 targets.

ATXN3 may therefore interplay with both the ubiquitylation and neddylation pathways. Currently, it is still unknown which residues or motifs are important for these proteases, and how they distinguish between ubiquitin and the remaining UBL proteins (substrate type specificity), or the conjugated target protein (target specificity). Clearly, more *in vitro* and *in vivo* analysis of DUBs and UBL proteases are necessary. Probably a combined strategy relying on the recognition of both the specific target, the nature of the attached UBLs and the domains present in the protease (e.g. UIM, UBA, PAZ-polyubiquitin associated zinc finger) determines their overall specificity (Amerik and Hochstrasser, 2004; Nijman *et al.*, 2005).

The interaction of ATXN3 with NEDD8 is dependent of the JD domain. In contrast, the interaction with Ub seems to be dependent of the presence of the UIM domains, although the catalytic centre is at the JD (Berke *et al.*, 2005; Mao *et al.*, 2005). The presence of the UIM positioned outside the catalytic domain may therefore confer differential specificity towards the ubiquitylated targets.

The importance of sequences outside the catalytic domains in the determination of the inherent specificity of the proteases has been reported. The deubiquitylating enzyme USP5 (aka isopeptidase T) comprises two UBA domains. *In vivo*, USP5 disassembles unanchored K48-linked chains (Amerik *et al.*, 1997); *in vitro*, the human USP5 showed comparable activity against all of the chains tested, Lys48-Ub₄, Lys63- and Lys29/6-Ub₄ and monoubiquitin; however, its isolated UBA2 domain showed substantial affinity for monoubiquitin. The ectopic expression of the UBA2 from HHR23A, which binds preferably Lys48-Ub₄, altered the specificity of the chimeric USP5, and biased its preference for the K48-Ub chains. Although the UBA2 domain of this DUB may not be the sole determinant of the enzyme's specificity, it may have a regulatory role in the protease activity (Raasi *et al.*, 2005).

The biophysical properties of both ATXN3 interactions need to be determined, and together with the analysis of the enzyme kinetics, may elucidate the substrate towards which ATXN3 shows a higher affinity, i.e., the specificity of the isopeptidase activity. Our preliminary results show that 6His-NEDD8 inhibits ATXN3's DUB activity against polyUb chains, *in vitro*. The competitive nature of the inhibition is not unexpected, as NEDD8 binds directly to JD, the catalytic domain of ATXN3.

A proposed model: linking E3 ligases, NEDD8, UPS and genotoxic stress

DUBs and E3 ligases are often found in protein complexes together. Several interactions have been reported: USP7 and HDM2 (Canning *et al.*, 2004); USP15 and Rbx1 (Hetfeld *et al.*, 2005), and USP20 and pVHL are just some of these. The ultimate example is A20, which possesses simultaneously DUB and E3-ligase activity (Wertz *et al.*, 2004). Independently of the purpose of these interactions - E3-ligase stabilization, differential regulation of the targets or the DUB itself, or definition of the target specificity - they seem to establish the proper conditions for the degradation of the right protein.

ATXN3 itself is known to be associated with some E3-ligases such as parkin (Tsai *et al.*, 2003), CHIP (Jana *et al.*, 2005), p300 which strongly ubiquitylates p53 (Grossman *et al.*, 2003) and PCAF, the p300-CBP-associated factor which has been recently described as an atypical E3 ligase (Li *et al.*, 2002; Linares *et al.*, 2007).

The model proposed in **Fig.22**, adapted from the work by Huang *et al.* (2006), fits many aspects discussed in this work.

In vivo, DDB2 has ubiquitin ligase activity, which is modulated by UV exposure, and, as referenced before, XPC is polyubiquitylated by DDB2 (Sugasawa *et al.*, 2005).

DDB is a heterodimeric complex, comprising DDB1 and DDB2. Although the mechanism remains poorly understood, DDB participates in the recognition steps of GGR-NER, together with the trimeric complex XPC/CETN2/HHR23B. DDB, itself, forms a complex with a SCF-like complex, which comprises cullin-4A, RBX1/ROC1, and is negatively regulated by CSN (COP9/signalosome), which can deconjugate both ubiquitin and NEDD8-modified cullin4A (Groisman *et al.*, 2003). Upon DNA damage, CSN dissociates from the SCF-like complex, allowing cullin4A to be neddylated, and thus the activation of the ubiquitin ligase complex. Therefore, in the basal conditions (**Fig.22A**), the DDB E3-ligase complex (DDB1, DDB2, CUL4A and ROC1 (E3-ligase RING domain)) is inactivated due to the interaction with CSN, the negative regulator of the complex.

Upon DNA damage (**Fig.22B**), the activated/neddylated DDB ligase complex translocates to the DNA lesion, recruiting the XPC/CETN2/HHR23 complex to the lesion site. During GGR-NER of low distorting lesions, the DDB complex helps the XPC complex to sense the damage spot (Fitch *et al.*, 2003a; Fitch *et al.*, 2003b). The polyubiquitylation of XPC is mediated by DDB2 (Sugasawa *et al.*, 2005); although the concomitant ubiquitylation of cullin4A and self-ubiquitylation of DDB2 is promoted by the same E3 ligase complex (**Fig.22C**), these proteins have different fates. Polyubiquitylation of XPC stabilizes it

and stimulates NER, whereas polyubiquitylated DDB2 is degraded, and the remaining ubiquitylated DDB ligase complex (polyubiquitylated CUL4A) is displaced from the lesion (**Fig.22D**). In the absence of ubiquitylation and inhibition of the proteasome, DDB2 is not degraded and the XPC binding to the damage site is compromised, as is NER efficiency (Wang *et al.*, 2005). Therefore, the DDB complex seems to not only to help XPC complex to sense the damage site, but also to promote its ubiquitylation.

The DNA repair mechanism comprises several proteins and multiple spots of regulation. The precise timing of protein association and dissociation during NER may be regulated by post-translational modifications. It is suggested by the authors that regulation of this pathway might involve negative regulators, as deubiquitylating enzymes (DUB), that are able to “reset” the mechanism.

It is the nature of the post-translational modifications in this model, polyubiquitylation, neddylation and sumoylation, which makes it worthy of attention.

Based on the known properties of ATXN3, and on the results from this work, there are several steps of NER, which ATXN3 may negatively regulate.

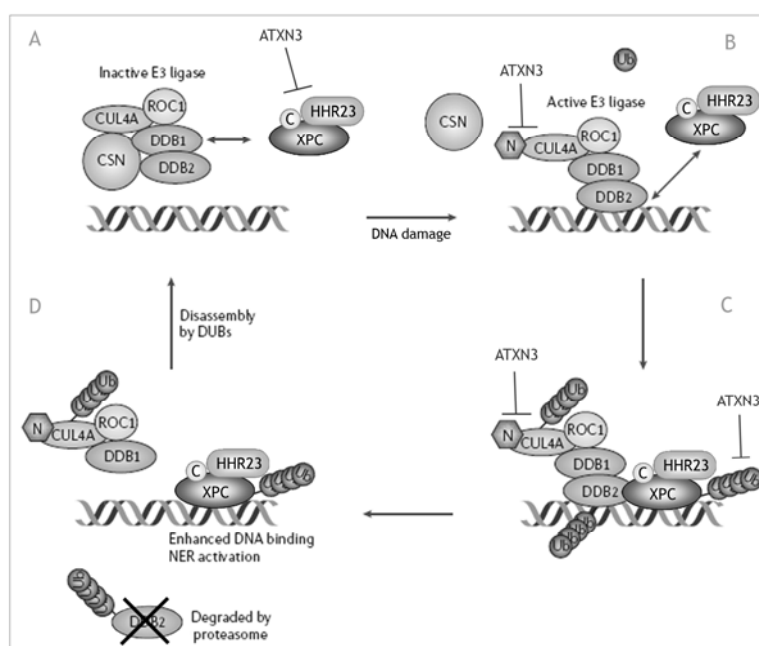


Fig. 22. Proposed model of how ATXN3 may be linking E3-ligases, neddylation, UPS and DNA repair.

Legend: C, centrin-2; CSN, COP9/signalosome; N, NEDD8; Ub, ubiquitin; DUBs, deubiquitylating enzymes. Adapted from (Huang and D'Andrea, 2006).

As depicted in Fig.22, ATXN3 is able to interact, through its JD domain, with both HHR23 proteins and XPC. ATXN3 may be competing with HHR23B for XPC, or vice-versa, thus disrupting the stabilisation of HHR23B/XPC/CETN2 complex.

The hypothesis that XPC may be a substrate of ATXN3 DUB activity should also be considered. The interaction with ATXN3 may be proofreading XPC's polyUb chain, destabilising XPC. Since the interaction with XPC occurs through the catalytic domain of ATXN3, reinforces this hypothesis. The competition for HHR23B, which shields XPC from degradation in the proteasome, may also be occurring at this stage.

Although the CSN seems to be the only negative regulator of CUL4A neddylation known to date in this pathway, the results obtained with this work suggest the possibility that ATXN3 may also deneddylate cullin4A. The neddylation of cullins stimulates the E3 ligase activity of SCF complexes; if ATXN3 would be able to deneddylate cullin4a, it would be promoting the deactivation of E3 ligase, the CSN binding to DDB complex and shutting down the mechanism. Further evidence support this possibility: murine ataxin-3 interacts directly with the C-terminal of mouse cullin-1 (MC Costa, personal communication), a region that is conserved in Cul4A; interestingly, cullins are typically neddylated at the C-terminal region of cullins. Moreover, recently it was reported that worm cul-1 may participate in NER regulation (Gao, M., personal communication). The authors knockdowned the expression of cul-1 in *C. elegans* and observed an increased ENU-induced germ line apoptosis. Since cullin-1 is a component of the Skp1/Cullin1/F-box (SCF) complex, it seems that the apoptosis induced by ENU is negatively regulated by neddylation through SCF. Altogether, all this evidence supports the possibility that, as a deneddylase, ATXN3 may be a negative regulator of NER.

Apart from the polyubiquitylation on XPC stabilization, XPC is also modified, by SUMO-1, upon UV irradiation (Wang *et al.*, 2005), in a DDB2-dependent manner. Interestingly, ATXN3 interacts also with SUMO-1. It would be interesting to evaluate if ATXN3 has any participation on the decision of whether or when, DDB2 ubiquitylates or sumoylates XPC.

The hypotheses raised still lack experimental validation. Nevertheless, we believe we have contributed to the further understanding of the normal function of ATXN3, and suggest it may be a factor that connects E3 ligases, neddylation, UPS and NER.

Conclusions

We have contributed to the understanding of the normal function of the protein involved in Machado-Joseph disease, ATXN3, by identifying and studying a new molecular interactor of normal ATXN3.

In **chapter 1**, we established a link between ATXN3 participating in the modulation of DNA repair, namely the GGR pathway:.

- In the study of direct protein-protein interactions of some putative interactors of normal ATXN3, we identified the XPC protein as a direct interactor of normal ATXN3.
- Immunofluorescence assays showed that the co-localisation of the two proteins, in mammalian cells, was enhanced upon UV radiation.
- No direct interaction could be observed between ATXN3 and the centrin-2 protein, the third component of NER's trimeric recognition complex. A transient interaction between the two proteins could not be ruled out.
- The conservation of some of the human interactions observed by the *C. elegans* protein homologue of ATXN3, atx-3, allow us to conclude that the atx-3 protein conserves a similar structure to its human counterpart, thus of biological relevance.
- *Atx-3* is an ENU-responsive gene, as wild-type animals (N2) showed a significant upregulation of *atx-3* expression, when exposed to a mutagenic agent, ENU.
- Absence of *atx-3* affects the expression levels of two potential DNA repair genes, ZK20.3 (RAD23 homologue) and *xpc-1*, and increases resistance to DNA-damaging conditions.

Consistent with a role for ATXN3 in UPS regulation, we showed in **chapter 2**, with *in vitro* and *in vivo* evidence, that ATXN3 interacts, through its JD, with the neural

precursor cell expressed developmentally down-regulated gene 8 (NEDD8), an ubiquitin-like protein implicated in cullin-dependent ubiquitylation.

The *C. elegans* homologue of ATXN3, atx-3, also interacts with NEDD8, indicating that atx-3 maintains the structural requirements for functionality, similar to the human protein's. Additionally, we gathered the first evidence that ATXN3 is potentially a deneddylase:

- We identified a novel molecular partner of normal ATXN3, NEDD8, an ubiquitin-like protein.
- The interaction between NEDD8 and ATXN3 is specific and dependent of its JD; in contrast, interaction with monomeric ubiquitin requires the full-length ATXN3.
- The specific interaction between the JD and NEDD8 led us to build a theoretical model of the complex, showing that monomeric Ub and NEDD8 could share the same binding site in ATXN3, which may adopt a substrate-like structure.
- Normal ATXN3 has deneddylase activity *in vitro*.
- The deneddylase activity of ATXN3 is dependent of the cysteine residue at position 14 (Cys14), as the mutant ATXN3C14A does not exert deneddylase activity *in vitro*, albeit the interaction with NEDD8 is maintained.

The biological implications of these findings are being further investigated, and hopefully will deepen our understanding of the MJD pathogenesis.

Appendix 1

Screening for proteins that interact with ataxin-3 using the yeast two-hybrid system

Introduction

Protein interactions are intrinsic to virtually all cellular process. In cells, long-lasting or transient protein interactions are engaged in the control and modulation of several cellular processes (Van Crielinge and Beyaert, 1999). At the starting point of this work, little was known about the ATXN3 protein, its physiological function, or the biological pathways it could be involved in. Most of the information came indirectly from the analysis of post-mortem material of MJD patients and lymphoblastoid or neuronal cell lines, all largely exploring the expression and localization of ATXN3. Information regarding the normal sub-cellular localisation of ATXN3 pointed out that it was present in the nucleus and associated with the inner nuclear matrix (Tait *et al.*, 1998), but also in the cytoplasm, within mitochondrial compartments (Trottier *et al.*, 1998). All the clues gathered from the other polyglutamine proteins, could only provide a more general scenario for these types of proteins, but a more specific protein network for ATXN3 was still unveiled. The study of typical ubiquitylated ATXN3-containing aggregates in MJD brains showed the presence of proteasome subunits and chaperones (Schmidt *et al.*, 2002), as well as CREB-binding protein (CBP),mastermind-like-1 or splicing factor SC35 (McCampbell *et al.*, 2000; Chai *et al.*, 2001).

The study of normal or altered protein interactions of a given disease-related protein may extend our understanding of that disease. The identification, characterization and manipulation of protein interactions engaged by the disease-causing protein may eventually, lead to a therapeutic strategy. This prompted our search for the ATXN3 biological interactome.

The first known direct interacting-partners of ATXN3, HHR23A and HHR23B, were identified based on the yeast two-hybrid (Y2H) methodology (Wang *et al.*, 2000). Confident that there were still more interactions to be unidentified, we set to perform an yeast two hybrid screening with normal ATXN3, in order to find new interacting partners that could give additional functional clues and, potentially, provide links to the biological pathway engaged by ATXN3. A Gal4-based yeast-two-hybrid system was used, in which a fusion protein comprising the DNA-binding domain of the yeast transcription factor Gal4 and ATXN3 (Gal4BD/ATXN3, bait) was used to screen a Gal4-activation domain (Gal4AD) fused to cDNAs from a human brain library (prey). Many eukaryotic transcription factors comprise physically separated transcription domains: the DNA binding domain and the activation domain. The full transcriptional activity may be

restored, if these transcriptional domains are brought into close proximity in the promoter region. Hybrid proteins comprising two interacting partners in fusion with binding domain and activation domain may activate the transcription of reporter genes under the control of a Gal4-responsive promoter. Hence, in the system used here, a positive interaction would be able to activate the expression of both reporter genes *GAL1-LacZ* and *GAL1_{UAS}-HIS3*, generating β -galactosidase expressing and histidine prototrophic (His⁺) yeast colonies.

Methods

Yeast strains

In the yeast two-hybrid assays, Y190 (*MAT α* , *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *trp1-901*, *leu2-3*, *112*, *gal4 Δ* , *gal80 Δ* , *cyh^r2*, *LYS2::GAL1_{UAS}-HIS3_{TATA}-HIS3*, *URA3::GAL1_{UAS}-GAL1_{TATA}-lacZ*) and Y187 (*MAT α* , *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *trp1-901*, *leu2-3*, *112*, *gal4 Δ* , *gal80 Δ* , *met⁻*, *cyh^r2*, *URA3::GAL1_{UAS}-GAL1_{TATA}-lacZ*) yeast strains were used for the library screening and mating assays, respectively (Harper *et al.*, 1993). The Y190 strain contains two different reporter genes, regulated by different GAL4-dependent promoters, reducing leakiness of gene expression. The *HIS3* reporter is under the control of *GAL1_{UAS}* and a minimal promoter, containing both TC (constitutive) and TR (regulated) *HIS3* TATA boxes (Flick and Johnston, 1990). The *LacZ* reporter is highly regulated by the intact *GAL1* promoter (including the *GAL1_{UAS}* and *GAL1* minimal promoter).

Yeast culture media

All media used for yeast growth and manipulation were prepared as described in the Yeast Protocols Handbook (Clontech). Selection media were prepared with all amino acids except tryptophan (W), leucine (L) or histidine (H or His) (SC-aa). These nutrients were added separately depending on the necessary selection medium. The leaky *HIS3* expression on the yeast strains was controlled by including 3-amino-1, 2, 4-triazole (AT) in the medium to suppress background expression of IGP dehydratase (*HIS3* gene product). The necessary concentration of AT for each yeast two-hybrid construct used was determined empirically.

Yeast two-hybrid bait constructs

The bait plasmids used for the library screen, pAS1-2/ATXN3 var2.1/14Q) and pAS1-2/ATXN3 var2.1/40Q) were prepared using classical cloning techniques (Sambrook and Russel, 2001). The complete coding sequences of isoform 1 ATXN3 (aa 1-364) (referred as ATXN3 var2.1) with a normal range of 14 or 40 glutamines residues were cloned in frame with GAL4BD domain of the pAS1-2 vector (kindly provided by Dr S.Elledge). The cDNA inserts were completely digested from initial pBluescript(SK) vector using *Bam*HI and *Xho*I restriction sites and cloned in frame into *Bam*HI and *Sall* site, a complementary *Xho*I site in pAS1-2 vector. An N-terminal fragment of ATXN3 pAS1/2.1N (aa 1-247) was also generated. The initial cDNA insert in pBluescript(SK) encoding full-length ATXN3 was completely digested with *Bam*HI and *Bgl*II was sub-cloned into *Bam*HI in order to be in frame in GAL4BD coding sequence of pAS1. These bait constructs have a *TRP1* gene that allows auxotrophic Y190 yeast strain to grow in media lacking tryptophan. The bait constructs were confirmed by automated sequencing and tested for its toxicity in the yeast and self-activation of the two reporter genes, *HIS3* and *LacZ*.

Self-activation assay

In order to assess the ability of the bait and prey constructs to self-activate Y190 reporter genes, pre-transformed yeasts were spread or plated as dots in selective drop-out media SC-WH or SC-LH, supplemented with a initial concentration of 20 mM AT, incubated at 30 °C, for five days, and then assayed for β -galactosidase expression. For the bait or prey constructs that showed initial self-activation properties, the proper AT concentration to eliminate this property were determined empirically.

Yeast protein extraction and analysis

To prepare protein extracts for immunoblotting, yeasts were grown in the respective drop-out media (SC-aa), to mid-log phase ($OD_{600}=0.5-1.5$), and proteins were extracted using the glass beads disruption protocol as described (Dunn and Wobbe, 1993). Briefly, a 600 μ l-yeast pellet was resuspended in three volumes of glass bead disruption buffer [20 mM Tris-HCl pH7.9, 1 mM EDTA, 10 mM $MgCl_2$, 5% glycerol, 300 mM $(NH_4)_2SO_4$, 1 mM DTT, 4 mM PMSF and yeast inhibitors concentrate (100 mM benzamidine, 10 mg/mL leupeptin, 10 mg/mL aprotinin)] and 150 μ L of acid-washed glass beads. Then, six cycles of 1 min vortexing/1 min ice incubation were performed. After centrifugation, supernatants were recovered and the pellets were resuspended with 400 μ L of

ressuspension buffer B (8 M urea, 0.1 M NaH₂PO₄, 10 mM Tris-Cl pH6.8). Proteins were diluted with sample buffer 2X or 4X, as necessary, and analysed by SDS-PAGE and immunoblotted.

Western blots: yeast expressed proteins

The expression of the bait and prey proteins was confirmed by western blot analysis. Protein extracts were obtained as described in the previous section. Proteins were separated by SDS-PAGE, transferred to a nitrocellulose or PVDF membrane (GE Healthcare) and immunoassayed. Generally, prey Gal4AD-fusion proteins were detected with anti-HA antibody (sc-7392, Santa Cruz Biotechnology), unless otherwise stated. The bait proteins were detected with a specific antibody. The blocking reaction of the membrane was generally performed with 5% SM/TBS-T (5% skimmed milk in Tris-buffered saline 1X (TBS), 0.05% Tween 20), for 1 hour, at 4 °C. Antibody dilutions were carried out as described in **Table A4**. Incubations of primary antibodies were performed overnight, at 4 °C, unless otherwise stated. Detection was carried out with ECL (GE Healthcare).

Human library screening

The human brain cDNA library used in the yeast two-hybrid screen, “Human brain MATCHMAKER cDNA library” in BNN132 *E. coli* (Clontech) and was kindly provided by Dr. Peter Heutink, Amsterdam. The cDNAs were primed with *Xho*I-(dT)₁₅ oligo from a pool of mRNAs obtained from a whole brain and directionally cloned into the *Xho*I-*Eco*RI sites of pACT2 vector. The library cDNA size range was 0.5-4.2 kb, with an average cDNA size of 2.0 kb. The library had been re-titered to 2.0x10⁸ cfu/mL, representing 3.5x10⁶ independent clones. The optimal transformation efficiency and the total number of transformants screened with each construct were determined; two serial dilutions (1/40 and 1/80) of the transformation, using three micrograms of the library DNA and each construct, were plated in SC-WL plates, yield 1.2x10⁴ - 1.5x10⁴ cfu/μg DNA, between the two bait constructs tested.

After the heat-shock, cells were plated in selective plates (SC-WLH/20 mM AT), incubated at 30 °C, for six days, scored, and then re-incubated for a total of ten days, to detect weaker interactions. The positive colonies were re-plated on selective medium, analysed for *LacZ* activation, and the transformants that became blue were selected to be further studied.

Yeast transformation

Y190 and Y187 were transformed using the lithium acetate method (Gietz and Woods, 2002). An average-size yeast colony (0.5-2 mm) of Y190 or Y187 was used to inoculate rich YPAD medium and was grown at 30 °C overnight; pre-transformed yeasts were grown in drop-out medium to keep selective pressure on the plasmid. Overnight cultures were diluted in YPAD to an $OD_{600}=0.3$ and then allowed to reach logarithmic growth, $OD_{600}= 0.5-0.6$. Harvested cells (2000 rpm) cells were washed twice with sterile water, and resuspended in 1.2 volumes of fresh made 100 mM lithium acetate (LiAc). The following reagents were added, in this order, to 50 μ L competent yeast suspension: 240 μ L of 50% PEG 3350 (m/v), 36 μ L LiAc 1 M, 50 μ L sheared salmon sperm DNA 2 mg/mL, x μ L of plasmid (1-5 μ g) and 50-x μ L of sterile water. Each tube was vortexed vigorously for 1 min, or until the pellet was completely resuspended, and then the cells were allowed to recover at 30 °C, for 30 min. Cells were then heat-shocked at 42 °C for 30-40 min. Finally, cells were briefly centrifuged (2000 rpm, 2 min), resuspended in 800 μ L of YPAD and plated onto the appropriate selective medium.

 β -galactosidase colony-lift filter assay

The β -galactosidase assay (Breedon and Nasmyth, 1985) was performed by colony-lift filter assay. The assay was either performed directly on fresh plates with single colonies or with dots of yeast transformants (WH^+ , LH^+ or WLH^+), grown on selective medium, supplemented with the sufficient AT concentration. A dry nylon membrane was placed over the grown plate surface to be assayed; when the membrane was evenly wetted, it was lifted and submerged in N_2 (I) for 10 s, to allow cell permeabilisation. Afterwards, it was thawed at room temperature and placed, colonies side up, in a filter paper pre-soaked with Z-buffer (16.1 mg/mL $Na_2HPO_4 \cdot 7H_2O$, 5.5 mg/mL $NaH_2PO_4 \cdot H_2O$, 0.75 mg/mL KCl, 0.25 mg/mL M_2SO_4 , pH 7.0), supplemented with 1 mg/mL X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) and 38.6 mM β -ME (final). Filters were incubated 1-24 hours at 30 °C, and then analysed for the presence of blue colonies ($LacZ^+$ transformants).

Yeast plasmid segregants

In order to evaluate if the putative positive HIS⁺/blue transformants obtained in the library screening were the result from a true positive interaction, dependent of both bait and library plasmid, it was necessary to obtain yeast plasmids segregants containing only the library “fished” plasmids. For this, the plasmids DNA mixture (bait + library cDNA plasmids) was isolated, using zymolyase method, then they were used to transform *E. coli*, from which they were subsequently isolated, and used to re-transform yeast cells. These prey segregants were tested for specificity on the activation of Y190 reporter genes when co-transformed with the bait plasmid or with the empty bait vector, pAS1.

Yeast plasmid DNA extraction by generation of spheroplasts

The H⁺/blue yeast transformants were grown overnight, at 30 °C, on the appropriate liquid selective medium. All centrifugations were carried out at 14000 rpm at 4 °C. Cells from overnight cultures (1.5 mL) were harvested, washed with 1 mL of Z-buffer and then re-centrifuged. Cells were then resuspended with 200 µL Z-buffer/0.1% β-mercaptoethanol/zymolyase and incubated 1 hour, at 37 °C. Cells were further lysed by adding 400 µL of lysis buffer (0.2 M NaOH/1% SDS) and incubated on ice for 5 min. Proteins were then precipitated with 3 M KCH₃COO pH 5.5 and separated by centrifugation. An equal volume of phenol:chloroform:isoamyl (25:24:1) was added to the collected supernatant and the DNA was precipitated from the aqueous phase with 2-propanol for 30 min at -20 °C. DNA was washed with 70% ethanol and air-dried. TE-dissolved DNA was used for transformation of *E. coli* by electroporation.

Yeast mating

For the yeast mating assay (Bendixen *et al.*, 1994), fresh average-size yeast colonies (0.5-2 mm) of each mating type were used to inoculate 500 µL of YPAD; inoculums were vigorously vortexed and incubated overnight at 30 °C with high shaking. Afterwards, 10 to 20 µL of the mating culture were spread or plated as dots (5-10 µL) in selective SC-WLH/AT plates and incubated, at 30 °C, for four to five days to allow selection of diploid cells with a positive interaction.

Results

Study of ATXN3 encoding bait constructs for yeast cell toxicity and self-activation properties

Three yeast two-hybrid bait constructs were prepared for the screening of a human brain cDNA library (**Table A1**). The pAS1-2 plasmid (kindly provided by Dr S. Elledge) was used for the construction of the expression vectors pAS1/2.1N, pAS/ATXN3 var2.1(14Q) and pAS1/ATXN3 var2.1(40Q). The bait plasmids encode for Gal4BD N-terminal-fusion proteins of (1) a N-terminal fragment of ATXN3 including 247 amino acids (Gal4BD-2.1N), (2) full-length ATXN3 protein with 14 glutamines (Gal4BD-ATXN3 var2.1(14Q)), and (3) full-length ATXN3 protein with 40 glutamines (Gal4BD-ATXN3 var2.1(40Q)). To determine if the constructs were appropriate for an Y2H screening or for direct interaction assays, their effect upon yeast growth (toxicity) was evaluated. Yeast were transformed with each bait construct and allowed to grow on selective media SD-W, during five days, at 30 °C; the average diameter was evaluated for the grown colonies, during this period. The fusion control interacting partners, Gal4BD-TSCII and Gal4AD-TSCI (van Slegtenhorst *et al.*, 1998), or Gal4BD-SNF4 and Gal4AD-SNF1 (James P *et al.*, 1996), were previously reported not to affect yeast growth. Both pAS1/2.1N and pAS1/ATXN3 var2.1(40Q) bait plasmids did not affect significantly the yeast growth (**Table A1**); however, yeast transformed with pAS1/ATXN3 var2.1(14Q) grew less in SC-W plates, denoting some toxicity of the GAL4BD-ATXN3 var2.1(14Q) protein over-expression in yeast.

The bait constructs were also evaluated for their ability to self-activate the transcription of the reporter genes, *GAL1_{UAS}-HIS3* and *GAL1-LacZ*. It should be noted that, Y190 reporter yeast strain is marginally prototrophic for histidine, but the addition of a competitive inhibitor of the *HIS3* product (IGP dehydratase), 3-aminotriazole (AT), strengthens the sensitivity of the reporter gene, rendering Y190 auxotrophic to histidine (Brent and Finley, 1997). Yeast transformed with the bait plasmids were plated in selective plates, SC-W-H, supplemented with 20 mM of 3-aminotriazole (SC-WH/20AT) and incubated, for five days. Self-activation of *GAL1-LacZ* expression induced by the bait plasmids was also evaluated, and the assay for β -galactosidase (β -gal) activity performed in the colonies grown in SC-WH/20AT.

Yeast transformants expressing Gal4BD-ATXN3 var2.1(14Q) and Gal4BD-ATXN3 var2.1(40Q) were unable to grow in selective SC-WH/20AT plates, but those expressing

Gal4BD-2.1N (ATXN3 N-terminal fragment), were able to significantly activate the *GAL1_{UAS}-HIS3* reporter leading to visible growth of large (1mm diameter) yeast colonies in SC-WH/20AT plates.

The β -galactosidase assays confirmed that none of the bait constructs encoding full-length ATXN3 could activate the Y190 reporter genes, whereas pAS1/2.1N bait could (**Table A1**). Ideally, the bait plasmids should not be able to autonomously activate the *GAL1_{UAS}-HIS3* and *GAL1-LacZ* reporter genes. Hence, the pAS1-2.1N was not used in the Y2H library screening. Later, the histidine auxotrophy of pAS1-2.1N transformants was restored with 140 mM 3-aminotriazole, and this bait plasmid was used in direct protein interactions assays; an interaction was considered positive when generating colonies in SC-WLH/140AT selective medium turned blue in β -gal assay.

Bait construct	SC-W	SC-WH/20AT	
		Growth ($\emptyset$)	β -gal assay
pAS1/2-1N	+++	+++ (1 mm)	B
pAS1/ATXN3 var2-1(14Q)	+	$\pm$ (<0.5 mm)	W
pAS1/ATXN3 var2-1(40Q)	+++	$\pm$ (<0.5 mm)	W

Table A1. Evaluation of bait toxicity in yeast growth and reporter gene self-activation. Yeast toxicity of each construct is reported in SC-W column, where the average diameter ($\emptyset$, mm) is indicated; the self-activation of the nutritional reporter gene (*GAL1_{UAS}-HIS3*) by the bait Gal4BD-fusions was evaluated in selective plates (SC-WH/20AT); the self-activation of the second reporter gene *GAL1-LacZ* was assessed by colony–lift filter β -galactosidase assay. Legend: +, growth; B, blue; W, white.

The expression of the bait constructs in yeast was confirmed by western blot (**Fig.A1**), using monoclonal anti-Gal4 DNA-BD antibody (Clontech) and specific polyclonal anti-MJD2.1 antibody produced in our lab (**Fig.A5** of Appendix 2). The full-length Gal4BD-ATXN3 var2.1(40Q) and the Gal4BD-2.1N fusion proteins were expressed and presented the expected molecular weights. However, the expression of the Gal4BD-ATXN3 var2.1(14Q) protein could not be detected, even though, the pAS1 vector used in the cloning process has a strong *ADH1* promoter, which induces high-level expression of sequences cloned downstream (Ammerer, 1983). Automated sequencing confirmed that the ATXN3 insert was in frame with the Gal4BD sequence; we assumed that the Gal4BD-ATXN3 var2.1(14Q) protein was correctly expressed albeit at very low levels, due to its toxicity in yeast. Previous studies showed that ATXN3 was able to interact with two

proteins, HHR23A and HHR23B, in the Y2H system (Wang *et al.*, 2000). To confirm that pAS1/ATXN3 var2.1(14Q) was indeed expressed in the yeast and, therefore, suitable for the two-hybrid assays regardless of its inherent toxicity, the direct interaction between Gal4BD-ATXN3 var2.1(14Q) and Gal4AD-HHR23B was tested. We observed a positive interaction between the two Gal4-fusion proteins, ATXN3 and HHR23B, showing that the ATXN3 bait constructs obtained by us could be used in the yeast library screening.

Screening for molecular interactors of ATXN3

In order to identify new human proteins that could interact with ATXN3, we screened a human brain cDNA library with both pAS1/ATXN3 var2.1(14Q) and pAS1/ATXN3 var2.1(40Q) plasmids encoding full-length ATXN3 with a normal-sized polyQ region.

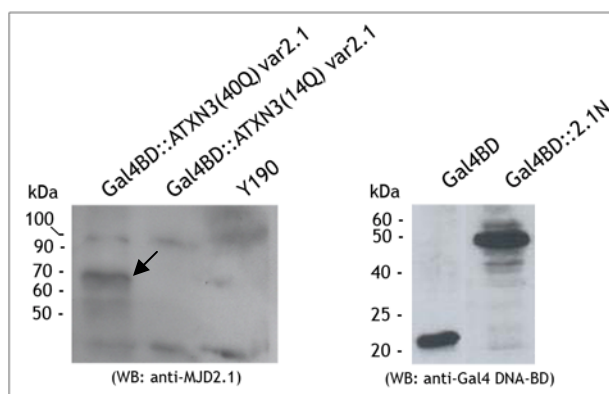


Figure A1. Western blotting analysis of bait Gal4BD-fusion proteins in Y190 yeast.

Protein extracts were resolved by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with polyclonal anti-MJD2.1, or monoclonal anti-Gal4 DNA-BD (Clontech). The Gal4BD-fusion proteins expressed in yeast showed the expected molecular weights: 47 kDa for the N-terminal fragment of ATXN3 (Gal4BD-2.1N) and 67 kDa for the Gal4BD-

ATXN3 var2.1(40Q) with; Gal4BD alone showed 20 kDa as expected. The Gal4BD-ATXN3 var2.1(40Q) fusion-protein could not be detected by western blot. Y190 indicates the lane corresponding to protein extracts of untransformed yeast. Detection was carried out with ECL (GE Healthcare).

Since the pAS1/ATXN3 var2.1(14Q) showed some toxicity in yeast cells, co-transformations of this bait plasmid with the library prey clones were done, rather than sequential transformations. The high transformation efficiency obtained for this construct (1.5×10^4 cfu/DNA), indicates that co-expression with a prey plasmid seems to decrease toxicity of pAS1/ATXN3 var2.1(14Q). As a positive control of the system, we used the direct interaction between the Gal4-fusions of tuberin (pGBT9/TSCII) and hamartin (pGAD/TSCI) involved in tuberous sclerosis, in which they are known to interact (van Slegtenhorst *et al.*, 1998).

Yeast co-transformed with pAS1/ATXN3 var2.1(14Q) or pAS1/ATXN3 var2.1(40Q) and with Gal4AD-tagged cDNA expression library were plated in SC-WLH/20AT selective plates. A total of 4.3×10^4 library cDNAs were screened with pAS1/ATXN3 var2.1(14Q), and 3.3×10^4 with pAS1/ATXN3 var2.1(40Q) bait plasmid. After a six day period of incubation, five His⁺ colonies were picked pAS1/ATXN3 var2.1(14Q) and four His⁺ were detected from pAS1/ATXN3 var2.1(40Q) screening plates, and re-streaked in fresh SC-WLH/20AT plate (masterplate). The original screening plates were re-incubated for another four days, to allow the growth of transformants comprising weaker interactions. After a ten day period of incubation, a total of ten and six His⁺ prototrophics were obtained from the library screenings, with pAS1/ATXN3 var2.1(14Q) and pAS1/ATXN3 var2.1(40Q), respectively. These 16 His⁺ co-transformants were replated in fresh SC-WLH/20AT and then evaluated for the activation of the *LacZ* reporter using the β -gal assay; all the preys exhibited β -galactosidase activity, and were considered true His⁺/blue positive clones (**Fig.A2**).

Isolation and identification of the putative prey interactors

To identify and characterise the protein interactors of ATXN3, isolated in the library screening, the prey plasmids were purified from yeast. Since more than one prey plasmid could be present in the same His⁺/blue positive clone, it was necessary to extract yeast library cDNAs and generate yeast segregants.

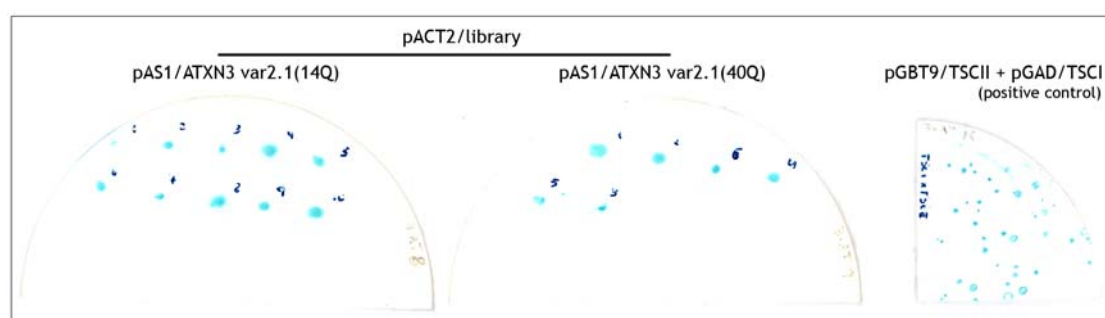


Figure A2. Yeast two-hybrid interactions of ATXN3 identified in the library screen. Positive interactions were identified by colony growth in selective SC-WLH/20AT medium and β -galactosidase assay. A total of 16 of putative interactors were identified using normal-sized ATXN3 var2.1. Interaction between tuberin (TSCII) and hamartin (TSCI) was used as positive control of the assay.

His⁺/blue positive clones were grown in liquid SC-WL and the cDNA prey plasmids were extracted from these cultures, using zymolase method and then subsequently electroporated into HB10 *E.coli*. Three colonies of each bacterial transformation were picked, and the prey plasmids were digested with *Bgl*III for insert excision. Comparison of the digestion patterns of the prey plasmids led to the selection of several cDNA clones (e.g. 1a, 1b and 1c sub-clones). The cDNA plasmids presenting different digestion patterns were selected, in order to further understand the difference obtained.

A total of 14 different prey plasmids were defined from the initial His⁺/blue clones obtained from the screen with pAS1/ATXN3 var2.1(14Q); 10 prey plasmids were chosen from the restriction digestion analysis of cDNA from the His⁺/blue clones isolated in the pAS1/ATXN3 var2.1(40Q) screening. All prey plasmids were re-transformed in yeast, and none of these prey segregants showed yeast toxicity or self-activated the expression of *HIS3* and *LacZ* reporter genes by itself. Hence, the direct interactions between each prey segregant and the original bait constructs were assessed. Only four out of the 14 yeast segregants expressing a prey plasmid (sub-clones) retrieved from screening performed with pAS/ATXN3 var2.1(14Q) bait were able to reproduce the initial results, indicating that, six out of the initial ten His⁺/blue clones identified (**Fig.A2**) represented false positive interactions. The four prey segregants, which reproduced the initial positive interactions with the pAS1/ATXN3 var2.1(14Q), also exhibited positive interaction with pAS1/ATXN3 var2.1(40Q); yeast co-transformation of these prey plasmids and the pGBT9/TSCII control bait produced no positive interaction, reinforcing the specificity of the His⁺/blue positive interactions found.

For further confirming, yeast mating assays were performed between Y187 (*Mat α*) expressing each of the prey plasmid and Y190 (*Mat α*) expressing Gal4BD/ATXN3 var2.1(14Q). Two of the mating diploids were negative for the expression of both reporter genes (His⁻/white).

In the case of the yeast segregants expressing prey plasmids isolated from the library screening performed with pAS/ATXN3 var2.1(40Q), all, with the exception of one, reproduced positive interactions. Hence, five out of six His⁺/blue clones isolated from the initial screening were potentially true positive interactors of ATXN3.

In conclusion, from the initial 16 putative interactors selected from the library screening with Gal4BD/ATXN3 bait plasmids, only seven were found to potentially

encode a true interactor of normal-sized ATXN3. For simplicity, a flowchart of the work performed is presented in **Figure A3**.

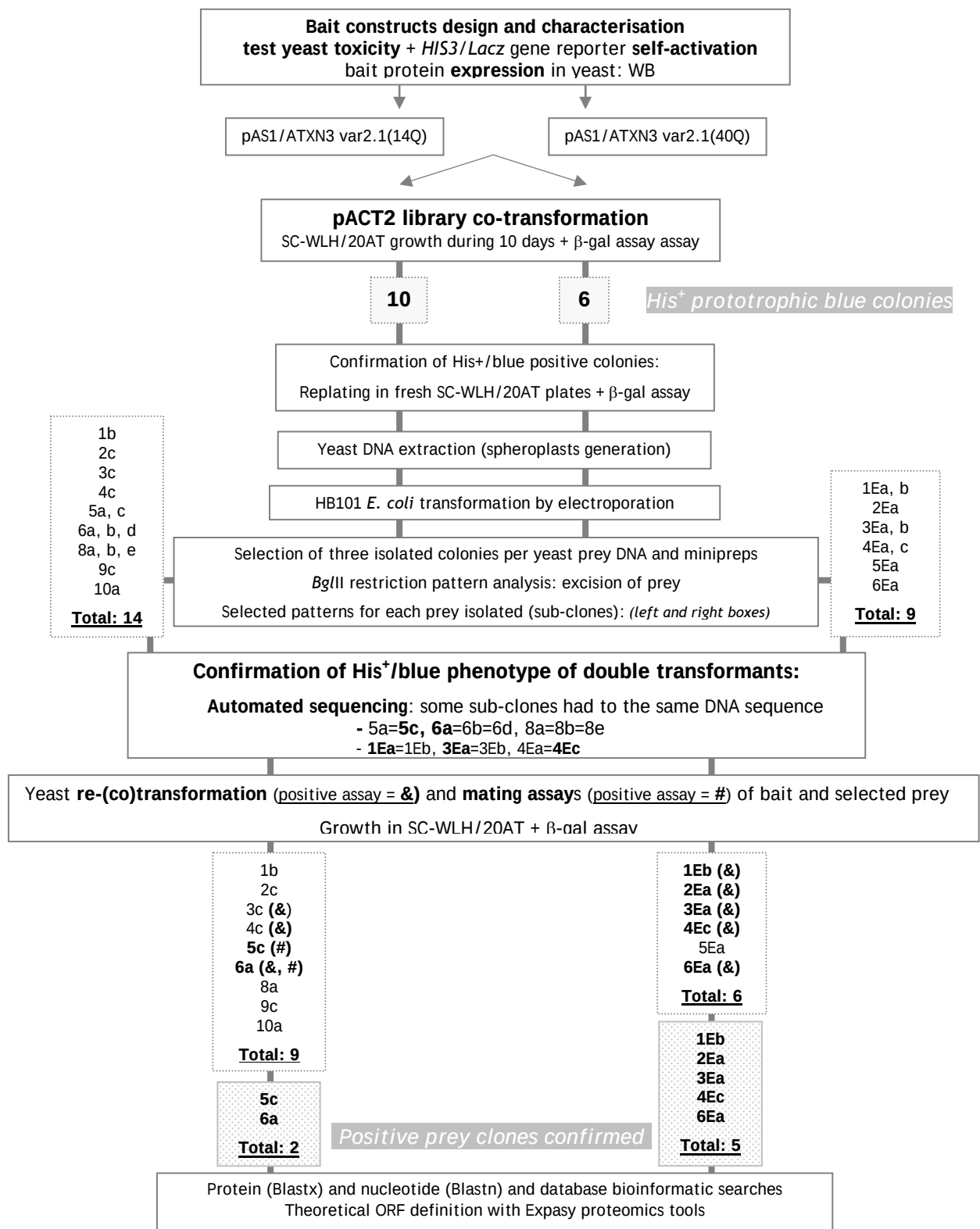


Figure A3. Flowchart representing the yeast two-hybrid analysis performed in this work. Two bait constructs encoding normal ATXN3 were designed, characterized and used to screen a yeast two-hybrid human brain cDNA library. Sixteen positive clones were isolate; further analysis confirmed that, seven of these could encode true positive interactors of normal ATXN3. Automated sequencing and bioinformatic searches were used to further identify, and characterized the DNA and/or protein sequences of each of these seven positive prey clones.

Sequence analysis of prey plasmids

Automated sequencing of all prey plasmids revealed that most sequences originated from the initial His⁺/blue clones corresponded to the same sequence. Using the BLASTx program, the protein databases were blasted with the translated prey, in order to identify the protein encoded. Some of the sequences did not show any similarity with any known protein sequence, at the time of the analysis. In these cases, a BLASTn search was performed. Initially, a control test for the quality of the cDNA library used was performed; PCR-amplification of the library DNA with specific primers for the coding sequence of HHR23 protein revealed that this gene was indeed represented in the library. However, none of the prey sequences identified corresponded to HHR23 encoding sequences. The results are summarized in **Table A2**.

The BLASTn search of the prey sequences, revealed some of the alignments to be potentially interesting, such as the human *PPP2R2B* gene implicated in spinocerebellar ataxia type 12 (SCA12) (Holmes *et al.*, 1999) (prey clone **4Ec**). *PPP2R2B* gene encodes for a brain-specific regulatory subunit B, isoform β of protein PP2A, a serine/threonine phosphatase involved in cell cycle regulation, cell morphology, development and regulation of specific signal transduction (Janssens and Goris, 2001). The PPA2 had been suggested to modulate GSK-3 β –dependent phosphorylation pathways in Wnt signalling (Ikeda *et al.*, 2000). Interestingly, GSK-3 β another serine/threonine phosphatase, has been recently reported to interact directly with ATXN3, leading to its phosphorylation (Fei *et al.*, 2007).

The prey clone **2Ea** aligned with the 3'-UTR of WW-domain containing protein 2 (WWP2) encoding sequence. WWP2 protein is a member of the E3 HECT-type ubiquitin ligase family (Xu *et al.*, 2004). Interestingly, WWP2 has been reported to interact with atrophin-1 (Wood *et al.*, 1998) and more recently this interaction has been replicated in an high-throughput screening for protein involved in hereditary ataxias and disorders with Purkinje cells associated-degeneration. Close proximity between the ATXN3- and ATN1-specific interaction clusters, the latter comprising WWP2 protein (Lim *et al.*, 2006), was observed.

The prey clone **5c** isolated with the Gal4-ATXN3 var2.1 (14Q) bait, aligned with the 3' untranslated region of the gene encoding NAP1L1, a nucleosome assembly protein 1-like 1, which seems to be involved in DNA replication, cellular proliferation and chromatin remodelling, acting like an histone chaperone (Park *et al.*, 2005).

Bait	Prey	Bioinformatic data	Encoded prey
	Code name, bp	Results (BLASTn program)	ORF = number of aa
ATXN3 var2-1(14Q)	5c , 395	NAP1L1 variant 2, mRNA: 3'-UTR alignment	+1 = 55 aa-STOP +2 = 33 aa-STOP +3 = 7 aa-STOP
	6a , 406	<i>Alu contamination Warning</i>	+1 = 33 aa-STOP +2 = STOP +3 = 39 aa-STOP
ATXN3 var2-1(40Q)	1Ea , 539	Protein G subunit $\beta 1/\gamma 2$ interacting factor 3, partial cds	+1 = 6 aa-STOP +2 = 3 aa-STOP +3 = 12 aa STOP
	2Ea , 569	WW domain containing E3 ubiquitin protein ligase 2 (WWP2), variant 1 and 2, mRNAs: 3'-UTR alignment	+1 = 17 aa-STOP +2 = 2 aa-STOP +3 = 32 aa-STOP
	3Ea , 544	Na ⁺ /K ⁺ ATPase subunit $\beta 1$ (ATP1B1), mRNA	+1 = 9 aa-STOP +2 = 4 aa-STOP +3 = 168 aa (from 95 to 262)
	4Ec , 558	Phosphatase protein 2 (PPA2), regulator subunit B, isoform β (<i>PPP2R2B</i>), neuronal isoform, mRNA	+1 = 6 aa-STOP +2 = 132 aa (from 273 to 404)
	6Ea , 597	Proteasome subunit 26S non-ATPase, 12 (PSMD12), p55 regulatory subunit, transcript 1, mRNA	+1 = 5 aa-STOP +2 = 138 aa (from 40 to 174)

Table A2. Prey plasmids selected from the library screening. In the table are displayed the prey cDNA clones obtained from the library screening with pAS1/ATXN3 var2-1 (14Q) and pAS1/ATXN3 var2-1 (40Q) bait plasmids; a code name was assigned to each prey (second column). All seven preys generating His⁺/blue positive clones were sequenced; each sequence determined (length, bp in the second column) was analysed against nucleotide and/or protein sequence databases, using the BLAST programs. The number of amino acids encoded by each cDNA prey plasmid, considering the three open reading frames (ORF), is presented also (fourth column). The theoretical DNA sequence translations were performed with ExPASy proteomics tools (<http://expasy.org/tools/#translate>).

NAP1L1 has been reported to interact with p300 (Asahara *et al.*, 2002), a known interactor of ATXN3 (Li *et al.*, 2002). However, the alignments corresponded to the 3'-untranslated region of the gene encoding NAP1L1 protein.

The most promising alignment was obtained with BLASTn for the **3Ea** sequence. The analysis identified a sodium/potassium-transporting ATPase subunit $\beta 1$ (Na^+/K^+ ATPase subunit $\beta 1$). However, when we determined the aminoacidic sequence encoded by frame +1, the frame in which the cDNA prey clone was actually being translated in yeast, we recurrently found that it did not correspond to the coding sequence of the gene identified by the BLASTn search, i.e., the alignments identified by BLASTn were not corresponding the fusion proteins being expressed. Some BLASTn alignments were obtained also with untranslated regions of databases sequences or even with *Alu* elements, which are genomic, transcriptionally silent sequences (Hagan *et al.*, 2003). We explored the possibility that the library had some systematic construction error inducing a frameshift by reverse-sequencing all the preys identified; we could not identify such a systematic alteration, like an insertion or deletion, in the upstream sequence of the prey vector (pACT2) that could explain the truncated peptides found.

Discussion

Several methodologies have been established in order to assess protein-protein interactions. Among the high-throughput tools for the study of protein-protein interactions, the yeast two-hybrid system (Y2H) is an *in vivo* technology that enables the identification of interacting proteins, confirm putative interactions, and define the directly involved interacting domains (Fields and Song, 1989). Being a scalable assay, it makes possible the screening of interactions among a large pool of encoded proteins, in a rather fast way (Fields, 2005).

The most striking advantage of the Y2H assays is the immediate access to the gene encoding the interacting, “fished” proteins. This is also a sensitive method, able to detect stable as well as transient interactions. As an *in vivo* tool, proteins tested by the Y2H, are more likely to be in their native conformations, thus enabling the detection of physiological significant interactions. The major drawback associated with this technique is the high percentage of false positives (and negative) ~50% (Deane *et al.*,

2002) among the interactions retrieved. The Y2H detects protein interactions depicted by (1) overexpressed bait and prey proteins (2) fused to transcriptional activators, (3) in yeast nucleus. Therefore, Y2H may identify protein interactions potentially occurring in an unnatural environment and the absence of some post-transcriptional modifications which may not occur in the yeast may lead to protein interactions with no biological significance or, in contrast, lead to false negative results.

Since its first version, the Y2H system has been constantly improved. The incorporation of multiple independent reporter genes to measure transcription activation, the design of different DNA-binding sequences at the promoters of the different yeast reporter genes, the use low-copy number vectors circumventing the protein overexpression and, importantly, the retesting interacting pairs in fresh yeast, allowed a significant improvement of the data quality gathered in the Y2H experiments (Cusick *et al.*, 2005). Despite the skepticism regarding Y2H, when it is coupled to complementary and confirmatory experiments as the GST-pull-down, co-immunoprecipitation and co-localisation assays of the endogenous proteins, the improved Y2H systems are a valuable tool and still largely applied in protein-protein interactions studies.

The complete genome sequencing of several organisms enabled the high throughput analyses and led to the comprehensive determination of all protein-protein interactions characteristic of an organism, at a given time, the interactome. For organisms as *Sccharomyces cerevisiae* (Uetz *et al.*, 2000; Ito *et al.*, 2001), *Helicobacter pylori* (Rain *et al.*, 2001), *C. elegans* (Walhout *et al.*, 2000; Li *et al.*, 2004), *Drosophila melanogaster* (Giot *et al.*, 2003) and *Homo sapiens* (Colland *et al.*, 2004; Rual *et al.*, 2005) drafts of the protein-protein interactions maps were possible using integrated high throughput Y2H-based screens coupled with other experimental procedures. Moreover, the definition of the interactome of polyQ proteins as huntingtin (Goehler *et al.*, 2004; Kaltenbach *et al.*, 2007) and ataxin-1 (Lam *et al.*, 2006) has recently successfully been achieved applying a Y2H-based approach.

Here, we performed a yeast two screening in order to identify molecular partners of ataxin-3 in human brain tissue using two bait plasmids encoding proteins of different polyQ lengths. Considering the high titre of the re-amplified cDNA library used and the suitability of the plasmids prepared, it was expected that some novel interaction(s) would be identified. Unfortunately, this was not the case. From the initial His⁺/blue clones isolated in the screening, 56% (9 out of 16) were false positive, i.e., were unable

to reproduce the screening interactions observed with the baits when re-tested by co-transformation and mating assays. Although high, this value is, unfortunately, consistent with the literature (Deane *et al.*, 2002).

The remaining seven clones showed consistent interactions, and interestingly, some with both bait proteins. With the subsequent bioinformatic analysis against protein or nucleotide databases, however, we were confronted with very disappointing results. The prey sequences isolated were recurrently interacting through small peptide fragments, which did not correspond to the open reading frame of the genes found to correspond to the prey sequence after a BLASTn search. In most of the preys, soon after the Gal4BD, the frame had a premature stop codon. No sequence alteration was identified upstream the multiple cloning site of pACT2 that could, somehow, be causing the frameshifts of the prey sequences isolated. Of course, if the bait choice and its characterisation are critical for the success of a yeast two-hybrid screening, the quality of the cDNA library used should not be undervalued and this was the main pitfall of our study. The fact that we found a cDNA sequence that aligned significantly with *Alu* elements, led us to suspect that the cDNA library was contaminated with genomic sequences, evidencing its poor quality. Albeit the number of the independent clones screened should have been higher, two normal-sized ATXN3 proteins were used, which in a sense could be considered as if the library was screened twice, independently, and less false positives should be expected. After automated sequencing, we observed that most of the clones selected corresponded to the same cDNA sequence. Again, this fact led us to suspect about the quality of the library used, namely its high redundancy, where a cloning bias towards the more frequent cDNA transcripts cannot be ruled out.

Hence, and since we had validated our prey constructs, we considered it more strategical to address a set of candidates interactors based on current hypothesis set for ataxin-3 function (described in chapter 1 and chapter 2).

Appendix 2

Supporting results and material

Table A3. Nucleotide sequences of the primers used along this work. The attB-primers were used with the Gateway cloning system.

Primer name	Sequence 5' → 3'
attB1-HHR23A	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGGCCGTCACCATCACGCTG
attB2-HHR23A	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACTCGTCATCAAAGTTCTG
attB1-HHR23B	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGCAGGTCACCTGAAGACC
attB2-HHR23B	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAATCTTCATCAAAGTTCTG
attB1-MJD	GGGGCAAGTTTGTACAAAAAAGCAGGCTGGATGGAGTCCATCTTCCACGAG
attB1-221cATXN3	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGTTAGACGAAGATGAG
attB2-MJD1.1	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATTTTTTCTTCTGTTTT
attB2.1-XPC-D1	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAATAGAGGCTGGTCTTTTTCAT
attB1-XPC	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGGCTCGGAAACGCGCGCCGGC
attB2-XPC	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACAGCTTCTCAAATGGGAACAG
attB1.2-XPC-D2	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGGCCGTCACCATCACGCTG
attB1-P8HHR23B	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGACGGTGAAAGCACTGAAAGAGAAG
attB1.2-ZK20.3	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGGTTTTGTCCGTTACATT
attB2.1-ZK20.3	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATTCCTCATCGAGGTTGCTGAAAATAAAG
CETN2-F1	GTACACGTCGGTTGCCTAAC
CETN2-R1	TTCTTCACGCTTGTGTGCTC
attB2-CETN2	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAATAGAGGCTGGTCTTTTTCAT
attB1.2-CETN2	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGGCCTCCAACCTTAAGAAGG
attB2-MJD2.1-JD(199)	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGTTGTGCTAATCTTCTCCA
SL1	GGTTTAATTACCCAAGTTTGAG
ZK20.3-F1	TGAAGATCAGACGATTGCGG
ZK20.3-R1	AATAAAGTTGATAGCGGCTTCCTCG
attB1-NEDD8	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGCTAATTAAAGTGAAGAC
attB2-NEDD8	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACTGCCTAAGACCACCTC
attB1-Ub	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGCAGATCTTCGTGAAGA
attB2-Ub	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAACCACCTCTCAGACGCAGG
xpc-R1	TAGCGATCGATGCTTTGG
xpc-R2	ATCCGACAACGCTGGAATG
atx-3-1R	ACTTTTGGCGGTACAACCTGG
atx-3-1F	AAAATCCTGCGATGGTGGAC
ceact-1F	GTCGGTATGGGACAGAAGGA
ceact-1R	GCTTCAGTGAGGAGGACTGG

Constructs	Assay		Fusion Protein	Western blot analysis				WB	
	Toxicity	Self-activation		Primary antibody	Dilution (%SM)	HRP-conjugated secondary antibody	Dilution (%SM)		
Baits									
pAS1 _G	-	± (20 mM AT)*	Gal4BD	anti-GAL4BD	1:500 (1%)	anti-mouse	1:10000 (1%)	+	
pAS1/JD	-	-	Gal4BD/JD	anti-HA	1:200 (1%)	anti-mouse	1:2000 (1%)	+	
pAS1/MJD2-1N	-	+ (140 mM AT)*	Gal4BD/2.1N	anti-GAL4BD	1:500 (1%)	anti-mouse	1:10000 (1%)	+	
pAS1/ATXN3 var2.1(14Q)	+	-	Gal4BD/ATN3 var2.1(14Q)	anti-MJD2.1	1:2000 (3%)	anti-rabbit	1:10000 (3%)	-	
pAS1/ATXN3 var2.1(40Q)	-	-	Gal4BD/ATN3 var2.1(40Q)	anti-MJD2.1	1:2000 (3%)	anti-rabbit	1:10000 (3%)	+	
pAS1 _G /ATXN3 var1.1(14Q)	+	+	Gal4BD/ATN3 var1.1(14Q)	anti-MJD1.1	1:3000 (1%)	anti-rabbit	1:5000 (1%)	+	
pAS1 _G /atx-3	-	-	Gal4BD/atx-3	anti-303JSP	1:2500 (1%)	anti-rabbit	1:5000 (1%)	+	
pAS1 _G /NEDD8	-	-	Gal4BD/NEDD8	-	-	-	-	ND	
Preys									
pACT2 _G	-	± (20 mM AT)*	Gal4AD	anti-HA	1:200 (1%)	anti-mouse	1:5000 (1%)	+	
pACT2 _G /JD	-	+	Gal4AD/JD	-	-	-	-	ND	
pACT2 _G /HHR23B	-	-	Gal4AD/HHR23B	anti-HA	1:200 (1%)	anti-mouse	1:2500 (1%)	+	
pACT2 _G /HHR23Bdel21	-	-	Gal4AD/HHR23Bdel21	anti-HA	1:200 (1%)	anti-mouse	1:5000 (1%)	+	
pACT2 _G /HHR23A	-	-	Gal4AD/HHR23A	anti-HA	1:200 (1%)	anti-mouse	1:5000 (1%)	+	
pACT2 _G /XPC-D1	±	-	Gal4AD/XPC-D1	-	-	-	-	ND	
pACT2 _G /XPC-D2	±	-	Gal4AD/XPC-D2	-	-	-	-	ND	
pACT2 _G /CETN2	-	-	Gal4AD/CETN2	anti-HA	1:100 (1%)	anti-mouse	1:5000 (1%)	+	
pACT2 _G /ZK20.3	-	-	Gal4AD/ZK20.3	anti-ZK20.3	1:1000 (1%)	anti-rabbit	1:5000 (1%)	+	
pACT2 _G /Ub	-	-	Gal4AD/Ub	-	-	-	-	ND	
pACT2 _G /NEDD8	-	-	Gal4AD/NEDD8 ^{a)}	anti-HA	1:100 (1%)	anti-mouse	1:2000 (1%)	+	

Table A4. List of all yeast two hybrid bait and prey constructs made and used in this work. For all constructs, the potential toxicity for yeast cells or self-activation properties were analysed. The expression of the Gal4BD- and Gal4AD-fusion proteins in the yeast was also confirmed. ATXN3 bait plasmids pAS1/2.1N, pAS1/ATXN3 var2.1(14Q) and pAS1/ATXN3 var2.1(40Q) (light gray) were described and characterized for the yeast two hybrid screening. Blocking reactions were performed overnight, at 4 °C, unless stated otherwise. **Legend:** *, optimised aminotriazole (AT) concentration to eliminate the construct's self-activation property; X_G, construct obtained with Gateway cloning technology; -, absence; +, presence; ND, not determined; SM, skimmed milk; a) blocking was performed with 1% SM/TBS-T 1h, 4 °C.

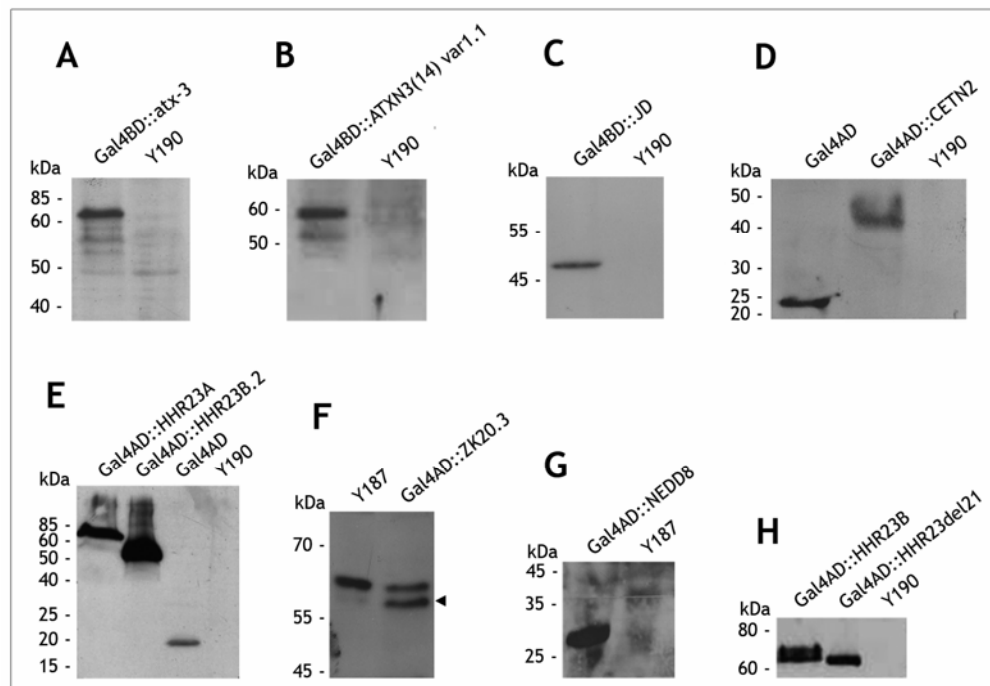


Figure A4. Western blotting analysis of bait and prey Gal4-fusion proteins. Protein extracts were analysed by SDS-PAGE, transferred to nitrocellulose or PVDF membrane and immunoblotted with anti-MJD1.1 (B), anti-HA (C, D, E, G, H; Santa Cruz Biotechnology), anti-303JSP (A) or anti-ZK20.3 (F). The yeast expressed fusion proteins showed the expected molecular weights (MW): NEDD8, 30 kDa; HHR23B, 64 kDa; Gal4AD-ZK20.3, 56 kDa; Gal4BD protein, 20 kDa; Gal4AD protein, 16 kDa. For the remaining Gal4-fusion proteins we found discrepancies between the observed and the expected (*parenthesis*) MWs in the western blots performed: atx-3, 65 (56) kDa; atx3 (14Q) var 1.1, 59 (62) kDa; human CETN2, 40 (36) kDa; HHR23A, 62 (54) kDa; HHR23B.2, 62 (60) kDa and JD (josephin domain), 47 (43) kDa. Lanes of Y190 or Y187 correspond to protein extracts of non-transformed yeast. Detection was carried out with ECL (GE Healthcare).

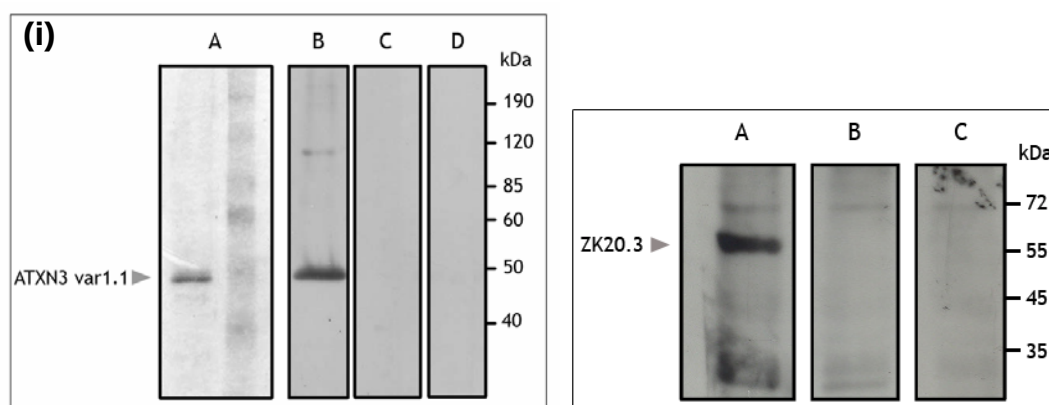


Figure A5. The novel primary anti-MJD2.1 and anti-ZK20.3 antibodies are highly specific. (i) The new anti-MJD2.1 antibody recognizes with high specificity the full-length 6His-ATXN3 var1.1. For characterization of the antibody, the recombinant 6His-tagged ATXN3 var1.1 was separated on a 10% SDS-PAGE gel and proteins were either stained in-gel with coomassie blue (A) or transferred to a nitrocellulose membrane and immunoblotted with anti-MJD2.1 dilutions (B, C, D). B, anti-MJD2.1 at 1:10000; C, anti-MJD2.1 antibody was pre-incubated with recombinant full-length 6His-ATXN3 var1.1 protein for 1 hour at 37 °C and then used to prepare a 1:10000 antibody dilution; D, immunoblotting was performed only with secondary antibody dilution, 1:30000 (anti-rabbit). **(ii)** The new antibody anti-ZK20.3 recognizes specifically the *C. elegans* homologue of RAD23 protein, ZK20.3. Assays to determine anti-ZK20.3 specificity were performed as follows: 500 µg of N2 protein extract were separated in a SDS-PAGE; proteins were transferred to a nitrocellulose membrane and incubated with anti-ZK20.3 dilutions. A, anti-ZK20.3 at 1:10000; B, anti-ZK20.3 was pre-incubated with 50 µg peptide-antigen, for one hour at 37 °C, and then used to prepare an 1:10000 dilution; in C, the membrane was incubated only with secondary antibody, 1:10000 (anti-rabbit). Both ATXN3 var1.1 and ZK20.3 proteins were detected at higher molecular weights than the expected, 42 kDa and 40 kDa, respectively. These abnormal migrations at approximately 50 and 60 kDa, respectively, may result from post-translational modifications of the proteins.

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