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The role of frameshifting and transcriptional dysregulation in spinocerebellar ataxia type-3

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Ce mémoire intitulé:
The role of ribosomal frameshifting and transcriptional dysregulation in spinocerebellar ataxia type-3

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Abstract

Spinocerebellar ataxia type-3 is a late-onset autosomal dominant progressive neurodegenerative disorder caused by a CAG trinucleotide repeat expansion in the *MJD1* gene. The pathology of SCA3 is characterized by the presence of nuclear inclusions (NIs) within neurons of selectively affected brain regions. One of the contributing factors to neuronal toxicity is the production of polyalanine proteins through a ribosomal -1 frameshift within the CAG-repeat during translation. To further investigate ribosomal frameshifting, we established a cellular model using three full-length MJD1 constructs based on the cDNA of the *MJD1* gene coding for various polyglutamine repeat lengths (CAG9, CAG100, and CAA96). We confirmed that with these constructs, -1 frameshifting occurs exclusively with the expanded-CAG repeat construct (CAG100), as it appears to be dependent on the energy requirements necessary to unfold secondary mRNA structures of the long CAG repeats. The presence of toxic polyalanine proteins produced by frameshifting most likely contributes to the pathogenesis of SCA3. Through the use of our newly established cellular model and *Drosophila* model, we also investigated transcriptional dysregulation through impaired RNA polymerase II (RNAPII) function. Our results show that transcriptional impairment in SCA3 appears to be independent of RNAPII activity alterations. In conclusion, we confirmed the production of frameshifted polyalanine proteins in a full-length ataxin-3 cellular model, but require further studies to understand the mechanism of transcription dysregulation involved in the pathogenesis of SCA3.

Key words: Spinocerebellar ataxia type-3 (SCA3), neurodegeneration, ataxin-3, nuclear inclusions, polyglutamine, polyalanine, frameshifting, RNA polymerase II, neurotoxicity, triplet expansion.

Résumé

L'ataxie spinocérébelleuse de type-3 (SCA3) est une maladie neurodegenerative autosomique dominante observée chez des individus d'âge adulte qui est causée par une expansion de séquences répétées de trinucleotide CAG dans le gène *MJD1*. La pathologie de SCA3 est caractérisée par la présence d'inclusions nucléaires (INs) dans les neurones des régions cérébrales distinctement affectées. Un des facteurs qui contribue à la neurotoxicité observée est la production de protéines contenant des polyalanines qui font suite à un changement -1 du cadre de lecture ribosomique à l'intérieur des séquences de CAG répétées, pendant la traduction. Afin de mieux étudier ce changement, nous avons établi un modèle cellulaire en utilisant trois constructions de MJD1 basées sur la séquence complète de cDNA du gène *MJD1* exprimant trois longueurs de répétitions de polyglutamine (CAG9, CAG100, et CAA96). À l'aide de nos constructions, nous avons confirmé que le changement de cadre de lecture ne se produit que lorsque la construction de CAG longue est utilisée (CAG100); ce changement semble être relié à l'énergie nécessaire au dépliement de structures d'ARN secondaire particulières aux longue répétions de CAG. Il est probable que la présence des protéines contenant des polyalanines toxiques contribue à la pathogenèse de SCA3. Aussi, en utilisant les modèles cellulaire et de drosophile que nous avons établi, nous avons testé un mécanisme de déregulation transcriptionelle lié à la fonction altérée de la polymérase II d'ARN (RNAPII). Nos résultats démontrent que dans la maladie SCA3, l'altération transcriptionelle semble être indépendante des modifications fonctionnelles de RNAPII. En conclusion, nous avons confirmé l'implication des protéines de polyalanine dans notre

modèle de la maladie, mais d'autres études seront nécessaires pour mieux comprendre la dérégulation transcriptionelle dans la pathogénèse de SCA3.

Mots clés: ataxie spinocérébelleuse de type-3 (SCA3), neurodegeneration, ataxine-3, inclusions nucléaires, polyglutamine, polyalanine, changement de cadre de lecture, ARN polymérase II , neurotoxicité, expansion de triplet.

Table of Contents

List of Figures

List of Tables

Abbreviations

ACA	adenine, cytosine, adenine
Ala	alanine
Asn	asparagine
ATPase	adenosine triphosphatase
CAA	cytosine, adenine, adenine
CAG	cytosine, adenine, guanine
CBP	CREB-binding protein
CDK	cyclin-dependent kinase
CREB	cAMP response element binding protein
CTD	C-terminal domain
CTD4H8	Purified mouse anti-RNA polymerase II monoclonal antibody
DAPI	4',6 diamidino-2-phenylindole
DM	Dystrophia myotonica 1
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside tri-phosphate
DRB	5,6-dichloro-1β-d-ribofuranosyl-benzimidazole
DRPLA	Dentatorubropallidoluysian atrophy
ECL	enhanced chemiluminescence
EGFP	enhanced green fluorescent protein
EYA	eyes absent protein
Fcp1	TFIIF-associated CTD phosphatase
FRAXE	Fragile X syndrome E

FRAXF	Fragile X syndrome A
FRDA	Friedreich ataxia
GCA	guanine, cytosine, adenine
GCG	guanine, cytosine, guanine
Gln	glutamine
HA	hemagglutinin
HD	Huntington's disease
HDAC	histone deacetylase
HDL2	Huntington disease-like 2
His	histidine
HRP	horseradish peroxidase
Hsp	Heat shock protein
II_A	hyperphosphorylation state
II_O	hypophosphorylation state
JD	Josephin domain
MAML1	mastermind-like 1
MAT1	'menage à trois' 1
MJD	Machado-Joseph disease
mRNA	messenger ribonucleic acid
MTOC	microtubule organizing center
Myc	myelocytomatosis virus
N2a	mouse neuroblastoma cells
NB	nuclear bodies

NGS	normal goat serum
NI	nuclear inclusion
NT	non-transfected
OPMD	oculopharyngeal muscular dystrophy
ORF	open reading frame
P/CAF	p300/CBP-associated factor
PABPN2	poly(A)-binding nuclear protein 2
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PML	Promyelocytic leukemia protein
Pp1	type 1 protein phosphatase
PQBP-1	polyglutamine binding protein-1
PS2	polyclonal RNA polymerase II CTD (phospho S2) antibody
pTEFb	positive-acting transcription elongation factor-b
RIPA	radioimmunoprecipitation
RNAPII	RNA polymerase II
RPIILS	RNA polymerase II large subunit
SAHA	suberoylanilide hydroxamic acid
SBMA	Spinobulbar muscular atrophy
SCA	Spinocerebellar ataxia
Scp1	small CTD phosphatase
Ser	serine
siRNA	small interfering RNA

SMN	survival of motor neurons protein
Sp1	Specificity protein 1 transcription factor
$TAF_{II}130$	TBP-associated factors
TBP	TATA-binding protein
TFIIF	Transcription initiation factor IIF
Thr	threonine
tRNA	transfer ribonucleic acid
UIM	ubiquitin-interacting motifs
YAC	yeast artificial chromosome
YSPTSPS	tyrosine/serine/proline/threonine/serine/proline/serine

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Chapter 1: Introduction

1.1 Trinucleotide repeat expansion diseases

Trinucleotide repeat expansion diseases are due to unstable trinucleotide sequences which are repeated several times within a stretch of DNA (Cummings and Zoghbi, 2000). The number of repeats is polymorphic in a normal population and no phenotype is observed until their expansion reaches a pathogenic length unique to each gene (Jasinska *et al.*, 2003). These diseases are categorized into two subclasses depending on the location of the repeat with respect to the gene. The first subclass, accounting for seven diseases, has its repeats in non-coding sequences of DNA (Jasinska *et al.*, 2003; Di Prospero and Fishbeck, 2005). The second subclass, consisting of up to nine diseases, is caused by an expansion of a CAG trinucleotide repeat within the coding region of the causative gene (Pearson *et al.*, 2005). This group includes spinobulbar muscular atrophy (SBMA), Huntington's disease (HD), the spinocerebellar ataxias (SCAs) (1, 2, 3, 6, 7, 17), and dentatorubropallidoluysian atrophy (DRPLA) (Pearson *et al.*, 2005). Because the CAG trinucleotide encodes the amino acid glutamine, these nine diseases are commonly referred to as polyglutamine diseases (Table 1.1).

Trinucleotide repeats are unstable in both somatic and germ cells (Pearson *et al.*, 2005). The exact cause of the triplet expansion is unknown, but most likely involves one or more of the following processes: formation of unusual DNA structures and DNA slippage during lagging-strand synthesis; aberrant repair of DNA mutagenic intermediates such as

double-strand or single-strand breaks, or endogenous DNA damage; or, recombination within the repeats by intrachromosomal strand annealing (Pearson *et al.*, 2005).

Three possible mechanisms of pathogenicity caused by triplet repeat expansions have been identified. The first, the gain-of-function mechanism for dominantly inherited diseases, such as in the expanded CAG repeat disorders, is based on abnormal protein products with expanded polyglutamine tracts which lead to misfolding and novel altered functions (La Spada and Taylor, 2003; Perutz *et al.*, 1999). The second, the loss-of-function mechanism for recessively inherited diseases, such as in fragile X syndrome and Friedrich ataxia, is based on transcriptional interference of the affected gene leading to reduced levels of their protein products (Pieretti *et al.*, 1991; Bardoni and Mandel, 2002). The third is also a gain-of-function mechanism for dominantly inherited diseases, but is based on abnormal interactions at the RNA level of the affected gene leading to disrupted processing and nuclear accumulation of the transcript, such as in the case of myotonic dystrophy (Wang *et al.*, 1995; Taneja *et al.*, 1995).

Table 1.1 Trinucleotide repeat expansion disorders. Adapted from Cummings and Zhoghbi, 2000; Di Prospero and Fischbeck, 2005; Everett and Wood, 2004; Ranum and Day, 2002.

Disease	Gene	Locus	Protein	Repeat size	
				Normal	Disease
Non-coding repeats					
Friedreich ataxia (FRDA)	*FXN*	9q13-q21.1	Frataxin	7-34	>100
Fragile X syndrome A (FRAXF)	*FMR1*	Xq27.3	Fragile X mental retardation 1 protein	6-53	>230
Fragile X syndrome E (FRAXE)	*FMR2*	Zq28	Fragile X mental retardation 2 protein	6-35	>200
Dystrophia myotonica 1 (DM)	*DMPK*	19q13	Dystrophia myotonica protein kinase	5-37	>50
Spinocerebellar ataxia 8 (SCA8)	Antisense to *KLHL1*	13q21	Undetermined	15-91	100-155
Spinocerebellar ataxia 12 (SCA12)	*PPP2R2B*	5q31q33	Regulatory subunit of the protein phosphatase PP2A	7-28	66-78
Huntington disease-like 2 (HDL2)	*JPH3*	16q24.3	Junctophilin 3	6-27	51-57
Polyglutamine disorders					
Spinal and bulbar muscular atrophy (SBMA)	*AR*	Xq13q21	Androgen receptor	9-36	38-62
Huntington's disease (HD)	*IT15*	4p16.3	Huntingtin	6-35	36-121
Dentatorubral-pallidoluysian atrophy (DRPLA)	*DRPLA*	12p13.31	Atrophin 1	6-35	49-88
Spinocerebellar ataxia 1 (SCA1)	*SCA1*	6p23	Ataxin 1	6-44	39-82
Spinocerebellar ataxia 2 (SCA2)	*SCA2*	12q24.1	Ataxin 2	15-31	36-63
Spinocerebellar ataxia 3 (Machado-Joseph disease) (SCA3/MJD)	*SCA3/MJD*	14q32.1	Ataxin 3	12-40	55-84
Spinocerebellar ataxia 6 (SCA6)	*CACNA1A*	19p13	α_{1A}-voltage-dependent calcium channel subunit	4-18	21-33
Spinocerebellar ataxia 7 (SCA7)	*SCA7*	3p12p13	Ataxia 7	4-35	37-306
Spinocerebellar ataxia 17 (SCA17)	*TBP*	6p27	TATA box binding protein	25-42	45-63

1.2 Pathogenesis of polyglutamine disorders

Nine neurodegenerative disorders (SBMA, HD, DRPLA and SCAs 1, 2, 3, 6, 7, 17), are caused by CAG repeat expansions within the coding sequence of the respective genes. Aside from their expanded polyglutamine tract, the mutant proteins do not share any homology, but the diseases have similar features and probably share common mechanisms of pathogenesis (Cummings and Zhoghbi, 2000).

The first similarity is the mode of inheritance. With the exception of SBMA, which is X-linked, all of these diseases are inherited in an autosomal dominant fashion.

Secondly, the progression and manifestations of the diseases are characterized by similar events. Neurodegeneration develops progressively, appears in midlife, and causes increasing neuronal dysfunction which eventually leads to neuronal loss 10-20 years following the onset of symptoms (Zoghbi and Orr, 2000). Also, the age of onset is an exponential function that is inversely dependent on the number of the repeats; that is, longer expansions lead to an earlier age of onset with more severe manifestations (Zoghbi and Orr, 2000). Polyglutamine disorders also display one of the fundamental features of neurodegenerative diseases: neuronal cell death is apparent only towards adulthood, despite expression of the mutant protein throughout life. This suggests that accumulation of the toxic product or cumulative cellular damage reaches a threshold beyond which the neuron cannot compensate (Paulson, 1999).

Thirdly, another phenomenon observed in affected families is genetic anticipation: over successive generations, the diseases present an earlier age of onset and progress more rapidly due to successive expansions of the CAG tract during transmission (Zoghbi and Orr, 2000).

Fourthly, in all of these diseases, only neuronal cells are specifically targeted by the mutant proteins. Moreover, only a selected subset of neurons (specific for each disorder) is affected by apoptosis, despite widespread expression of mutant protein in the nervous system and throughout the body (Paulson *et al.*, 1997a; Reddy and Housman, 1997; Zoghbi and Orr, 2000).

Fifthly, neuronal accumulation of mutant protein in nuclear or cytoplasmic inclusions is a common feature to all polyglutamine diseases (Ross and Poirier, 2004; Michalik and van Broeckhoven, 2003). Mutant polyglutamine-containing proteins aggregate in vulnerable neurons and these aggregates are also positive for ubiquitin and proteasome components (Cummings and Zhoghbi, 2000). Though the presence of these nuclear inclusions may appear to be the cause of neuronal dysfunction, this link remains uncertain as several reports have indicated that they are neither necessary nor sufficient to initiate polyglutamine-mediated disease (Cummings and Zhoghbi, 2000). Nevertheless, the commonalities of inclusion formation in many unrelated neurodegenerative diseases might reflect a shared coping response to diffuse toxic proteins and indicate a common pathological mechanism (Ross, 1997).

And finally, transcriptional dysregulation appears to represent a unifying pathogenic mechanism in polyglutamine disorders, as profound transcriptional changes are often noted in each case (Evert *et al.*, 2003).

In spite of the many similarities among polyglutamine disorders, as reflected by the abundant publications looking into common mechanisms of toxicity, it is important to note that each disease is nonetheless distinct and characterized by unique properties. For example, each is caused by a different gene, located on different chromosomes. Furthermore, each possesses a characteristic critical threshold for glutamine repeat number, below which the disease does not occur (Gusella and Macdonald, 2000). Also, the brain regions affected are not identical (Margolis, 2003). Therefore, the protein context and neuronal cell-type affected in the various disorders present differences unique to each disease and must be investigated individually.

1.3 Characteristics of spinocerebellar ataxia type-3 (SCA3)

1.3.1 Clinical and pathological features

SCA3 is a neurodegenerative disorder also called Machado-Joseph disease (MJD). The clinical features of SCA3 patients include cerebellar ataxia, oculomotor abnormalities, spasticity, peripheral neuropathy, and cognitive disturbances (Dürr *et al.*, 1996). Core features include progressive ataxia, dysarthria, postural instability, nystagmus, eyelid retraction, and facial fasciculations. Dystonia is often prominent in younger patients (Sudarsky and Coutinho, 1995). Five subtypes of the disease have been identified; the

most common, type 2, is associated with early adulthood to middle-age onset (~20-45 years) and its clinical manifestations are cerebellar and pyramidal signs (Margolis, 2003).

The pathology of SCA3 is associated with neurodegeneration occurring mainly in the basal ganglia (substantia nigra), brain stem, spinal cord, pontine nuclei, and cerebellum with preservation of cerebellar cortex and inferior olive (Dürr *et al.*, 1996; Paulson *et al.*, 1997a; Sudarsky and Coutinho, 1995). The neuropathy involves both sensory and motor neurons in myelinated and unmyelinated fibers (Margolis, 2003).

Currently, there is no treatment or cure for SCA3, but identification of the mutant gene has been useful for diagnosis and genetic counselling (Colomer Gould, 2005).

1.3.2 Molecular genetics of SCA3

This disease is an autosomal dominantly-inherited ataxia originally described in people of Azorean/Portuguese descent. The disorder has subsequently been identified in many other countries including Japan, Brazil, Australia, and China (Sudarsky and Coutinho, 1995).

The cause of SCA3, the most common dominantly inherited ataxia, was identified to be an unstable expansion of a CAG repeat in the human *MJD1* gene (Kawaguchi *et al.*, 1994), which had been previously mapped to chromosome 14q24.4-q32.1 (Takiyama *et al.*, 1993). The *MJD1* gene encodes the cytoplasmic protein ataxin-3 of 42 kDa with

ubiquitous expression throughout the body. Despite this widespread expression, pathological sites are selective to certain brain regions only (Paulson *et al.*, 1997a).

The number of normal CAG repeats ranges from 10-51, whereas the expanded CAG length is from 55-86; however, intermediate repeat lengths of 53 and 54 have been observed in SCA3 patients who display an abnormal phenotype (van Alfen *et al.*, 2001; Zhou *et al.*, 1997). These expanded repeats are highly unstable and may expand from one generation to the next, which explains the anticipation observed in patients (Galvao *et al.*, 2004). Large changes in CAG repeat number are also more frequently associated with paternal transmissions, suggesting the expansion occurs during the pre-meiotic proliferative stages of spermatogonial divisions (Galvao *et al.*, 2004; Pearson *et al.*, 2005).

An interesting observation is that despite being an autosomal dominant disorder, clinical studies have shown that SCA3 patients homozygous for the mutation display more severe symptoms at an earlier age than heterozygotes (Lerer *et al.*, 1996; Sobue *et al.*, 1996; Zlotogora, 1997). This suggests that in heterozygotes, the second non-mutant allele may act protectively to reduce the age at onset or that dosage of repeat-bearing proteins may be important for pathogenesis.

1.3.3 Cellular features of the disease

1.3.3.1 Misfolded polyglutamine proteins

The polyglutamine proteins produced by the *MJD1* gene with expanded-CAG repeats are prone to misfolding (Jana and Nukina, 2004). The first line of cellular defence against these proteins involves molecular chaperones, which participate in refolding abnormal peptide conformations (McClellan and Frydman, 2001). Secondly, if this system fails, the defective proteins are tagged with ubiquitin and targeted for degradation by the proteasome complex (Goldberg, 2003; Ciechanover and Brundin, 2003). Thirdly, if the misfolded proteins cannot be removed, they form aggregates which the cell attempts to remove by autophagy (Ravikumar *et al.*, 2004).

Lastly, it was observed that when mutant ataxin-3 binds to the nuclear matrix, this causes a conformational change which exposes the polyglutamine tract (Perez *et al.*, 1999). Upon exposure of the expanded tract, more aggregation and binding of other polyglutamine-containing proteins occur. If removal of these aggregates is unsuccessful, they ultimately grow in size and form into larger nuclear inclusions (NIs), a hallmark of polyglutamine diseases (Jana and Nukina, 2004).

1.3.3.2 Inclusions in SCA3 affected brain regions

In SCA3 patients, the presence of visible nuclear inclusions (NIs) of various sizes was detected in brain tissue (Paulson *et al.*, 1997a). Inclusions containing mutant ataxin-3 protein were observed in regions such as the cerebral cortex, striatum, thalamus, and

lateral geniculate body (Yamada *et al.*, 2004). Interestingly, NIs were even detected in regions of the nervous system that showed no obvious neuronal loss (Yamada *et al.*, 2001a).

1.3.3.3 Formation of inclusions

Nuclear inclusions are most likely a manifestation of misfolding and aggregation of the mutant protein, and as a consequence, lead to aberrant protein-protein interactions within affected cells (Chai *et al.*, 2001). Studies suggest that NIs in polyglutamine diseases arise from nuclear bodies (NBs), which are intranuclear structures that function in transcriptional regulation, growth suppression and apoptotic cell death, and are found distributed throughout the nucleus in a speckled pattern (Seeler and Dejean, 1999; Zhong *et al.*, 2000). The appearance of large macroscopic inclusions characteristic of SCA3 is preceded by the presence of insoluble, micro-aggregates of mutant ataxin-3 (Chai *et al.*, 2001). These authors found that in SCA3, expanded polyglutamine co-localized with promyelocytic leukemia protein (PML), an essential component of NBs (Takahashi *et al.*, 2003). This suggests that NBs are gradually enriched in misfolded mutant proteins and eventually lose their characteristic structure due to the excessive accumulation of mutant proteins.

1.3.3.4 Mechanism of aggregation

Perutz *et al.* (1994) proposed that the glutamine repeats act as polar zippers: they are capable of linking β-strands together into sheets by networks of hydrogen bonds between

their main-chain amides and polar side chains; this cross-linking would allow for the joining of complementary proteins, resulting in the formation of insoluble aggregates. The expansion of glutamines might cause the mutated protein to acquire non-specific or increased affinity for each other or for other glutamine-containing proteins, thereby causing pathological defects (Perutz *et al.*, 1994; Stott *et al.*, 1995). However, this cross-linking in the presence of the mutant protein context was not as apparent (Perutz *et al.*, 1994).

Alternatively, Green H. (1993) proposed that aggregation was based on the activity of a transglutaminase enzyme which links the amino groups of lysine residues in other proteins through isopeptide bonds to form covalently bonded aggregates. In fact, huntingtin, a polyglutamine disease protein, was shown to be a substrate for brain transglutaminase and its aggregation was dependent on Ca^{2++} levels, which are linked to impulse-conduction in neurons (Kahlem *et al.*, 1996; Kahlem *et al.*, 1998).

Neither of these processes has been demonstrated *in vivo*. Whichever the mechanism may be, it appears that the proteins are destabilized and drawn into the nucleus, where ubiquitination and aggregation occur.

1.3.3.5 Components of inclusions

Inclusions are composed of not only the mutant protein, but also other cellular factors which might provide insights into the aberrant protein-protein interactions that occur prior to cell death. Of interest is the presence of the wild-type ataxin-3 protein (with a

non-pathogenic polyglutamine length) within inclusions. Studies have found that wild-type ataxin-3 can accumulate in eosinophilic nuclear inclusions, or Marinesco bodies, in the absence of expanded polyglutamines under stress conditions such as aging; in this case, wild-type ataxin-3 may play a role in the ubiquitination of other proteins (Fujigasaka *et al.*, 2000). On the other hand, it can also be recruited to mutant protein-containing inclusions in the presence of pathologically expanded polyglutamine (Paulson *et al.*, 1997b; Uchihara *et al.*, 2001).

As the inclusions contain misfolded proteins, it is common to also detect the presence of molecular chaperones, ubiquitin (a cellular modification used to tag proteins for degradation), and proteasomal components, part of the cell's protein degradation machinery (Paulson *et al.*, 1997b; Chai *et al.*, 1999b).

1.3.3.6 Fate of inclusions

Several reports have shown that inclusions are highly ubiquitylated and that the proteasome complex redistributes into polyglutamine aggregates formed by the disease protein ataxin-3; this suggests proteasome function is closely linked to aggregate formation (Chai *et al.*, 1999b; Schmidt *et al.*, 2002). In addition, it was also observed that proteasomal inhibitors promoted aggregation of mutant ataxin-3, suggesting an involvement of the protein surveillance machinery in suppression of aggregation (Chai *et al.* 1999b).

Evidence from SCA3 patient brains, obtained by immunohistochemical analysis of neuronal NIs in pontine neurons, revealed the presence of chaperones in the Hsp40 family along with subunits of the proteasomal proteolytic machinery. As this machinery was detected in only a fraction of the pontine neurons, this suggests proteasomal perturbations in specific regions in these brains (Schmidt *et al.*, 2002). Overall, these results suggest that inclusions are ultimately destined to undergo proteasome-mediated degradation.

Though the ubiquitin-proteasomal pathway is frequently referred to as the only pathway responsible for the clearance of protein aggregates, studies have shown that the autophagy-lysosomal pathway is also involved in the removal of the aggregate-prone polyglutamine and polyalanine proteins (Ravikumar *et al.*, 2002).

1.3.3.7 The role of inclusions

The role of inclusions in polyglutamine diseases has been extensively examined in many cases. Studies have demonstrated that inclusions can be harmful, coincidental, or even protective (Sisodia, 1998). The formation of NIs may be the underlying cause of neuronal dysfunction in polyglutamine diseases; however, these disruptions do not invariably bring about cell death (Davies *et al.*, 1997). In many of the cases studied, the correlation between the presence of nuclear inclusions and cell death was poor, suggesting that inclusions are not necessarily the primary toxic insult (Everett and Wood, 2004).

Moreover, it has been reported that in Huntington's disease, inclusion formation actually promotes cell survival in neurons (Arrasate *et al.*, 2004). These results indicate that inclusion formation might protect neurons by decreasing the levels of toxic diffuse and soluble forms of mutant protein. Evidence in SCA7 demonstrated that extensive inclusion formation leads to reduced symptoms, but is nonetheless insufficient to halt the disease, as the system is eventually overwhelmed and results in neuronal dysfunction (Bowman *et al.*, 2005). When aggregates were present in one brain region (the pons), the size and shape of the neuronal nuclei were more preserved than in neurons without inclusions. This suggests that inclusions are not necessarily toxic to neurons of diseased brains (Uchihara *et al.*, 2002). There is also a debate over whether large aggregates or small invisible aggregates directly cause cell death (Zhoghbi and Orr, 2000).

Notwithstanding, the presence of NIs is critical, as it likely represents a biological marker for the underlying pathogenic process (Chai *et al.*, 2001). Aggregation could help explain why polyglutamine toxicity only affects neurons. Since these cells are post-mitotic, the aggregate load is not reduced when the nuclear membrane disintegrates, as it happens in dividing cells, where the aggregates are distributed to the cytoplasm of daughter cells (Michalik and Van Broeckhoven, 2003). It was shown in a SCA3 cell culture model that cell cycle arrest enhances toxicity (Yoshizawa *et al.*, 2000); also, in a fly SCA3 model, cell death was observed only in post-mitotic cells of the eyes, not the diving cells of imaginal discs (Warrick *et al.*, 1998). As neurons are known to be arrested in G_0 phase, these results possibly explain why they are more vulnerable than

other types of cells in SCA3. The selective death of specific neurons, however, remains unexplained.

1.3.4 Structure of ataxin-3 protein

The ataxin-3 protein, coded by the *MJD1* gene, is an intracellular protein of 42 kDa with mostly cytoplasmic expression, but is also found in neuronal nuclei of SCA3 patients (Paulson *et al.*, 1997a) and is therefore predicted to contain a nuclear localization signal (Tait *et al.*, 1998). The protein contains an amino (N)-terminal Josephin domain (JD), two ubiquitin (Ub)-interacting motifs (UIMs), a polyglutamine stretch and a short variable C-terminal tail (Goto *et al.*, 1997) (Figure 1.1). A rare splice variant containing a third putative UIM in the carboxyl (C)-terminal was also identified by the same group. The JD appears to contain a catalytic cysteine protease with deubiquitinase activity (Scheel *et al.*, 2003). The UIM is a conserved 15- amino acid motif for binding polyubiquitin chains (Chai *et al.*, 2004) and plays an important role in both the catalytic activity of ataxin-3 and the deubiquitinating reaction as a chain-editing enzyme that shortens Ub chains (Mao *et al.*, 2005). Therefore, the UIM can bind to ubiquitinated proteins that accumulate when the proteasome is inhibited and the JD can function to de-ubiquitinate these proteins or even itself in *trans* (Berke *et al.*, 2005).

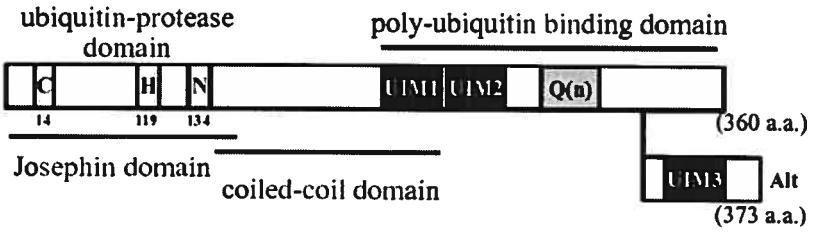


Figure 1.1 Structure of the ataxin-3 protein containing an N-terminal Josephin domain with a predicted ubiquitin-specific protease with catalytic triad (Cys^{14}, His^{119}, and Asn^{136}), a coiled-coil domain, a C-terminal ubiquitin-interacting motifs (UIMs), and a polyglutamine tail Q(n). Berke SJS *et al.*, (2005) J. Biol. Chem. 280:32026-32034.

1.3.5 Functions of ataxin-3 protein

1.3.5.1 Protein maintenance activity

The physiological function of ataxin-3 is still unknown. There is strong evidence pointing towards its involvement in the ubiquitin-proteasome system (see Figure 1.1). Ubiquitin-dependent pathways are responsible for degradation of most cytosolic proteins, including misfolded and damaged proteins, and are therefore critical for proper protein quality control (Ciechanover, 1994).

Studies demonstrate that the ataxin-3 protein can bind polyubiquitin chains of four or more ubiquitin tags through its UIM and that binding is equally effective in both wild-type and pathological ataxin-3 (Burnett *et al.*, 2003). Moreover, UIMs are primarily responsible for inhibiting degradation through binding of ubiquitylated proteins and thus preventing access to the proteasome (Burnett and Pittman, 2005). The decreased degradation by the proteasome is, therefore, indirectly mediated by alteration of the substrate via ataxin-3 and not through a direct effect on the proteasome itself (Burnett and

Pittman, 2005). The ataxin-3 protein also has ubiquitin protease activity that removes polyubiquitin chains, with cysteine-14 being the catalytic site on the protein, and also binds an active site inhibitor of ubiquitin protease (Burnett *et al.*, 2003).

More recently, Warrick *et al.*, (2005), demonstrated that normal ataxin-3 was recruited to NIs and helped to reduce accumulation of pathogenic protein in a *Drosophila* SCA3 model. Furthermore, this protective activity was mediated by the ubiquitin-proteasomal pathway. In addition, this intrinsic activity was even able to suppress toxicity of other polyglutamine disease proteins, such as huntingtin in Huntington's disease and ataxin-1 in SCA1. These results may explain the fact that although SCA3 is an autosomal dominant disorder, homozygotes display a more severe phenotype due to the cumulative effects of the loss of the protective function of the wild-type protein in conjunction with the gain of a novel function of the mutant protein when both alleles are mutated.

As for the role of mutant ataxin-3 in SCA3 pathology, evidence by Berke *et al.* (2005) shows that the proteasome efficiently degrades both normal and expanded ataxin-3 proteins and that proteasome inhibition by soluble expanded ataxin-3 does not appear to play a major role in SCA3 pathogenesis. In general, the presence of a polyglutamine expansion does not dramatically impair proteasome activity under basal conditions, but does significantly impair its ability to respond to stress and increases stress-induced protein aggregation following stress (Ding *et al.*, 2002), suggesting the pathology is complex and involves other cellular disruptions.

Although more clues are being revealed about the proteasomal inhibitory functions of this protein, it is not yet clear how essential wild-type ataxin-3 function is to the pathway, and whether mutant ataxin-3 directly or indirectly alters its function, if at all. Initial reports suggest that the expansion modulates wild-type ataxin-3 protease activity and/or the range of its substrates, but whether or not this function is linked to the disease pathology has yet to be determined (Burnett *et al.*, 2003). It is further hypothesized that pathological ataxin-3 and misfolded denatured proteins, coupled with decreased proteasome activity due to aggregation, would increase the likelihood of this continued process and, over time, would result in inadequate degradation of many cellular regulators resulting in cellular dysfunction (Burnett *et al.*, 2003).

Another protein maintenance function of the endogenous ataxin-3 has to do with its association with histone deacetylase 6 (HDAC6), a protein that binds to polyubiquitinated substrates (Kawaguchi *et al.*, 2003), and the ATPase motor protein dynein that transports various cellular cargos along cytoskeletal microtobules towards the center of the cell (Vallee *et al.*, 2004). The association of these three proteins - ataxin-3, HDAC6, and dynein - is responsible for trafficking aggregated proteins within the cell to form aggresomes (Burnett and Pittman, 2005).

Therefore, it appears that wild-type ataxin-3 is first recruited to bind and trim ubiquiting chains on misfolded ubiquitylated proteins, shielding them from the proteasome; then its deubiquitin activity and UIMs are required for aggresome formation and for ataxin-3 to associate with dynein and HDAC6 to facilitate transport of the misfolded protein to the

microtubule organizing center (MTOC) for formation of aggresomes (Burnett and Pittman, 2005).

1.3.5.1 Transcriptional regulator activity

The second proposed function of ataxin-3 is its involvement in transcriptional regulation. Research by Li *et al.*, (2002) has shown that endogenous ataxin-3 interacts with key regulators of transcription and acts to repress transcription via multiple mechanisms. The polyglutamine-containing C-terminus of the protein is able to repress transcription when targeted to chromatin. Furthermore, the glutamine rich C-terminus binds to the transcriptional coactivators CREB-binding protein (CBP), p300, and PCAF, all of which have intrinsic histone acetyltransferase activity (Cho *et al.*, 1998); ataxin-3 was shown to inhibit their ability to activate transcription. As an indication towards a pathological effect, the expanded polyglutamine repeats of ataxin-3 bind to transcription coactivators with a higher affinity than the normal length polyglutamine repeats protein.

This group also identified a second mechanism of transcriptional repression by full-length ataxin-3: the N-terminus of the protein was shown to bind and mask H3 and H4 histones and prevent acetylation by p300, thereby decreasing transcriptional accessibility. Despite what is known about its function, the specific targets of ataxin-3 in pathogenesis of SCA3 have yet to be determined. It is believed, however, that transcriptional dysregulation is implicated in the disease process.

1.4 Objectives and thesis outline

The purpose of this study is to gain further insight into specific aspects of the pathology of SCA3 at the cellular level. Following this introductory chapter (Chapter 1), this manuscript is composed of three experimental chapters, each with specific objectives designed to focus on one aspect of the disease. The first objective is to develop and characterize SCA3 model systems to be used in the following chapters (Chapter 2). The second objective is to build on the theory of ribosomal frameshifting in SCA3 pathogenesis, an aspect that has been previously reported by our lab (Chapter 3). The third objective is to investigate one mechanism of transcriptional dysregulation in the disease progression (Chapter 4). The last chapter is a general conclusion which summarizes this study and discusses possible therapeutic applications (Chapter 5).

Chapter 2: Model systems of SCA3

2.1 Introduction: established models of SCA3

2.1.1 Mouse models

Mouse models are the preferred choice for the study of human diseases because they are a good represention of the mammalian systems; in addition, they are small and short-lived, breed quickly to generate relatively large numbers, and can be used for invasive procedures (Colomer Gould, 2005). Transgenic mice expressing mutant full-length and truncated (C-terminal fragments containing the glutamine tract) versions of the ataxin-3 protein have been generated for research purposes (Ikeda *et al.*, 1996; Cemal *et al.*, 2002; Goti *et al.*, 2004). Particularly, the mice expressing the truncated version displayed massive degeneration of the cerebellum and developed ataxia by four weeks of age. In contrast, expression of full-length mutant ataxin-3 in mice failed to show any pathology (Ikeda *et al.*, 1996). From this model, researchers proposed that, since the truncated version was toxic, proteolytic cleavage of the mutant protein might occur to liberate the polyglutamine tract, which leads to death of neurons. Further evidence by Wellington *et al.* (1998) demonstrated that ataxin-3 can be cleaved by a caspase. The generation of a third mouse model led to different results through widespread expression of human yeast artificial chromosome (YAC) constructs encompassing the *MJD1* locus; cerebellar ataxia and neurodegeneration were observed in the absence of a cleaved ataxin-3 fragment (Cemal *et al.*, 2002). A fourth mouse model developed by Goti *et al.* (2004) was established via the expression of full-length human mutant *MJD1a* cDNAs. These

animals displayed abnormal behaviour with prominent neuronal nuclear inclusions in selective brain regions and in the spinal cord. Unique to this last model, however, was the finding that not only the mutant ataxin-3, but also its putative-cleavage fragment, were enriched in nuclear fractions of brain homogenates. Altogether, these mouse models have been important for the elucidation of the mechanisms of disease pathogenesis and progression in human.

2.1.2 Invertebrate models: *Drosophila*

The fruit fly, *Drosophila melanogaster*, has been used as an experimental organism for many human neurodegenerative diseases. The first polyglutamine disease modeled in *Drosophila* was SCA3. Warrick *et al.* (1998) expressed a truncated ataxin-3 protein (containing 12 amino acids of ataxin-3 upstream of the glutamine repeat, the expanded glutamine repeat, and the 43 amino acids comprising the carboxy-terminus of the protein) in fly eyes and observed both retinal degeneration and nuclear inclusions in the photoreceptor cells of developing eye discs. Furthermore, they showed that the deleterious effect of the expanded mutant protein could be prevented by co-expression of P35, an antiapoptotic agent, which partially restored pigmentation of the eye. Another group, Ghosh and Feany (2004), also modeled SCA3 in flies, but examined neurotoxicity in brain regions in an attempt to determine whether common pathways of toxicity govern neurodegeneration in the eye and brain. Using the same truncated ataxin-3 protein as the previous group, expression in this case showed cell-type vulnerability and demonstrated retina-specific effects. Expression of expanded ataxin-3 in the brain caused toxicity to Kenyon cells, the projection neurons of the mushroom bodies, structures implicated in

learning and memory. Using this model, the authors were able to show that overexpression of the molecular chaperone Hsp70 can suppress polyglutamine-induced neurodegeneration. In addition, antiapoptotic therapies were shown to be beneficial to the toxicity associated with SCA3 and possibly other neurodegenerative disorders. More recently, Warrick *et al.*, (2005) expressed a full-length ataxin-3 construct in flies and found that ataxin-3 accumulated in ubiquitinated inclusions and that the full-length protein is more selectively toxic to the nervous system. Each of these *Drosophila* models has provided unique insights to various aspects of the human disease.

2.1.3 *In vitro* or cellular models

Cell models are often used to study specific aspects of neurodegenerative diseases because they are versatile and easy to manipulate; however, these models are unable to reproduce the breadth of pathological events that happen in the human brain and thus remain of limited interest. Several groups were able to model SCA3 in cell culture to illustrate the cellular expression and interactions of the mutant protein. Full-length ataxin-3 expression appears in the cytoplasm and nucleus, where the protein is associated with the nuclear matrix (Ikeda *et al.*, 1996; Paulson *et al.*, 1997b; Tait *et al.*, 1998). The mutant full-length ataxin-3 also forms aggregates in these cell models, but with less efficiency and less toxicity than the truncated version. Also, transfected cells show indentations of the nuclear envelope and undergo spontaneous non-apoptotic cell death (Ikeda *et al.*, 1996; Paulson *et al.*, 1997b; Evert *et al.*, 1999). The importance of nuclear localization of the protein for aggregate formation was shown using full-length ataxin-3 which was recruited from the cytoplasm into nuclear inclusions by a non-related

glutamine-rich repeat (Perez *et al.*, 1998). Furthermore, the involvement of the proteasomal degradation system was also identified in cell models and its co-localization to inclusions was demonstrated by others (Chai *et al.*, 1999b). It was further shown that Hsp40 chaperones suppress ataxin-3 aggregation in neural and non-neural cells and that this reduction in aggregation was associated with a decrease in neurotoxicity (Chai *et al.*, 1999a). This evidence suggests a toxic role for aggregates in this cellular model. And finally, a novel aspect of SCA3 was revealed in a Cos-1 cell culture model by Gaspar *et al.* (2000) and Toulouse *et al.* (2005) in our lab; it was demonstrated that ribosomal frameshifting occurs in the long CAG repeats of the truncated *MJD1* constructs which leads to the production of polyalanine-containing proteins. It is believed that these proteins, which were shown to be more toxic, play a role in pathogenesis of the disease. Toulouse *et al.* demonstrated this toxicity by using two constructs with different repeats, CAG and CAA, both of which code for polyglutamine; however, only the CAG construct can frameshift into the -1 frame to produce a polyalanine-encoding protein. Therefore, the practicality and simplicity of these cellular models are essential as a first step for studying disease processes in a controlled and reproducible environment.

2.2 Objectives

The objective of this chapter is to establish a full-length cellular SCA3 model by generating three full-length MJD1 constructs of different repeat lengths and types, tagged in the three reading frames, and to characterize their expression in Cos-1 cells. Aside from using the full-length of the gene, the tags used in this model will be more easily detectable than the ones used in our previous model established by Toulouse et al.,

(2005). Secondly, using a *Drosophila* model also expressing full-length ataxin-3 previously established in our laboratory, we aim to characterize the phenotype of these flies so they can be used for further experiments and to test potential treatments.

2.3 Materials and Methods

2.2.1 Cellular model: generation of plasmid constructs

The original full-length *MJD1* cDNA was obtained from a previous construct (Ichikawa *et al.*, 2001). Modifications were made to generate the constructs used by Toulouse *et al.*, (2005). Subsequent alterations were made by others in our lab. Here, we will only describe the modifications made to generate the constructs used in this study.

The CAG9 expression construct was generated from a previously made construct in pEGFP vector. The sequence was amplified by PCR using primers 2491 pMJD16(5_HindIII_1)F (5'-AGAGACGAGAAGCCTACTTTAAGCTTCAGCA-GCAACAGCAGCAGCAACAGCA-3') and 2492 pMJD16(3_EcoRI_v3)R (5'-GGATGTGAATTCCTGTCCTGATAGGTCCCGCTGCTGCT-3').

Full-length human *MJD-1* cDNAs with either 98 CAG or 94 CAA repeats from a previously generated construct in pBSII (Bluescript vector) were amplified by PCR using primers 2637 MJD(BZ07BamHI)F (5'-CGGTACCCGGGATCC-AGACAAATAA-3') and 2638 MJD(BZ07_TAG-TAC)R (5'-CGCGTCGACTCAGTGATGATGGTGGTG-ATGATGATCCATTGAGGT-3').

The PCR reaction conditions were the following: 10ng DNA template, 5 μL 10X PCR buffer, dNTPs (25 mM each), Primers (100 ng/μL each), 0.5 μL Native *Pfu* (Pyrococcus furiosus) DNA polymerase enzyme (Stratagene), and sterile water to a final volume of 50 μL. The touchdown PCR settings were 94°C for 45 sec, followed by 25 cycles of 94°C for 45 sec, 63°C (decreasing at 0.5°C per cycle) for 45 sec, 72°C for 90 min, followed by 10 cycles of 94°C for 45 sec, 58°C for 45 sec, 72°C for 90 min, and finished with 72°C for 5 min. Products were visualized on 1% agarose gel. Bands were excised and purified using QIAEX II Gel Extraction Kit (Qiagen). Purified band products were resuspended in 20 μL of H_2O and 2 μL of this product was run on a 1% agarose gel to confirm desired products were obtained.

Addition of 3' A-overhangs post-amplification for subcloning was carried out by mixing the remaining 18 μL with 3 μL of 10XPCR buffer, 3.5 μL dNTP, and *Taq* (Thermus aquaticus) polymerase (Stratagene) and incubating at 72°C for 15 min. The TOPO TA Cloning Kit (Invitrogen) was used for the rest of the steps. The TOPO Cloning Reaction was done using 4 μL of the DNA with 3' A-overhangs, 1 μL of salt solution, and 1 μL of the pCR2.1-TOPO vector. Reactants were mixed gently and incubated at room temperature for 30 min. Transformation using the One Shot DH5α-T1 Competent Cells was done by adding 2 μL of the TOPO Cloning reaction to 50 μL of the competent cells and mixed gently. Cells were then placed on ice for 30 min, heat-shocked at 42°C for 30 sec, placed on ice for 5 min, and 300 μL of S.O.C. medium was added. Cells were plated at 2 volumes (50 μL and 350 μL) onto Luria-Bertani (LB) Medium plates (with 50 μg/mL ampicillin, 40 μL IPTG, and 40 μL X-Gal) and incubated overnight at 37°C.

Analysis of positive clones was performed by selecting white colonies and culturing them in 3 mL of LB (with 50μg/mL ampicillin) overnight at 37°C. Isolation of plasmid DNA was done using the Qiafilter Plasmid Mini Kit (Qiagen). Transformants were analysed by restriction enzyme digestion with enzymes XhoI (Invitrogen) and SacI (New England BioLabs) for construct CAG9 (reaction conditions: 14 μL H_2O, 2 μL Buffer NEB2 (New England BioLabs), 0.5 μL XhoI, 0.5 μL ScaI, and 3 μL DNA), and BamHI (Invitrogen) and SalI (New England BioLabs) for constructs CAG100 and CAA96 (reaction conditions: 13.8 μL H_2O, 2 μL Buffer NEB3 (New England BioLabs), 10 mg/mL bovine serum albumin (BSA), 0.5 μL BamHI, 0.5 μL SalI, and 3 μL DNA). Reagents were incubated for 3 hrs at 37°C and analyzed on 1% agarose gel. Positive transformants were also analyzed by sequencing (2 μg of plasmid DNA in 50 μL H_2O with M13F and M13R primers).

Positively selected DNA plasmids in TOPO vector (for CAG100 and CAA96 constructs) were then subjected to restriction enzyme digestion to cut out the insert of interest (using 2 μg of DNA with enzymes BamHI (Invitrogen) and Sal I (New England BioLabs)), and so was the final pEGFP-N1vector (Clontech Laboratories) (using 200 ng/μL of vector with enzymes BglII (New England BioLabs) and SalI (New England BioLabs)). Confirmation of digestion was visualized on 1% agarose gel. Ligation was carried out by combining the 3 μL digested vector, 6 μL insert, 4 μL 5X buffer, 6 μL H_2O, and 1 μL T4 Ligase enzyme (New England BioLabs), and incubating the reaction at room temperature for 4 hrs. Transformation was done by adding 3 μL of ligation product to 50 μL of

competent *E. coli* cells, placed on ice for 30 min, heat-shocked for 30 sec at 42°C, placed on ice for 2 min, followed by addition of 450 μL of LB and incubation at 37°C for 1 hr. Cells were then plated at 2 volumes (50 μL and 450 μL) onto kanamycin plates (0.025 mg/ml) and grown overnight at 37°C. Positive colonies were selected as previously described above and grown in 3 mL LB with kanamycin. DNA plasmids were extracted same as previously, and confirmation of insert incorporation was done by restriction enzyme digestion with NheI (New England BioLabs) and ApaI (New England BioLabs) enzymes. Positive colonies were then grown in 400 μL LB with kanamycin overnight and isolation of plasmid DNA was done using Qiafilter Plasmid Maxi Kit (Qiagen). All plasmids were confirmed by sequencing.

Additional steps were required to generate the CAG9 construct. The plasmid DNA from the CAG9 construct in TOPO vector was subjected to DNA precipitation and PCR with *Pfu* polymerase with primers 2491 and 2492 to amplify the insert (same conditions as previously described). This PCR product and the CAG100 construct in pEGFP vector were subjected to restriction enzyme digestion with EcoRI (Invitrogen) and HindIII (New England BioLabs) to generate the insert (with sticky ends) and vector, respectively. The insert and vectors were ligated, transformed, selected, and confirmed (same as previously).

Upon sequence verification of the three constructs (CAG9, CAG100, and CAA96), it was noticed that the EGFP tag was in the -1 frame and not in the main reading frame. Corrections were made by digesting the constructs with BamHI (Invitrogen) (2 μL DNA,

0.5 μL BamHI 2 μL Buffer React 3 (Invitrogen)), and 15.5 μL H_2O), and incubating at 37°C overnight. Blunt ends were filled in to add 4 nucleotides (to place the tag in the main frame) using 3 μL DNA, 3 μL 10X Buffer React 3 (Invitrogen), 0.5 μL Klenow (Invitrogen), 5 μL dNTP (1.25mM), and H_2O to a final reaction volume of 30 μL. The reaction was incubated at 25°C for 30min, stopped by adding 5 μL EDTA (0.5M) and incubated at 75°C for 20min. Constructs were then subject to ligation, transformation, selection, and sequence confirmation (same as previously).

All final constructs generated contained appropriate repeat lengths, EGFP and Myc epitope in the mainframe, hemagglutinin (HA) tag in the -1 frame and His epitope in the +1 frame (Figure 2.1A).

2.2.2 Cell culture and transient transfections

All cell lines were cultured at 37°C in a humid atmosphere enriched with 5% CO_2. Cos-1 and HeLa cells were grown on tissue culture dishes and maintained in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% fetal bovine serum (Gibco-BRL) and 1% Penicillin/Streptomycin (Gibco-BRL).

For transient transfections, Cos-1 cells were plated at a density of 150,000 cells per well on coverslips in six-well plates 24 h before transfection. For protein extraction, cells were plated directly in six-well plates. Subsequently, the medium was removed and 800 μL of Opti-MEM was added to each well and returned to the 37°C incubator. In parallel, for each transfection 1ug of DNA was diluted in 100 μL of Opti-MEM and 6 μL of

PLUS Reagent from the Lipofectamine PLUS Reagent kit (Invitrogen Life Technologies). The mixture was allowed to stand for 15 min at room temperature. In a second tube, 5 μL of Lipofectamine Reagent was diluted into 100 μL of Opti-MEM. The two solutions were combined, mixed gently and incubated at room temperature for 15 min. For each transfection, the Lipofectamine PLUS Reagent - DNA complex mix solution was overlayed onto the cells and incubated for 3 hrs at 37°C. After this period, 2 mL of DMEM supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin was added. Cells were returned to the 37°C incubator for an additional 48 hrs.

2.2.3 Fluorescent visualization

Forty-eight hours after transfection, Cos-1 cells were washed in phosphate-buffered saline (PBS), fixed for 15 min in 4% paraformaldehyde and washed three times in PBS and coverslips were mounted on slides using DAPI (4',6 diamidino-2-phenylindole) Mounting Medium for Fluorescence (Vector Laboratories), and the localization of full-length ataxin-3-EGFP fusion proteins was observed directly with a fluorescence microscope (Leica DM6000).

2.2.4 Quantification of inclusions

To quantitate the presence of inclusions, we counted all cells that contained inclusions in random microscope fields at 200x magnification. Data are expressed as the percentage of transfected cells containing inclusions.

2.2.5 Statistical analysis

Analysis of the statistical significance of the frequency of inclusions in cells transfected with the different constructs was performed using the One-way Anova of variance test.

2.2.6 Cellular protein extraction and Western blot analysis

Forty-eight hours after transfection, Cos-1 cells were washed in ice-cold PBS and solubilized for 20 min on ice in non-denaturing RIPA (radioimmunoprecipitation) buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40 (20 mM Tris-HCl pH 8, 137mM NaCl, 10% glycerol, 1% nonidet P-40, 2 mM EDTA)), 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate) supplemented with protease inhibitors (Roche) and phosphatase inhibitors (10 mM sodium fluoride and 1 mM sodium orthovanadate), and passed through a 23 gauge needle. Samples were centrifuged at 10 000g for 5 min at 4°C, the supernatant was recuperated and protein concentration measured as follows: Protein concentrations were determined by the Bradford method using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad), O.D. values were plotted against a BSA (New England BioLabs) standard curve and protein concentrations were calculated.

Thirty micrograms of each protein extract were aliquoted, boiled for 10 min in 1XSDS sample buffer (2.4 mL of 1M Tris-Cl pH 6.8, 3 mL of 20% SDS, 3 ml of 100% Glycerol, 1.6 mL of β-mercaptoethanol, 0.0006g Bromophenol blue), electrophoresed on an 8% polyacrylamide gel (SDS-PAGE), and transblotted to a nitrocellulose membrane (Bio-Rad). Membranes were blocked for 2 hrs at 4°C in 5% milk (instant skim milk powder)

in PBS-T (0.1% Tween-20 in PBS), incubated overnight at 4°C with primary antibodies (Mouse monoclonal anti-ataxin-3 antibody (1:500) (Chemicon International), and Mouse anti-actin monoclonal antibody (1:10 000) (Chemicon International)) in 5% milk, washed three times for 10 min in PBS-T, and incubated for 1 h at 4°C with secondary antibody (Anti-mouse IgG HRP-conjugated antibody (1:10 000)(Cell Signaling Technology)), followed by three 10 min washes in PBS-T. Immunodetection was performed using the enhanced chemiluminescence (ECL) system (Perkin Elmer Precisely). Membranes were exposed to Kodak BioMax MR Films (GE Healthcare).

2.2.7 *Drosophila* stocks and transgenes

These transgenic flies have been previously characterized within the context of a parallel ataxin-3 study in our lab (Gaspar *et al.*, manuscript in preparation). Here, were will only describe the procedures used to obtain and characterize the *Drosophila* lines necessary for the present study. Flies bearing CAG92 and CAA96 transgenic constructs in a homozygous state were grown at 25°C (or 29°C as stated in the results section) on normal fly food. Adult male flies of these backgrounds were crossed to virgin *gmr-GAL4* flies to obtain lines driving the expression of the transgenic protein in the developing eye. For a control cross, male flies of a w^{1118} background (the original fly stock used for microinjection of the *MJD1* constructs) were crossed with virgin *gmr-GAL4* flies to yield a cross of +/*gmr-GAL4* genotype. Full-length MJD1 constructs used to make transgenic flies are shown in figure 2.1B.

2.2.8 Protein extraction from fly heads

For each fly line (control, CAG92, and CAA96), 30 heads were collected per sample, added to RIPA lysis buffer supplemented with protease and phosphatase inhibitors (same as in cells), homogenized, sonicated twice for 10 sec each, and spun at 10 000g for 5 min at 4°C. The supernatant was transferred to a new tube and protein concentration measured as before. Western blot analysis was performed similarly as in cells.

2.2.9 *Drosophila* model: phenotype characterization

For phenotypic characterization, adult transgenic flies were observed upon eclosion (emergence of the adult insect from the pupal case) and compared to control genotype fly lines. Adult flies at day 10 from each construct and control were mounted onto a slide with nail polish, observed on a Leica MZ 12 dissecting microscope and photos were taken on a Leica DFC 320 camera and digital images assembled using Adobe Photoshop 8.0.

2.4 Results

2.4.1 Distribution and subcellular localization of ataxin-3 in Cos-1 cells

Using the constructs generated, we were able to obtain expression of the ataxin-3 protein in Cos-1 cells. The subcellular localization of the enhanced green fluorescent protein (EGFP)-tagged, full-length ataxin-3 was analyzed by fluorescence microscopy. The wild-type form of ataxin-3 (CAG9) showed expression in both the cytoplasm and the

nucleus (Figure 2.2). Similarly, both expanded polyglutamine ataxin-3 constructs (CAG100 and CAA96) showed diffuse nuclear and cytoplasmic expression. In addition, cells expressing these two expanded constructs also contained inclusions of various sizes both in the nucleus and perinuclear region, a cellular feature common to other polyglutamine diseases. Furthermore, some nuclei of the expanded ataxin-3 (CAG100) expressing cells were of irregular form, most likely representing dying cells. In other cellular models, expression of full-length ataxin-3 with normal and expanded repeats remained diffusely distributed in transfected cells, with most expression found in the cytoplasm and only a small amount in the nucleus (Paulson *et al.*, 1997b; Perez *et al.*, 1998; Paulson et al., 1997a). These differences in subcellular localization probably illustrate that the process is complex and varies depending upon the cell type and other cell type-specific factors (Perez *et al.*, 1998). This being said, our full-length cellular model system, which shows aggregate formation, is in keeping with the mouse and *Drosophila* models expressing the full-length ataxin-3 protein; and it also reproduces the observations made in patients.

2.4.2 Characterization of nuclear inclusions

Inclusion formation in this model was limited to the expanded MJD1 constructs only, and was not detected with the construct of normal repeat length (Figure 2.2). Among cells expressing the expanded form, not all contained inclusions. Inclusions were observed in 24.0% and 14.6% of transfected cells for the CAG100 and CAA96 constructs, respectively. The CAG100 expressing cells are thus significantly more prone to form large visible aggregates than their CAA96 expressing counterpart $F(1,20) = 4.43$ ($P<$

0.05). Both these frequencies are considerably higher than those from other studies, where full-length expanded ataxin-3 was expressed in different cell types and obtained either no aggregate formation in 293T cells (Perez *et al.*, 1998) or aggregates in less than 1% of transfected HeLa cells (Chai *et al.*, 1999b). Furthermore, Toulouse *et al.*, (2005) also experimented with several cell types in their cellular model; both aggregation and frameshifting varied according to cell type. These discrepancies most likely reflect cell-type specific differences, since it is known that aggregate formation and subsequent recruitment of nuclear proteins to form inclusions can vary widely and thus may contribute to selective toxicity in neurons observed in polyglutamine diseases (Chai *et al.*, 2001). These observations underscore the importance of modeling the disease in diverse cell types and model systems, in order to obtain maximum information on the pathological processes involved.

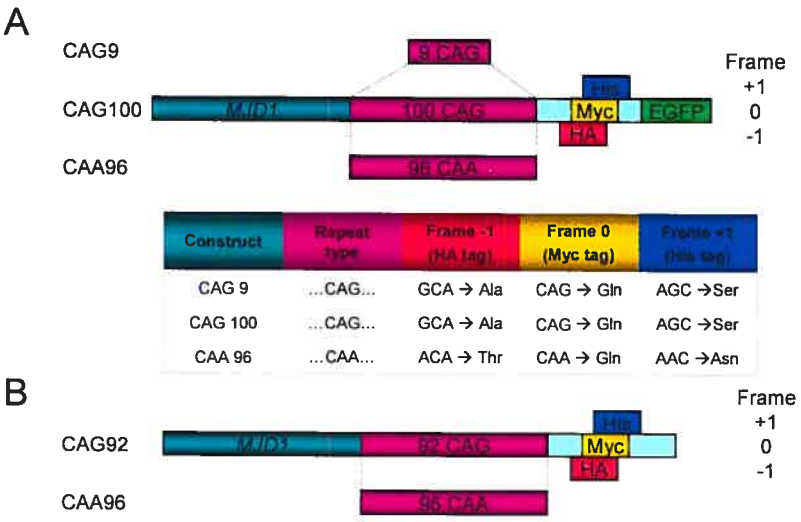


Figure 2.1 Schematic representation of full-length MJD1 constructs. (A) Full-length ataxin-3 protein with various polyglutamine repeat lengths in an EGFP-vector and tagged with three epitopes (HA, Myc, and His), one in each of the reading frames used in the cellular model. (B) Full-length MJD1 constructs used to generate transgenic flies.

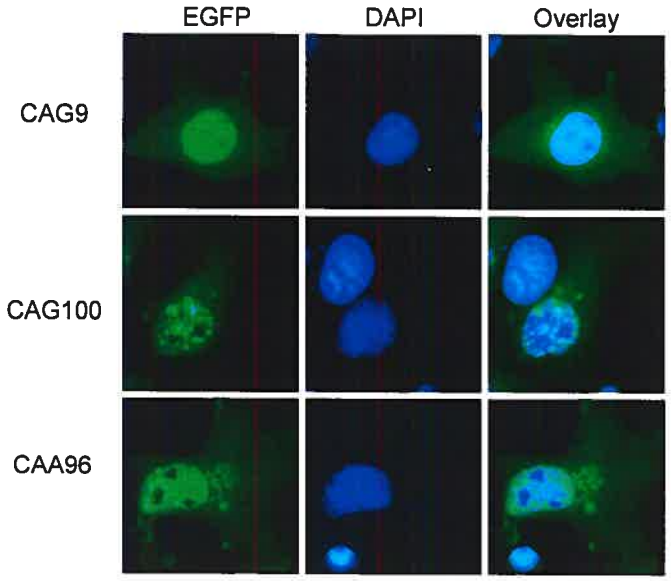


Figure 2.2 Diffuse nuclear and cytoplasmic expression of full-length MJD1 constructs. In Cos-1 cells expressing the normal ataxin-3 protein, CAG9, no aggregates are formed. In cells expressing the mutant ataxin-3 protein, CAG100 and CAA96, polyglutamine inclusions form within the nucleus and in the perinuclear region.

2.4.3 Phenotypic characterization of transgenic flies

Expression of the polyglutamine expanded transgene CAG92 in flies disrupted eye morphology (rough eye phenotype) and pigmentation when directed to the developing eye by using a specific promoter (Figure 2.3). We observed moderately abnormal eyes with the CAG92 fly at 25°C, while at 29°C (a temperature which leads to higher expression of the transgene) we observed severe discoloration of eye pigmentation and rough eye phenotype in flies expressing CAG92. It is important to emphasize that this phenotypic presentation is progressive and ultimately results in almost complete ablation of the eye. Epon embedded sections prepared from the heads of these flies show severe degeneration and loss of ommatidium cells of the eye (Gaspar *et al.*, manuscript in preparation). The CAA96 transgenic fly did not show any phenotypic abnormality in either the eye morphology or pigmentation and was indistinguishable from the non-transgenic, control fly. The reason for this difference may be due to the inability of the CAA96 construct to -1 frameshift during translation of the protein and generate a more toxic polyalanine species, as it happens with the CAG92 construct. This mechanism has been previously explored by our group and will be further discussed in Chapter 3 of this manuscript (Gaspar *et al.*, 2000; Toulouse *et al.*, 2005; Gaspar *et al.*, manuscript in preparation). Alternately, an mRNA toxicity mechanism exclusive to CAG and not to CAA may partly explain these observations, and is currently under investigation in our lab.

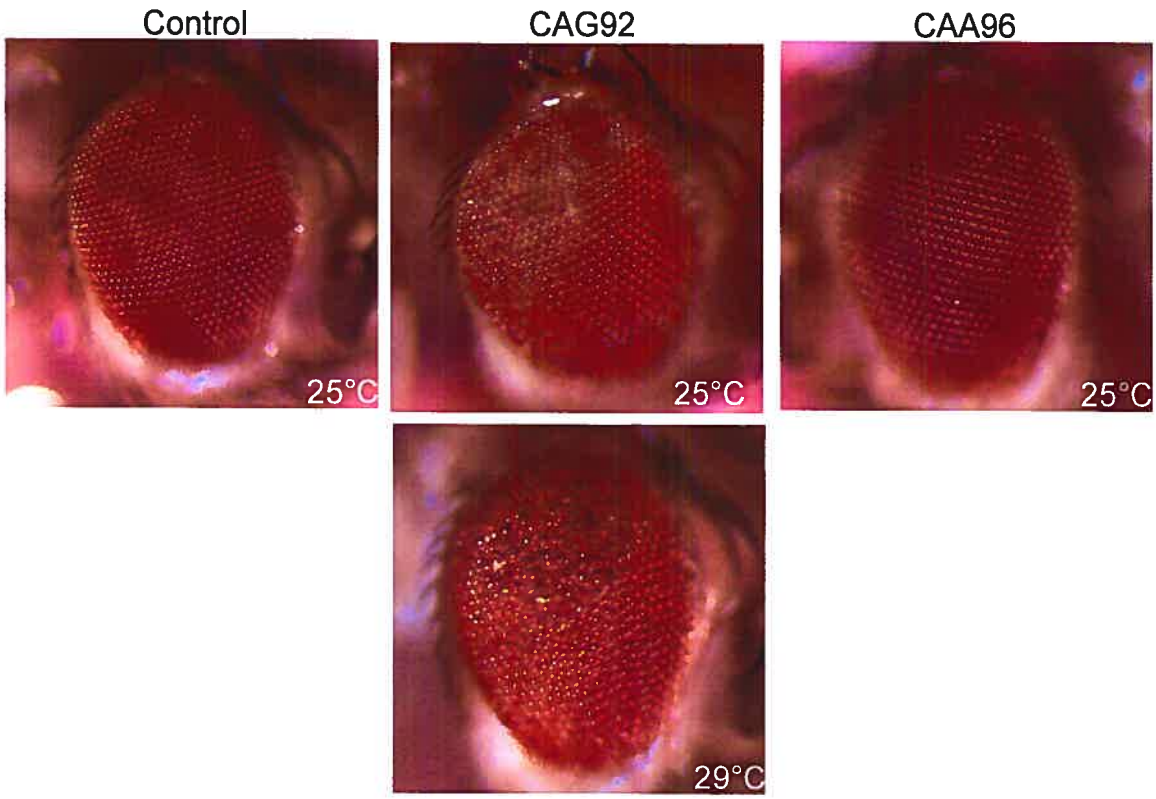


Figure 2.3 Expanded-CAG polyglutamine protein causes adult eye degeneration. Dissecting microscope pictures of the external eyes of adult flies expressing CAG92 or CAA96 in the eye at day 10, compared to control flies. The eyes of CAG92 expressing flies show moderate pigment degeneration compared to control and CAA96 expressing flies cultured at 25°C, while at 29°C (leading to higher expression of the transgene), they show severe discoloration of eye pigmentation and rough eye morphology. The eyes of CAA96 expressing flies displayed no degeneration and appear normal, as in control flies.

2.4.4 Protein expression of ataxin-3 in cells and flies

Western blot analysis was used to confirm expression of the various constructs in transfected cell and fly head protein extracts (Figure 2.4). Using an anti-ataxin-3 antibody, proteins of the expected molecular weights were detected. The endogenous ataxin-3 protein migrated at ~42 kDa in the Cos-1 cell extracts, but is absent in the fly as invertebrates lack the functional homologue of the ataxin-3 protein. In Cos-1 cells, proteins from the wild-type CAG9 construct were detected at ~70 kDa, and proteins from the expanded constructs, CAG100 and CAA96, were detected at ~85 kDa for both.

As for the transgenic flies with similar levels of expression, many observations were made from Western blots. Firstly, the polyglutamine expanded proteins (CAG92 and CAA96) both migrated at their theoretical size of 55 kDa. Secondly, the presence of other bands, most likely minor ataxin-3 isoforms, was also detected at ~60 kDa and ~75 kDa. Thirdly, and more interestingly, extracts of the CAG92 expressing fly also contained insoluble components which remained trapped in the stacking gel during SDS-PAGE. These trapped proteins most likely represent aggregates which are known to be highly insoluble and migrate as high molecular weight complexes (Paulson *et al.*, 1997b). The insoluble components were also observed in the CAA96 fly extract, but at a much lower level. The aggregates of the CAG92 extract most likely contain not only insoluble polyglutamine proteins, but also non-polar, polyalanine proteins, as will be explained later in Chapter 3. Fourthly, in the CAG92 fly, an extra band appears at ~40 kDa which could possibly be a cleavage product of the polyglutamine protein. In the SCA3 mouse model developed by Goti *et al.* (2004), a putative-cleavage fragment of 37 kDa was also

detected in the brains of the polyglutamine expanded ataxin-3 transgenic mice. The identification and characterization of this ~40 kDa band will be performed in order to confirm if it is indeed a cleavage product of ataxin-3 (Gaspar *et al.*, manuscript in preparation).

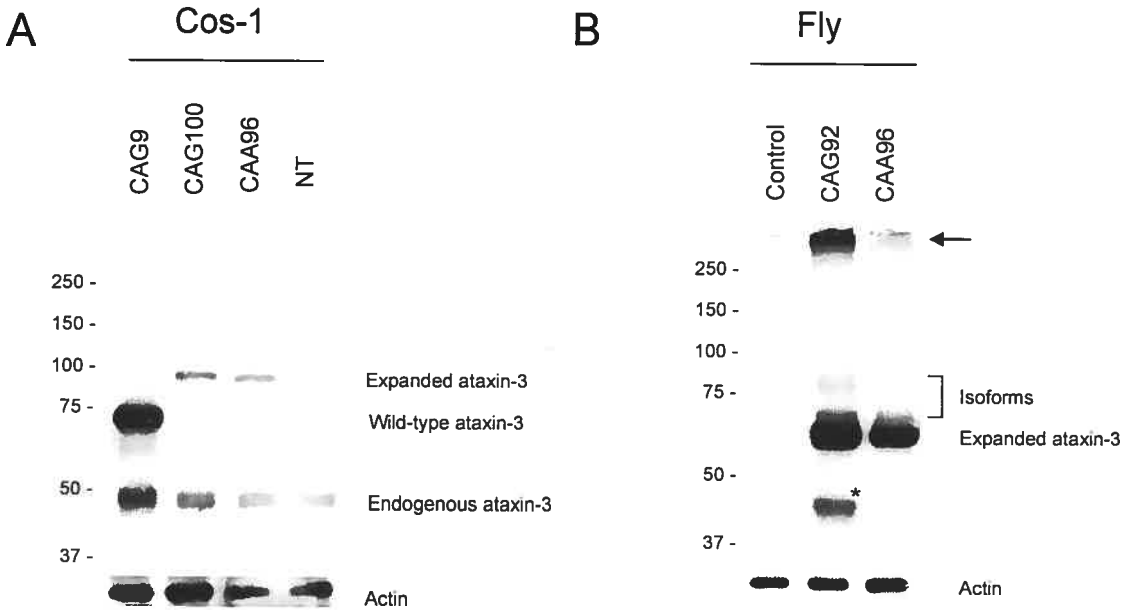


Figure 2.4 Expression of ataxin-3 protein from (A) lysates of transiently transfected Cos-1 cells and (B) fly head extracts. Shown are Western blots using ataxin-3 antibody. (A) Endogenous ataxin-3 protein was detected at a size of ~42 kDa along with the transfected full-length ataxin-3 EGFP-fused protein at ~70 kDa (CAG9), and expanded proteins at ~85 kDa (CAG100 and CAA96). (B) Flies lack the functional homolog of ataxin-3 and show only the size of the transgenic ataxin-3 construct at ~55 kDa. Other minor ataxin-3 isoforms were also detected at ~60 kDa and 75 kDa. The presence of insoluble mutant-ataxin-3 aggregates (arrow) remained in the stacking gel in the fly extracts. A possible cleavage fragment (*) was also detected in the CAG92 fly extract. Non-transfected (NT).

2.5 Discussion

We have generated normal and expanded full-length constructs of the *MJD1* cDNA and successfully expressed them in Cos-1 cells to establish a cellular SCA3 model. With the expanded MJD1 constructs (CAG100 and CAA96), we were able to reproduce key features of SCA3 disease, such as nuclear and cytoplasmic expression with the presence of intranuclear inclusions. In some of the CAG100-expressing cells with very large inclusions, we were also able to observe abnormal nuclear morphology, such as blebbing, characteristic of apoptotic cells. Similar nuclear stress features were also detected by Toulouse *et al.*, (2005), though necrotic morphology was also observed by Evert *et al.*, (1999). These abnormal cells likely represent a later stage of the disease where the inclusions become large and the cell is overwhelmed by their accumulation, thus inducing an apoptotic response. In fact, recent evidence showed that full-length polyglutamine-expanded ataxin-3 activates a mitochondrial apoptotic pathway and induces neuronal death by promoting mitochondrial release of apoptogenic proteins (Tsai *et al.*, 2004; Chou *et al.*, 2006).

Our results are, however, comparable to a recently reported cellular model, which shows that expression of the full-length ataxin-3 protein is largely diffusely distributed within the cells, irrespective of the polyglutamine length (Haacke *et al.*, 2006). Aggregates in this case were detected in 5-10% of cells transfected with the polyglutamine expanded ataxin-3 construct, which is a lower frequency of aggregation than the one we obtained here. It is important to re-emphasize the different cell types used (Cos-1 cells in this study, mouse neuroblastoma cells (N2a) by Haacke *et al.* (2006), 293T cells by Perez *et*

al., (1998), and HeLa by Chai *et al.*, (1999a)) and slightly different constructs due to epitope tags which likely contribute to observable differences.

Initial studies in the field claim that polyglutamine proteins must be proteolytically cleaved in order to translocate to the nucleus and exert their toxic effect (Zoghbi and Orr, 1999). In keeping with this hypothesis, polyglutamine-containing fragments of the ataxin-3 protein have been detected in neuronal inclusions in human brains (Schmidt *et al.*, 1998; Goti *et al.*, 2004). Conversely, several other studies failed to detect any proteolytic cleavage in SCA3 patient brains (Berke *et al.*, 2004, Chou *et al.*, 2006) or transgenic mice models (Cemal *et al.*, 2002). It is clear that a wide range of anti-ataxin-3 antibodies spanning the whole protein are required to determine if proteolytic cleavage plays a role in SCA3 neurotoxicity. *In vitro* models of SCA3 have shown that at least one caspase is able to release a polyglutamine-containing fragment from ataxin-3 (Wellington *et al.*, 1998; Berke et al., 2004); however, a direct relationship in SCA3 between caspase cleavage of a polyglutamine protein and pathogenesis has yet to be demonstrated *in vivo* for SCA3 (Zoghbi and Orr, 1999). In another polyglutamine disease, Huntington's disease, the role of a cleavage fragment in disease progression and NI formation is also controversial. Studies showed that caspase-1 cleaves mutant and wild-type huntingtin *in vitro;* further *in vivo* studies provide functional mechanistic evidence of caspase-1 activation mediated by exon 1 of mutant huntingtin (Ona *et al.*, 1999). Using dominant mutants of caspase-1, they observed that NI formation, specific neuroreceptor abonormalities, and more importantly, symptomatic onset and mortality was delayed, thus prolonging survival in their transgenic mouse model. As stated

previously, the presence of a cleavage fragment in SCA3 and its implication in disease progression is currently being investigated in our lab (Gaspar *et al.*, manuscript in preparation).

Most recently, Haaecke *et al.*, (2006) demonstrated in a cellular model that the introduction of a cleavage site into the full-length ataxin-3 protein increased the number of aggregates and that this was also essential for the sequestration of full-length non-pathogenic ataxin-3. If proteolytic processing of the ataxin-3 protein occurs in our system, the presence of an endogenous cleavage fragment could explain why we observed a higher percentage of cells with inclusions when compared to other studies. Though this particular cleavage fragment could not be detected by Western blot in our cellular model, the finding by Haaecke *et al.* is in keeping with the potentially cleaved ataxin-3 fragment in our fly model. Detection of this fragment in cell culture could simply be a matter of optimizing the conditions of the protein extraction procedure, as was the case for detecting it in our fly model. On the other hand, the differences between an *in vivo* and an *in vitro* system are many and obvious, which could explain any discrepancies observed between the two systems.

In conclusion, although cellular models are versatile and practical for manipulation, they do present drawbacks, especially in studies where massive overexpression of the mutant proteins triggers cell death within days rather than years as seen in the patients. Consequently, these models do not reproduce the processes leading to the slow advancing dysfunction and degeneration of neurons in the human diseases (Michalik and Van

Broeckhoven, 2003), but rather provide insightful clues to certain aspects of protein mechanisms and interactions. Thus, animal models are required to finely dissect the mechanisms of pathology and to provide deeper insight into the disease. In mouse models, for example, researchers were able to reproduce progressive neuronal dysfunction, an occurrence which more closely and accurately mimics the human disease than cellular models (Michalik and Van Broeckhoven, 2003). Despite the practical uses of mouse models in polyglutamine disorders, they too possess limitations as the same mutant protein might not lead to similar devastating phenotypes observed in patients. Another limitation includes the lifespan of the mouse, which might not be long enough to complete the full cascade of changes that follows the initiating event (Gusella and MacDonald, 2000). Nevertheless, all these models are valuable for investigating the pathogenic triggers, as well as for identifying early biochemical targets, even if they may not faithfully illustrate the final stages of cell death (Gusella and MacDonald, 2000).

As for *Drosophila* models of SCA3, such as the one we established, these are especially important as they allow for the study of the gain-of-function hypothesis in neurodegeneration, since invertebrates lack functional homologues of the protein in question (Michalik and Van Broeckhoven, 2003). Drug screening in flies also has its advantages such as low cost, small size, short lifespan, and vast numbers of flies that can be tested at the same time which is important for statistical analysis. This organism also represents a rapid and inexpensive way to obtain pharmacological candidates for testing in mammalian systems (Muqit and Feany, 2002). The best advantage of *Drosophila* models is its potential for large-scale screening methods through mutagenesis in order to

define novel gene activities that can modify the physiological effect of a toxic disease protein *in vivo* (Fortini and Bonini, 2000). The benefits of modeling human diseases in a fly model are relevant due to homologous cellular pathways which are involved in pathogenesis in both organisms. For instance, we and others obtain a progressive degenerative phenotype when we overexpress a foreign mutant human *MJD1* cDNA in the fly; this demonstrates that the two organisms share common pathways and mechanisms. However, caution should also be taken as flies might not model pathology in the same way as in humans due to activation of non-relevant pathways that are specific to invertebrates (Muqit and Feany, 2002). Nevertheless, *Drosophila* models are essential for investigations on the mechanism of disease, and as such, have widely been used to study several other neurodegenerative diseases.

Chapter 3: The role of frameshifting in SCA3

3.1 Introduction: ribosomal frameshifting in SCA3

3.1.1 Similarities between SCA3 and OPMD

Pathological expansions of triplet repeats extend beyond polyglutamine-encoding CAG repeats. Short expansions of polyalanine-encoding GCG repeats have been identified in several genes, namely the poly(A)-binding nuclear protein 1 (PABPN1) gene, which causes oculopharyngeal muscular dystrophy (OPMD) (Brais *et al.*, 1998). In fact, in this particular disease, a very small polyalanine expansion is sufficient for protein aggregation and toxicity (Brais *et al.*, 1998). The presence of inclusions in both SCA3 and OPMD led to the idea that they might share pathophysiological features. Both polyglutamine and polyalanine inclusions are formed similarly through aggregation of the misfolded protein and formation of fibrillar macromolecules (Calado *et al.*, 2000). Other similarities between the two diseases include their late age of onset and specific cell-type degeneration despite widespread expression of mutant protein (Reddy and Housman, 1997; Ruegg *et al.*, 2005). There is also the presence of ubiquitinated protein in the aggregates and the involvement of chaperone and proteasome pathways in both cases (Abu-Baker *et al.* 2003, Gaspar *et al.*, 2000).

3.1.2 Previous studies on frameshifting in SCA3

Based on the similarities between the two diseases, Gaspar *et al.*, (2000) hypothesized that a -1 frameshift error could occur in the CAG tract of *MJD1* which would result in an

alternate alanine-encoding GCA frame. The presence of frameshifted polyalanine species was indeed detected in lymphoblastoid cells and in the nuclear inclusions of pontine neurons from MJD patients (Gaspar *et al.*, 2000). In addition to the *in vivo* results, an *in vitro* model also showed that frameshifts did occur and that their frequency increased with the size of the CAG repeat. Furthermore, when replacing polyglutamine repeats with polyalanine repeats in the same protein, toxicity increased.

A subsequent investigation was carried out by Toulouse *et al.*, (2005) who found that frameshifting was confined exclusively to the –1 frame and that it only occurred in polyglutamine repeats encoded by a CAG codon. The authors showed that the frameshifting error occurs during translation and further evidence was also provided on the increased toxicity of frameshifted polyalanine peptides.

3.1.3 Mechanism of programmed ribosomal frameshifting

The proposed mechanism of translational errors in SCA3 is programmed ribosomal frameshifting. In many viruses, this method is a common occurrence used by the translating ribosome to change reading frames in order to obtain several proteins from the same mRNA (Harger *et al.*, 2002).

The ribosome is a molecular machine which reads the open reading frame (ORF) of the mRNA to faithfully and efficiently translate it into polypeptides. Non-standard coding mechanisms have also been identified: programmed ribosomal frameshifting can occur where the translating ribosome is induced to slide forward one nucleotide (+1 frameshift)

or backwards (-1 frameshift). By doing so, multiple ORFs are utilized from the same mRNA transcript (Shigemoto *et al.*, 2001). Programmed -1 ribosomal frameshifing is common to viral genomes and requires two basic sequence elements: a slippery heptamer sequence and an mRNA structural element, such as a pseudoknot structure or a stem-loop downstream from the slippery sequence (Shigemoto *et al.*, 2001). The 'simultaneous-slippage model' proposes that the repetitive nature of the first *cis*-acting mRNA signal, the 'slippery sequence', allows for the ribosome-bound tRNAs to realign in the -1 frame (Jacks *et al.*, 1988). The second element, the mRNA secondary structure, interacts with the ribosome and causes the ribosome to pause over the slippery sequence. This stalled complex is associated with a helicase which attempts to unwind the secondary structure, but is stereochemically unable to do so. As a result, strain is placed on the tRNA connected to the ribosome resulting in breakage of codon-anticodon interaction. As the tRNA structure is relaxed, it is allowed to re-pair with the mRNA, but this time in the -1 position (Namy *et al.*, 2006). Other *cis*-acting factors important to frameshifting include the spacing between the slippery sequence and secondary structure (Farabaugh, 1996). Though investigations of the mechanism of frameshifting were based on viral models, cases have also been identified in mammalian genes which also use -1 programmed ribosomal frameshifting (Shigemoto *et al.*, 2001).

Another type of -1 frameshifting can also occur in the absence of the slippery sequence. This 'hungry codon' hypothesis requires special structural features of some tRNAs, such as doublet decoding (the recognition of a 2-base codon by a 2-base anticodon), that allow them to promote disruption of the reading frame. This type of decoding might occur as a

result of depletion of particular aminoacyl-tRNAs in cells starved of a particular amino acid (Farabaugh, 1996). It was also shown that the nature of the downstream secondary structure could also act to increase frameshifting in response to aminoacyl-tRNA limitations (Atkinson *et al.*, 1997).

Though it is not known which of these frameshifting mechanisms occurs in SCA3 affected cells, the expanded CAG repeat is known to form long stable hairpin structures in the transcript, meeting the secondary structure requirement in both cases (Michlewski and Krzyzosiak, 2004).

3.1.4 Frameshifting in a full-length context

As mentioned earlier, frameshifting was identified as a contributor to pathogenesis of SCA3 in other models, but all studies so far were performed using a truncated version of the mutant protein (Toulouse *et al.*, 2005). Though it is known that truncated mutant proteins containing the polyglutamine tract are more toxic to neurons and peripheral cells, it is also important to note that the typical gradual central nervous system pathology in these diseases is only seen when full-length proteins are expressed. Therefore, the specific protein context of each mutant protein also contributes to the late onset of polyglutamine diseases and selective neuronal vulnerability (Zoghbi and Orr, 2000). Evidence has shown that the unique protein composition of each expanded polyglutamine protein plays an important role in specifying the details of the pathogenesis, as studies using polyglutamine proteins in the absence of normal protein context are not able to replicate the specific features of disease (Chai *et al.*, 2001). In particular, the

composition of nuclear inclusions differs depending on the protein context of the expanded polygltutamine domain (Chai *et al.*, 2001).

Additionally, results have shown that full-length ataxin-3 proteins were much less efficient at forming aggregates than did C-terminal ataxin-3 fragments with an extended polyglutamine segment, which emphasizes the influence of the full-length sequence context on the aggregation properties of ataxin-3 (Haacke *et al.*, 2006). Therefore, it is important for future studies to examine frameshifting in a full-length context in order to fully understand its contribution to the progression of the disease.

3.2 Objective

In the second part of this study, the objective is to confirm that ribosomal frameshifting does occur using the established full-length ataxin-3 cellular model in an attempt to elucidate its contribution to SCA3 pathogenesis.

3.3 Materials and Methods

3.3.1 Immunocytochemistry

Cells were seeded the day before at a density of 150,000 cells per well on coverslips in 6-well plates. Forty-eight hours after transfection, cells were washed in PBS, fixed for 15 min in 4% paraformaldehyde, washed twice in PBS, permeabilized with 0.2% Triton X-

100 PBS, washed twice in PBS, and blocked for 1 hr with 10% normal goat serum (NGS). The primary antibody (Rabbit anti-HA epitope/fusion tag antibody (Alpha Diagnostic International)) was diluted 1:500 in 2% NGS and incubated at 37°C for 30 min before use. Coverslips were then incubated with the diluted primary antibody at room temperature overnight. The secondary antibody (Rhodamine (TRITC)-conjugated AffiniPure Goat Anti-Rabbit IgG, Fragment Specific (Jackson ImmunoResearch Laboratories)) was diluted 1:300 in 2% NGS and incubated at 37°C for 30 min before use. After washing coverslips three times in PBS, they were incubated in the corresponding diluted secondary antibodies for 1 hr. Cells were washed 5 times in PBS and mounted using DAPI Mounting Medium for Fluorescence (Vector Laboratories). Cells were viewed by using fluorescence microscopy (Leica DM6000) under 400x magnification. The digitally stored images were combined and displayed using OpenLab software and assembled using Adobe Photoshop 8.0.

3.3.2 Quantification of frameshifting

To quantitate frameshifting, we scored all cells that were EGFP and HA-positive in five random microscope fields at 200x magnification. Data are expressed as the percentage of cells with frameshifted proteins (HA-positive) over transfected cells (EGFP-positive).

3.3.3 Statistical analysis

Analysis on frequency of frameshifting was carried out using the One-way Anova of variance test.

3.4 Results

3.4.1 Generation of a cellular frameshifting reporter system

In order to further understand frameshifting within large CAG repeats, we used the full-length MJD1 constructs generated in Chapter 2 of this study in a cellular model as a frameshifting reporter system. In order to do so, we made use of the epiptope tags at the C-terminus of each construct. Three epitopes were present on all constructs and were specific for each reading frame: a Myc epitope in the main frame (polyglutamine), an HA epitope in the –1 frameshifted reading frame (polyalanine), and a His-tag epitope in the +1 frameshifted reading frame (polyserine) (Figure 2.1). It was ensured that a translation initiation site was present only in the main frame so that initiation could not occur in any of the other reading frames. As mentioned earlier, the two CAG repeat lengths were: CAG9 (wild-type) or CAG100 (expanded polyglutamine). In both of these cases, if frameshifting to the –1 frame occurs, the ribosome would shift to read a GCA repeat which codes for alanine proteins. The third construct, CAA96, also codes for expanded polyglutamine, but it differs from the CAG100 construct in that if frameshifting were to occur into the –1 frame, the ribosome would instead read an ACA repeat which codes for threonine.

3.4.2 Detection of frameshifting in a cellular model

The described constructs were transfected into Cos-1 cells and monitored through immunocytochemistry performed using an antibody against the –1 frame (anti-HA epitope). Frameshifting was monitored at 48 hrs post-transfection since it was reported to peak during this period when using the truncated form of ataxin-3 (Toulouse *et al.*, 2005).

Since the constructs express EGFP in the main frame, expression of the main frame ataxin-3 protein could be observed directly under fluorescent illumination.

The presence of –1 frameshifted HA-positive species was observed only in cells with polyglutamine inclusions (CAG100 and CAA96) and not in the wild-type MJD1 construct (CAG9) (Figure 3.1). These frameshifted polyalanine species in the CAG100-transfected cells were expressed as small aggregates which were localized perinuclearly and cytoplasmically and did not completely co-localize with the polyglutamine inclusions. As expected, cells expressing only the -1 frameshifted species were never observed. Though frameshifting to the +1 frame was not examined in this study, it had never been observed in other studies (Toulouse *et al.*, 2005).

It was also observed that the nuclear morphology of CAG100 transfected cells which contained polyalanine species was irregular compared to polyalanine-lacking cells. As for the CAA96 transfected cells, which cannot frameshift into alanine-containing proteins, the nuclear morphology did not display irregularities and was indistinguishable from the CAG9 transfected cells. Therefore, as nuclear morphology is an indication of toxicity, it appears that the production of polyalanine proteins through frameshifting might contribute to cellular toxicity.

3.4.3 Frequency of -1 frameshifting in cells

As not all cells expressing the expanded polyglutamine protein displayed frameshifted ataxin-3 molecules, it was important to establish the frequency of its occurence in order

to determine what the contribution to toxicity might be. In order to determine how frequently frameshifting occurs in the different constructs, transfected cells were counted from several random fields and scored for the presence of HA staining. The frequency of ribosomal frameshifting, or slippage, was calculated as the number of HA positive cells over the number of cells transfected (Figure 3.2). Of the transfected cells, 18.1% of CAG100 and 3.8% of CAA96 expressing cells were positive for the HA staining. Using the One-way ANOVA test of variance, the results show that the difference between the frequencies of ribosomal slippage observed in the CAG100 construct and CAA96 is highly significant. $F(1,20) = 8.47$ ($P<0.01$). Moreover, no significant difference was visible when comparing cells expressing CAG9 with cells expressing CAA96. Therefore, it was confirmed that when using the full-length MJD1 construct, frameshifting does not occur with normal CAG repeat lengths (CAG9). It only occurs significantly with expanded CAG repeat tracts (CAG100) and not with CAA repeat tracts (CAA96). These results are in accordance with those obtained by Toulouse *et al.*, (2005), who also demonstrated using a truncated ataxin-3 construct that expanded-CAG repeats frameshifted significantly more than expanded-CAA repeats.

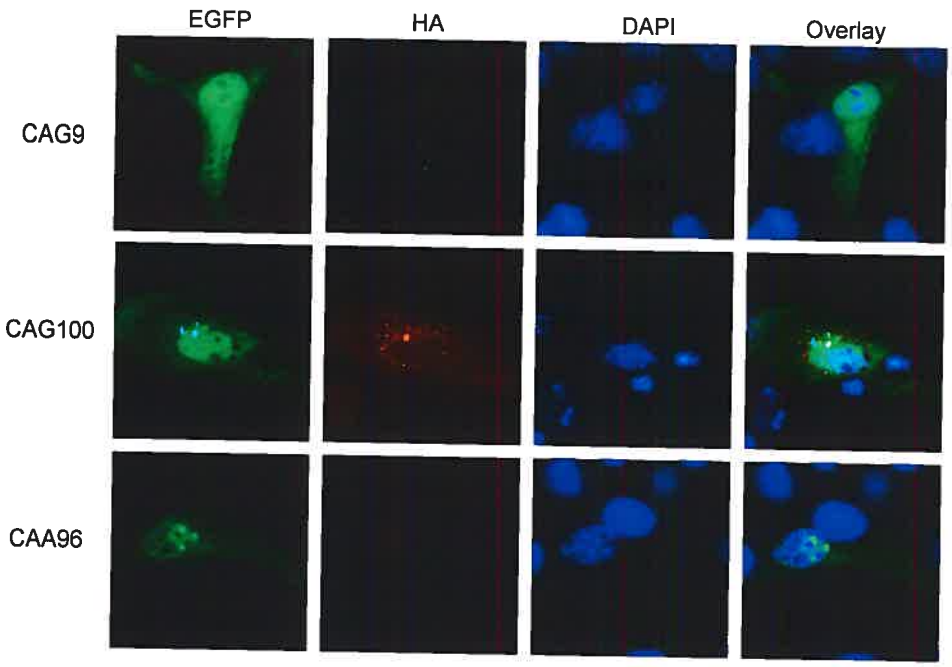


Figure 3.1 Frameshifted polyalanine products are detected in CAG100. Immunocytochemistry on transiently transfected Cos-1 cells with wild-type (CAG9), expanded MJD1 (CAG100), and expanded MJD1 (CAA96) constructs. The presence of -1 frameshifted species was detected by HA-antibody. Nuclear staining was done using DAPI.

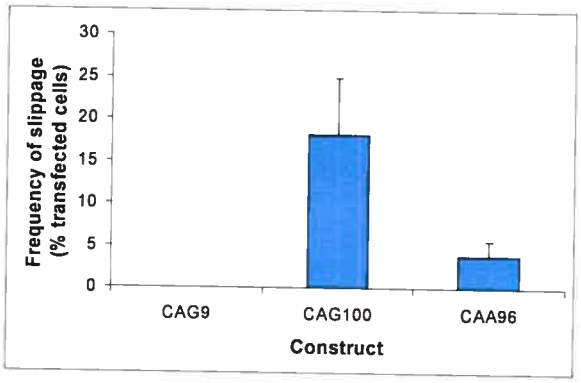


Figure 3.2 Frequency of ribosomal slippage to the –1 frame generating polyalanine proteins occurs significantly more in the CAG100 than in the CAA96 construct. Cell count from immunocytochemistry on transiently transfected Cos-1 cells 48h post-transfection. Frequency of slippage was calculated as number of polyalanine expressing cells (HA-positive) over number of transfected cells (EGFP-positive). Results are presented as the mean of five fields counted ± SE.

3.5 Discussion

In this section of the study, we used the full-length ataxin-3 cell model established in chapter 2 and were able to confirm that ribosomal slippage to the –1 frame does occur within the SCA3 CAG repeats to produce polyalanine proteins. The presence of the polyalanine species in perinuclear and cytoplasmic regions suggests that the proteasome degradation pathway might be overwhelmed and that the cell succumbs to the polyalanine toxicity. This phenomenon is specific only to the expanded CAG construct as it does not occur at a significant level in the expanded CAA construct and it is undetectable with the short CAG construct.

Despite clearly being able to detect the presence of frameshifted polyalanine proteins by immunocytochemistry, the task of quantifying its presence by Western blot analysis remains elusive in this study. Though it has been detected in one cell culture model (Toulouse *et al.*, 2005), the reported study used a truncated ataxin-3 construct in which frameshifting occurs at a much higher frequency (47% of cells). Full-length ataxin-3 proteins do not frameshift as often, leading to lower levels of polyalanine species content and perhaps lending to the difficulty in detection by immunoblotting we have experienced. Other explanations include difficulty in extraction of the insoluble polyalanine species, which might require more stringent extraction conditions (currently underway in our lab).

The reason why frameshifting does not occur to a large extent in CAA96, when compared to CAG100, is most likely due to the nature of the mRNA and its secondary

conformation. Secondary structure and energy requirement analysis comparing the two repeats show that only the CAG repeat forms stable hairpins, whereas the CAA repeat forms a single large loop (Toulouse *et al.*, 2005). Therefore, only in the presence of an expanded CAG repeat would the ribosome be stalled and induced to frameshift to the -1 frame.

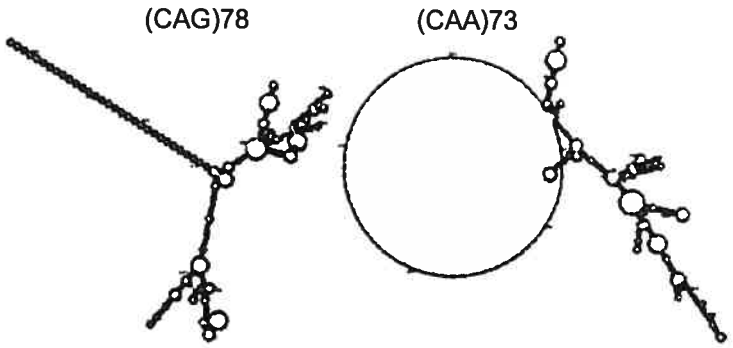


Figure 3.3 Secondary structure predictions for polyglutamine encoding repeats. Only the mRNA of long CAG repeats forms a hairpin whereas the CAA repeats only forms a large single loop, as predicted by the MFOLD program. Toulouse et al., (2005) Hum Mol Genet. 14(18):2649-60.

Although the frequency of frameshifting with a normal CAG length (less than 40) was reported to be ~ 0.7% of the total population of cells, or 5% of transfected cells, in other studies (Toulouse *et al.*, 2005), in our current model, frameshifting was not detected at all in the wild-type construct. Furthermore, when using the truncated version in the previous study, frameshifting occurred in a greater fraction of cells with the expanded CAG construct by comparison to expressing the full-length version (47% vs. 18%, respectively). This was to be expected, as truncation of the protein appears to produce toxic effects faster than when the polyglutamine domain is flanked by the entire protein (Ikeda *et al.*, 1996). Also, it was shown that in the *Drosophila* model developed by Warrick *et al.*, (2005), the ataxin-3 wild-type protein has an intrinsic protective role in

polyglutamine toxicity. In this study, when a normal full-length ataxin-3 protein was co-expressed with a pathogenic full-length ataxin-3 protein in the fly eye, these transgenic flies showed dramatically improved retinal structure and retained vision in comparison to flies bearing the pathogenic allele alone, which showed degeneration of the eye and were blind. Furthermore, the restoration of the eye phenotype mediated by normal ataxin-3 protein was attributed to a delay in nuclear inclusion formation by the pathogenic protein and was dependent on the normal protein's ubiquitin-associated activities. From what was shown by this group, we can conclude that in the absence of all the functional domains of the ataxin-3 protein, it would not be possible to assess its protective role in mediating disease progression, which could reduce or delay the occurrence of frameshifting in our case into the more toxic polyalanine frame.

The contribution of frameshifting to toxicity was inferred by examining the nuclear morphology of affected cells. Only the expanded-CAG repeat induced an abnormal nuclear morphology that suggests cells are undergoing apoptosis. The expanded-CAA nuclear morphology was comparable to the normal CAG repeat. These results also correlate with what we observed in our *Drosophila* model (from chapter 2), where cell toxicity in the eye was seen only in the CAG92 and not the CAA96-expressing flies, which were indistinguishable from control flies. Our previous studies of toxicity used inducible stable transfectants, light scattering properties of cells, and propidium iodide staining to quantify cell toxicity. Though they were performed using a truncated version of the ataxin-3 protein, these studies showed that there was an increase in cell death over

time in both expanded polyglutamine constructs, but more notably in the expanded-CAG construct due to the presence of polyalanine proteins (Toulouse *et al.*, 2005).

Polyalanine polypeptides are known to be toxic. Polyalanine peptides form β-pleated sheet fibrillar macromolecules (Blondelle *et al.*, 1997) and these stable oligomers are extremely resistant to chemical denaturation and enzymatic degradation (Forood *et al.*, 1995). Physiological evidence for this toxicity includes the presence of the PABPN1 mutant protein in inclusions which are also more resistant to salt extraction (Calado *et al.*, 2000). Other studies have shown that polyalanine aggregates are associated with toxicity and cell death (Rankin *et al.*, 2000).

As for polyalanine toxicity in comparison to polyglutamine, there are important differences which must be considered to fully understand their respective involvement in the disease process. It is likely that polyalanine and polyglutamine expansions interact with different subsets of proteins due to different chemical properties, since glutamine is a polar amino acid and alanine is a non-polar amino acid (Rankin *et al.*, 2000). Aggregates of expanded ataxin-3 have previously been shown to be of fibrillar form (Chow *et al.*, 2004). Similarly, aggregates of polyglutamine expanded ataxin-7 were also fibrillar, but in contrast, when its polyglutamine tract was replaced by a polyalanine tract, researchers observed small, numerous, insoluble microaggregates rather than a few large structured aggregates of soluble polyglutamine proteins (Latouche *et al.*, 2006) which correlate with what we observed here with the HA staining in expanded-CAG constructs. Furthermore, the constructs expressing the polyalanine tail led to significantly greater

neuronal loss than polyglutamine containing constructs of the same protein (Latouche *et al.*, 2006). This provides further evidence of the way these proteins containing alanine polymers accumulate and the resulting toxicity of polyalanine aggregates.

Therefore, all these combined reports, together with our results, provide strong evidence pointing towards the increased toxicity of polyalanines over polyglutamines (Rankin *et al.*, 2000; Gaspar *et al.*, 2000; Toulouse *et al.*, 2005; Latouche *et al.*, 2006). In a recent study investigating the toxicity of polyalanines, researchers found that at high doses, polyalanines were indeed harmful to cells; surprisingly, however, at low doses polyalanines could protect against polyglutamine toxicity (Berger *et al.*, 2006). This group generated transgenic flies expressing either short (normal) or long (pathogenic) polyalanines fused to a nuclear localization signal. When the pathogenic transgene was expressed in the nervous system, they observed abnormal behaviour manifestations and reduced survival to adulthood in comparison to normal flies. And, when the pathogenic transgene was expressed in the eye, they observed an abnormal eye phenotype which was associated with inclusion formation, thus verifying the toxic effects of polyalanines. On the other hand, the effect of polyalanines at low doses was shown to be protective against polyglutamine toxicity. When this same group used a cell model to co-transfected a pathogenic polyalanine peptide at a low dose with an N-terminal fragment of the huntingtin protein (the mutant protein in Huntington's disease) containing a pathogenic length of polyglutamines, they observed a decreased toxicity accompanied by a decrease in inclusion formation. This protective, anti-apoptotic effect was mediated by induction

of a heat-shock response by polyalanine. Their results were also confirmed *in vivo* in a *Drosophila* model (Berger *et al.*, 2006).

Further detailed investigations of our cellular model would be required to determine the level of polyalanine expression in frameshifted cells that also contain polyglutamine proteins, in order to test whether this protective effect is also present in SCA3. It would be difficult to relate this effect to SCA3 patient brain tissue as the amount of polyalanine proteins in affected neurons would be difficult to quantify in order to compare its toxicity at low and high doses. Nevertheless, this polyalanine protective effect investigated by Berger *et al.*, appears to be independent of the protein context and could add to the previously reported protective effect of normal ataxin-3 (Warrick *et al.*, 2005).

In conclusion, we developed a system to investigate a unique aspect of the pathology of SCA3 and possibly other CAG-repeat diseases. In light of the evidence showing differences in disease manifestations in a full-length protein context, this new cellular frameshifting model should more accurately represent cellular processes in the disease progression; furthermore, it will be used to explore potential therapeutic treatments. Likewise, our SCA3 fly model (mentioned in Chapter 2), also generated using the full-length gene tagged in three frames, is currently being used to investigate the contribution of frameshifting to disease developement in a living organism (Gaspar *et al.*, manuscript in preparation). This *in vivo* model will complement well what we have shown in the cellular model presented here in an attempt to understand the pathogenesis of SCA3 in humans.

Chapter 4: Transcriptional dysregulation in polyglutamine disorders

4.1 Introduction: the study of transcriptional impairment

4.1.1 Sequestration of transcriptional activators by inclusions

An early component of polyglutamine toxicity appears to be altered gene expression at the transcriptional level. In order to discern the mechanisms by which this process might occur, researchers have looked into factors involved in transcriptional activity. The study of transcriptional regulator involvement in polyglutamine diseases has mainly focused on cellular proteins which possess glutamine repeats, as these domains would be prone to forming homotypic glutamine-glutamine interactions with the pathogenic polyglutamine repeats (Michalik and Van Broeckhoven, 2003). Due to the nuclear localization of both transcription factors and mutant polyglutamine proteins, there is heightened interest in identifying aberrant interactions between these two types of proteins (Chai *et al.*, 2001). Examples of glutamine-rich transcriptional regulators include cAMP-response element binding protein (CBP), TATA-binding protein (TBP), TBP-associated factors ($TAF_{II}130$), and Specificity protein 1 (Sp1); these have all been identified as factors which interact directly with mutant polyglutamine proteins and are sequestered into inclusions in cellular and animal models of these diseases, and in patients' tissue (Michalik and Van Broeckhoven, 2003). Consequently, the suppression of functional activity of these elements could repress transcription and contribute to pathogenesis (McCampbell *et al.*, 2000). Though it is possible that depletion of these nuclear factors

from the nuclear matrix could attenuate transcription, there is also reason to believe it might not be the sole cause of this observation. For example, the lack of histone acetyltransferase activity by CBP could be supplemented by any of the other coactivators with the same activity (Okazawa *et al.*, 2003). Also, some types of inclusion bodies (such as those in SCA3) are dynamic and allow for import and export of proteins, which suggests that nuclear proteins are not permanently sequestered in inclusions (Chai *et al.*, 2002). Therefore, in light of these observations, the sequestration hypothesis may require further revision in order to explain transcriptional repression by inclusions.

4.1.2 Inhibition of histone acetyltransferase activity

Another mechanism of transcriptional dysfunction involves harmful interactions between the expanded polyglutamine tracts and the acetyltransferase domains of several histone acetylases, such as CBP, p300, and P/CAF (Steffan *et al.*, 2001). As these enzymes are responsible for the acetylation of histones, they function to maintain chromatin in a transcriptionally active state. Impairment of their actions thereby compromises transcription and contributes to polyglutamine pathogenesis. As further proof, researchers have successfully shown that by blocking the action of histone deacetylases, thereby increasing acetylation of histones, neurodegeneration can be reduced in *Drosophila* and cellular models of polyglutamine toxicity (McCampbell *et al.*, 2001; Steffan *et al.*, 2001). Thus, the decrease in histone acetylation could be a common mechanism of transcriptional repression in polyglutamine disorders.

4.1.3 Direct co-repressor activity

An additional mode of transcription dysregulation in expanded CAG disorders could be mediated by the mutated protein in question. It has been found that several of the polyglutamine disease proteins can function directly to repress transcriptional activity. For example, in SCA1, the AXH domain of the wild-type ataxin-1 protein is responsible for transcriptional repression and participates in protein aggregation (de Chiara *et al.*, 2005). Another example includes atrophin-1, the mutant protein in DRPLA, which also has the role of repressing transcription as part of its normal function (Wood *et al.*, 2000). Therefore, perturbations of the normal function of these mutant proteins would suggest that altered transcription may be a major cause of cellular dysfunction in polyglutamine diseases.

4.1.4 Transcriptional impairment factors in SCA3

As for SCA3, a multitude of transcriptional changes are to be expected in the presence of mutant ataxin-3 protein, not only through aberrant interactions with important transcription factors, but also through loss of normal transcriptional regulator activity of the protein.

Firstly, transcription factors, especially those containing a glutamine domain, have been shown to be recruited to inclusions and therefore could impair transcription due to a suppression of their normal activity. Transcription factors that have been studied in SCA3 and are believed to be involved in cellular dysfunction in this disorder include TBP, $TAF_{II}130$, Sp1, CREB and CBP (Yamada *et al.*, 2000); these have all been found to

co-localize with nuclear inclusions in SCA3 brain tissue (Shimohata *et al.*, 2000). In the case of the transcription activator CBP, it is known to be implicated in neuronal responses to nerve growth factors (Liu *et al.*, 1998); the lack of its functional activity probably diminishes its ability to mediate signals critical for neuronal survival (McCampbell *et al.*, 2000). These results suggest that nuclear depletion of essential transcription factors containing a glutamine-repeat domain could be the cause for neurodegeneration in affected cells.

This glutamine-specific sequestration even seems to be the case in invertebrate models. In *Drosophila*, eyes absent (EYA) protein is a transcriptional activator important in eye development, but more importantly it contains a polyglutamine tract near the N-terminus (Bonini *et al.*, 1993). By virtue of this polyglutamine domain, it too is recruited into nuclear inclusions (Perez *et al.*, 1998).

On the other hand, transcription factors lacking a polyglutamine domain are also recruited to inclusions. Transcriptional activators or co-activators MAML1 (Chai *et al.*, 2001), and PML (Yamada *et al.*, 2001b) all appear to be targeted to long polyglutamine tracts of ataxin-3 and are recruited to inclusions.

Secondly, transcriptional impairment is believed to be involved in SCA3, since altered expression of several genes is observed in the presence of mutant ataxin-3 protein. Gene profiling of mutant ataxin-3 expressing cells and SCA3 patient brain tissue shows differentially expressed genes involved in the inflammatory reactions, nuclear

transcription, and cell surface-associated processes (Evert *et al.*, 2003). Expanded ataxin-3 protein also causes transcriptional dysregulation of apoptotic factors Bax and Bcl-X_L by either direct interaction with transcription factors or through sequestration of crucial transcription factors linked to these apoptotic molecules, thus inducing neuronal cell death in SCA3 (Chou *et al.*, 2006). It is not known, however, whether these observed changes occur due to the presence of the mutant protein, or if they simply reflect consequences of the pathogenesis.

Finally, direct repressor activity by the mutant protein could also bring about SCA3 pathology. As mentioned earlier, ataxin-3 is a histone-binding protein with two independent co-repressor activities (Li *et al.*, 2002). Any perturbation of its endogenous function could lead to transcriptional impairment.

4.1.5 Transcriptional impairment through altered RNA polymerase II function

RNA polymerase II (RNAPII) is the central enzyme responsible for generating mRNA transcripts. As it is a critical molecule for transcription, any disturbance of its function would be predicted to altered gene expression. The functional activity of RNAPII is of interest since it can be affected by cellular stress, such as in the presence of misfolded proteins and inclusions. Nuclear factors which bind to polyglutamine disease proteins and are shown to interact with RNAPII include polyglutamine binding protein-1 (PQBP-1), CA150, 'survival of motor neurons' protein (SMN), CBP, and TBP (Okazawa, 2003); the last two of which have been shown to be sequestered in SCA3 inclusions. Studies in

other polyglutamine diseases, such as SCA1 and Huntington's disease, have reported altered activity of the RNAPII (Okazawa *et al.*, 2002; Luthi-Carter *et al.*, 2002). Though it is not known by which mechanism the function of RNAPII is affected in SCA1 and Huntington's disease, it is quite possible that it could be one shared by other polyglutamine diseases and, therefore, there is good reason to believe that RNAPII could be involved in SCA3 pathology as well.

4.1.6 Structure and function of RNAPII

The human RNAPII is composed of 12 subunits (Kershnar *et al.*, 1998). The largest subunit of the mammalian RNA polymerase II consists of a C-terminal domain (CTD) which is comprised of an evolutionarily conserved consensus sequence, Tyr-Ser-Pro-Thr-Ser-Pro-Ser (YSPTSPS) (Corden, 1990). The number of repeats varies depending on the species: 20 in yeast, 45 in flies and 52 in mammals (Palancade and Bensaude, 2003). Five of the seven amino acids of the consensus sequence can be phosphorylated (Jones *et al.*, 2004) and phosphorylation correlates with the level of activity of the polymerase: initiating polymerases are hypophosphorylated (II_O), whereas elongating polymerases are hypherphosphorylated (II_A) (Cadena and Dahmus, 1987; Payne *et al.*, 1989).

Phosphorylation patterns differ during the transcriptional process: Ser5 phosphorylation is associated with the promoter at initiation, whereas Ser2 phosphorylation is associated with elongation in the coding region (Kormanitsky *et al.*, 2000). The kinases responsible for phosphorylation include CDK9/cyclin T1 (pTEFb), which phosphorylates Ser 2 (Marshall and Price, 1995), and CDK7/cyclinH/MAT1, which phosphorylates Ser5

(Rodriguez *et al.*, 2000; Schroeder *et al.*, 2000). Their actions are opposed by the following phosphatases: TFIIF-associated CTD phosphatase (Fcp1), Ssu72, type 1 protein phosphatases (Pp1), and small CTD phosphatase (Scp1) (Yeo *et al.*, 2003; Palancade and Bensaude, 2003).

In addition to regulating transcription, CTD phosphorylation also mediates binding partners involved in mRNA processing steps (Proudfoot *et al.*, 2002). CTD phosphorylation is important in mRNA synthesis as Ser5 phosphorylation mediates recruitment and activation of the 5' capping enzyme and promoter clearance (Ho and Shuman, 1999), and Ser2 phosphorylation is involved in recruitment of 3'RNA processing factors and efficient 3' cleavage and polyadenylation (Ahn *et al.*, 2004; Ni *et al.*, 2004). Another role for phosphorylation of this domain is to recruit chromatin modifying enzymes (Palancade and Bensaude, 2003).

The RNAPII phosphoisoforms II_A and II_O co-exist in dynamic equilibrium, regardless of transcriptional state of the cell (Palancade and Bensaude, 2003). In conditions of cellular stress, such as exposure to toxins or UV radiation, the levels of RNAPII in the hyperphosphorylated form increase (Palancade and Bensaude, 2003), most likely to augment transcription of essential survival genes. As polyglutamine toxicity is associated with cellular stress and transcriptional disturbances, we sought to investigate RNAPII's functional activity through its phosphorylation level in affected SCA3 cells.

4.2 Objective

The third objective of this study is to determine if transcriptional dysregulation by RNAPII plays a role in SCA3 using both the established cellular and the *Drosophila* model.

4.3 Materials and Methods

4.3.1 Immunocytochemistry

Immunocytochemistry was performed as previously described in Cos-1 cells transfected with all three constructs. The primary antibody used for immunocytochemistry was RNA polymerase II H5 monoclonal antibody (1:500) (Covance Research Products) with the secondary antibody Alexa Flor 488 donkey anti-mouse (1:300) (Molecular Probes).

4.3.2 Quantification of inclusions

To quantitate the expression of phosphorylated RNAPII, we counted all transfected cells that were positive for H5 staining in six random microscope fields at 200x magnification. Data are expressed as the percentage of transfected cells with H5 expression.

4.3.3 Statistical analysis

Analysis of the frequency of phosphorylated RNAPII expression in cells was performed using the One-way Anova of variance test.

4.3.4 Protein extraction and Western blot analysis

Forty-eight hours after transfection, Cos-1 and HeLa cells transfected with all three constructs were harvested the same as described in chapter 2. Western blots were also performed same as previously described in chapter 2, except membranes were blocked and antibodies diluted in 5% BSA (Fisher Scientific) in PBS-T (0.1% Tween-20 in PBS). The primary antibodies used in this section were Polyclonal RNA polymerase II CTD repeat YSPTSPS (phospho S2) antibody (1:1000) (abcam), Purified mouse anti-RNA polymerase II monoclonal antibody [CTD4H8](1:1000) (BD Biosciences), and Mouse anti-actin monoclonal antibody (1:10 000) (Chemicon International). The secondary antibodies used were Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (1:10000) (Jackson ImmunoResearch Laboratories) for the first primary antibody and Anti-mouse IgG HRP-linked antibody (1:10 000)(Cell Signaling Technology) for the other primary antibodies.

4.4 Results

4.4.1 Expression of phosphorylated RNAPII in cells

Immunocytochemistry was carried out using the monoclonal H5 antibody, which is specific for the hyperphosphorylated form of RNAPII at Ser2, but is also known to bind at Ser5 phosphorylation site (Jones *et al.*, 2004). This antibody was used to detect the presence of RNAPII molecules engaged in active mRNA synthesis (Palancade and Bensaude, 2003). Expression of phosphorylated RNAPII occurs throughout the nucleus with larger punctuated nuclear structures that match transcription foci (Figure 4.1). It has

been reported that these 'dots' overlapped with bromouridine incorporation, indicating that they represent active sites of *in situ* transcription (Zeng *et al.*, 1997). In our system, staining of H5 in all three constructs - normal (CAG9) and expanded forms (CAG100 and CAA96) - all showed similar expression patterns. In an attempt to determine if the number of cells which expressed phosphorylated-RNAPII differed between the constructs, the total number of transfected cells were counted from several fields and scored for positive H5 expression; however, results showed that they did not significantly differ (Table 4.1). As for nuclear inclusions present in the expanded forms, they rarely co-localized with the transcription foci, indicating that hyperphosphorylated RNAPII is not recruited to the inclusions.

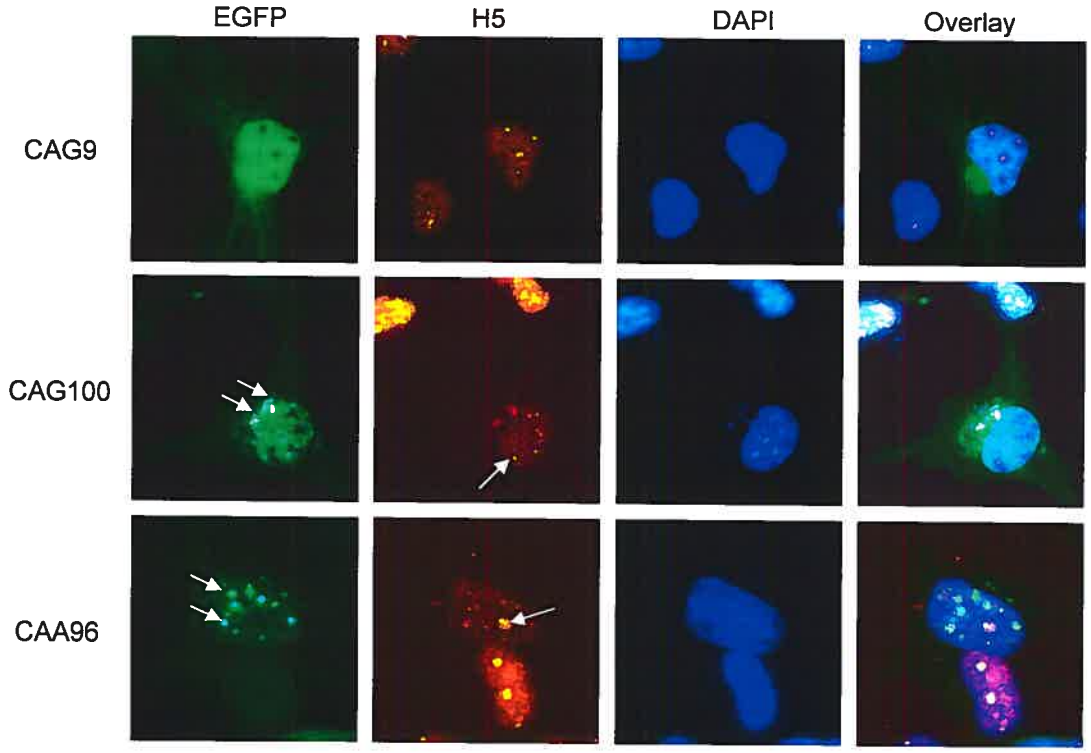


Figure 4.1 Detection of phosphorylated RNA polymerase II by H5-antibody (orange) by immunocytochemistry on transiently transfected Cos-1 cells. H5 staining shows nuclear distribution with punctuated transcriptional foci (arrows in H5) which do not co-localized with nuclear inclusions (arrows in EGFP) (green). DAPI staining of nuclei (blue) is shown in the third column of panels and the last column of panels show merged images of EGFP, H5, and DAPI staining.

Table 4.1 Percentage of transfected cells with phosphorylated RNAPII expression.

Construct	Percentage of transfected cells (%)
CAG9	69.3
CAG100	48.9
CAA96	46.9

4.4.2 Quantification of RNAPII phosphorylation at the protein level

Western blot analysis was performed on Cos-1 and HeLa cells transfected with the three constructs described previously using a polyclonal antibody against the phosphorylated Ser2 of the CTD repeat of the RNAPII (PS2) and another monoclonal antibody against both the hyper- and hypophosphorylated Ser5 form of the CTD repeat (CTD4H8) (Figure 4.2). The results with the PS2 antibody show that in both cell lines there is no noticeable difference in the levels of expression of activated form of RNAPII in the mutant ataxin-3 construct (CAG100) compared to the others. The same results were produced using the Ser5 specific antibody (CTD4H8). In this case, the higher molecular weight band (II_O) represents the hyperphosphorylated form at 240 kDa, while the smaller band (II_A) represents the hypophosphorylated form at 210 kDa. There does not appear to be any shift between the two II_A to II_O forms in either constructs.

Using the same two antibodies on Western blots obtained from our transgenic fly model, similar results were obtained. Both transgenic flies (CAG92 and CAA96), as well as the control fly, showed the same level of phosphorylated RNAPII expression. Therefore, since we do not see a difference in the phosphorylation state between wild-type and mutants, we do not have reason to believe that the functional activity of RNAPII is compromised in SCA3.

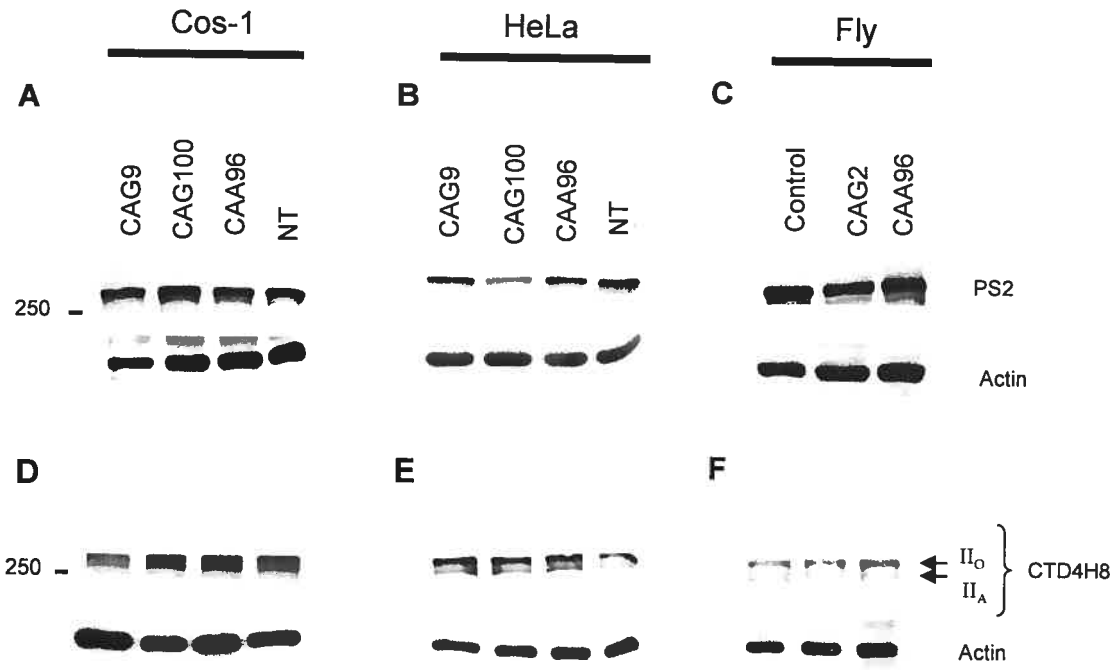


Figure 4.2 Phosphorylated RNA polymerase II protein levels do not show differential expression in mutants. Western blot on transiently transfected Cos-1 cells (A, D), HeLa cells (B, E), and transgenic flies (C, F). The PS2 antibody recognizes the phospho-Ser 2 of the CTD consensus sequence, YSPTSPS; the CTD4H8 antibody recognizes both the hypophosphorylated (II_A) and hyperphosphorylated (II_o) form at position Ser 5 of the CTD repeat. Non-transfected (NT).

4.5 Discussion

In this chapter of the study, we sought to investigate a functional link between SCA3 and altered transcription. In particular, we investigated transcriptional dysregulation as a mechanism of pathology by specifically analyzing RNAPII function. We investigated the activity of RNAPII by using antibodies specific for the two main phosphorylation sites on the serine residues. Our results show that neither the active RNAPII is recruited to nuclear inclusions formed by the mutant protein, nor is the level of phosphorylation of RNAPII affected in our polyglutamine mutants. Therefore, we do not believe that the transcriptional activity of the enzyme is impaired in the presence of mutant ataxin-3 protein. Though results from some studies suggest that the level of phosphorylation may not correlate exactly with transcriptional activity, it is known that hyperphosphorylation occurs together with initiation of transcription and the elongating RNAPII (Zeng *et al.*, 1997).

Reports in other polyglutamine diseases such as SCA1 have found that a ternary complex containing mutant ataxin-1 and PQBP-1 did not sequester RNAPII into inclusion bodies, but did reduce levels of the active species (Okazawa *et al.*, 2002). In that study, since the RNAPII was also not sequestered to inclusion bodies, it was suggested that the pathology could be mediated by the soluble and not the inclusion-forming fraction of the mutant polyglutamine protein. By Western blot analysis, the authors observed a decrease of phosphorylated RNAPII in cells which expressed both PQBP-1 and mutant ataxin-1 in transgenic mouse brains. The interaction between these three proteins substantially represses basal transcription.

In another study, researchers investigated RNAPII function in Huntington's disease (HD), a polyglutamine disease with many similar features to SCA3 (Luthi-Carter *et al.*, 2002). In HD transgenic mice, RNA polymerase II large subunit (RPIILS) protein levels were decreased in cerebellum of mutant mice, whereas they were found to be increased for RPIILS mRNA levels in the same brain region. However, RPIILS protein levels were increased in striatum and cortex. Contrary to results in SCA1, RPIILS did co-localize with huntingtin protein in the majority, but not all, of the cells which contained nuclear and cytoplasmic aggregates. However, it is unknown if sequestration of RPIILS is sufficient to influence transcription of key neuronal genes. It is also unknown if the interaction is direct or through other transcriptional proteins that are sequestered.

Furthermore, even though it has been reported that sequestration of some transcription factors could lead to the transcriptional suppression of specific genes, results in SCA1 and HD show that the presence of inclusion bodies is not essential for repressing general transcription (Hoshino *et al.*, 2004). It is therefore more plausible that mutant proteins disturb transcription by affecting nuclear factors in the nucleoplasm outside of inclusion bodies (Hoshino *et al.*, 2004).

Therefore, as RNAPII function does not appear to be altered in SCA3, this illustrates that transcriptional dysregulation as an underlying cause for cellular dysfunction does not occur through the same mechanism for all polyglutamine diseases. Transcriptional impairment is still believed to be one of the causes of SCA3 pathogenicity, as several

transcription factors have been implicated in this disease. Further investigation of transcriptional dysregulation in SCA3 should consider other transcription factors implicated in polyglutamine diseases, or look at splicing factors and mRNA processing factors, as they also have dynamic relocalization patterns within the nucleus which are dependent upon transcriptional activity of the cell (Zeng *et al.*, 1997). Since transcriptional impairment could be responsible for the degeneration observed in affected neurons, and since we have shown that in SCA3 this impairment seems to be independent of RNAPII activity alterations, characterizing the mechanisms by which it might occur would be important to understand the cellular pathology as well as to identify potential therapeutic targets for SCA3 and other polyglutamine disorders.

Chapter 5: General conclusion

5.1 Summary of this study

In the work presented here, we accomplished the following:

(1) We generated three full-length MJD1 constructs of different polyglutamine repeat lengths and types, and tagged them in each reading frame.

(2) We characterized the expression of these constructs in Cos-1 cells to establish a cellular model of SCA3.

(3) We also characterized a *Drosophila* SCA3 model, previously established in our laboratory, in order to use the model for further experiments.

(4) We confirmed the occurrence of ribosomal frameshifting in our full-length SCA3 model.

(5) We investigated a mechanism of transcriptional dysregulation in SCA3 using both our cellular and *Drosophila* SCA3 models and concluded that transcriptional impairment occurs independently of altered RNAPII function.

These results are relevant and important for several reasons. Firstly, developing a cellular model of a human disease is important for the purpose of dissecting key features of this pathology. Our cellular SCA3 model, together with models developed by others in the field, is an essential tool to help understand aberrant processes leading to cellular dysfunction, which cannot easily be studied otherwise. Furthermore, it has been shown that each SCA3 model published so far has unique features and adds to the knowledge of the disease mechanism in SCA3 and other expanded-CAG repeat disorders. The

Drosophila SCA3 model we characterized will enable the *in vivo* study of disease features and allow for the testing of other hypotheses, as we have done here. Experiments using our fly model complemented our cellular model, as the basic responses to cellular insults are conserved between invertebrates and humans (Driscoll and Gerstbrein, 2003); also, dissection of the mechanism of toxicity in the fly will provide better insight into the pathology of SCA3, as the *in vivo* model is one step closer to human subjects. Overall, cellular and animal models will be most advantageous for the identification of molecular targets and drug testing of emerging therapeutic strategies; they can also be used in genetic screens for modifier genes and modulating phenotypes, which can be used to prevent or improve clinical manifestations of the neurodegenerative processes of polyglutamine disorders.

Secondly, confirming the occurrence of ribosomal frameshifting using full-length MJD1 constructs in our SCA3 model provides sturdier support for the implication of polyalanines in SCA3 disease progression. Ribosomal frameshifting is a novel aspect of polyglutamine disorders, whose consequences have harmful implications which extend beyond SCA3, as this mechanism could also be present in numerous other CAG-repeat disorders. Detailed investigation of polyglutamine and polyalanine toxicity in SCA3 will lead to a better understanding of other proteinopathies caused by these proteins.

Lastly, several studies have shown that altered gene expression is implicated in the pathology of many neurodegenerative disorders. As transcriptional dysfunction is one of the earliest components of polyglutamine toxicity, understanding how it might occur

would be most beneficial to the development of therapies which could prevent any of the initial pathological manifestations at the cellular level.

5.3 Opportunities for therapeutic intervention

Although much progress has been made in elucidating the molecular and cellular mechanisms that underlie the pathology of SCA3 and other related disorders, effective treatments have not yet been developed. Research on the development of therapeutic strategies is currently underway in several of the areas described below.

5.3.1 Misfolded protein

Targeting abnormal protein conformation by pharmacological means would be useful for disease prevention as it would be applicable to all polyglutamine diseases (Gusella and MacDonald, 2000) and even polyalanine diseases, since they both involve misfolded proteins. The use of mouse and fly models has shown that many of the proteins involved in neurodegenerative diseases can take on misfolded conformations when mutated (Zoghbi and Botas, 2002). Furthermore, protein misfolding underlies many unrelated neurological diseases and therefore perturbations of its clearance might be a common pathogenic event (Zoghbi and Botas, 2002).

5.3.2 Cellular defense system

The cellular defense mechanism can be enhanced using drugs which activate chaperones and stimulate proteasome activity selectively (Ross and Poirier, 2004). Chaperone overexpression can confer protection by inhibiting signal transduction leading to cell

death, by suppressing stress kinases (Gabai *et al.*, 1997), preventing caspase activation (Zhou *et al.*, 2001), or by coating misfolded proteins and preventing them from interacting with other factors (Zoghbi and Botas, 2000). In fact, both Hsp70 and Hsp40 were identified as modifiers of SCA3 and were shown to improve disease phenotypes in a *Drosophila* model (Warrick *et al.*, 1998).

5.3.3 Modulation of aggregation

As the debate over whether toxicity is mediated through the soluble or insoluble forms of the mutant protein is still ongoing, it also remains unclear whether aggregates would be a good target for therapies. Yet, the use of aggregation-inhibiting and promoting compounds would be an alternative therapeutic target for these diseases (Gusella and MacDonald, 2000). Chemical chaperones, synthetic peptides, intracellular antibodies and certain chemicals, such as the azo-dye Congo red, can prevent oligomerization, aggregation and also disintegrate existing aggregates, leading to significant improvement of motor performance and extended lifespan (Michalik and Van Broeckhoven, 2003; Sanchez *et al.*, 2003). In fact, inhibitors such as Congo red and cytamine were shown to reduce aggregation by 33% and 66%, respectively, in a Huntington's (HD) cellular model. These compounds were further validated in a *Drosophila* model, in which rescue of neuronal degeneration was demonstrated (Apostol *et al.*, 2003). Of interest to our study is the use of cytamine, since it prevents aggregation through inhibition of transglutaminase, an enzyme shown to be required for the formation of polymers in HD (Kahlem *et al.*, 1998). If indeed aggregation in SCA3 is dependent on transglutaminase

activity, this drug could distinguish the effects of aggregates formed by glutamines versus alanines.

Another method might be to target the nuclear localization signal of the affected proteins, since they accumulate within the nucleus in SCA3. It has been shown in SCA1 that when the mutant protein is expressed in the cytoplasm rather than in the nucleus, neither aggregation nor disease occurs (Klement *et al.*, 1998). These applications would be important to many other neurodegenerative diseases such as Alzheimer's, amyotrophic lateral sclerosis and Parkinson's disease, which also involve the gradual accumulation of mutant proteins in the cell nucleus (Zoghbi and Orr, 2000).

5.3.4 Mutant protein expression

The level of mutant protein can be reduced at the mRNA level through RNA interference (Ross and Poirier, 2004). This effect has been investigated in SCA3, where a small interfering RNA (siRNA) was engineered to silence expression of the disease allele which differed from wild-type alleles by a single nucleotide polymorphism (Miller *et al.*, 2003; Li *et al.*, 2004).

5.3.5 Cleavage fragments

If in fact cleavage fragments are involved in SCA3 pathogenesis, decreasing their concentration below a critical level could be explored as a goal for therapy (Goti *et al.*, 2004). This has been the case in Huntington's disease, where inhibition of the caspase-1 enzyme prevented cleavage and led to recovery of the phenotype (Ona *et al.*, 1999).

5.3.6 Apoptotic inhibitors

The successful use of other caspase inhibitors such as zVAD-fmk and minocycline, used to prevent the activation of the apoptotic pathway in HD models (Ona *et al.*, 1999), could be applicable to other polyglutamine diseases. Also important to the study of apoptotic inhibitors is the effect of contagious apoptosis, whereby neighbouring cells are exposed to triggering factors that are similar to those that affect a cell that is dying, as the dying neuron releases proapototic factors (Friedlander, 2003). Using inhibitors of apoptosis will not only slow the death of one particular cell, but also restrict the production of diffusible toxic factors. This would be important in controlling the number of neuronal cells affected by apoptosis.

5.3.7 Transcriptional dysregulation

Given that the transcriptional machinery is impaired in many polyglutamine disorders, one possibility for treatment would be through administration of histone deacetylase inhibitors, such as suberoylanilide hydroxamic acid (SAHA). This chemical has been shown to be effective in *Drosophila* and mouse models of Huntington's as it dramatically improved the motor impairment of transgenic mice (Hockly *et al.*, 2003). Another example was done using genetic reduction of *Sin3A* corepressor activity in a *Drosophila* model which alleviated transcriptional repression (Steffan *et al.*, 2001). Though RNAPII function was not impaired in SCA3 (as shown by our results), other polyglutamine disorders could benefit from the use of drugs, such as 5,6-dichloro-1-[beta]-D-ribofuranosylbenzimidazole (DRB) or isoquinolinesulfonamide derivatives (H8 and H7)

(Dubois *et al.*, 1994), which modulate phosphorylation levels of RNAPII and could be an effective therapy for this type of transcriptional dysregulation.

5.4 Conclusion

The research presented in this manuscript along with all other findings published in the field, contribute to a better understanding of Machado-Joseph disease (SCA3). Hopefully, this work will be applicable to finding a cure for not only this disorder, but also for other related diseases with the ultimate goal of improving a patient's quality of life.

References

Abu-Baker A, Messaed C, Laganiere J, Gaspar C, Brais B, Rouleau GA. (2003) Involvement of the ubiquitin-proteasome pathway and molecular chaperones in oculopharyngeal muscular dystrophy. Hum Mol Genet. 12(20):2609-23.

Ahn SH, Kim M, Buratowski S. (2004) Phosphorylation of serine 2 within the RNA polymerase II C-terminal domain couples transcription and 3' end processing. Mol Cell. 13(1):67-76.

Apostol BL, Kazantsev A, Raffioni S, Illes K, Pallos J, Bodai L, Slepko N, Bear JE, Gertler FB, Hersch S, Housman DE, Marsh JL, Thompson LM. (2003) A cell-based assay for aggregation inhibitors as therapeutics of polyglutamine-repeat disease and validation in Drosophila. Proc Natl Acad Sci U S A. 100(10):5950-5.

Arrasate M, Mitra S, Schweitzer ES, Segal MR, Finkbeiner S. (2004) Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. Nature. 431(7010):805-10.

Atkinson J, Dodge M, Gallant J. (1997) Secondary structures and starvation-induced frameshifting. Mol Microbiol. 26(4):747-753

Bardoni B, Mandel JL. (2002) Advances in understanding of fragile X pathogenesis and FMRP function, and in identification of X linked mental retardation genes. Curr Opin Genet Dev. 12(3):284-93.

Berger Z, Davies JE, Luo S, Pasco MY, Majoul I, O'Kane CJ, Rubinsztein DC. (2006) Deleterious and protective properties of an aggregate-prone protein with a polyalanine expansion. Hum Mol Genet. 15(3):453-65.

Berke SJ, Chai Y, Marrs GL, Wen H, Paulson HL. (2005) Defining the role of ubiquitin-interacting motifs in the polyglutamine disease protein, ataxin-3. J Biol Chem. 280(36):32026-34.

Blondelle SE, Forood B, Houghten RA, Perez-Paya E. (1997) Polyalanine-based peptides as models for self-associated beta-pleated-sheet complexes. Biochemistry. 36(27):8393-400.

Bonini NM, Leiserson WM, Benzer S. (1993) The eyes absent gene: genetic control of cell survival and differentiation in the developing Drosophila eye. Cell 72(3):379-95.

Bowman AB, Yoo SY, Dantuma NP, Zoghbi HY. (2005) Neuronal dysfunction in a polyglutamine disease model occurs in the absence of ubiquitin-proteasome system impairment and inversely correlates with the degree of nuclear inclusion formation. Hum Mol Genet. 14(5):679-91.

Brais B, Bouchard JP, Xie YG, Rochefort DL, Chretien N, Tome FM, Lafreniere RG, Rommens JM, Uyama E, Nohira O, Blumen S, Korczyn AD, Heutink P, Mathieu J, Duranceau A, Codere F, Fardeau M, Rouleau GA. (1998) Short GCG expansions in the PABP2 gene cause oculopharyngeal muscular dystrophy. Nat Genet. 18(2):164-7.

Burnett B, Li F, Pittman RN. (2003) The polyglutamine neurodegenerative protein ataxin-3 binds polyubiquitylated proteins and has ubiquitin protease activity. Hum Mol Genet. 12(23):3195-205.

Burnett BG, Pittman RN. (2005) The polyglutamine neurodegenerative protein ataxin 3 regulates aggresome formation. Proc Natl Acad Sci U S A. 102(12):4330-5.

Cadena DL, Dahmus ME. (1987) Messenger RNA synthesis in mammalian cells is catalyzed by the phosphorylated form of RNA polymerase II. J Biol Chem. 262(26):12468-74.

Calado A, Tome FM, Brais B, Rouleau GA, Kuhn U, Wahle E, Carmo-Fonseca M. (2000) Nuclear inclusions in oculopharyngeal muscular dystrophy consist of poly(A) binding protein 2 aggregates which sequester poly(A) RNA. Hum Mol Genet. 9(15):2321-8.

Cemal CK, Carroll CJ, Lawrence L, Lowrie MB, Ruddle P, Al-Mahdawi S, King RH, Pook MA, Huxley C, Chamberlain S. (2002) YAC transgenic mice carrying pathological alleles of the MJD1 locus exhibit a mild and slowly progressive cerebellar deficit. Hum Mol Genet. 11(9):1075-94.

Chai Y, Berke SS, Cohen RE, Paulson HL. (2004) Poly-ubiquitin binding by the polyglutamine disease protein ataxin-3 links its normal function to protein surveillance pathways. J Biol Chem. 279(5):3605-11.

Chai Y, Koppenhafer SL, Bonini NM, Paulson HL. (1999a) Analysis of the role of heat shock protein (Hsp) molecular chaperones in polyglutamine disease. J Neurosci. 19(23):10338-47.

Chai Y, Koppenhafer SL, Shoesmith SJ, Perez MK, Paulson HL. (1999b) Evidence for proteasome involvement in polyglutamine disease: localization to nuclear inclusions in SCA3/MJD and suppression of polyglutamine aggregation in vitro. Hum Mol Genet. 8(4):673-82.

Chai Y, Shao J, Miller VM, Williams A, Paulson HL. (2002) Live-cell imaging reveals divergent intracellular dynamics of polyglutamine disease proteins and supports a sequestration model of pathogenesis. Proc Natl Acad Sci U S A. 99(14):9310-5

Chai Y, Wu L, Griffin JD, Paulson HL. (2001) The role of protein composition in specifying nuclear inclusion formation in polyglutamine disease. J Biol Chem. 276(48):44889-97.

Cho H, Orphanides G, Sun X, Yang XJ, Ogryzko V, Lees E, Nakatani Y, Reinberg D. (1998) A human RNA polymerase II complex containing factors that modify chromatin structure. Mol Cell Biol. 18(9):5355-63.

Chou AH, Yeh TH, Kuo YL, Kao YC, Jou MJ, Hsu CY, Tsai SR, Kakizuka A, Wang HL. (2006) Polyglutamine-expanded ataxin-3 activates mitochondrial apoptotic pathway by upregulating Bax and downregulating Bcl-xL. Neurobiol Dis. 21(2):333-45.

Chow MK, Paulson HL, Bottomley SP. (2004) Destabilization of a non-pathological variant of ataxin-3 results in fibrillogenesis via a partially folded intermediate: a model for misfolding in polyglutamine disease. J Mol Biol. 335(1):333-41.

Ciechanover A, Brundin P. (2003) The ubiquitin proteasome system in neurodegenerative diseases: sometimes the chicken, sometimes the egg. Neuron. 40(2):427-46.

Ciechanover A. (1994) The ubiquitin-proteasome proteolytic pathway. Cell. 79(1):13-21.

Colomer Gould VF. (2005) Mouse models of Machado-Joseph disease and other polyglutamine spinocerebellar ataxias. NeuroRx. 2(3):480-3.

Corden, JL (1990) Tails of RNA polymerase II.Trends Biochem. Sci., 15, 383-387.

Cummings CJ, Zoghbi HY. (2000). Fourteen and counting: unraveling trinucleotide repeat diseases. Hum Mol Genet. 9(6):909-16.

Davies SW, Turmaine M, Cozens BA, DiFiglia M, Sharp AH, Ross CA, Scherzinger E, Wanker EE, Mangiarini L, Bates GP. (1997) Formation of neuronal intranuclear

inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. Cell. 90(3):537-48.

de Chiara C, Menon RP, Dal Piaz F, Calder L, Pastore A. (2005) Polyglutamine is not all: the functional role of the AXH domain in the ataxin-1 protein. J Mol Biol. 354(4):883-93.

Ding Q, Lewis JJ, Strum KM, Dimayuga E, Bruce-Keller AJ, Dunn JC, Keller JN. (2002) Polyglutamine expansion, protein aggregation, proteasome activity, and neural survival. J Biol Chem. 277(16):13935-42.

Di Prospero NA, Fischbeck KH. (2005) Therapeutics development for triplet repeat expansion diseases. Nat Rev Genet. 6(10):756-65.

Driscoll M, Gerstbrein B. (2003) Dying for a cause: invertebrate genetics takes on human neurodegeneration. Nat Rev Genet. 4(3):181-94.

Dubois MF, Nguyen VT, Bellier S, Bensaude O. (1994) Inhibitors of transcription such as 5,6-dichloro-1-b-D ribofuranosylbenzimidazole (DRB) and isoquinolinesulfonamide derivatives (H-8 and H-7*), promote the dephosphorylation of the C terminal domain (CTD) of RNA polymerase II largest subunit. J. Biol. Chem. 269, 13331-13336.

Dürr A, Stevanin G, Cancel G, Duyckaerts C, Abbas N, Didierjean O, Chneiweiss H, Benomar A, Lyon-Caen O, Julien J, Serdaru M, Penet C, Agid Y, Brice A. (1996) Spinocerebellar ataxia 3 and Machado-Joseph disease: clinical, molecular, and neuropathological features. Ann Neurol. 39(4):490-9.

Everett CM, Wood NW. (2004) Trinucleotide repeats and neurodegenerative disease. Brain. 127(11):2385-405.

Evert BO, Vogt IR, Vieira-Saecker AM, Ozimek L, de Vos RA, Brunt ER, Klockgether T, Wullner U. (2003) Gene expression profiling in ataxin-3 expressing cell lines reveals distinct effects of normal and mutant ataxin-3. J Neuropathol Exp Neurol. 62(10):1006-18.

Evert BO, Wullner U, Schulz JB, Weller M, Groscurth P, Trottier Y, Brice A, Klockgether T. (1999) High level expression of expanded full-length ataxin-3 in vitro causes cell death and formation of intranuclear inclusions in neuronal cells. Hum Mol Genet. 8(7):1169-76.

Farabaugh PJ. (1996) Programmed translational frameshifting. Annu Rev Genet. 1996;30:507-28.

Forood B, Perez-Paya E, Houghten RA, Blondelle SE. (1995) Formation of an extremely stable polyalanine beta-sheet macromolecule. Biochem Biophys Res Commun. 211(1):7-13.

Fortini ME, Bonini NM. (2000) Modeling human neurodegenerative diseases in Drosophila: on a wing and a prayer. Trends Genet. 16(4):161-7.

Friedlander RM. (2003) Apoptosis and caspases in neurodegenerative diseases. N Engl J Med. 348(14):1365-75.

Fujigasaki H, Uchihara T, Koyano S, Iwabuchi K, Yagishita S, Makifuchi T, Nakamura A, Ishida K, Toru S, Hirai S, Ishikawa K, Tanabe T, Mizusawa H. (2000) Ataxin-3 is translocated into the nucleus for the formation of intranuclear inclusions in normal and Machado-Joseph disease brains. Exp Neurol. 165(2):248-56.

Gabai VL, Meriin AB, Mosser DD, Caron AW, Rits S, Shifrin VI, Sherman MY. (1997) Hsp70 prevents activation of stress kinases. A novel pathway of cellular thermotolerance.J Biol Chem. 272(29):18033-7.

Galvao R, Mendes-Soares L, Camara J, Jaco I, Carmo-Fonseca M. (2004) Triplet repeats, RNA secondary structure and toxic gain-of-function models for pathogenesis. Brain Res Bull. 56(3-4):191-201.

Gaspar C, Jannatipour M, Dion P, Laganiere J, Sequeiros J, Brais B, Rouleau GA. (2000) CAG tract of MJD-1 may be prone to frameshifts causing polyalanine accumulation. Hum Mol Genet. 9(13):1957-66.

Ghosh S, Feany MB. (2004) Comparison of pathways controlling toxicity in the eye and brain in Drosophila models of human neurodegenerative diseases. Hum Mol Genet. 13(18):2011-8.

Goldberg AL. (2003) Protein degradation and protection against misfolded or damaged proteins. Nature. 426(6968):895-9.

Goti D, Katzen SM, Mez J, Kurtis N, Kiluk J, Ben-Haiem L, Jenkins NA, Copeland NG, Kakizuka A, Sharp AH, Ross CA, Mouton PR, Colomer V. (2004) A mutant ataxin-3 putative-cleavage fragment in brains of Machado-Joseph disease patients and transgenic mice is cytotoxic above a critical concentration. J Neurosci. 24(45):10266-79.

Goto J, Watanabe M, Ichikawa Y, Yee SB, Ihara N, Endo K, Igarashi S, Takiyama Y, Gaspar C, Maciel P, Tsuji S, Rouleau GA, Kanazawa I. (1997) Machado–Joseph disease gene products carrying different carboxyl termini. Neurosci Res. 28(4):373-7.

Green H. (1993) Human genetic diseases due to codon reiteration: relationship to an evolutionary mechanism. Cell. 74(6):955-6.

Gusella JF, MacDonald ME. (2000) Molecular genetics: unmasking polyglutamine triggers in neurodegenerative disease. Nat Rev Neurosci. 1(2):109-15.

Haacke A, Broadley SA, Boteva R, Tzvetkov N, Hartl FU, Breuer P. (2006) Proteolytic cleavage of polyglutamine-expanded ataxin-3 is critical for aggregation and sequestration of non-expanded ataxin-3. Hum Mol Genet. 15(4):555-68.

Harger JW, Meskauskas A, Dinman JD. (2002) An "integrated model" of programmed ribosomal frameshifting. Trends Biochem Sci. 27(9):448-54.

Ho CK, Shuman S. (1999) Distinct roles for CTD Ser-2 and Ser-5 phosphorylation in the recruitment and allosteric activation of mammalian mRNA capping enzyme. Mol Cell. 3(3):405-11.

Hockly E, Richon VM, Woodman B, Smith DL, Zhou X, Rosa E, Sathasivam K, Ghazi-Noori S, Mahal A, Lowden PA, Steffan JS, Marsh JL, Thompson LM, Lewis CM, Marks PA, Bates GP. (2003) Suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, ameliorates motor deficits in a mouse model of Huntington's disease. Proc Natl Acad Sci U S A. 100(4):2041-6.

Hoshino M, Tagawa K, Okuda T, Okazawa H. (2004) General transcriptional repression by polyglutamine disease proteins is not directly linked to the presence of inclusion bodies. Biochem Biophys Res Commun. 313(1):110-6.

Ichikawa Y, Goto J, Hattori M, Toyoda A, Ishii K, Jeong SY, Hashida H, Masuda N, Ikeda H, Yamaguchi M, Sugai S, Aze Y, Narumiya S, Kakizuka A. (1996) Expanded polyglutamine in the Machado-Joseph disease protein induces cell death in vitro and in vivo. Nat Genet. 13(2):196-202.

Jacks T, Madhani HD, Masiarz FR, Varmus HE. (1988) Signals for ribosomal frameshifting in the Rous sarcoma virus gag-pol region. Cell. 55(3):447-58.

Jana NR, Nukina N. (2004) Misfolding promotes the ubiquitination of polyglutamine-expanded ataxin-3, the defective gene product in SCA3/MJD. Neurotox Res. 6(7-8):523-33.

Jasinska A, Michlewski G, de Mezer M, Sobczak K, Kozlowski P, Napierala M, Krzyzosiak WJ. (2003) Structures of trinucleotide repeats in human transcripts and their functional implications. Nucleic Acids Res. 31(19):5463-8.

Jones JC, Phatnani HP, Haystead TA, MacDonald JA, Alam SM, Greenleaf AL. (2004) C-terminal repeat domain kinase I phosphorylates Ser2 and Ser5 of RNA polymerase II C-terminal domain repeats. J Biol Chem. 279(24):24957-64.

Kahlem P, Green H, Djian P. (1998) Transglutaminase action imitates Huntington's disease: selective polymerization of Huntingtin containing expanded polyglutamine. Mol Cell. 1(4):595-601.

Kahlem P, Terre C, Green H, Djian P. (1996) Peptides containing glutamine repeats as substrates for transglutaminase-catalyzed cross-linking: relevance to diseases of the nervous system. Proc Natl Acad Sci U S A. 93(25):14580-5.

Kawaguchi Y, Kovacs JJ, McLaurin A, Vance JM, Ito A, Yao TP. (2003) The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. Cell. 115(6):727-38.

Kawaguchi Y, Okamoto T, Taniwaki M, Aizawa M, Inoue M, Katayama S, Kawakami H, Nakamura S, Nishimura M, Akiguchi I, *et al.* (1994) CAG expansions in a novel gene for Machado-Joseph disease at chromosome 14q32.1. Nat Genet. 8(3):221-8.

Kershnar E, Wu SY, Chiang CM. (1998) Immunoaffinity purification and functional characterization of human transcription factor IIH and RNA polymerase II from clonal cell lines that conditionally express epitope-tagged subunits of the multiprotein complexes. J Biol Chem. 273(51):34444-53.

Klement IA, Skinner PJ, Kaytor MD, Yi H, Hersch SM, Clark HB, Zoghbi HY, Orr HT. (1998) Ataxin-1 nuclear localization and aggregation: role in polyglutamine-induced disease in SCA1 transgenic mice. Cell. 95(1):41-53.

Kormanitsky, P, Cho EJ and Buratowski S (2000). Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription. Genes Dev. 14, 2452-2460.

La Spada AR, Taylor JP. (2003) Polyglutamines placed into context. Neuron. 38(5):681-4.

Latouche M, Fragner P, Martin E, El Hachimi KH, Zander C, Sittler A, Ruberg M, Brice A, Stevanin G. (2006) Polyglutamine and polyalanine expansions in ataxin7 result in different types of aggregation and levels of toxicity. Mol Cell Neurosci. 31(3):438-45.

Lerer I, Merims D, Abeliovich D, Zlotogora J, Gadoth N. (1996) Machado-Joseph disease: correlation between the clinical features, the CAG repeat length and homozygosity for the mutation. Eur J Hum Genet.4(1):3-7.

Li F, Macfarlan T, Pittman RN, Chakravarti D. (2002) Ataxin-3 is a histone-binding protein with two independent transcriptional corepressor activities. J Biol Chem.277(47):45004-12.

Li Y, Yokota T, Matsumura R, Taira K, Mizusawa H. (2004) Sequence-dependent and independent inhibition specific for mutant ataxin-3 by small interfering RNA. Ann Neurol. 56(1):124-9.

Liu YZ, Chrivia JC, Latchman DS. (1998) Nerve growth factor up-regulates the transcriptional activity of CBP through activation of the p42/p44(MAPK) cascade. J Biol Chem. 273(49):32400-7.

Luthi-Carter R, Hanson SA, Strand AD, Bergstrom DA, Chun W, Peters NL, Woods AM, Chan EY, Kooperberg C, Krainc D, Young AB, Tapscott SJ, Olson JM. (2002) Dysregulation of gene expression in the R6/2 model of polyglutamine disease: parallel changes in muscle and brain. Hum Mol Genet. 11(17):1911-26.

Mao Y, Senic-Matuglia F, Di Fiore PP, Polo S, Hodsdon ME, De Camilli P. (2005) Deubiquitinating function of ataxin-3: insights from the solution structure of the Josephin domain. Proc Natl Acad Sci U S A. 102(36):12700-5.

Margolis RL. (2003) Dominant spinocerebellar ataxias: a molecular approach to classification, diagnosis, pathogenesis and the future. Expert Rev Mol Diagn. 3(6):715-32.

Marshall NF, Price DH. (1995) Purification of P-TEFb, a transcription factor required for the transition into productive elongation. J Biol Chem. 270(21):12335-8.

McCampbell A, Taye AA, Whitty L, Penney E, Steffan JS, Fischbeck KH. (2001) Histone deacetylase inhibitors reduce polyglutamine toxicity. Proc Natl Acad Sci U S A. 98(26):15179-84.

McCampbell A, Taylor JP, Taye AA, Robitschek J, Li M, Walcott J, Merry D, Chai Y, Paulson H, Sobue G, Fischbeck KH. (2000) CREB-binding protein sequestration by expanded polyglutamine. Hum Mol Genet. 9(14):2197-202.

McClellan AJ, Frydman J. (2001) Molecular chaperones and the art of recognizing a lost cause. Nat Cell Biol. 3(2):E51-3.

Michalik A, Van Broeckhoven C. (2003) Pathogenesis of polyglutamine disorders: aggregation revisited. Hum Mol Genet. 12:R173-86.

Michlewski G, Krzyzosiak WJ. (2004) Molecular architecture of CAG repeats in human disease related transcripts. J Mol Biol. 340(4):665-79.

Miller VM, Xia H, Marrs GL, Gouvion CM, Lee G, Davidson BL, Paulson HL. (2003) Allele-specific silencing of dominant disease genes. Proc Natl Acad Sci U S A. 2003 100(12):7195-200.

Muqit MM, Feany MB. (2002) Modelling neurodegenerative diseases in Drosophila: a fruitful approach? Nat Rev Neurosci. 3(3):237-43.

Namy O, Moran SJ, Stuart DI, Gilbert RJ, Brierley I. (2006) A mechanical explanation of RNA pseudoknot function in programmed ribosomal frameshifting. Nature. 441(7090):244-7.

Ni Z, Schwartz BE, Werner J, Suarez JR, Lis JT. (2004) Coordination of transcription, RNA processing, and surveillance by P-TEFb kinase on heat shock genes. Mol Cell;13(1):55-65.

Okazawa H. (2003) Polyglutamine diseases: a transcription disorder? Cell Mol Life Sci. 60(7):1427-39.

Ona VO, Li M, Vonsattel JP, Andrews LJ, Khan SQ, Chung WM, Frey AS, Menon AS, Li XJ, Stieg PE, Yuan J, Penney JB, Young AB, Cha JH, Friedlander RM. (1999) Inhibition of caspase-1 slows disease progression in a mouse model of Huntington's disease. Nature. 399(6733):263-7.

Palancade B, Bensaude O. (2003) Investigating RNA polymerase II carboxyl-terminal domain (CTD) phosphorylation. Eur J Biochem. 270(19):3859-70.

Paulson HL, Das SS, Crino PB, Perez MK, Patel SC, Gotsdiner D, Fischbeck KH, Pittman RN. (1997a) Machado-Joseph disease gene product is a cytoplasmic protein widely expressed in brain. Ann Neurol. 41(4):453-62.

Paulson HL, Perez MK, Trottier Y, Trojanowski JQ, Subramony SH, Das SS, Vig P, Mandel JL, Fischbeck KH, Pittman RN. (1997b) Intranuclear inclusions of expanded polyglutamine protein in spinocerebellar ataxia type 3. Neuron. 19(2):333-44.

Paulson HL. (1999) Protein fate in neurodegenerative proteinopathies: polyglutamine diseases join the (mis)fold. Am J Hum Genet. 64(2):339-45.

Payne JM, Laybourn PJ, Dahmus ME. (1989) The transition of RNA polymerase II from initiation to elongation is associated with phosphorylation of the carboxyl-terminal domain of subunit IIa. J Biol Chem. 264(33):19621-9.

Pearson CE, Nichol Edamura K, Cleary JD. (2005) Repeat instability: mechanisms of dynamic mutations. Nat Rev Genet. 6(10):729-42.

Perez MK, Paulson HL, Pendse SJ, Saionz SJ, Bonini NM, Pittman RN. (1998) Recruitment and the role of nuclear localization in polyglutamine-mediated aggregation. J Cell Biol.143(6):1457-70.

Perez MK, Paulson HL, Pittman RN. (1999) Ataxin-3 with an altered conformation that exposes the polyglutamine domain is associated with the nuclear matrix. Hum Mol Genet. 8(13):2377-85.

Perutz MF, Johnson T, Suzuki M, Finch JT. (1994) Glutamine repeats as polar zippers: their possible role in inherited neurodegenerative diseases. Proc Natl Acad Sci U S A. 91(12):5355-8.

Pieretti M, Zhang FP, Fu YH, Warren ST, Oostra BA, Caskey CT, Nelson DL. (1991) Absence of expression of the FMR-1 gene in fragile X syndrome. Cell. 66(4):817-22.

Proudfoot NJ, Furger A and Dye MJ. (2002) Integrating mRNA processing with transcription. Cell 108: 501-512.

Rankin J, Wyttenbach A, Rubinsztein DC. (2000) Intracellular green fluorescent protein-polyalanine aggregates are associated with cell death. Biochem J. 348:15-9.

Ranum LP, Day JW. (2002) Dominantly inherited, non-coding microsatellite expansion disorders. Curr Opin Genet Dev. 2002 Jun;12(3):266-71. Review.

Ravikumar B, Duden R, Rubinsztein DC. (2002) Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. Hum Mol Genet. 11(9):1107-17.

Ravikumar B, Vacher C, Berger Z, Davies JE, Luo S, Oroz LG, Scaravilli F, Easton DF, Duden R, O'Kane CJ, Rubinsztein DC. (2004) Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat Genet. 36(6):585-95.

Reddy PS, Housman DE. (1997) The complex pathology of trinucleotide repeats. Curr Opin Cell Biol. 9(3):364-72.

Rodriguez CR, Cho EJ, Keogh MC, Moore CL, Greenleaf AL, Buratowski S. (2000) Kin28, the TFIIH-associated carboxy-terminal domain kinase, facilitates the recruitment of mRNA processing machinery to RNA polymerase II. Mol Cell Biol. 20(1):104-12.

Ross CA, Poirier MA (2004) Protein aggregation and neurodegenerative disease. Nat. Med. 10: S10-S17.

Ross CA. (1997) Intranuclear neuronal inclusions: a common pathogenic mechanism for glutamine-repeat neurodegenerative diseases? Neuron. 19(6):1147-50.

Ruegg S, Lehky Hagen M, Hohl U, Kappos L, Fuhr P, Plasilov M, Muller H, Heinimann K. (2005) Oculopharyngeal muscular dystrophy - an under-diagnosed disorder? Swiss Med Wkly. 135(39-40):574-86.

Sanchez I, Mahlke C, Yuan J. (2003) Pivotal role of oligomerization in expanded polyglutamine neurodegenerative disorders. Nature. 421(6921):373-9.

Scheel H, Tomiuk S, Hofmann K. (2003) Elucidation of ataxin-3 and ataxin-7 function by integrative bioinformatics. Hum Mol Genet. 12(21):2845-52.

Schmidt T, Landwehrmeyer GB, Schmitt I, Trottier Y, Auburger G, Laccone F, Klockgether T, Volpel M, Epplen JT, Schols L, Riess O. (1998) An isoform of ataxin-3 accumulates in the nucleus of neuronal cells in affected brain regions of SCA3 patients. Brain Pathol. 8(4):669-79.

Schmidt T, Lindenberg KS, Krebs A, Schols L, Laccone F, Herms J, Rechsteiner M, Riess O, Landwehrmeyer GB. (2002) Protein surveillance machinery in brains with spinocerebellar ataxia type 3: redistribution and differential recruitment of 26S proteasome subunits and chaperones to neuronal intranuclear inclusions. Ann Neurol. 51(3):302-10.

Schroeder SC, Schwer B, Shuman S, Bentley D. (2000) Dynamic association of capping enzymes with transcribing RNA polymerase II. Genes Dev. 14(19):2435-40.

Seeler JS, Dejean A. (1999) The PML nuclear bodies: actors or extras? Curr Opin Genet Dev. 9(3):362-7.

Shigemoto K, Brennan J, Walls E, Watson CJ, Stott D, Rigby PW, Reith AD. (2001) Identification and characterisation of a developmentally regulated mammalian gene that utilises -1 programmed ribosomal frameshifting. Nucleic Acids Res. 29(19):4079-88.

Shimohata T, Nakajima T, Yamada M, Uchida C, Onodera O, Naruse S, Kimura T, Koide R, Nozaki K, Sano Y, Ishiguro H, Sakoe K, Ooshima T, Sato A, Ikeuchi T, Oyake M, Sato T, Aoyagi Y, Hozumi I, Nagatsu T, Takiyama Y, Nishizawa M, Goto J, Kanazawa I, Davidson I, Tanese N, Takahashi H, Tsuji S. (2000) Expanded polyglutamine stretches interact with TAFII130, interfering with CREB-dependent transcription. Nat Genet. 26(1):29-36.

Sisodia SS. (1998) Nuclear inclusions in glutamine repeat disorders: are they pernicious, coincidental, or beneficial? Cell. 95(1):1-4.

Sobue G, Doyu M, Nakao N, Shimada N, Mitsuma T, Maruyama H, Kawakami S, Nakamura S. (1996) Homozygosity for Machado-Joseph disease gene enhances phenotypic severity. J Neurol Neurosurg Psychiatry. 60(3):354-6.

Steffan JS, Bodai L, Pallos J, Poelman M, McCampbell A, Apostol BL, Kazantsev A, Schmidt E, Zhu YZ, Greenwald M, Kurokawa R, Housman DE, Jackson GR, Marsh JL, Thompson LM. (2001) Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in Drosophila. Nature. 413(6857):739-43.

Stott K, Blackburn JM, Butler PJ, Perutz M. (1995) Incorporation of glutamine repeats makes protein oligomerize: implications for neurodegenerative diseases. Proc Natl Acad Sci U S A. 92(14):6509-13.

Sudarsky L, Coutinho P. (1995) Machado-Joseph disease. Clin Neurosci. 3(1):17-22.

Tait D, Riccio M, Sittler A, Scherzinger E, Santi S, Ognibene A, Maraldi NM, Lehrach H, Wanker EE. (1998) Ataxin-3 is transported into the nucleus and associates with the nuclear matrix. Hum Mol Genet. 7(6):991-7.

Takahashi J, Fujigasaki H, Iwabuchi K, Bruni AC, Uchihara T, El Hachimi KH, Stevanin G, Durr A, Lebre AS, Trottier Y, de The H, Tanaka J, Hauw JJ, Duyckaerts C, Brice A. (2003). PML nuclear bodies and neuronal intranuclear inclusion in polyglutamine diseases. Neurobiol Dis. 13(3):230-7.

Takiyama Y, Nishizawa M, Tanaka H, Kawashima S, Sakamoto H, Karube Y, Shimazaki H, Soutome M, Endo K, Ohta S, Kagawa Y, Kanazawa I, Mizuno Y, Yoshida M, Yuasa T, Horikawa Y, Oyanagi K, Nagai H, Kondo T, Inuzuka T, Onodera O, Tsuji S. (1993) The gene for Machado-Joseph disease maps to human chromosome 14q. Nat Genet. 4(3):300-4.

Taneja KL, McCurrach M, Schalling M, Housman D, Singer RH. (1995) Foci of trinucleotide repeat transcripts in nuclei of myotonic dystrophy cells and tissues. J Cell Biol. 128(6):995-1002.

Toulouse A, Au-Yeung F, Gaspar C, Roussel J, Dion P, Rouleau GA. (2005) Ribosomal frameshifting on MJD-1 transcripts with long CAG tracts. Hum Mol Genet. 14(18):2649-60.

Tsai HF, Tsai HJ, Hsieh M. (2004) Full-length expanded ataxin-3 enhances mitochondrial-mediated cell death and decreases Bcl-2 expression in human neuroblastoma cells. Biochem Biophys Res Commun. 324(4):1274-82.

Uchihara T, Fujigasaki H, Koyano S, Nakamura A, Yagishita S, Iwabuchi K. (2001) Non-expanded polyglutamine proteins in intranuclear inclusions of hereditary ataxias--triple-labeling immunofluorescence study. Acta Neuropathol (Berl). 102(2):149-52.

Uchihara T, Iwabuchi K, Funata N, Yagishita S. (2002) Attenuated nuclear shrinkage in neurons with nuclear aggregates--a morphometric study on pontine neurons of Machado-Joseph disease brains. Exp Neurol. 178(1):124-8.

Vallee RB, Williams JC, Varma D, Barnhart LE. (2004) Dynein: An ancient motor protein involved in multiple modes of transport. J Neurobiol. 58(2):189-200.

van Alfen N, Sinke RJ, Zwarts MJ, Gabreels-Festen A, Praamstra P, Kremer BP, Horstink MW. (2001) Intermediate CAG repeat lengths (53,54) for MJD/SCA3 are associated with an abnormal phenotype. Ann Neurol. 49(6):805-7.

Vonsattel JP, Myers RH, Stevens TJ, Ferrante RJ, Bird ED, Richardson EP Jr. (1985) Neuropathological classification of Huntington's disease. J Neuropathol Exp Neurol. 44(6):559-77.

Wang J, Pegoraro E, Menegazzo E, Gennarelli M, Hoop RC, Angelini C, Hoffman EP. (1995) Myotonic dystrophy: evidence for a possible dominant-negative RNA mutation. Hum Mol Genet. 4(4):599-606.

Warrick JM, Morabito LM, Bilen J, Gordesky-Gold B, Faust LZ, Paulson HL, Bonini NM. (2005) Ataxin-3 suppresses polyglutamine neurodegeneration in Drosophila by a ubiquitin-associated mechanism. Mol Cell. 18(1):37-48.

Warrick JM, Paulson HL, Gray-Board GL, Bui QT, Fischbeck KH, Pittman RN, Bonini NM. (1998) Expanded polyglutamine protein forms nuclear inclusions and causes neural degeneration in Drosophila. Cell 93(6):939-49.

Wellington CL, Ellerby LM, Hackam AS, Margolis RL, Trifiro MA, Singaraja R, McCutcheon K, Salvesen GS, Propp SS, Bromm M, Rowland KJ, Zhang T, Rasper

D, Roy S, Thornberry N, Pinsky L, Kakizuka A, Ross CA, Nicholson DW, Bredesen DE, Hayden MR. (1998) Caspase cleavage of gene products associated with triplet expansion disorders generates truncated fragments containing the polyglutamine tract. J Biol Chem. 273(15):9158-67.

Wood JD, Nucifora FC Jr, Duan K, Zhang C, Wang J, Kim Y, Schilling G, Sacchi N, Liu JM, Ross CA. (2000) Atrophin-1, the dentato-rubral and pallido-luysian atrophy gene product, interacts with ETO/MTG8 in the nuclear matrix and represses transcription. J Cell Biol. 150(5):939-48.

Yamada M, Hayashi S, Tsuji S, Takahashi H. (2001) Involvement of the cerebral cortex and autonomic ganglia in Machado-Joseph disease. Acta Neuropathol (Berl). 101(2):140-4.

Yamada M, Sato T, Shimohata T, Hayashi S, Igarashi S, Tsuji S, Takahashi H. (2001a) Interaction between neuronal intranuclear inclusions and promyelocytic leukemia protein nuclear and coiled bodies in CAG repeat diseases. Am J Pathol. 159(5):1785-95.

Yamada M, Tan CF, Inenaga C, Tsuji S, Takahashi H. (2004) Sharing of polyglutamine localization by the neuronal nucleus and cytoplasm in CAG-repeat diseases. Neuropathol Appl Neurobiol. 30(6):665-75.

Yamada M, Tsuji S, Takahashi H. (2000) Pathology of CAG repeat diseases. Neuropathology. 20(4):319-25.

Yeo M, Lin PS, Dahmus ME, Gill GN. (2003) A novel RNA polymerase II C-terminal domain phosphatase that preferentially dephosphorylates serine 5. J Biol Chem. 278(28):26078-85.

Yoshizawa T, Yamagishi Y, Koseki N, Goto J, Yoshida H, Shibasaki F, Shoji S, Kanazawa I. (2000) Cell cycle arrest enhances the in vitro cellular toxicity of the truncated Machado-Joseph disease gene product with an expanded polyglutamine stretch. Hum Mol Genet. 9(1):69-78.

Zeng C, Kim E, Warren SL, Berget SM. (1997) Dynamic relocation of transcription and splicing factors dependent upon transcriptional activity. EMBO J. 17;16(6):1401-12.

Zhong S, Muller S, Ronchetti S, Freemont PS, Dejean A, Pandolfi PP. (2000) Role of SUMO-1-modified PML in nuclear body formation. Blood. 95(9):2748-52.

Zhou H, Li SH, Li XJ. (2001) Chaperone suppression of cellular toxicity of huntingtin is independent of polyglutamine aggregation. J Biol Chem. 276(51):48417-24.

Zhou YX, Takiyama Y, Igarashi S, Li YF, Zhou BY, Gui DC, Endo K, Tanaka H, Chen ZH, Zhou LS, Fan MZ, Yang BX, Weissenbach J, Wang GX, Tsuji S. (1997) Machado-Joseph disease in four Chinese pedigrees: molecular analysis of 15 patients including two juvenile cases and clinical correlations. Neurology 48:482-5.

Zlotogora J. (1997) Dominance and homozygosity. Am J Med Genet. 68(4):412-6.

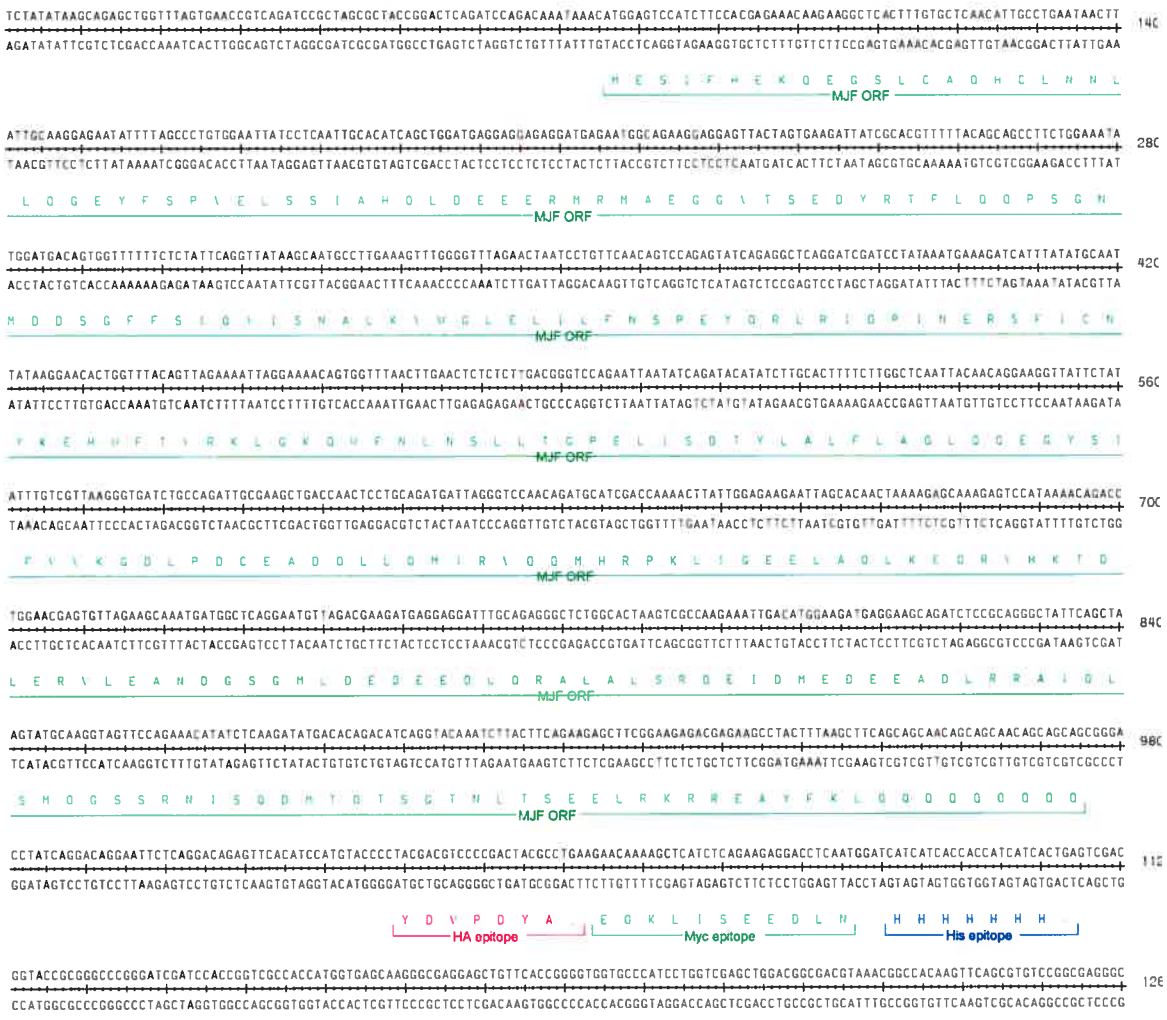
Zoghbi HY, Botas J. (2002) Mouse and fly models of neurodegeneration. Trends Genet. 18(9):463-71.

Zoghbi HY, Orr HT. (1999) Polyglutamine diseases: protein cleavage and aggregation. Curr Opin Neurobiol. 9(5):566-70.

Zoghbi HY, Orr HT. (2000) Glutamine repeats and neurodegeneration. Annu Rev Neurosci. 23:217-47.

Appendix I: Construct sequences

CAG9 sequence



CAG100 sequence

CAA96 sequence